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Electrophysiological studies to counter anthelmintic resistance: studies in somatic muscle and pharynx of A. suum

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Electrophysiological studies to counter anthelmintic resistance: Studies in somatic muscle and pharynx of *A. suum*

by

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DEDICATION

To my parents, family, relatives, friends, well-wishers…

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From my childhood days, existence of life on the Earth and its diversity always fascinated me. Whenever I tried to comprehend the beauty of life, its origins, its intricacies and its mysteries, I always had overwhelming feelings. I realized that one way of paying respect to this life, is to live our own life in a contented way filled with gratitude, humbleness, empathy and compassion.

ABSTRACT

Anthelmintic drugs apart from a regular treatment of worm infections, find their common use in prophylaxis. The prophylactic usage in farm animals and mass drug administrations (MDA) in humans are common due to an unavailability of effective vaccines. The anthelmintic resistance occurs when an anthelmintic drug is repeatedly used resulting in selection of resistant parasites. Reports of anthelmintics resistance are extensive in farm animals. Emerging reports of resistance in human parasites raises a serious concern. Under the present scenario, research as a part of my graduate program is aimed at countering anthelmintic resistance. I have addressed this aim by working on three different approaches / goals that are arranged into individual chapters in my dissertation. In my first goal (chapter 3), I have proposed a mechanism to potentiate cholinomimetic anthelmintics like levamisole. Levamisole acts on the nicotinic receptors of the somatic (body) muscle of the parasite to produce paralysis. Presently, levamisole resistance is reported in major livestock producing areas across the world. Using *Ascaris suum* a round worm of pigs as a model parasite, I have studied a mechanism to potentiate the levamisole and thus counter levamisole resistance in parasitic worms. I have used electrophysiological methods on the somatic muscle of *A. suum* to study the levamisole response. I have observed that, a brief application of AF2 caused potentiation of levamisole responses. AF2 neuropeptide has been isolated in abundant quantities across nematode species and shown to be excitatory on neuromuscular system of nematodes. I have proposed that AF2

receptors are attractive targets in order to potentiate other cholinomimetic anthelmintics like levamisole, pyrantel and oxantel.

The second goal (chapter 4) explores the study of anthelmintic combination Startect®. Combination of anthelmintic drugs is generally employed to slow the onset of resistance in parasites and to achieve synergism in therapy. Startect® consists of derquantel, a new anthelmintic drug that is combined with abamectin to treat resistant parasitic worms in sheep. Derquantel and abamectin have been hypothesized to interact synergistically to control worm infections. I have tested this hypothesis in isolated tissues (somatic muscle and pharynx) of parasitic nematode *A. suum*. In this study, I have concluded that these two drugs produced a greater than additive effect on the somatic muscle nicotinic receptors but, did not interact on the pharyngeal muscle glutamate receptors.

In the third goal (chapter 5) I have identified nicotinic receptor population in the pharynx of the parasitic worm *A. suum* and proposed them as novel drug targets. In my study, current cholinomimetic drugs did not act on the nicotinic receptors of pharynx. This demonstrated that they are novel targets. As current literature on pharyngeal nicotinic receptors is limited, I have explored their pharmacological properties. I have observed that the nicotinic receptors of pharynx are distinct from the nicotinic receptors of vertebrate host. Hence, these receptors are attractive targets for selective drug targeting of parasites and to minimize the side effects in vertebrate hosts.

Key words: levamisole, potentiation, AF2, abamectin, derquantel, interaction, pharyngeal nAChRs

CHAPTER 1. GENERAL INTRODUCTION

1.1 Introduction

Parasitism is an association between the parasite and the host, benefiting the parasite while harming the host. Endoparasites like helminths reside in different tissues and organs of the host. Helminth parasites infect more than a billion people worldwide. The word “helminth” is derived from the Greek word for “worms”. Helminths fall under three main groups namely: flatworms (platyhelminths, include flukes and tapeworms), thorny headed worms (acanthocephalans) and roundworms (nematodes). Gastro Intestinal (GI) nematodes namely round worms, hook worms and whipworms, are soil transmitted. These GI nematode infections are endemic to a majority of tropical countries (Albonico et al., 2004; Savioli and Albonico, 2004; Hotez et al., 2007a). The high global prevalence of helminth infection in humans and livestock has resulted in debility, reduced productivity and severe economic losses (de Silva et al., 2003; Kaplan, 2004b). The drugs that are used to control helminth infections are called “anthelmintics”. Currently, as there are no effective vaccines available, the treatment and the prophylaxis of worm infections rely mainly upon anthelmintic drugs. Anthelmintics act on the parasitic worms by selectively disrupting their physiology. GI nematode worms reside and maintain their site of predilection in the host gut by constantly moving against peristaltic waves of the GI tract. The movements of worms resemble sinusoidal waves which are facilitated by rhythmic contractions and relaxations of the worm somatic muscle. Worms use their muscular pharynx to constantly feed on the ingesta of the host gut in order to meet

energy demands. Pharyngeal peristalsis helps in ingestion of the food and also aids in digestion. Both movement of the worm and the pharyngeal peristalsis is assisted by rhythmic activations of excitatory neurons and inhibitory motor neurons innervating the somatic or pharyngeal muscle groups. The cholinergic transmission is responsible for the excitatory neuromuscular transmission in somatic muscle as well as pharyngeal muscle. However, the inhibitory transmission differs in somatic muscle and pharyngeal muscle of the worm. The inhibitory neuromuscular transmission is GABAergic in somatic muscle while, glutamatergic in pharyngeal muscle. The neurotransmitters (ACh, GABA or glutamate) released at the neuromuscular junctions activate the post synaptic receptors on the somatic muscle (nAChRs or GABA gated chloride channels) and the pharynx (nAChRs or glutamate gated chloride channels).

Broadly, anthelmintics are classified into two main groups based on their mode of action. First group contains anthelmintics that target the membrane ion-channels of the nematode somatic muscle (example levamisole, pyrantel) or pharynx (example ivermectin). The action on ion channels is rapid in onset (less than 4 hours). The anthelmintic drugs which mimic ACh when they act on the nAChRs of the somatic muscle are referred as cholinergic anthelmintics (example levamisole, pyrantel). Release of endogenous neurotransmitters at the neuromuscular junction leads to receptor binding and the action of neurotransmitter is quickly terminated by active reuptake or enzymatic destruction. However, there are no enzymes in the worm which can inactivate or breakdown the anthelmintic drugs. This result in anthelmintic

drugs to exist in their active form result in the worm, persistent activation of ion channels leading to disruption of the resting membrane potential.

Second group contains anthelmintics that affect the parasite by inhibiting biochemical pathways and/or enzymes. This group includes benzimidazoles like albendazole (inhibit polymerization of microtubules), closantel (inhibits chitinase), diethylcarbamazine (inhibit lipooxygenase), melarsomine (binds to –SH group on enzymes like glutathione reductase), nitazoxanide (inhibit pyruvate-ferredoxin oxidoreductase), nitroscanate (inhibits cholinesterase and uncouple oxidative phosphorylation). The drugs belonging to this group act gradually (1-4 days) resulting in weakening the parasite and eventually its removal from the host (Martin and Robertson, 2000, 2007).

At therapeutic doses, anthelmintics are selective on the parasite over the host and hence, effective in treating worm infections. Treatment with anthelmintics against parasitic worms fails during anthelmintic resistance. Anthelmintic resistance refers to the ability of parasites to survive treatments that are generally effective at the recommended therapeutic doses. Resistance developed to one anthelmintic drug affects the efficacy of other drugs that target the same receptors. The situation becomes alarming when anthelmintic resistance develops to multiple groups of drugs that act on different receptors on the parasite. Intensive use of an anthelmintic agent to control worm infections in herds has led to the selection of genetically resistant parasites. This situation is also true in some human parasitic infections in countries where mass drug administration (MDA) is commonly used. Resistance has reduced the efficacy of many of the currently used anthelmintics

thus, limiting our drug options to treat worm infections (James *et al*., 2009). Further, anthelmintic resistance is also a global concern due to the high prevalence of infections in both humans and livestock (Geerts and Gryseels, 2000; Kaplan, 2004b; Jones and George, 2005). Hence, the development of anthelmintic resistance needs to be countered either by increasing the potency of existing anthelmintics or identifying potential target sites on the worm for the development of new anthelmintic drugs.

1.2 Thesis Organization

I have organized my thesis dissertation into chapters to cover the review of literature together with my research attempts to counter the problem of anthelmintic resistance. In chapter 2, I have reviewed the background information pertaining to the life cycle, ion channels (of somatic muscle and pharynx) and neuropeptides of the parasitic nematode *Ascaris suum*. The emphasis is placed on the ion channels which are the targets of anthelmintic drugs. In addition, how the ion channels in turn are modulated by neuropeptides secreted by nematodes and how we can explore the possibility of utilizing neuropeptide receptors for controlling worm infections. At relevant places, I have included additional information from the model nematode *Caenorhabditis elegans* and the vertebrate hosts for meaningful comparisons. My PhD study includes three projects which employ the current clamp technique. Each project is reported as an individual chapter (Chapter 3, 4 and 5).

The published work in chapter 3 has been reproduced from the journal *Molecular and Biochemical Parasitology* (2010). A background for my project in chapter 3 was

initiated based on the observations made by Trailovic *et al* (2005) on the AF2 receptor responses in somatic muscle of *A. suum*. AF2 is an excitatory neuropeptide found to be abundant across the phylum nematoda. Trailovic *et al* (2005) showed that a brief application of neuropeptide AF2, produces long-lasting potentiation of the membrane potential and the contraction responses of the somatic muscle to ACh. They proposed a model to explain their observations that AF2 stimulates the voltage-activated calcium channels (VACCs) and elevates the cytosolic calcium from the sarcoplasmic stores (Trailovic *et al*., 2005). In subsequent findings, Verma *et al* (2007)confirmed that AF2 caused an increase in the voltage activated calcium currents in *A. suum* somatic muscle. Based on these observations, I have explored the possibility that since AF2 potentiates the ACh response, then does it have any effect on the responses to cholinergic anthelmintics like levamisole? Cholinergic anthelmintics like levamisole mimic ACh when they act on the nAChRs of the *Ascaris* somatic muscle. I have taken the research further by testing the hypothesis that AF2 potentiates levamisole similar to ACh in the somatic muscle of *A. suum*. In a current clamp study, I have examined the interactions of levamisole receptor, AF2 neuropeptide receptor and ryanodine receptors (RyRs) which modulates the cytosolic calcium levels.

Chapter 4 consists of a Pfizer project to test interaction of two anthelmintic drugs derquantel and abamectin present in a novel anthelmintic combination Startect®. The novel anthelmintic combination Startect® has been released recently into the market. These drugs have been hypothesized to act synergistically on the parasitic worms to produce the anthelmintic effect. I have tested this hypothesis on the

somatic muscle and the pharynx of our model parasite *A. suum*. I have used current clamp technique to study the responses to the individual drugs and also their combination on the somatic muscle and the pharynx of *A. suum*.

In chapter 5, I have pharmacologically characterized a novel population of nAChRs on the pharynx of *A. suum*. Here, I have proposed pharyngeal nAChRs of nematodes as unexploited drug target that has the potential to be screened for developing new anthelmintic drugs. Here, I tested the hypothesis that the pharyngeal nAChRs of *Ascaris* are pharmacologically distinct from the known nAChRs of the somatic muscle the worm or nAChRs of the vertebrate host.

In chapter 6, I have included a general discussion summarizing the significance of my research findings and the future studies in this area. In appendix, I have included a published review paper (Robertson *et al*., 2008) elaborating the electrophysiological techniques employed on the somatic muscle preparation of *A. suum*. This paper includes the electrophysiological techniques namely current clamp, voltage clamp and patch clamp, the analysis of the recordings and their interpretation.

CHAPTER 2. LITERATURE REVIEW

2.1 Nematode parasites

Nematodes are among the numerically dominant metazoans on our planet Earth. They are the most ubiquitous, numerous and diverse phylum inhabiting terrestrial, marine and freshwater environments. Plat (1994) estimated that for every five animals living on our planet, four are nematodes! So far, more than 26,000 nematode species have been identified of which, about 16,000 are parasitic species. New species of nematode parasites are still being identified and recorded (Okulewicz *et al*., 2005). Nematodes diverged from the arthropods and chordates lineage over 1200 million years ago (Wang *et al*., 1999). The word “Nematoda” comes from the Greek words “nematos”, meaning thread, and “eidos”, meaning form. Nematodes are known to infect plants, invertebrates and vertebrates. The vast majority of nematodes are free-living microbivores, while many species have adopted parasitic lifestyle. Parasitic nematodes have been known for a long time and the earliest recorded literature dates back to Egyptian papyrus from 1500 BC. Nematode parasites are also mentioned by the ancient Greeks namely Aristotle and Hippocrates (the father of scientific medicine). Some nematode parasite depends on the host for transport to a new food source (phoretic association). Some nematodes use the host as a source of food upon its death (necromantic association).

Most plants and animals have parasitic nematode species adapted uniquely to exploit their food, other resources in addition to gaining shelter. The reasons that ensures success for the parasitic life of the nematode includes,

a) Exterior shell covering the eggs protect from the environment.

b) Cuticle surrounds the larval and the adult stages protects from the host environment. c) Dormant facultative diapause employed by some nematodes (like dauer stage of *C. elegans*) during the harsh period.

d) Biochemical adaptations of the larval and adult stages once they are in the host or the intermediate host.

e) Implementation several reproductive strategies (sexual or hermaphrodite).

Some nematodes are being used as model parasites to study extensively to understand their lifecycle, ecology and physiology in order to effectively control or manage them (Blaxter and Bird, 1997).

2.2 Nematode body structure

Nematodes are bilaterally symmetrical worms possess body architecture analogous to a *tube within a tube*. The outer tube is formed by the body wall while, the inner tube is formed by digestive tract. Between these two tubes, a fluid filled body cavity called the pseudocoelom lies that contain the reproductive tract. Unlike a true coelom the pseudocoelom does not possess a cellular lining or peritoneum. Since there is no developed vascular system, the circulation of nutrients in the pseudocoelom is assisted by changes in hydrostatic pressure. The hydrostatic pressure of pseudocoel (16-225mm Hg with a mean of 70mm Hg in *Ascaris suum*)

is gained or lost during somatic muscle contractions/relaxations when nematodes move around (Harris, 1957). The body wall has three layers: outer cuticle, middle hypodermis and an inner layer of somatic muscle cells (see Fig 1).

2.2.1 Cuticle

The cuticle is a non-cellular complex structure which surrounds the entire body of the worm forming a barrier between the nematode and its environment. At the posterior end of some male nematodes the cuticle forms the bursa, a flap like extension used for grasping the female during copulation. Bursa is absent in male *Ascaris* but, rather contains curved tail with two spicules. The cuticle allows the passage of water molecules and ions. The rigid cuticle maintains the integrity of the body and also protects against host digestive enzymes or immune system. Fig 1.B and C shows the cuticle providing attachment to hypodermis and to body-wall muscle (Singh, 1978; Johnstone, 2000; Page *et al*., 2006). The cuticle is not an inert structure rather; it contains enzymes to help moulting. The cuticle contains negatively charged pores with a radius of 1.5 nm allows transport of organic acids (both volatile and nonvolatile fatty acids). These organic acids are produced as a result of anaerobic metabolism of carbohydrates in the somatic muscle of the worm. The structural part of cuticle formed by a collagenous extracellular matrix containing cross-linked collagens and insoluble proteins called cuticulins. The cuticle is secreted by the underlying layer of hypodermis. In nematodes, the synthesis of

cuticle occurs four times at the end of each larval stage between L1 to L2, L2 to L3, L3 to L4 and L4 to immature adults.

2.2.2 Hypodermis

The hypodermis contains an enzyme protocollagen-prolinehydroxylase responsible for synthesizing the external cuticle. Electrophysiological studies have reported large conductance anion channels present on the hypodermal membrane facing the cuticle and somatic muscle membrane. These ion channels are shown to conduct organic acids generated from anaerobic metabolism of carbohydrates towards the cuticle for excretion (Blair *et al*., 2003). The functions of hypodermis include formation / dissolution of cuticle (during moulting), active exchange of water and small molecules while providing anchorage for somatic muscle (Fetterer and Wasiuta, 1987). The cellular hypodermis protrudes into the body cavity forming four longitudinally thickened areas known as cords. There are dorsal, ventral and two lateral cords. The dorsal and ventral cords contain longitudinal nerve trunks while the lateral cords contain excretory canals (Fig1.B). Internal to the hypodermis we find an obliquely striated layer of somatic muscle.

2.2.3 Somatic muscle

The somatic musculature of parasitic nematode *Ascaris suum* consists of longitudinal muscles which carry oblique striations, Fig 1.C (Rosenbluth, 1965a, 1965b; Stretton, 1976). There are about 10^5 muscle cells present in the worm which

are innervated by 250 neurons. The somatic muscle layer is divided by two lateral lines on either side of the worm resulting in dorsal and ventral muscular layers (del Castillo *et al*., 1989). Each half is innervated by individual nerve cords namely dorsal and ventral nerve cords present underlying the musculature. Somatic muscle cells have contractile and non-contractile structures. The contractile structure of the somatic muscle is called the spindle region. The spindle region consists of muscle filaments which can contract upon depolarization of the muscle cell. In addition, the contractile spindle anchors the muscle cell to the underlying hypodermis hence in turn to the cuticle. The non-contractile structures of the muscle cell include arms which reach nerve cord for innervation and a bag region containing nucleus, cell organelles and glycogen stores. A constricted neck region (well defined in *Ascaris*) is formed when the muscle bag connects to the spindle region of muscle cell before giving rise to arms. The arm extends all the way towards the nerve cord and branches into finger like projections just before receiving innervation from the nerve cord.

2.2.4 Arm

The arm arises as a thin cytoplasmic extension of the muscle cell near the neck region (Fig.1.c). Generally, innervation of vertebrate muscle cell gets the innervation from the nerve process that branches out from the nerve cord. Strangely, in nematodes the muscle sends out arms which branches into finger like projections at the end to receive innervation from the nerve cord (Rosenbluth, 1965b; Stretton,

1976). Muscle bags send more than one arm to the nerve cord (average 2.7 arms/ muscle cell in *Ascaris*). A syncytium is formed by the aggregation of fingers (average of 3 fingers/arm) arising from numerous arms of several muscle cells which surrounds the nerve cord (Rosenbluth, 1965b; Del Castillo *et al*., 1967; del Castillo *et al*., 1989). The impulses are conducted at the level of syncytium surrounding the nerve cord with a 50 nm space forms the neuromuscular junction (NMJ). The arm acts as a cable to carry the impulses all the way to bag region and finally conveyed to spindle region for contraction / relaxation. Before joining the syncytium the muscle arms belonging to different muscle bags cross over. The cross over region contains tight junctions which, allows signals from the syncytium to be shared between the muscle cells. The tight junction functions to synchronize the electrical activity to aid in better signal transmission over muscle cells that lies far away from the nerve cord (del Castillo *et al*., 1989).

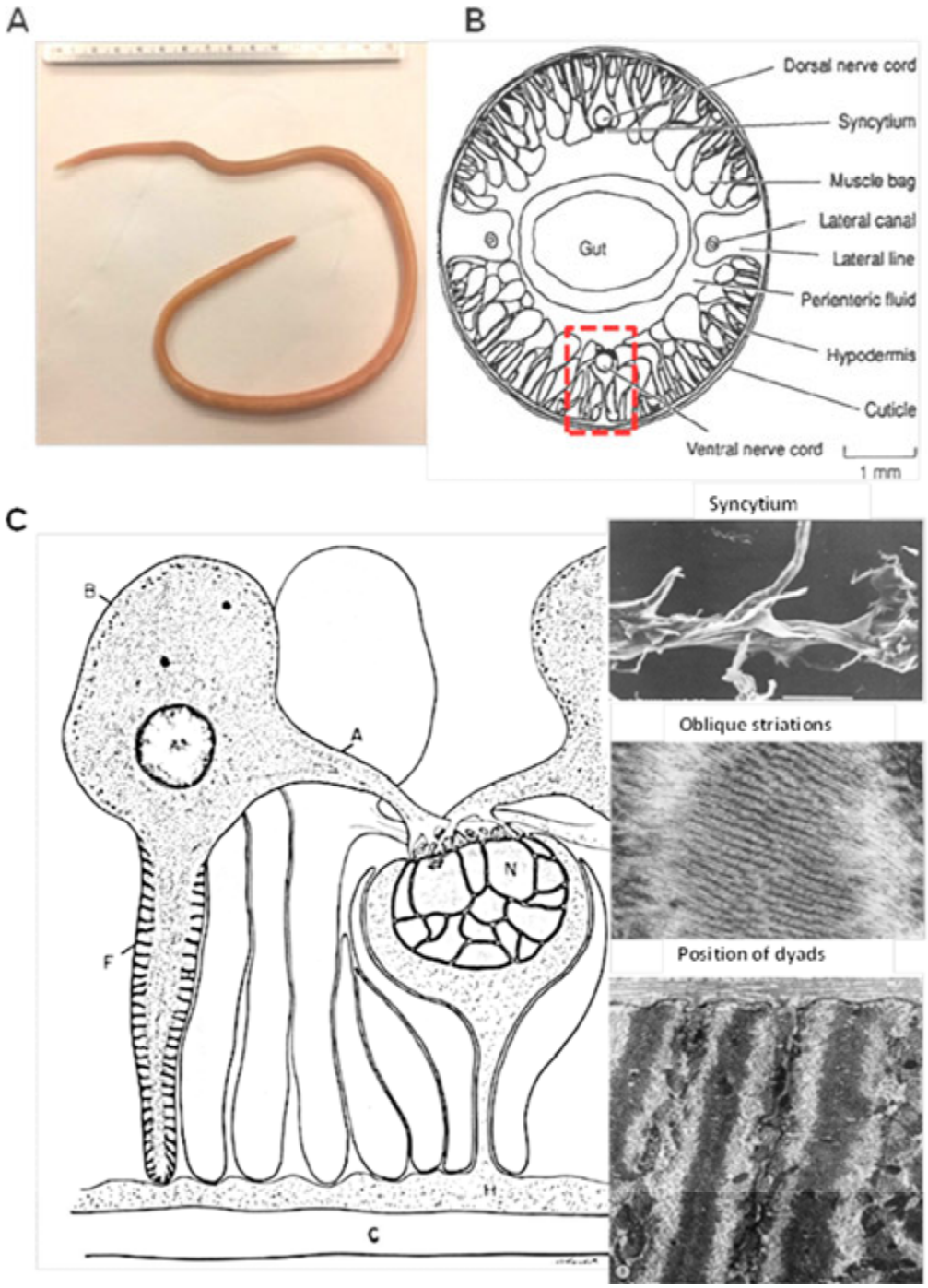

Fig. 1. A. A photograph of female *A. suum* worm. A 15 cm scale placed just above the worm. **B.** Diagram of cross section of the worm depicting the position of nerve

cords, lateral canals, muscle cells, hypodermis and cuticle (Martin *et al*., 1996b). The area with in the rectangular interrupted box if further magnified in **C** to show the architecture of muscle and its innervation. **C.** Diagram of muscle cell (Rosenbluth, 1965b) showing the bag region, B, spindle containing contractile filaments, F, which are oriented obliquely. The spindle makes contact with hypodermis, H covered externally by cuticle, C. The arm, A arising near the neck region reaches for the nerve cord, N, forming a syncytium. The neuromuscular junctions are formed between syncytium and nerve cord. The photographs on the right side shows the syncytium (top), oblique striated filaments (middle) and position of dyads formed by a cisternae of sarcoplasmic reticulum meeting the t-tubule shown in white arrow within the photograph (Rosenbluth, 1965a, 1969; del Castillo *et al*., 1989).

2.2.5 Syncytium

Syncytium formed by the interlacing of fingers to forms a closely woven network around the nerve cord (Fig.1.C). At the neuromuscular junctions (NMJs), the nerve cord forms neuronal extensions or spines which make synaptic contact with the muscle syncytium. At the NMJ neurotransmitters released from the spines of nerve cord interact with the post synaptic receptors of the syncytium (Del Castillo *et al*., 1963b; del Castillo *et al*., 1989). The action potential is then carried to the bag region and the spindle region via the arms. Muscle syncytium is also the place where spontaneous activity / spikes are generated due to a tonic release of neurotransmitter from the spines of nerve cord.

2.2.6 Bag region of muscle cell

Unlike the contractile spindle region of the muscle cell which is firmly attached to the hypodermis, the bag region of the somatic muscle floats freely facing the pseudocoele (Fig.1.C). The muscle bag contains nucleus, fibrillar bundles of cytoskeleton and mitochondria. The muscle bag is filled with glycogen granules as an energy reserve for the worm during starvation (Rosenbluth, 1965b). In addition to synaptic receptors found on the syncytial membrane at the NMJs, there are receptors found extra-synaptically on the bag region of the muscle cell. The existence of extra-synaptic receptors raises the possibility of autocrine or paracrine functions of the muscle cells. Unlike the synapse, receptors on the bag region of the muscle are easily accessible to electrophysiological techniques. Many electrophysiological studies aimed at knowing the receptors of the muscle cell are resulted from the study of extra-synaptic receptors of the bag region (Martin *et al*., 1991). Two microelectrode current clamp or voltage-clamp has been used to study the responses of ion channels to drugs at the whole cell level. In these techniques two microelectrodes are placed in the bag region and the drugs are perfused locally over the bag region to study the cell responses. For studying ion channels at the single channel level using the patch clamp, the somatic muscle preparations were treated with collagenase to derive membrane vesicles. These vesicles budding from the muscle bag membrane are then patched on to patch pipette to study the electrical properties of the single channels (Robertson and Martin, 1993; Levandoski *et al*., 2005; Qian *et al*., 2006).

2.2.7 The contractile spindle

The contractile apparatus of the muscle cell are similar to skeletal muscle of vertebrates but they are formed by obliquely striated filaments (Fig.1.c). These contractile filaments are restricted to the spindle region of the muscle cell. The contractile filaments form an angle of 6 degrees with the long axis of the worm resulting in oblique striations. The contractile filaments of the spindle are firmly attached underneath to the hypodermis. The organization includes A-zone (Anisotropic zone containing thick filaments), I- zone (Isotropic zone, containing thin filaments). In vertebrates, a typical Z-line present in the middle of I-zone which demarcates the length of the sarcomere. But, *Ascaris* muscle does not contain Z-line. Instead has Z-line counterparts, referred to as dense bodies. These dense bodies are closely associated with the sarcolemma (plasma membrane of muscle cell) on one side and with a cisterna of the sarcoplasmic reticulum on the other side (Rosenbluth, 1965a). The cisternae are analogous to terminal cisternae of vertebrate muscle which are formed as enlarged areas of sarcoplasmic reticulum surrounding the t-tubule. The cisternae of sarcoplasmic reticulum are gated by ryanodine receptors (RYRs) which mediate a rapid rise in intracellular calcium for muscle contraction. An action potential arising from the NMJ travels to the muscle spindle along the sarcolemma which extends deeper into the muscle filaments as transverse tubules (t-tubules). T-tubules allow depolarization of the membrane to reach the interior of muscle cell for contraction. Voltage gated calcium channels of the t-tubule lie in opposition to the cisternae containing RYRs for facilitating calcium

induced calcium release. Calcium induced calcium release occurs as a sequel to the opening of voltage gated calcium channels present on the t-tubule resulting in contraction of the muscle. The contraction of the muscle cell depends on ATP. Contraction is terminated when ATP gets broken down and cytosolic calcium is rapidly taken into the sarcoplasmic reticulum.

2.3 Comparison of neuromuscular system of *Ascaris* and vertebrates

Muscle cells of nematodes are unusual compared to the vertebrate neuromuscular system. Unlike vertebrates, muscles send processes (arms) to the nerve for receiving innervation from the nerve cord. The somatic muscle of *Ascaris* is similar to vertebrate smooth muscle which is roughly spindle shaped cell outline containing a nucleus and depends on extracellular calcium for contractions. The presence of cross striations is similar to vertebrate skeletal muscle, but these striations are oblique instead of being parallel to the long axis. *Ascaris* somatic muscle shows similarities to cardiac muscle by possessing dyads instead of triads of vertebrate skeletal muscle. The dyads of *Ascaris* somatic muscle are a combination of a terminal cisternae and a t-tubule while triads are a result of two terminal cisternae surrounding a t-tubule, Fig.1.C (Rosenbluth, 1969). Even the properties of somatic muscle syncytium are similar to vertebrate cardiac muscle in terms of auto rhythmicity or automatic production of spike potentials.

2.4 Nervous system in *Ascaris suum*

The nervous system in *Ascaris* was described as early as the beginnings of the 20th century by Goldschmidt (1910). Eighty years later, advances in biochemistry, immunology, electrophysiology and molecular biology have led to the accumulation of knowledge on the structure and function of the nervous system of the worm. Stretton *et al* (1992) described the *Ascaris* nervous system further as an anatomically simple nervous system that contains only 298 neurons but chemically complex as it secretes a variety of neurotransmitters and neuropeptides.

Among 298 neurons, 200 are found to be associated with the circum-esophageal nerve ring coupled with associated ganglia and also the posterior ganglia (posterior 5mm). In *Ascaris,* the circum-esophageal nerve ring is considered as the brain located about 2- 5 mm from the anterior tip. The posterior ganglia are found caudally about 5mm from the posterior tip of the worm (Fig 2.B). The nerve cords namely one dorsal and two sub ventral nerve cords arise from the nerve ring and travel in the anterior direction referred as pharyngeal / enteric nervous system. On the other hand, the somatic nervous system arises from the nerve ring to run posteriorly covering the entire length of the worm (Fig 2.C). The somatic nervous system is formed by two main nerve cords, dorsal and ventral nerve cord. The ventral nerve cord contains neuronal cell bodies (about 100 neurons) while, the dorsal nerve cord mainly contains the axonal fibers instead of entire neuronal cells. The neuronal cell bodies of the ventral nerve cord communicate with the dorsal nerve cords via commissures each formed by 1-2 axons (Stretton *et al*., 1992;

Martin *et al*., 1996b). Since all the neurons are concentrated in the ventral nerve cord the axons from the commissures make synapses with dorsal and ventral somatic musculature.

These neuronal cell bodies of the somatic nervous system of *Ascaris* are broadly classified as motor neurons, interneurons and sensory neurons. Motor neurons derive their name based on their site of innervation (dorsal or ventral muscle) and also on their function (excitatory or inhibitory). Dorsal motor neurons of the nerve cord innervate dorsal musculature while ventral motor neurons innervate ventral musculature. Each motor neuron morphologically has an axonal region which makes multiple neuromuscular synapses while, the dendritic region receives synaptic input from other neurons. The excitatory neurons which excite dorsal musculature are referred to as dorsal excitatory (namely DE1, DE2 and DE3) and those which excite ventral musculature are called ventral excitatory (V1 and V2). These excitatory neurons contain the enzyme cholineacetyltransferase, responsible for the synthesis of ACh, an excitatory neurotransmitter (Johnson and Stretton, 1985). The inhibitory neurons which inhibit the dorsal musculature referred as dorsal inhibitory (DI) and that which inhibit the ventral musculature are called ventral inhibitory (VI). These inhibitory neurons have shown GABA immunoreactivity synthesis and release of GABA, an inhibitory neurotransmitter (Johnson and Stretton, 1987). The excitatory motor neurons of the dorsal and ventral nerve cord are also connected by interneurons. The sensory neurons are specialized to sense environmental cues and to convey the information further to the motor neurons.

The repeating segmental pattern arrangement of excitatory and inhibitory motor neurons has been described in *Ascaris* (Walrond *et al*., 1985; Stretton *et al*., 1992; Martin *et al*., 1996b). Fig 2.C shows, each repeating segment is made up of 7 anatomical types of motor neurons: dorsal excitatory (DE1, DE2 and DE3), dorsal inhibitory (DI), ventral excitatory (V1 and V2), ventral inhibitory (VI). Among these types, some of the motor neurons namely DE1, VI, V1 and V2 are paired. Hence, there are a total of 11 motor neurons in each segment. In addition to motor neurons, there are 6 large interneurons all along the length of ventral cord to innervate excitatory motor neurons of the dorsal and ventral muscle. Each segment also has three commissures or communicating axons on the right side and one on the left side of the worm.

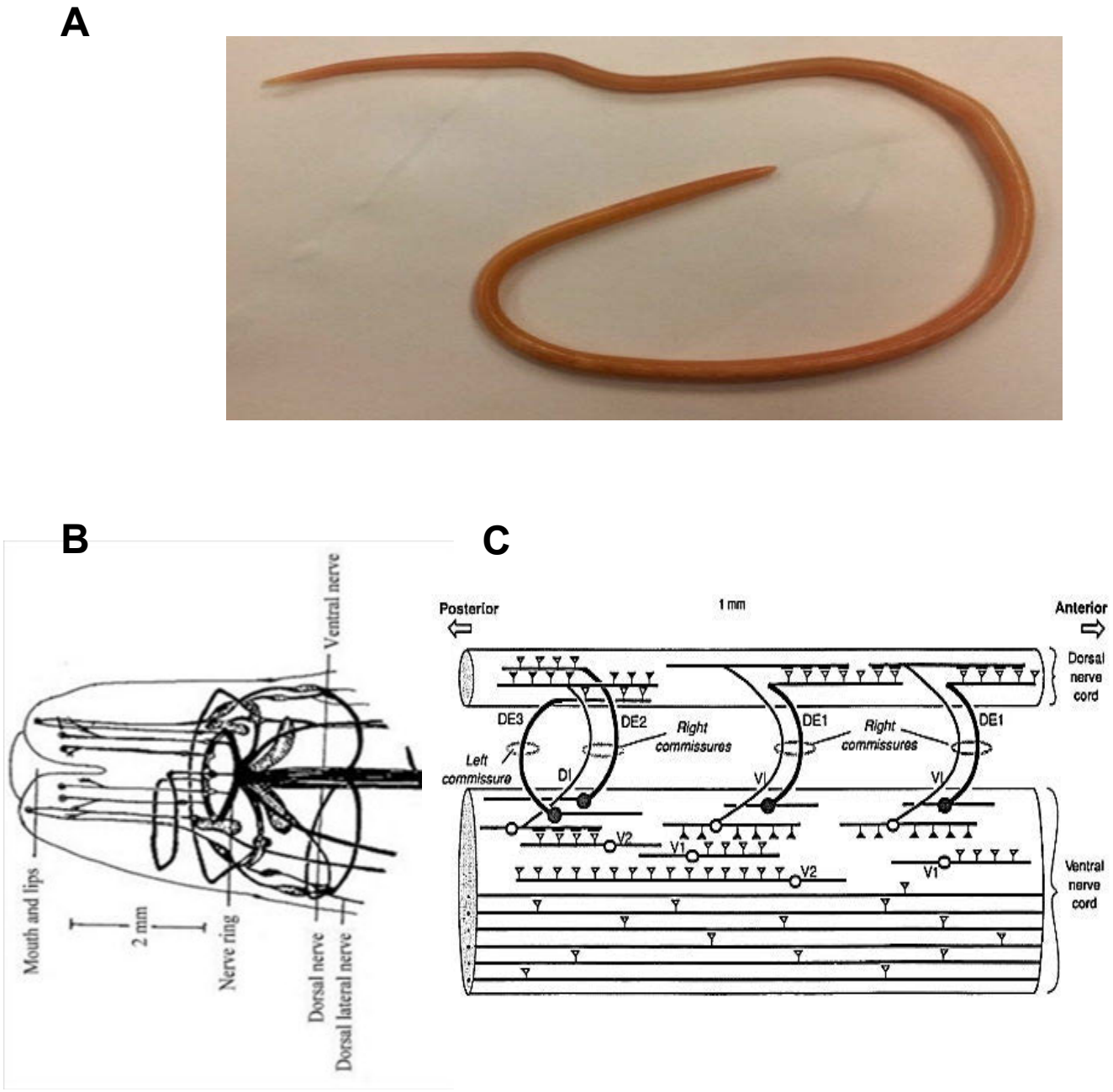

Fig. 2. A. Photograph of a female *A. suum* worm. **B.** The head region of *Ascaris* showing the mouth region containing lips, the position of nerve ring and origin of dorsal and ventral nerve cord (Crompton and Joyner, 1979). **C.** The repeating motor neuron segments in *Ascaris* (Martin *et al*., 1996b). The cell bodies of motor neurons

(excitatory and inhibitory) arranged in the ventral nerve cord. The commissures originating from the ventral nerve cord project into dorsal nerve cord.

2.5 Resting membrane potential of *Ascaris* muscle cells

The resting membrane potential *Ascaris* muscle cells was -30 to -35 mV with a resting conductance of 2 to 3 μS from electrophysiological recordings in Artificial Perienteric Fluid, APF (DeBell et al., 1963; Del Castillo et al., 1963b; del Castillo et al., 1964a; Brading and Caldwell, 1971a; Wann, 1987; del Castillo et al., 1989). The resting membrane potential was also dependent on the temperature of the bath solution. Lowering the bath temperature to 20°C caused the resting potential to reach −29.9 mV while an increase in the temperature of the bath to 37°C caused the resting potential to reach more negative values, −33.8 mV (Wann, 1987). The internal ionic concentrations of *Ascaris* somatic muscle observed were, K^+ 99.4 ± 2.8 mM, Na^+ 48.6 ± 2.4 mM, Cl^-13.7 ± 1 mM as reported by Brading and Caldwell (1971a). The resting membrane potential of *Ascaris* showed relative sensitivity to changes in the extracellular concentrations of anions rather than cations. A relative insensitivity of resting membrane potential was observed to changes in the composition of extracellular Na^+ and K^+. Changing the extracellular concentration of Cl^- affected the resting membrane potential. A ten fold increase in extracellular chloride concentration resulted in 12 mV change in resting membrane potential.

Under normal conditions, the resting membrane potential remains stable, occassionally interrupted by rhythmic spontaneuous activity originating from the syncytium (Brading and Caldwell, 1971a).

2.6 Rhythmic spontaneous activity of the muscle cell

The rhythmic spontaneuous activity of the *Ascaris* muscle cell includes spikes, slow waves and a modulatory waves (Fig 3 A, B and C). Spontaneuous activity is generated at the level of muscle syncytium and is conducted all along the arms to reach the bag region and later to the contractile spindles (Weisblat, 1976; Weisblat and Russel, 1976). External application of d-tubocurarine did not completely block the firing of spike potentials and confirmed these spontaneous active preparations are myogenic in origin (Del castillo et al., 1963a). The syncitium in spontaneously active preparations is maintained in depolarized state by continuous release of small quantities of ACh. Excitatory (cholinergic) and inhibitory (GABAergic) nerve fibers control the membrane potential of the syncitial membrane and hence, the frequency of spike firing. Spike potential amplitudes observed from the muscle bags were dependent on the proximity of the muscle cell to nerve cord, the concentration of extracellular calcium and the temperature of the bath (DeBell and Sanchez, 1968). Depolarizations as high as 60 mV were recorded from the *Ascaris* muscle bag region which had a resting membrane potential of -30mV.

Spikes were described as brief repeating action potentials had an amplitude of greater than 5mV. Spikes were observed as single action potentials which lasted less than 0.5sec. Occassionally several single action potentials joined to form

clusters that lasted upto 1.5 seconds. The spikes in *Ascaris* show complexity in shape, amplitude and duration, Fig 3.C (Weisblat, 1976). They are dependent on extracellular Ca^{++} and not Na^{+} and they were blocked by lanthanum and cobalt (Weisblat, 1976). Spike potential is thought to involve activation of voltage activated calcium currents during their rapid rise and voltage-activated potassium currents during their rapid decline (Thorn and Martin, 1987; Martin *et al*., 1992) and calcium-activated chloride currents during slow decline before reaching recovery (Turner, 2001).

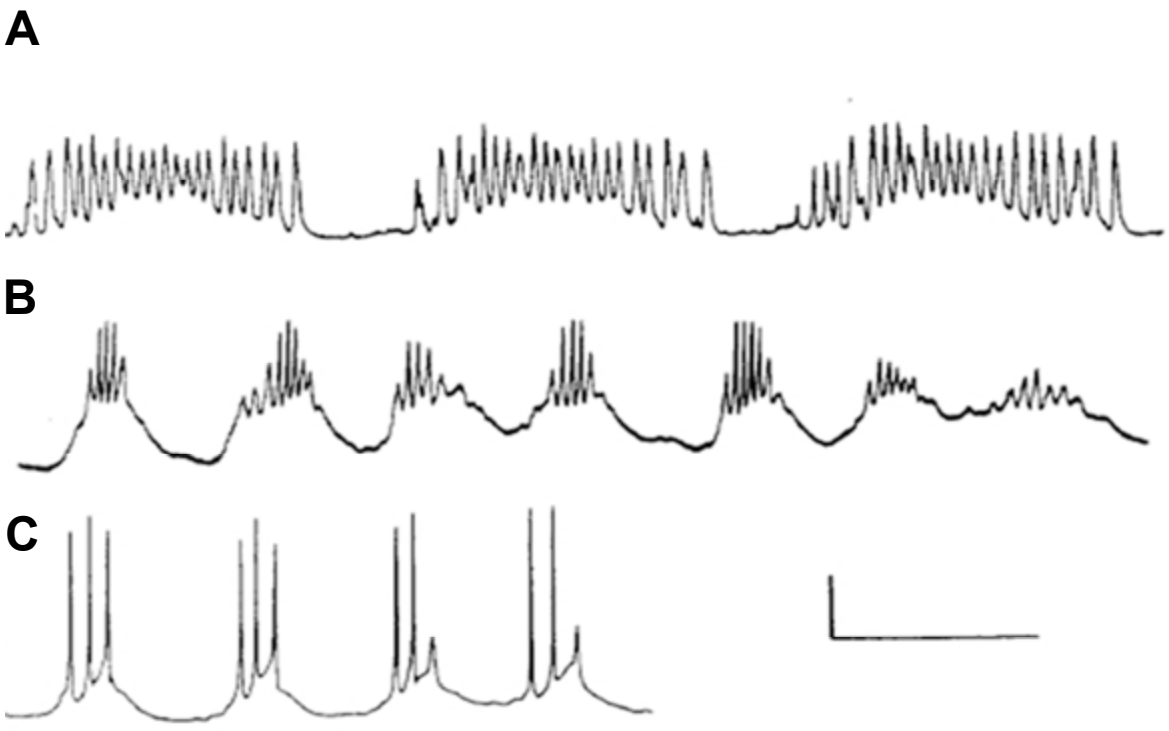

Figure 3. shows three different spontaneous activities observed in the *Ascaris* somatic muscle cells placed in APF (Weisblat, 1976). **A.** Modulatory waves which is further expanded in time scale to observe slow waves (**B** and **C**). Many spikes are

seen on the slow wave in **C**. The calibration 10 mV x 12 s in **A**, 10 mV x 1 s in both **B** and **C**.

Apart from spikes, *Ascaris* muscle also produces slow waves (100-1000 ms) shown in Fig 3. B and long lasting modulatory waves (3-20 ms) shown in Fig. 3. A. The slow waves were dependent on both sodium and calcium. The slow waves and spikes are modulated at the level of muscle syncytium through the involvement of neurotransmitters namely GABA and ACh (del Castillo *et al*., 1989). The syncytial membrane acts like an amplifier to boost the signal generated by the nervous system to further transmit the signals through the arms to the bag and the contractile spindle. Piperazine (100 μM, 5 min) is known to decrease the amplitude of the spike potentials and quietens the preparations by causing hyperpolarization (up to 15mV) of the muscle cell. The actions of piperazine is similar to the effects of the inhibitory neurotransmitter (GABA) or activation of inhibitory fibres of the ventral nerve cord (Del castillo et al., 1963a; Del Castillo et al., 1963b; Delcastillo et al., 1963; del Castillo and Morales, 1967b; del Castillo et al., 1989).

2.8 Anaerobic metabolism resulting in excretion VFAs

Gastrointestinal nematodes metabolize carbohydrates through anaerobic metabolism to excrete large quantities of organic acids as their end-products (Komuniecki *et al*., 1987). These include acetate, propionate, butyrate, 2-

methylbutyrate, valerate and 2-methylvalerate. Major quantities of organic acids are excreted from the somatic muscle cells. Electrophysiological studies have revealed presence of a large conductance, voltage-sensitive, calcium dependent chloride channel on the somatic muscle membrane (Dixon *et al*., 1993; Valkanov *et al*., 1994; Valkanov and Martin, 1995; Robertson and Martin, 1996) and also on hypodermis (Blair *et al*., 1998). These ion channels not only permeable chloride ions but also organic acids. The mechanisms that underlie excretion of organic acids by nematodes have been studied and modeled. From the model, organic acids produced by muscle cells diffuse outward through the calcium dependent chloride channel to accumulate in pseudocoelomic fluid that surrounds the muscle. The organic acids once accumulated in the pseudocoelom get eliminated through the intestine (Harpur and Popkin, 1973) and the small canals in the lateral line of the hypodermis (Thompson, 1996). The other important route is to diffuse across the calcium dependent chloride channels of the hypodermis which is thought to form a low resistance pathway. Organic acids after crossing the hypodermis exit the worm through pores found in the cuticle (Ho *et al*., 1990; Sims *et al*., 1992).

2.9 Locomotion in Nematodes

The outer cuticle in nematodes maintains a constant diameter of the body while allowing flexibility along the longitudinal axis. Circular muscle is absent in nematodes. The somatic muscle consists of obliquely arranged longitudinal muscle fibers. The contraction occurring in dorsal and ventral musculature sequentially,

never simultaneously, results in body bends. Along the length of the worm at any position a bend is generated as a result of contraction of dorsal or ventral muscles with simultaneous relaxation of opposing muscles. This generates a sinusoidal wave containing alternating series of dorsal and ventral bends (dorsoventral, 2-D plane). The electrical activation by the dorsal and ventral nerve cords on to the somatic muscle happens sequentially but never overlaps. The waves are generated both in forward or reverse directions and allows worms to swim in both directions as seen in *C.elegans* (Stretton *et al*., 1992).

2.10 Host invasion

Parasitic nematodes get into the host ecosystem either by direct ingestion of the eggs (*Ascaris*) or through an arthropod vector (*Brugia*, *Onchocerca*) or actively invading through the skin (*Ancylostoma*). Despite a varied routes of infection, the parasite once enters the host has to adapt quickly to the internal environment of the host. The host internal environment includes temperature, pH, osmotic pressure and other site specific factors (like bile, trypsin in small intestine). Although, these factors provide a barrier to some extent, it is also thought to give unique cues to the parasite that are not found in the intermediate host or in the external environment. The cues initiate behavioral, biochemical changes that allows the parasite to develop, integrate and adapt to the host internal environment (Roberts and Modha, 1997). The life cycle of *Ascaris suum,* the round worm of pigs starts once eggs are ingested by the host (Fig.4). The infective eggs are oval shaped, thick-shelled

protected with a sticky external coat to survive in the external environment. The eggs are laid in the one-celled stage which later develops into the infective stage (L2) inside the egg. The hatching of the egg in the small intestine of the host leads to the release of second stage (L2) larvae. If the host (pig) ingests infected paratenic hosts (earth worms and rodents) then, L2s are released during the digestion in the small intestine. The L2 juveniles after hatching penetrate the intestinal mucosa to enter into the portal circulation of the liver (within 24 hours). In liver, L2s moult into L3 juveniles and later enters into the systemic circulation. From the blood stream, the larvae traverse to the lungs via pulmonary arteries (by 4 to 6 days after infection). The L3 larvae penetrate the lung tissue to reach pulmonary capillaries and later into alveoli of the lung (2 weeks post infection). If the larvae dies within the lungs results in a serious "*Ascaris* pneumonia". The larvae ascend the bronchial tree to the reach the throat, and are swallowed during coughing. The L3 larvae once re-ingested and arrive in to the small intestine to complete their development (L3 to L4 to adults). It takes about 2-3 months for this cycle to complete starting from ingestion of the infective eggs to the adult *Ascaris*. The adult worms can reproduce and live 1- 2 years in the intestine. The egg production is intermittent and up to 200,000 eggs per day are produced by female worms which are then passed in the host feces. The contaminated feces with eggs form a route of infection to other hosts or paratenic hosts. These eggs survive for a long time in the farm as a reservoir of infection to the neighboring hosts.

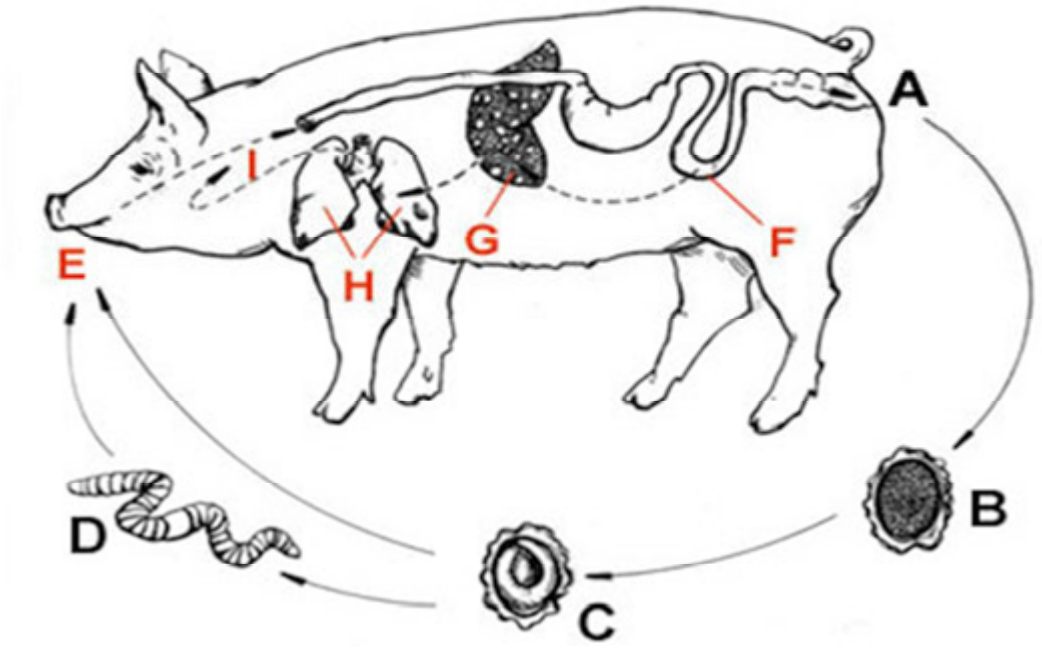

Fig 4. A. The pig parasitic round worm eggs are passed in feces of the vertebrate host (pig). These eggs **B** are rough, oval, thick shelled with a sticky coat. The egg develops into L1 and later into L2 shown in **C**. The development of L1 and L2 occur within the eggshell. These larvae when ingested by earth worms or dung beetles, the causes hatching of the egg to release L2 in the paratenic host **D**. Either egg or paratenic host forms source of infection when ingested by the vertebrate host **E**. L2 larvae released in small intestine **F**, migrates to liver **G** and the L2 larvae molt to L3 in liver. L3 larvae migrate from liver to lung **H** further migrates to trachea **I** and later get re ingested to reach small intestine. In the small intestine, L3 molts to L4 and to immature adult worms. The above diagram is taken from http://cal.vet.upenn.edu/.

2.11 Nematode neurotransmitters and their receptors

2.11.1 Acetylcholine

Acetylcholine (ACh) was identified as a neurotransmitter in 1920's in vertebrates. Three decades later ACh was isolated from *Ascaris*. Later ACh was found to produce contractions of *A. suum* somatic muscle (Baldwin and Moyle, 1949; Mellanby, 1955). ACh was later suggested to be present at the neuromuscular junctions of *Ascaris* somatic muscle (Del castillo *et al*., 1963a; Del Castillo *et al*., 1967). Identification of the enzyme cholineacetyltransferase in excitatory motor neurons confirmed ACh was indeed the excitatory neurotransmitter (Johnson and Stretton, 1985). When ACh was perfused on the muscle bags of *Ascaris,* it elicited depolarization and increase membrane conductance (Colquhoun *et al*., 1991). Later, Colquhoun *et al* described the relative potency of ACh by comparing to vertebrate nicotinic and muscarinic agonists on the *Ascaris* muscle. The rank order potency includes,

Metahydroxy phenyl propyl trimethylammonium (HPPT) > Dimethyl phenyl piperazinium (DMPP) > ACh > carbachol > nicotine > trimethylammonium (TMA) > muscarone > furtrethonium > arecoline.

Similarly, the rank order potency of vertebrate nicotinic and muscarinic antagonists to inhibit the ACh responses were tetraphenylphosphonium (TPP) > quinacrine > pancuronium ~ curare > trimethaphan > atropine > chlorisondamine ~ decamthonium > hexamethonium > dihydro-β-erythroidine.

Classically in vertebrates ACh activates two distinct acetylcholine receptors; nicotinic (activated by nicotine) and muscarinic (activated by muscarine). Nicotinic receptors are ligand gated ion channels while, muscarinic receptors (mAChRs) belong to G-protein coupled receptors (GPCRs).

2.11.2 Nicotinic acetylcholine receptors (nAChRs)

The nicotinic acetylcholine receptors (nAChRs) mediate synaptic transmission at the neuromuscular junction of vertebrates and invertebrates (Changeux and Edelstein, 1998). Changeux *et al* (1970) first purified nAChR from the electric eel (*Electrophorus electricus).* A decade later sequence information of nAChR was described (Sumikawa *et al*., 1982; Miledi *et al*., 1983). Apart from neurons of vertebrates, the nAChRs are also found to be present in non-neuronal cells namely epithelial cells of bronchi, endothelial cells of the arteries, macrophages, and keratinocytes of the skin (Macklin *et al*., 1998; Bruggmann *et al*., 2002). This implies a much broader functional significance of nAChRs than envisioned earlier. Neuronal nAChRs are also involved in learning and memory (Cordero-Erausquin *et al*., 2000). Selective drugs targeting neuronal nAChRs have been proposed to benefit the treatment of disorders like schizophrenia (Pereira *et al*., 2002), Parkinson's disease, Alzheimer's disease and in the control of pain (Rashid and Ueda, 2002).

All nAChRs are made up of 5 subunits arranged around a central pore forming a non-selective cation channel (Fig. 5). Each subunit has extracellular N and C-terminals, four transmembrane spanning domains (M_1 to M_4) with M_2 lining the pore

and a cytoplasmic domain between M3 and M4 for anchoring and modulation of the ion channel. The subunits include α-subunits which differ from non-α subunits by having vicinal (neighboring) cysteines on the extracellular N-terminal loop. The vicinal cysteines are important for ACh binding to open the channel. The nAChR pentameric ion channel is formed by two or more alpha subunits and three or less non alpha subunits. There is a cys-loop formed by disulphide bonds between two cysteines separated by 13 highly conserved amino acid residues. The cys-loop is unique to the α-subunits not found in non α-subunits. The α-subunits of nAChRs, GluCls and GABA gated chloride channels contain cys-loop, hence they are referred to as cys-loop receptors (Zouridakis et al., 2009).

In vertebrates nAChRs can be divided into skeletal muscle and CNS/neuronal nAChR types. The adult skeletal muscle nAChRs are made up of two alpha subunits, and 3 non-α subunits. These non-α subunits include β, γ and δ subunits (Mishina et al., 1986; Boyd, 1997; Hogg et al., 2003). The alpha subunits are essential for binding of ACh and there are a minimum of two ACh binding sites on the nAChR. The neuronal nAChR can be homomeric where the channel is made up of single type of alpha subunits (example α7 nAChRs) or it can heteromeric which is made up of two alpha subunits (example α2β3 nAChRs) or more than two alpha subunits (example α3β2 nAChRs). The homomeric or heteromeric receptors differ from each other in their pharmacological properties (Boyd, 1997; Paterson and Nordberg, 2000; Robertson and Martin, 2007).

Interestingly, the genomics of *C. elegans* reveals an extensive and diverse nicotinic receptor gene family. There are about 27 nAChR subunit genes encoding nAChR

subunits in *C.elegans* forming the largest gene family when compared to 17 nAChR subunit genes in mammals and birds. The functional significance of possessing a large number of genes encoding nAChRs is yet to be elucidated. In *Ascaris*, originally the nicotinic acetylcholine receptors are thought to be present only at the synapse(Del castillo et al., 1963a), but later it was identified that the receptors are present extrasynaptically on the bag region of the muscle (Brading and Caldwell, 1971a; Martin, 1982a; Harrow and Gration, 1985).

In *C.elegans* based on the sequence similarity, subunit proteins of nAChR have been classified into five groups. These include UNC-29 group, UNC-38 group, ACR-8 group, ACR-16 group and DEG-3 group (Mongan et al., 1998; Sattelle et al., 2002; Jones and Sattelle, 2004). Among the subunits, alpha subunits of nAChRs of the *C. elegans* somatic muscle include ACR-8, ACR-16, UNC-38, UNC-63 and LEV-8. The non-alpha subunits of nAChRs of the *C. elegans* muscle include LEV-1 and UNC-29. Apart from the nAChRs that are present on the *C. elegans* muscle, nAChRs are also expressed in sensory neurons which includes DEG-3 group (Fleming et al., 1997; Culetto et al., 2004; Rand, 2007).

Electrophysiological studies reveal two pharmacologically distinct nAChRs on the body muscle of *C. elegans*; levamisole sensitive receptors and nicotine sensitive receptors. These nAChRs receptors were expressed in *Xenopus* oocytes and studied under voltage clamp. For reconstituting a levamisole sensitive (nicotine insensitive) in *Xenopus* oocytes, 8 genes were found be required. Among them, 5 genes encode for the levamisole receptor subunits which include; three α subunits namely *lev-8, unc-38* and *unc-63,* two non- α subunits namely *lev-1* and *unc-29*

(Fleming et al., 1997; Culetto et al., 2004). Three genes encoding for ancillary proteins namely, *ric-3, unc-50* and *unc-74* were found to be essential for expressing levamisole sensitive nAChRs. The RIC-3 is a transmembrane protein of endoplasmic reticulum acts as chaperone to promote receptor folding, assembly or maturation. The UNC-50 encodes a transmembrane protein of Golgi complex, prevents lysosomal targeted destruction of the subunits of levamisole sensitive nAChR during intracellular trafficking. The UNC-74 is thioredoxin containing protein found to be required for trafficking the nAChR subunits to the synapses. For further reading please refer to Boulin *et al* (2008).

Interestingly enough, two subunits of the levamisole sensitive receptors of *Ascaris* namely, UNC-38 and UNC-29 subunits were expressed in *Xenopus* oocytes. The expression did not involve addition of genes *ric-3, unc-50* and *unc-74* that encode for ancillary proteins. When cRNAs encoding UNC-38 and UNC-29 subunits were added in the ratio of 1:5 resulted in a nAChR where levamisole was more potent agonist than nicotine. When the ratios of cRNAs encoding UNC-38 and UNC-29 subunits were reversed to 5:1, resulted in a nAChR where nicotine was more potent agonist than levamisole (Fleming et al., 1997; Williamson et al., 2009).

The nicotine sensitive (levamisole insensitive) nAChRs in *C. elegans* are homomers made up of five ACR-16 alpha subunits (Francis *et al*., 2005; Touroutine *et al*., 2005). Loss of function studies involving mutations of ACh-16 subunit resulted in loss of the muscle nicotine sensitive receptor in *C.elegans*.

Unlike *C. elegans* muscle that possesses pharmacologically distinct nicotine sensitive or levamisole sensitive nAChRs, the story is different in the parasitic

nematode *Ascaris.* Electrophysiological studies have revealed 3 nAChR subtypes in *A. suum* somatic muscle. This pharmacological characterization is based on the preferential selectivity of agonists namely, nicotine (N-subtype), levamisole (L-subtype) and bephenium (B-subtype). Unlike pharmacologically distinct *C.elegans* muscle nAChRs, the three subtypes of nAChRs in *Ascaris* somatic muscle show an overlapping pharmacology. A high concentration of a nAChR agonist (for example levamisole) can activate all the 3 subtypes of *Ascaris* muscle nAChRs. This overlapping pharmacology has been revealed in *Ascaris* muscle contraction studies (Robertson *et al*., 2002) and single channel recordings from the bag region of the muscle (Levandoski *et al*., 2005; Qian *et al*., 2006). Single channel recordings on the bag region of the muscle showed three mean conductance states of nAChRs of *Ascaris* muscle; small (25pS, N-subtype), intermediate (35pS, L-subtype) and large (45pS, B-subtype). Further experiments extended the observations on other cholinergic anthelmintics and antagonists on *Ascaris* muscle. The N-subtype was activated by oxantel, methyridine; L-subtype by pyrantel and competitively antagonized by paraherquamide; B-subtype was competively antagonized by paraherquamide and derquantel. The anthelmintic thenium was found to be less selective between L or B subtypes (Robertson *et al*., 2002; Martin *et al*., 2003; Martin *et al*., 2004; Martin and Robertson, 2007).

Interestingly, nAChRs that gate anions namely chloride has also been described in *C.elegans*. They are referred to as ACh gated chloride channels (ACCs). There are 4 subunits has been identified namely ACC-1, ACC-2, ACC-3 and ACC-4. Some of these subunits namely, ACC-1s or ACC-2s can be assembled into homo pentamers

in *Xenopus*. The pattern of expression and function of these ion channels is yet to be understood (Putrenko *et al*., 2005).

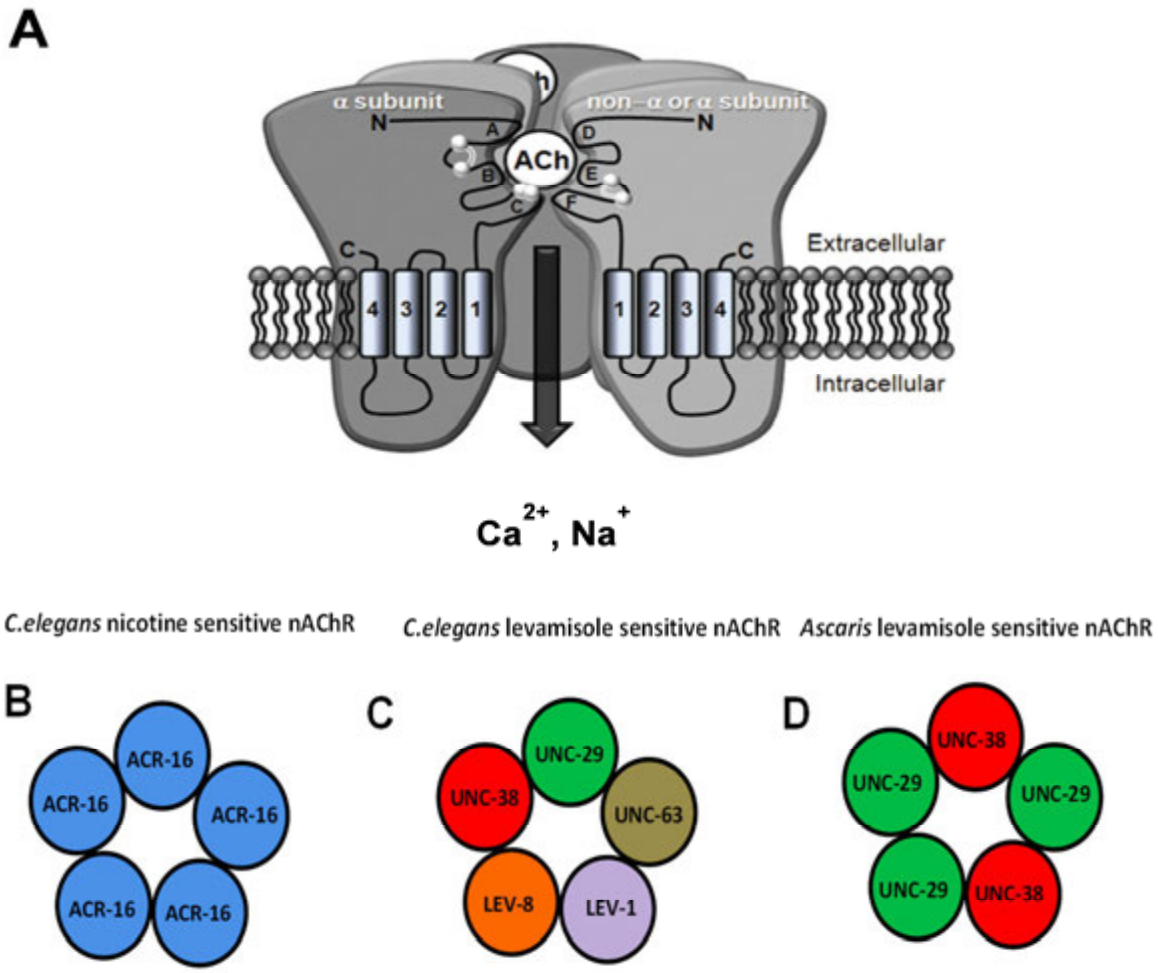

Fig 5. A. The cartoon represents nicotinic acetylcholine receptor containing five subunits. The α-subunits contain vicinal cysteines that are important for ACh binding (Sattelle et al., 2002; Jones and Sattelle, 2010). The cys loop is also seen on the α-subunit. **B.** The subunit composition of homo pentameric *C. elegans* nicotine sensitive nAChR. The subunit composition of hetero pentameric

levamisole sensitive nAChR from *C. elegans* **(C)** and *Ascaris* **(D)** (Raymond *et al*., 2000; Boulin *et al*., 2008; Williamson *et al*., 2009; Wolstenholme, 2011).

Ryanodine receptor in nematodes

Apart from the nAChRs which are present on the muscle cell membrane permeable to calcium, there are intracellular ryanodine receptors (RYRs) on the sarcoplasmic reticulum. Ryanodine receptors function as calcium channels of the sarcoplasmic reticulum to release calcium in to the cytosol in response to an influx of calcium through nAChRs on the membrane. RYRs gate the sarcoplasmic reticulum, a calcium reservoir of the nematode somatic muscle. Ryanodine receptors were first identified as they were bound strongly to the alkaloid ryanodine (Bennett *et al*., 1996). Hence, these receptors were named after the alkaloid ryanodine as RYRs. Unlike nAChRs which are pentamers, ryanodine receptors are homotetramers made up of UNC-68 subunits encoded by a single gene *unc-68* in *C. elegans* (Maryon *et al*., 1996). The addition of ryanodine to *C. elegans* produced phenotypes with incomplete hypercontractive paralysis (Kim *et al*., 1992). Interestingly a similar effect has not been observed with ryanodine on the *Ascaris suum* muscle strips. In *C. elegans* the action of ryanodine on ryanodine receptors have been observed using single channel studies when the UNC-68 subunits were expressed on planar lipid bilayers. In these studies, ryanodine locked the RYRs in a sub-conductance state (partially open) emptying the sarcoplasmic calcium stores (Kim *et al*., 1992).

In addition to calcium, there are other ligands like caffeine which acts as agonist at RYRs. The contribution of RYRs to muscle contractions has been studied in

C.elegans.

These studies infer that RYRs play a supportive role but not a main role in excitation-contraction coupling (Kimball *et al*., 1996; Maryon *et al*., 1996). The UNC-68 null mutants in *C. elegans* were found to have nearly normal body muscle although, carried some defects associated with pharyngeal pumping, reduced locomotor activity and slow growth when compared to that of wild-type (Maryon *et al*., 1996). A reduction in the RYRs function (unc-68 null mutants) in parasitic nematodes has been predicted to reduce the response to treatment with nicotinic anthelmintics like levamisole, pyrantel and also contribute for drug resistance (Puttachary *et al*., 2010). Worms that are resistant to levamisole accompanied by a reduced RYR function might still survive in spite of some locomotory defects.

2.11.3 G-protein coupled ACh receptors (GARs)

The existence of G-protein coupled ACh receptors (GARs) in *C. elegans* was first reported in 1983 (Culotti and Klein, 1983). Subsequently alternative splicing revealed that there are 3 genes that encode GARs, *gar-1* (Park *et al*., 2000), *gar-2* (Suh *et al*., 2001) and *gar-3* (Park *et al*., 2003). Green fluorescence protein (GFP) constructs showed the expression pattern of GARs in *C. elegans*. GAR-1 was found to be expressed in sensory neurons in the head and also in the posterior ventral microtubule cell (PVM) neuron. Atypical pharmacological characteristics of GARs have been observed. GAR-1s were coupled to the inhibitory subunit of Gi proteins and showed binding to atropine, but not to scopolamine. GAR-2 coupled to the Gi

family was found to be expressed in sensory neurons in head, ventral cord motor neurons and hermaphrodite specific motor neurons (HSN). The pharmacology showed GAR-2 bound neither to atropine nor to scopolamine. GAR-3s were expressed in pharyngeal muscles (highest in the terminal bulb), ventral cord motor neurons and the SAB interneurons. GAR-3s showed pharmacology similar to conventional vertebrate muscarinic receptors such as binding to scopolamine and carbachol. GAR-3s in *C. elegans* play a role in regulation of membrane potential and excitation-contraction coupling in pharyngeal muscle. GAR-3s were found to be required for normal feeding behavior as they regulate pharyngeal pumping (Steger and Avery, 2004; Rand, 2007).

The parasitic nematode *Ascaris* GARs have been found to be expressed in head and tail of adult worms (Kimber *et al.*, 2009). Though, *A. suum* GAR-1 (AsGAR-1) showed a high structural homology with *C.elegans* GAR-1, they exhibited an atypical pharmacology. The rank order potency of agonists of AsGAR-1 showed ACh (EC_{50} 20.3 μM) > carbachol ~ arecoline > oxotremorine > bethanechol > pilocarpine. The rank order potency series of antagonists on AsGAR-1 includes promethazine > mianserine > atropine > propranolol > spiperone > pirenzepine > cimetidine > scopolamine > diphenhydramine. Atypical pharmacology of AsGARs coupled with their role in sensory perception, locomotion, pharyngeal pumping and reproduction have made them attractive drug targets.

2.11.4 γ-amino butyric acid (GABA)

γ-Amino butyric acid (GABA) was identified in the mammalian central nervous system in the 1950's and was initially described as factor-I (I represents inhibitory action on neuronal activity). A decade later factor-I was identified as amino acid neurotransmitter GABA. GABA was synthesized from the decarboxylation of glutamate catalyzed by the enzyme glutamic acid decarboxylase (Bowery and Smart, 2006). Application of GABA (5-10 μM) resulted in hyperpolarization of the *Ascaris* muscle (10 to 20 mV) and muscle relaxation (Martin, 1982a; Martin et al., 1991; Martin, 1993) accompanied by an increased chloride conductance (Holden-Dye *et al*., 1989). Application of GABA also abolished rhythmic spontaneous action potentials in the *Ascaris* somatic muscle (Martin, 1980). Apart from the neuromuscular junctions in nematodes, similar to nAChRs, GABA gated chloride channels were found extrasynaptically on *A. suum* somatic muscle bags (Martin, 1980). The anthelmintic drug piperazine mimicked GABA in its action (del Castillo *et al*., 1964c). But, single channel recordings on *Ascaris* muscle involving GABA and piperazine showed some difference in mean open-times of GABA gated chloride channel. GABA produced mean open times of 32 ms while piperazine induced much shorter mean open-times of 14 ms on the GABA channels (Martin, 1985).

GABA immunoreactivity was concentrated in the inhibitory motor neurons in *Ascaris* somatic muscle (Johnson and Stretton, 1987). In *C.elegans*, GABA produced inhibition of somatic muscle contractions and foraging rather interestingly, excited

enteric muscles that are involved in defecation (McIntire *et al*., 1993). Holden-Dye *et al* (1989) found dihydromuscimol, a GABA agonist in vertebrates to be more potent (7.5 times) than GABA in *Ascaris*. But potent vertebrate $GABA_A$ antagonists namely pitrazepine, securinine, bicuculline, RU5135and SR95531 produced little effect on GABA receptors. These observations suggested that although there were some similarities of *Ascaris* GABA receptors to the vertebrate $GABA_A$ receptor, they possessed different pharmacology (Holden-Dye et al., 1989; Martin et al., 1991).

2.12 Nematode neuropeptides

Nematodes possess anatomically simple nervous system that comprise about 300 neurons. But, they are chemically complex in terms of their secretory products. Nematode nervous system is known to secrete more than 250 different neuropeptide signaling molecules (Marks and Maule, 2010). Neuropeptides are short sequences of amino acids synthesized, co-localized in neurons similar to the neurotransmitters and are released at the nerve terminals. Specific genes are involved in the production of neuropeptides. Neuropeptides are synthesized in neuronal cell bodies as pro-peptide precursors and then encapsulated in vesicles for further processing. The matured (dense core) vesicles are then transported along the axon to reach nerve terminals for their release. The neuropeptides released at the nerve endings modulate synaptic activity either by directly binding to their own receptors or indirectly affecting neurotransmitter receptors by modulating their functions (Li and Kim, 2008). The neuropeptides fall into two major groups: the

insulin-like peptides (ILPs) and the FMRFamide (Phe-Met-Arg-Phe-NH2)-like peptides (FLPs). Other minor groups of neuropeptides include non-insulin and non-FLP peptides are included in a separate group called neuropeptide-like proteins (NLPs). Nematodes possess largest family of genes among the invertebrates that encode more than 250 distinct neuropeptides. Currently, in *C. elegans*, 38 *ins*, 43 *nlp*, and 32 *flp* genes have been documented. Similar complexity is predicted to exist in parasitic nematodes (McVeigh *et al*., 2006; McVeigh *et al*., 2008; Marks and Maule, 2010).

Insulin-like growth factors are key hormones in mammals regulating metabolism, growth and differentiation. Interestingly enough, nematodes possess more than 76 insulin-like peptides (ILPs). Some of their known functions in *C. elegans* include fat metabolism, aging and diapause (Kawano *et al*., 2000; Kawano *et al*., 2003). In addition to ILPs in nematodes there are about 124 neuropeptide like proteins (NLPs) encoded by 43 *nlp* genes. These NLPs belong to a diverse group that possess little similarity among each other (McVeigh *et al*., 2006). NLPs are implicated in multiple *C. elegans* social behaviors (de Bono and Bargmann, 1998). In the present review, I have focused on FLPs especially the neuropeptide AF2 that I have used in my research.

2.12.1 FMRFamide like peptides (FLPs)

Nematode FMRFamide-like peptides (FLPs) are among the diverse regulatory neuropeptides seen in invertebrates. An older term FMRFamide is still continued in its usage which refers to the common sequence of Phe-Met-Arg-Phe-amide at their C-terminal encoded by specific FLP. After identifying several FLPs, the common sequence found to end in Arg-Phe-NH2 (–RFamide) peptides. In contrast to the vertebrate–RF amides which have restricted distributions with focused roles, nematode FLPs have broader roles in nervous system by influencing muscle, motorneurons, behaviour and sensory functions (McVeigh *et al*., 2006). The *flp* gene complements are largely comparable across the nematodes. Some neuropeptides isolated from *Ascaris* or *C. elegans* or other invertebrates exert their effects in other nematodes across the phylum (Mousley *et al*., 2004; Mousley *et al*., 2005). In *C. elegans* so far 30 distinct genes designated as *flp-1* to *flp-28, flp-32* and *flp-33* have been identified.

In parasitic nematodes, PCR-based cDNA analysis of expressed sequence tags identified 290 distinct FLPs (Mousley *et al*., 2005). Immunocytochemical localization studies of neuropeptides revealed FLP immunoreactivity in all main nematode neural structures. In *Ascaris* the immunoreactivity studies indicated the expression in nerve ring, somatic and enteric nervous systems including motor, sensory and interneurons (Cowden *et al*., 1993; Brownlee *et al*., 1996). In the nomenclature of FLPs, the first letter refers to species from which FLP was first isolated. For example in neuropeptide AF2 and PF1, the first letters A- refers to *A. suum* while,

P- refers to *Panagrellus redivivus*. The second letter refers to type of neuropeptide, in this case FMRFamide like peptides (FLPs). The number refers to the chronological order of discovery within the species of nematode. For example, AF2 is a heptapeptide, A-referring to *Ascaris*, F-refers to FLP neuropeptide, 2-refers to the second neuropeptide isolated in *A. suum*. Selected FLPs and their actions on *Ascaris* muscle and pharyngeal peristalsis (induced by serotonin) have been listed in the Table 1.

Table 1. Modified from McVeigh *et al* (2006) showing responses to FLPs in *Ascaris* somatic and pharyngeal muscle.

Gene	Peptide sequence	Peptide name	Ascaris muscle	Ascaris pharyngeal peristalsis	Electrophysiology
flp-1	KPNFIRFa	PF4	Fast inhibitory		
	SDPNFLRFa	PF1	Slow inhibitory	↓	
	SDIGISEPNFLRFa	AF11	Slow inhibitory	↓	DE2↑R_{in}↓EPSP↑; DI↑R_{in}↓
flp-4	SGKPTFIRFa	AF5	excitatory		DE2↑Rin↓EPSP↑; DI↓Rin↓
flp-6	KSAYMRFa	AF8/PF3	Excitatory on ventral muscle. Inhibitory on dorsal muscle.	↓	DE2↓R_{in}↓EPSP↓; DI↑↓R_{in}↓
flp-8	KNEFIRFa	AF1	biphasic	↓	DE2↑R_{in}↓EPSP↑; DI↓R_{in}↓
flp-14	KHEYLRFa	AF2/PF5	biphasic	No effect	DE2↑R_{in}↑↓; DI– R_{in}↓
flp-18	AVPGVLRFa	AF3	excitatory	No effect	DE2↑R_{in}↑EPSP↑; DI– R_{in}↓
	GDVPGVLRFa	AF4	excitatory	No effect	DE2↑R_{in}↑EPSP↓; DI↓R_{in}↓
	GFGDEMSMPGVLRFa	AF10	excitatory		DE2↑R_{in}↑; DI– R_{in}↓

Table 1. Continued

flp-21	GLGPRPLRFa	AF9	excitatory		DE2↑EPSP↑; DI↓R_{in}↓
flp-29	ILMRFa	AF16		No effect	DE2– EPSP↓; DI–
	FDRDFMHFa	AF17	excitatory		DE2↓R_{in}↓EPSP↓ EPSPa↓; DI↓R_{in}↓

Table 1 abbreviations: These electrophysiological studies were conducted on dorsal excitatory (DE2), dorsal inhibitory (DI) motor neurons of *A. suum.* The effects on the somatic muscle are denoted here as hyperpolarizing (↓), depolarizing (↑) or negligible effect (–). The effects on pharyngeal peristalsis are denoted as increase (↑), decrease (↓) or no effect. The electrophysiology on motor neuronal (excitatory, DE2 or inhibitory, DI) input resistance (R_{in}) are denoted here as increase (↑), decrease (↓), biphasic (↓↑) effect. The frequency of excitatory postsynaptic potential frequencies (EPSPs) generated by motor neurons is denoted as increase (↑) or decrease (↓). EPSPa represents changes in the EPSP amplitude (McVeigh *et al*., 2006).

2.12.2 Processing FLPs and signal transduction

Specific *flp* genes control the transcription of FLPs, initiates the synthesis of large propeptides. This propeptides may contain a single copy of individual FLP peptides or even copies of multiple distinct FLP peptides. These propeptides containing an individual FLP are encoded along with glycine extensions which contain cleavage

sites. Processing of the immature propeptide happens on the way to the synapse. The N-terminal signal peptide sequence of the propeptide is important for directing the FLP to go through the secretory pathway for the synaptic release of the mature peptide. The C-terminal of the propeptide is necessary for the hydrolytic cleavage involving enzymes namely, subtilisin like pro-protein convertases (SPCs). The C-terminals of the propeptide are further acted upon by carboxy-peptidases which cleave some of the dibasic residues before entering into the next step of amidation. The amidation of C-terminal confers the activity of FLPs and those peptides which are not amidated are functionally inactive. Later, FLPs released from the nerve terminals into the synapse for interacting with specific FLP receptors of the post-synaptic membrane. The signal is terminated by enzymatic destruction of peptide by enzymes including neprilysin-like zinc metalloendopeptidases, aminopeptidases and deamidases (McVeigh *et al*., 2006).

2.12.3 FLP receptor studies

Fig 6 shows that FLPs mostly signal through specific G-protein coupled receptors (GPCRs). The GPCRs of FLPs contain five subtypes (X1-X5) coupled with different downstream second messenger pathways. The activation of GPCRs X1 and X2 by AF1 and AF2 results in Gα mediated activation of adenylate cyclase to elevate the cAMP levels (Reinitz *et al*., 2000; McVeigh *et al*., 2006). The elevated cAMP levels after application of AF2 have been shown to increase the glycogen metabolism (Rex *et al*., 2004a; McVeigh *et al*., 2006). AF1 and AF2 application on the somatic

muscle of *Ascaris* results in a biphasic response (initial hyperpolarization followed by excitation / contraction). AF2 after a brief application following a short duration wash has shown to produce long lasting potentiation of nAChR responses (Trailovic *et al*., 2005). However, elevated cAMP levels after application of some FLPs did not correlate with the contractions of the body muscle suggesting these events are independent of each other. PF1 activates X5- GPCR present on the hypodermal membrane and leads to Gα mediated activation of a calcium channel causing a calcium influx. The increased calcium levels in hypodermis further activate nitric oxide synthase resulting in increased nitric oxide production. The nitric oxide produced in hypodermis diffuses into the pseudocoelomic fluid. Nitric oxide from the pseudocoelomic fluid later enters the somatic muscle to produce muscle relaxation (McVeigh *et al*., 2006). GPCRs X3 and X4 are coupled to Gα that inhibits adenylate cyclase to decrease cAMP levels. GPCR X3s are activated by AF3 while, GPCR X4s are activated by AF5, AF7, AF11, AF17, AF19 and PF1. Activation of GPCR X3 caused a contraction of somatic muscle while, activation of GPCR X4 caused muscle relaxation resulting in increased body length of the worms.

Unlike other neuropeptides which activate GPCRs, PF4 neuropeptide activates ion channels similar to the neurotransmitters. PF4 binds to a chloride channel of the body muscle of *Ascaris* to cause hyperpolarization and relaxation. This action was found not inhibited by G-protein inhibitors. The hyperpolarization induced by PF4 is rapid and comparable in time course to the GABA gated chloride channel (Holden-Dye et al., 1997; Purcell et al., 2002b, a; McVeigh et al., 2006).

GPCRs of FLPs from *C. elegans* have been studied through heterologous expression. Chinese hamster ovary cells, human embryonic kidney cells or *Xenopus* oocytes were used as mediums to express these GPCRs for screening the putative ligands. The potency of ligands were studied based on the receptor binding resulting in cellular responses in calcium fluorescence based assays or GTP binding assays. These studies have helped to identify about 11 reported GPCRs. Further techniques in *C.elegans* involving gene silencing techniques such as RNA interference (RNAi) have identified about 60 GPCRs (McVeigh *et al*., 2006). FLPs have also been studied in bioassays for determining the effects on neuromuscular function in somatic, ovijector and pharyngeal muscle of *Ascaris*. In some studies in *Ascaris*, the FLPs were injected into the pseudocoelomic fluid to study their effects on whole worms and the changes in cAMP levels (Reinitz *et al*., 2000).

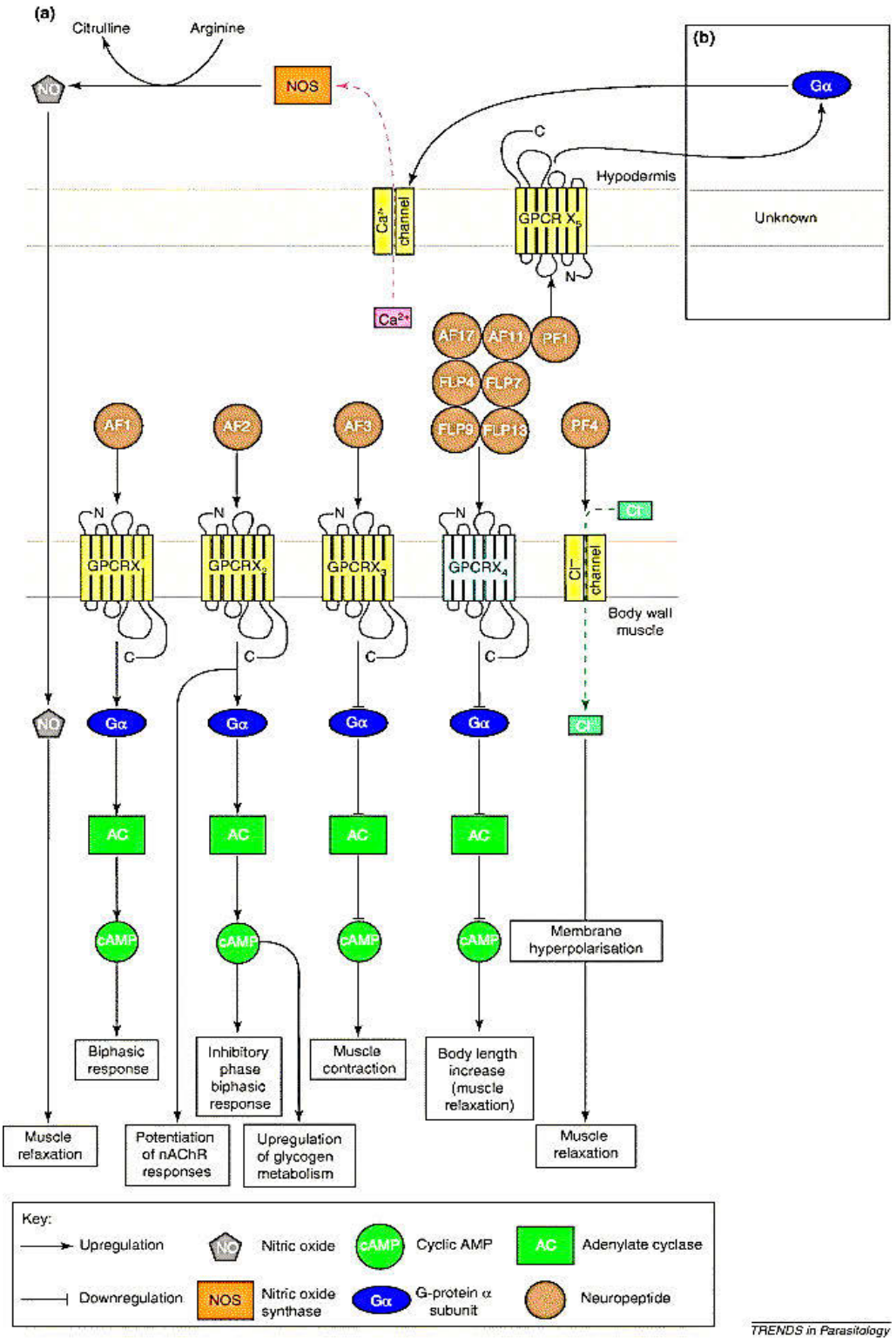

Fig 6. FLP signaling pathways from *A. suum* and *Ascaridia gali* modified from McVeigh *et al* (2006). A majority of FLPs act on GPCRs causing an

increase/decrease in cAMP except PF4 which acts on the chloride channel. Note that an increase in cAMP does not always correspond to muscle contraction. PF1 acts on the GPCR of hypodermis to cause an entry of calcium and results in the production of nitric oxide (NO). The nitric oxide produced escapes from hypodermis to the muscle cell to cause muscle relaxation.

2.12.4 Neuropeptide AF2

AF2 is a heptapeptide KHEYLRF-NH_2 (AF2) was first isolated from the head extracts of the parasitic nematode *A. suum* (Cowden and Stretton, 1993). Later it was also found in free living nematodes, nematodes *Panagrellus redivivus* (Maule *et al*., 1994), *C. elegans* (Marks *et al*., 1995) as well as in the parasitic nematode *Haemonchus contortus* (Keating *et al*., 1995). Among the free living or parasitic nematodes studied, AF2 was found to be the most abundant FLP.

AF2 was found to be an excitatory neuropeptide when tested in bioassays, somatic muscle contraction assays and electrophysiological studies. AF2 has been found to elevate cAMP levels in *A. suum* when injected into the pseudocoelomic fluid. AF2 produced a sustained elevation in cAMP levels (127 times higher than the control) and produced paralysis by shortening the body length of the worm. Sustained cAMP levels are thought to produce spatial and temporal effects on the muscle cells that receive input from the motor neurons and also the potentiate PKA activity (Reinitz *et al*., 2000; Kubiak *et al*., 2003; Thompson *et al*., 2003). In *Ascaris* muscle contraction assays application of AF2 induced a biphasic response which consisted of an initial

inhibitory phase followed by an extended excitatory phase. The extended excitatory effect included rhythmical contractions observed at nanomolar concentrations of AF2 (Cowden and Stretton, 1993; Pang *et al*., 1995). Increasing concentrations of AF2 (from nm to μM) reduced the initial inhibitory phase but increased the excitatory phase (Pang *et al*., 1995). AF2 application produced an increase in muscle tension response to ACh in *Ascaris* muscle (Keating, 1996). The effect of AF2 was tested on dorsal excitatory motor neurons type 2 (DE2) and dorsal inhibitory (DI) motor neurons. These two motor neurons eventually form a common final output on to the somatic muscle cells responsible for locomotion. On the DE2 motor neuron, AF2 produced strong depolarization (>25 mV) with a biphasic effect on the input resistance (initial decrease followed by an increase) with negligible change in the frequency of the excitatory junction potentials, EPSPs. The effect of AF2 persisted in the extracellular substitution of calcium with cobalt which suggested a direct effect of AF2 rather than a presynaptic effect involving release of neurotransmitters. In contrast to DE2 motorneurons, AF2 had no significant effect on dorsal inhibitory motor neurons. These effects correlated with direct injection of AF2 to pseudocoelomic fluid of the *Ascaris.* In these experiments AF2 produced rapid jerkiness or hyperactivity (Davis and Stretton, 2001). Davis and Stretton from this study concluded that the profound excitatory effect of AF2 is on DE2 motor neurons. In electrophysiological studies, a brief application of AF2 (1 μM, 2 min) followed by a wash (1 min), produced long lasting potentiation of ACh depolarizations (Trailovic *et al*., 2005). AF2 also produced an increase voltage activated calcium currents in *Ascaris* muscle (Verma *et al*., 2007). For drug discovery, targeting the AF2

receptors has gained momentum due to an abundance of AF2 across the nematode phylum and further profound effects on locomotion and cAMP levels.

2.13 *Ascaris* Pharynx

In addition to somatic muscle, the pharynx of *Ascaris* is also regarded as a valid drug target due to its role in feeding. The pharynx of *Ascaris* is important for feeding from the ingesta of the host gut. As the worms possess limited glycogen reserves, they continuously feed on the ingesta to maintain their energy demands. Starving and slow moving worms do not survive within the host gut as they are removed during bowel movements. An appropriate functioning of the pharynx is necessary for feeding and further for the survival of worms within the host gut.

2.13.1 Anatomy of *Ascaris* Pharynx

Pharynx in *Ascaris* is a muscular and glandular pumping organ with a tri-radiate lumen (Fig 7 A, B and C). Externally, the *Ascaris* pharynx resembles a cylindrical tube approximately 1 cm in length and slightly greater than 1 mm in width/diameter at its bulkiest end. The cuticle along with hypodermis and the somatic muscle layer surrounds the pharynx from outside. In addition, the interior of the pharyngeal lumen is derived from the cuticle (del Castillo and Morales, 1967a). The arrangement of cells (muscle cells and marginal cells) surrounding the inner lumen of the pharynx is analogous to the arrangement of segments of an orange. Unlike the somatic muscle

spindles of *Ascaris* which are obliquely striated, the pharyngeal muscle has non-striated radially oriented filaments similar to vertebrate smooth muscle. These non-striated myofibrils of the pharyngeal muscle cells extend radially between the external and internal surfaces of the pharynx (Reger, 1966). This radial arrangement of myofibrils allows the pharynx to functions like a pump analogous to the vertebrate diaphragm. A contraction will increase the size of the lumen thus decreasing the intra luminal pressure to enable suction. On the contrary, a relaxation reduces the size of the lumen thus increasing the intra luminal pressure to push the contents down into the intestine. There are two valves one at each end (anterior and posterior) that ensure unidirectional flow of the material within the pharynx. There are glandular cells that are found interspersed between the myoepithelial cells to secrete digestive enzymes. The intestine is attached to the pharynx and is separated by a valve. The intestine remains flaccid under a high pseudocoelomic pressure (average -70mm Hg). The pseudocoelomic pressure is essential for the maintaining tonicity of the somatic musculature of the worm. Under these conditions, the pharynx has to pump to override the pseudocoelomic pressure in order to pump the food materials into the intestine. Thus, the pharynx behaves like a pressure pump.

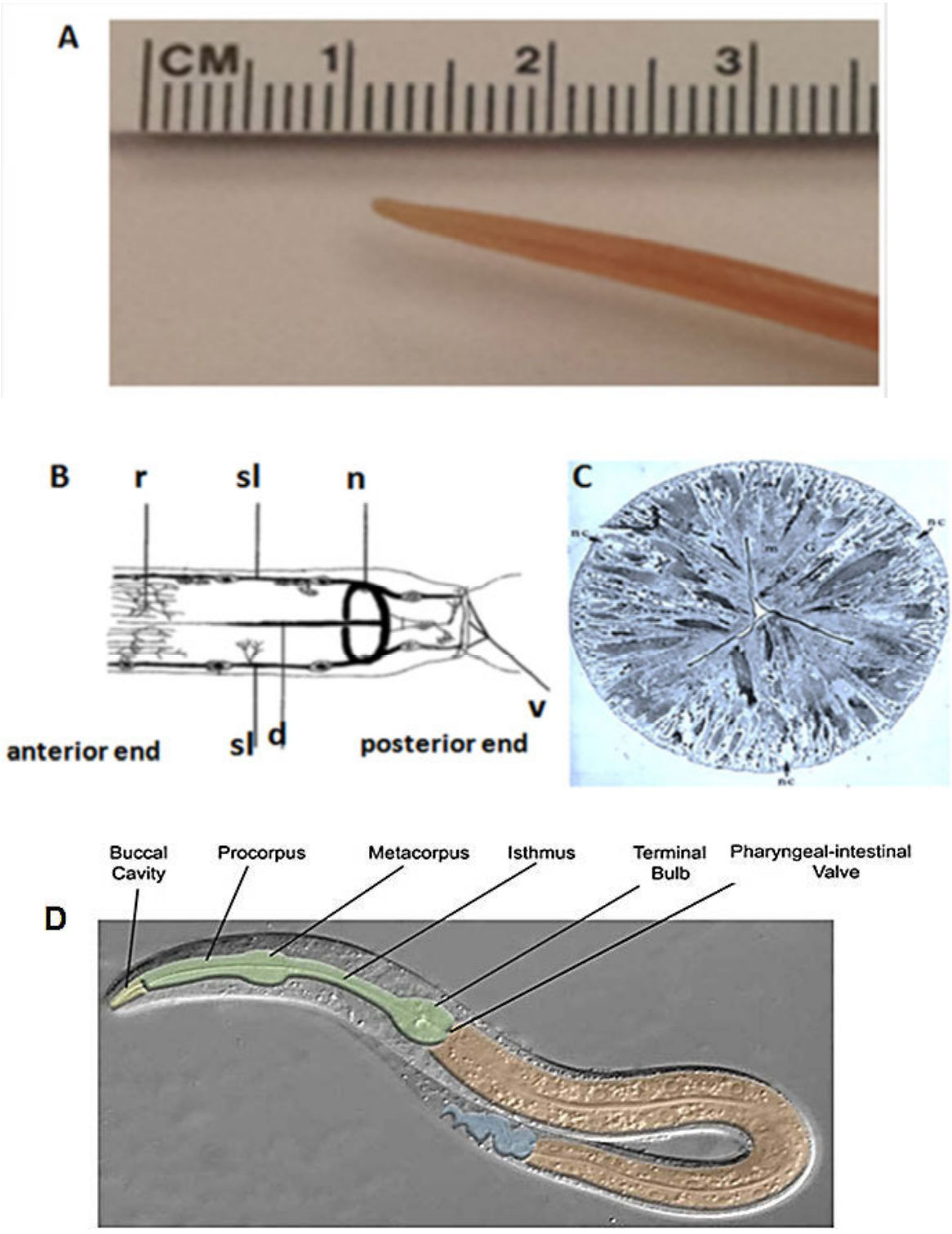

Fig. 7 A. Photograph of the head region of female *A. suum* worm (cm scale above the worm). **B.** A diagram of the head region of Ascaris showing the

enteric/pharyngeal nervous system. The two ends of pharynx, anterior end (towards the lips) and the posterior end (joins the intestine at the valve region, v). The pharyngeal nervous system originates from the nerve ring (n) travels anteriorly as two sub lateral nerve cords (sl) and a dorsal nerve cord (d) (Brownlee *et al*., 1996). These nerve cords (nc) can also be observed in the cross section of pharynx, **C**. Muscle cells, m, gland cell, G, are seen in the cross section of pharynx surrounding the pharyngeal lumen (del Castillo and Morales, 1967a). **B**. Observe the ramification (r) of nerve plexus over the pharynx. **D.** The pharynx of *C.elegans* showing different segments of pharynx namely procarpus, metacorpus (anterior bulb), isthmus, terminal bulb and the valve (Mango, 2007). Unlike *Ascaris* pharynx, *C.elegans* pharynx is visible due to transparent cuticle, contains distinct divisions.

2.13.2 Pharyngeal peristalsis in *Ascaris*

In contrast to the transparent pharynx of C.elegans, *Ascaris* pharynx is opaque and difficult to discern divisions. In addition, there are no distinct (anterior and posterior) bulbs visible in *Ascaris* pharynx (Mapes, 1965). The studies on *Ascaris* pharynx showed that the pharyngeal muscle contractions result in rhythmic peristalsis not a two stage pumping. A two stage pumping seen in *C. elegans* has characteristic contractions of anterior and terminal bulb followed by isthmic peristalsis. In the literature, we often find a common term “pharyngeal pumping”, which does not clearly distinguish pharyngeal muscle contractions of the *Ascaris* from the *C. elegans*. The more appropriate term for pharyngeal contractions in *Ascaris* is

pharyngeal peristalsis. The pharyngeal contractions in case of *C .elegans* involves distinct pharyngeal pumping (contractions of anterior and posterior bulb) followed by pharyngeal peristalsis (isthmus). The pharyngeal contractions in *C. elegans* are explained in later paragraphs. In *Ascaris*, pharyngeal peristalsis starts at the anterior tip of the pharynx and proceeds all the way to the end of pharynx. The anterior tip of *Ascaris* pharynx is considered as the pacemaker. This anterior tip initiates peristalsis either by myogenic activity or by enteric nervous system activity. The depolarization of pharyngeal muscles at the anterior tip of pharynx results in progressive contraction waves which moves posteriorly at an average speed of 4 cm/sec. The *Ascaris* pharynx at any stage of peristalsis never opens fully at both ends (anterior or posterior). Fig 8 shows, during pharyngeal peristalsis the pharyngeal lumen at the anterior end initially closes before it opens at the posterior end ensuring one way flow of food materials (Saunders and Burr, 1978). The peristaltic cycles have been recorded from *Ascaris* pharynx show 4 pumps/sec from an intact pharynx while, 2.5 pumps/sec from an isolated pharynx (Mapes, 1965; Saunders and Burr, 1978).

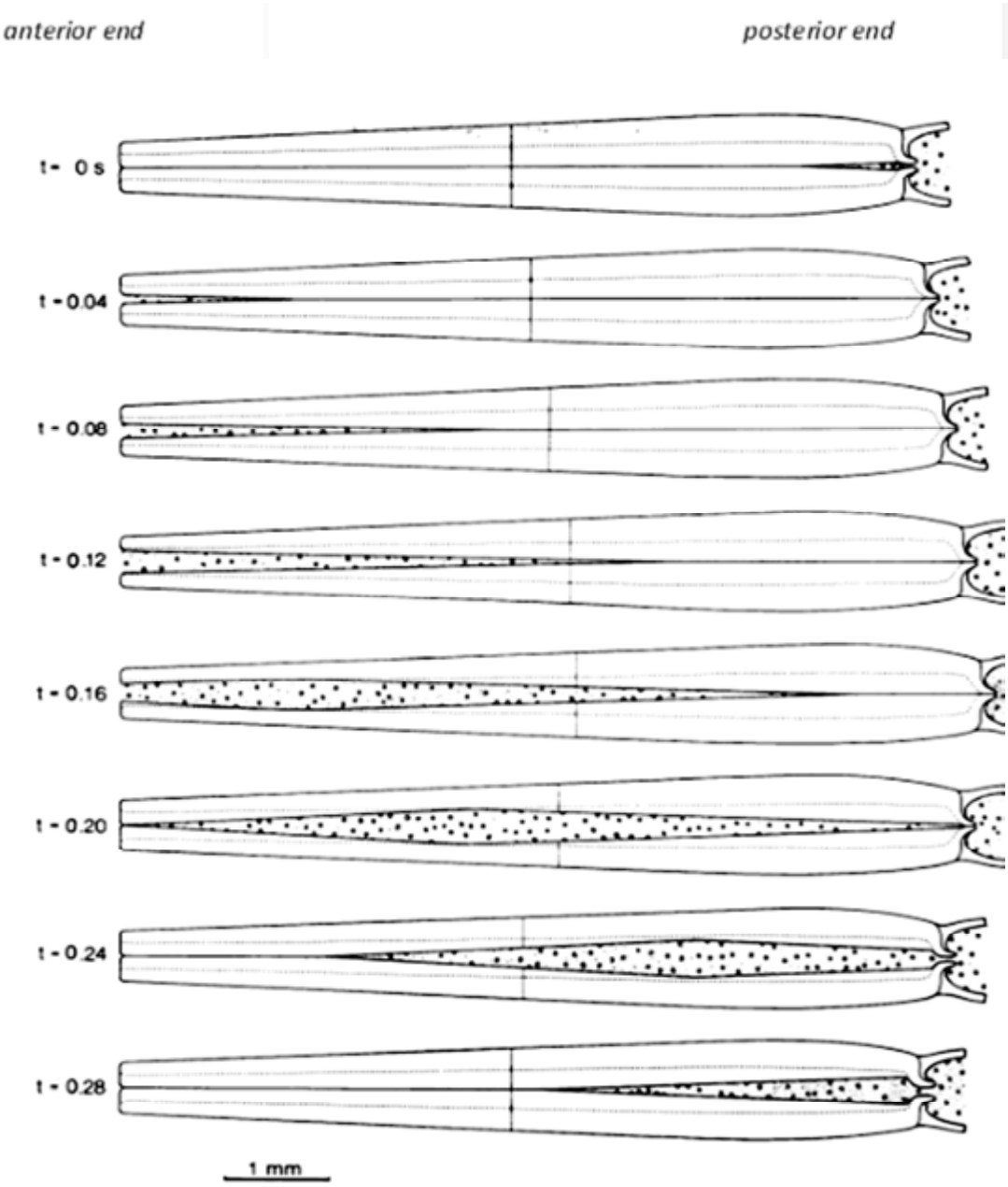

Fig 8. The diagram showing various stages observed during normal peristaltic cycle of *Ascaris* pharynx (Saunders and Burr, 1978). The different time points represented in seconds shows repeating cycle of pharyngeal peristalsis (approximately every 0.32 s). At any point pharynx never opens simultaneously at both anterior and posterior ends.

2.13.3 Membrane potential of *Ascaris* pharynx.

Electrophysiological studies have been made by exposing the pharynx by dissecting out cuticle and surrounding muscle layer in *Ascaris.* Electrophysiologically the whole *Ascaris* pharynx behaves like one single giant cell owing to the presence of numerous gap junctions (del Castillo and Morales, 1967a). *Ascaris* pharynx has a resting membrane potential of -40mV maintained mainly by extracellular organic anions found in the perienteric fluid. The inorganic anion chloride extracellularly failed to substitute for the removal organic anions (for example bicarbonate). The resting potential *Ascaris* was also found to be sensitive to pH. Acidification from pH 7 to 6 resulted in depolarization (of 20 mV) while an alkalizing to pH 8 resulted in hyperpolarization (of 4 mV). The observed relation between pH and resting potential has been linked to permeability of anions from the perienteric fluid of the worm (del Castillo and Morales, 1967b, a). By examining electron microscopy sections of pharyngeal muscle, Del Castillo and Morales (1967a) suggested existence of membrane spaces within the cytoplasm of pharyngeal muscle. These spaces are proposed to be equivalent to the tubular systems of the muscle cells in vertebrates that function to carry the flow of action potentials. Interestingly, when microelectrode tip enters into theses spaces the resting potential is either greatly reduced or absent as if the electrode has briefly moved out of the cell into the surrounding bath solution. The recorded signals from a microelectrode placed in these spaces resembled extracellular action potentials (del Castillo and Morales, 1967b, a).

The action potential in *Ascaris* pharynx was found to be a regenerative, all or none process in contrast to the somatic muscle the worm. The action potentials resulted in depolarization (positive overshoot) to reach a plateau. The depolarization is result of influx of cations namely sodium or calcium. From the plateau followed a repolarization caused by a potassium spike. Interestingly, the potassium spike goes intensely negative to produce negative membrane potential before reaching the resting membrane potential. This intense negative hyperpolarization was called a negative potassium spike. Depolarization can reach as high as 18mV with a marked hyperpolarization (a fast negative potassium spike as low as -108 mV) during the repolarization. Voltage-gated potassium channels are shown to be responsible for the negative regenerative hyperpolarizing spike. This voltage gated potassium channel has been found to close when the membrane is depolarized but, opens during rapid negative membrane potentials (Byerly and Masuda, 1979b). Unlike the somatic muscle of *Ascaris,* spontaneous activity (spikes) was occasionally observed in pharynx (del Castillo and Morales, 1967a).

2.13.4 Nervous system of *Ascaris* pharynx

We observed previously that somatic muscle cell of *Ascaris* extend their arms to the nerve cord to receive innervation forming syncytium that surrounds the nerve cord. In contrast, pharyngeal neurons are embedded below the pharyngeal muscle regions. The neuromuscular synapses are made when the nerve cord containing neurons extend their course. There are 3 longitudinal nerve cord arise from the

circumferential ring extend in the anterior direction to innervate the pharyngeal muscle. These nerve cords are referred to as dorsal and a paired sub-ventral cords (Saunders and Burr, 1978). The dorsal nerve is made up of two neuronal cell bodies when compared to sub ventral nerve cord which contains 4-6 neuronal cells (Goldschmidt, 1910; Brownlee *et al*., 1995; Brownlee *et al*., 1996). There are 2-3 cross connecting commissures joining the longitudinal cords together. The processes arise from the nerve cords spread across (ramifiy) the pharyngeal muscle to provide a widespread innervation. This widespread innervation is important for coordinating the muscular and glandular activity of the pharynx.

The pharyngeal nervous system is complex, involving cholinergic (excitatory), glutamatergic (inhibitory) and serotoninergic (excitatory) systems innervating the pharyngeal muscle. In addition, the pharyngeal nervous system has GABAergic components (excitatory) limited to the sensory neurons, and peptidergic secretory components (McIntire *et al*., 1993; Brownlee *et al*., 1996). The peptidergic components have been studied by immunoreactivity and show the presence of pancreatic polypeptide, peptide YY, gastrin and numerous invertebrate neuropeptides (Brownlee *et al*., 1996).

2.14 Anatomy of *C.elegans* pharynx

The pharyngeal muscle contractions of *C. elegans* pharynx have been well understood due to transparent nature of cuticle and pharynx (Fig 7.D). When compared to *Ascaris* pharynx, *C.elegans* pharynx has distinct divisions. The

pharynx of *C. elegans* is bilobed consisting of an anterior lobe and the posterior lobe. These two lobes are joined by a narrow tube called Isthmus where, the nerve ring is located. *C .elegans* pharynx is encased by a basement membrane on the exterior side. The pharynx can be further subdivided into six sections starting from the anterior end to the posterior end. These include the buccal cavity, procorpus, metacorpus (anterior bulb), isthmus, terminal bulb (posterior bulb) and pharyngeal-intestinal valve. Muscle regions of pharynx mediate rhythmic contractions. These rhythmic contractions aid in suction of bacteria into the pharyngeal lumen for grinding before passing them back into the intestines. At the anterior-most end the pharynx is connected to arcade cells which form the lips. The pharyngeal lumen is covered with cuticle which is continuous with the outer cuticle of the body. Just under the outer cuticle there are nerve endings that contain mechanoreceptors. The cuticle between isthmus and metacarpus forms a sieve which helps in trapping particulate matter after expelling excess of liquid and later to mix with digestive enzymes secreted by gland cells (Albertson and Thomson, 1976). The pharynx is made up of a total of 77 cells. Among them, the contractile areas of the pharynx are formed by 34 muscle cells and 20 neuronal cells. These 20 neuronal cells belong to the pharyngeal nervous system sometimes referred to as the enteric nervous system. The other cell groups include 9 marginal cells, 9 epithelial cells and 5 gland cells.

There are a total of 34 muscle cells divided into eight pharyngeal muscle cell segments (pm1,-8) which are positioned from the anterior to the posterior end of the

pharynx. The muscle cells of the pharynx towards the lumen form a tri-radiate symmetry similar to *Ascaris* pharynx. The pharyngeal muscle cells are joined to each other by marginal cells in turn connected by desmosomes or tight junctions. The pharynx has been functionally divided into anterior carpus (combination of procarpus and metacarpus) made up of pharyngeal muscles pm1 through pm4 forming the anterior half of the pharynx. The corpus is important for ingestion and trapping bacteria. The middle Isthmus is formed by pharyngeal muscle pm 5. The isthmus functions in peristalsis to regulate the flow of food from the corpus to the terminal bulb known as pharyngeal peristalsis. The terminal bulb of the pharynx is formed by pharyngeal muscles pm6 through pm8. The terminal bulb is important for grinding the bacteria.

Other cells of the *C. elegans* pharynx include, 9 marginal cells arranged in sets of 3 cells (mc1-3) embedded between the muscle cells. There are gap junctions between muscle cell and marginal cell to relay the signals. There are 5 gland cells which make up two cell types (g1-2) to secrete digestive enzymes. Towards the outer border of pharynx there are 9 epithelial cells which anchor the basement membrane of the pharynx to the outer cuticle.

The pharyngeal nervous system consists of 20 cells which include paired and unpaired neurons. The neurons of the pharynx are situated between the pharyngeal muscle and the basement membrane of hypodermis Based on their functions; neurons are classified into five types of motor neurons (M1-5, total seven cells), 6 types of interneurons (I1-6, total eight cells), two neuro-secretory motor neurons

(NSMs), one motor interneuron and two marginal cell neurons (Albertson and Thomson, 1976). The role of important motorneurons that regulates feeding behavior in *C.elegans* is discussed in further paragraphs.

2.14.1 The pharyngeal muscle action potential

The resting membrane potential of *C.elegans* is similar to *Ascaris* and ranges from -40mV to -50mV. Each pumping event is a consequence of a single muscle action potential. These action potentials are intrinsic to the pharyngeal muscle cells and are independent of the involvement of pharyngeal nervous system. Pumping of the pharynx in *C. elegans* is found to continue even after laser killing of the entire pharyngeal nervous system. Among all the other motor neurons, laser killing of M4 had observable effects. During rapid pumping, the MC motor neuron played a role in generating excitatory postsynaptic potential (EPSPs) on to the pharyngeal muscle (Raizen and Avery, 1994; Raizen *et al*., 1995). The action potentials generated by the pharyngeal muscle of *C. elegans* are similar to *Ascaris* pharyngeal muscle. These pharyngeal action potentials are also comparable to the ventricular myocardium in vertebrates (Raizen and Avery, 1994). Similar to *Ascaris* pharynx, *C.elegans* pharynx has three phases namely excitation phase, plateau phase, and repolarization phase.

The electrical activity of the pharynx of *C. elegans* has also been studied indirectly by making electropharyngeograms (EPGs). The EPGs represent the sum total of

electrical events that flow in and out during pharyngeal openings. Each excitation phase corresponds to a positive spike in the EPG while, the repolarization phase is seen as a negative spike (Raizen and Avery, 1994).

2.14.2 Feeding in *C.elegans* pharynx

C. elegans feeding behavior is classified as filter-feeding and differs from *Ascaris*. As the worm ingests liquid with suspended bacteria, the excess liquid is expelled out to concentrate the bacteria. The filter-feeding of *C. elegans* is explained in two motions of pharynx that is, pumping and the isthmus peristalsis. The pharyngeal lumen during the resting state is closed resembling a Y-shape. This Y-shape of the pharyngeal lumen is a result of relaxation of radially oriented pharyngeal muscle fibers. Pumping results in simultaneous contraction of the muscles of the corpus, anterior isthmus, and the terminal bulb to open the pharyngeal lumen. The opened pharyngeal lumen is a triangular shape due to contraction of radially oriented contractile filaments. The opening of the pharyngeal lumen allows filling with liquid containing suspended bacteria. During this period the posterior end of isthmus is closed. The relaxation of the carpus expels the fluid to retain the bacteria. The trapped bacteria are then passed on to terminal bulb for grinding by a process called isthmus peristalsis. Isthmus peristalsis occurs when the corpus (anterior bulb) and terminal bulbs relax. Isthmus peristalsis follows once after every fourth pumping. Before the next contraction and relaxation cycle, the terminal bulb grind the bacteria then, passes them into intestine once the pharyngo-intestinal valve is

opened (Albertson and Thomson, 1976; Avery and Horvitz, 1989; Avery and Shtonda, 2003).

2.14.3 Motorneuron controls during feeding

Although the nematode pharynx is independent and self-regulated, two nervous systems exert considerable control over its activity. These two nervous systems are the pharyngeal nervous system and the somatic nervous system. The somatic nervous system senses the sensory stimulation (presence of food) from the exterior and passes the information on to the pharyngeal nervous system. Severing the connections between these two nervous systems results in pharyngeal pumping which is unresponsive to external stimuli. Selective laser surgery of motor neurons has given some valuable information on their role in pharyngeal activity. This procedure has pointed to three motor neuron types necessary and sufficient for normal feeding namely M4, MC, and M3. When all pharyngeal neurons except the motor neurons M4, MC, and M3 are laser ablated, feeding is found to be nearly normal. Another important pharyngeal neuron type is the neurosecretory motor neuron (NSM). The NSM motorneuron functions in conveying the sensory information such as the presence of food to the worm (Avery and Horvitz, 1989; Raizen and Avery, 1994; Raizen *et al*., 1995; Avery and Shtonda, 2003).

I have described briefly the three important neuron types for the regulation of the pharyngeal nervous system of *C. elegans*.

M3 motor neurons are a bilaterally symmetric pair of motor neurons which send their input to the metacorpus. MC neurons generate inhibitory postsynaptic potentials (IPSPs) in the pharyngeal muscle which trigger repolarization and relaxation (Albertson and Thomson, 1976). The M3 motor neurons are essential for regulating the speed of relaxation and in the absence of M3 motor neurons the relaxation is delayed. The speed of relaxation is found to be important for effective trapping of bacteria (Avery, 1993a). The studies on M3 motor neurons suggest that it is an inhibitory motor neuron. The M3 motor neuron mediates its function through the neurotransmitter glutamate. Application of glutamate pulses over the pharyngeal muscle mimics responses similar to stimulation of the M3 motor neuron. In mutants of the *avr−15* (avermectin-resistant) gene which codes for glutamate-gated chloride channel subunits, both the M3 motor neuron transmission as well as the responses to glutamate were found to be absent (Dent *et al*., 1997). These glutamate channels were found to be avermectin-sensitive. When the avermectin sensitive glutamate channels from *C. elegans* are expressed in *Xenopus* oocytes, these channels were found to be irreversibly opened by avermectin (Cully *et al*., 1994).

M4 motorneurons sends its synapses on to the posterior half of the isthmus muscles. The M4 motor neurons control peristalsis of the isthmus and in its absence isthmic peristalsis was found to be absent. Worms where M4 neurons were laser killed swallow little or no food in the pharynx which resulted in failure to grow resembling starvation. Although these worms continue to pump but, the bacteria in the pharynx were found concentrated in the anterior isthmus and corpus. Further,

these worms show a stuffed pharynx due to paralysis of the posterior isthmus (Avery and Horvitz, 1989; Raizen and Avery, 1994; Raizen *et al*., 1995). Immunohistochemistry studies suggest the M4 motor neuron mediates its effects through acetylcholine as its neurotransmitter (Alfonso *et al*., 1993).

Neurosecretory motor neuron (NSM) are serotonergic neurons located in the anterior bulb (Albertson and Thomson, 1976). The effect of serotonin has been studied on *C.elegans* hermaphrodites. Serotonin increased pharyngeal pumping frequency, stimulated egg laying and decreased the movement of the worm (Avery and Horvitz, 1989). Exogenous application of serotonin stimulated pharyngeal pumping suggesting the presence of serotonergic receptors on the pharyngeal muscle. However, laser killing of NSM motorneurons was observed to have subtle effects on pharyngeal pumping in *C. elegans* suggesting other motor neuron (MC motorneuron) may compensate in the absence of NSM (Avery and Horvitz, 1989; Bargmann and Avery, 1995; Raizen *et al*., 1995).

MC motorneurons control the rate of pharyngeal pumping and their absence has shown to cause slow pumping. The MC motor neurons are found to be excitatory motor neurons. The MC motor neurons are found to initiate action potentials excitatory postsynaptic potential (EPSPs) in the pharyngeal muscle as well as to increase the frequency of pharyngeal pumping (Raizen *et al*., 1995). The MC motor neuron are termed as the pacemaker of the pharynx. Pharmacological and genetic studies in *C. elegans* suggest that acetylcholine is the neurotransmitter of MC motor neuron. Application of acetylcholine or cholinergic agonists excited the pharyngeal

muscle while curare competitively blocked the response (Raizen *et al*., 1995). MC motor neurons were difficult to stain in immuno-histochemical studies. Interestingly MC motor neuron synapses were found on the marginal cells not on the pharyngeal muscle inferring that the signal spreads through gap junctions. This is proposed to be analogous to purkinje fibers which conduct the impulses of the cardiac pacemaker to the myocardium (Albertson and Thomson, 1976).

2.14.4 Genes encoding pharyngeal muscle nAChRs in *C.elegans*

Even though, genome of *C. elegans* is fully sequenced, the literature available on the pharyngeal muscle nAChRs is limited. Only two genes among the genes regulating pharyngeal pumping code for the pharyngeal nAChR function. The genes coding for the pharyngeal nAChRs subunits and their expression studies is still an unexplored ground.

***eat-2* gene** encodes a ligand-gated ion channel subunit and most closely resembles a non-alpha-subunit of nicotinic acetylcholine receptors (nAChR). EAT-2 expression was found on the postsynaptic membrane of the pharyngeal muscle. The expression of EAT-2: GFP fusion protein was found near the junction of pharyngeal muscles pm4 (at the anterior bulb) and pm5 (part of the isthmus), which is also the site of the MC motor neuron and its synapse. In addition, *eat-2* genetically interacts with *eat-18*, expressed in pharyngeal muscle encoding a transmembrane protein required for proper function of pharyngeal nicotinic receptors (Raizen *et al*., 1995).

***eat−18* gene** encoding a transmembrane protein associated with the functioning of pharyngeal nicotinic receptors. EAT-18 is required for the pharyngeal muscle to respond to nicotine and also for MC motorneuron transmission. The *eat-18* null mutants were indistinguishable from the worms which had MC motor neuron ablated. These *eat-18* null mutants also resembled worms that carried partial loss-of-function mutations in the *cha−1* and *unc-17* genes necessary for cholinergic transmission. The gene *cha−1* encodes an enzyme cholineacetyltransferase that synthesizes acetylcholine. The gene *unc−17* encodes a synaptic vesicle acetylcholine transporter (VAChT) required for loading acetylcholine into synaptic vesicles in cholinergic neurons (Avery and Horvitz, 1989; Avery, 1993b, a; Raizen and Avery, 1994; Raizen et al., 1995).

eat-5 gene encodes a protein “innexin” which is expressed in pharyngeal muscle cell (pm4 and pm5). Innexins are required for synchronizing the pharyngeal muscle contractions by forming electrical connections (gap junctions) between pharyngeal muscle cells. The protein EAT-5 forms electrically permeable intercellular channels (gap junctions), with a predicted membrane topology analogous to that of connexin gap junctions found in vertebrates (Avery, 1993b; Avery et al., 1993).

2.14.5 Neurotransmitters controlling pharyngeal peristalsis / pumping

Glutamate

Glutamate is an excitatory neurotransmitter in the vertebrate nervous system but is both excitatory and inhibitory neurotransmitter in invertebrates. Glutamate is produced during transamination where an amino group of amino acid (for example alanine or aspartate) is transferred on to a α-ketoacid (α-ketoglutarate). The intermediates which are derived during the production of glutamate form substrates for glycolysis, gluconeogenesis and the citric acid cycle. Glutamate is stored in the synaptic vesicles at the presynaptic nerve terminals to activate glutamate receptors on the postsynaptic cell. Glutamate gates the cation (calcium) gated NMDA receptors in vertebrates. The NMDA receptors are involved in cognitive functions (learning and memory) in synapses found in hippocampus and neocortex. In invertebrates, glutamate receptors activate chloride channels (GluCls) to result in hyperpolarization. Glutamate transporters found on the presynaptic membranes and glial membranes rapidly remove glutamate from the synapse.

These GluCls are more closely related to mammalian glycine receptors than GABA gated chloride channels (Vassilatis *et al*., 1997; Dent, 2006). GluCls are involved in pharyngeal pumping, sensory perception and locomotion in nematodes. The GluCls are the targets of the macrocyclic lactone group (ivermectin, moxidectin) of anthelmintics, the biggest selling class of anthelmintics in veterinary medicine. These drugs are not only potent anthelmintics but also, insecticides and acaricides. The glutamatergic motor neurons (M3) innervate pharyngeal muscle (pm4) cells

(Albertson and Thomson, 1976). The glutamatergic motor neurons (M3) generate inhibitory post synaptic potential (IPSPs) to regulate the timing of muscle relaxation during pharyngeal pumping (Avery, 1993a; Raizen and Avery, 1994; Raizen et al., 1995).

Two genes *glc-1 and glc-2* encoding subunits encoding GluClα and GluClβ, have been identified in *C.elegans* by expression studies in *Xenopus* oocytes, Table 2 (Cully *et al*., 1994). The homomeric channel formed by expression GluClα in *Xenopus* revealed an ivermectin gated channel while expression of GluClβ revealed a glutamate gated ivermectin insensitive channel. The heteromeric glutamate-gated chloride channel (GluClα andβ) assembled was ivermectin sensitive glutamate gated chloride channel. GFP tagging revealed the expression of GluCls in pharyngeal muscle cells and neurons (mechanosensory neurons namely ALM, PLM and PVD) (Dent *et al*., 2000). Alternative splicing of the gene *avr-15,* responsible for avermectin resistance in *C.elegans* revealed two α subunits, GluClα2A and GluClα2B which are sensitive to ivermectin and glutamate. Both GluClα2 and the GluCl β were found to be expressed in pharyngeal muscle (pm4 and pm5). Alternative splicing of *avr-14,* another gene responsible for ivermectin resistance yielded GluClα3A and GluClα3B (Dent *et al*., 2000). Unlike the expression of *avr-14* and *avr-15*, which are widespread, *glc-2* (GluClβ) expression is limited to the pm4 of the pharyngeal muscle cells (Laughton *et al*., 1997) Expression of GluClα2 was wide-ranged including the nematode motor nervous system which explains how the effect of ivermectin application on the nematode affects the locomotory system.

Later, gene *glc-3* encoding GluClα4 was also identified which is predicted to be distant form α or β GluCl subunits. Glutamate activation of most of homomeric GluCl α receptors produces a rapid opening (EC_{50} was 1–2 mM) of the channel which resulted in rapid desensitization. However the desensitization was not observed in receptors containing homomeric GluClβ subunits (Cully *et al*., 1994; Vassilatis *et al*., 1997; Dent *et al*., 2000; Forrester *et al*., 2003; Wolstenholme and Rogers, 2005b). Interestingly, upon ivermectin treatment GluCl opening was slow and essentially irreversible (EC_{50} 0.1 to 10 μM) and these channels remained open even when the drug is removed. Ivermectin had no effect on homomeric GluClβ subunits (Cully *et al*., 1994; Wolstenholme and Rogers, 2005b). The application of glutamate and ivermectin has shown potentiation in the activation of GluCls (Cully *et al*., 1994; Forrester *et al*., 2004). The high-resolution structure of the GluCl shows the binding sites of avermectin anthelmintics. The slow effect of avermectins is due to initial entry of the drug through the plasma membrane and then binding to the pore region formed by the α-helixes of cys-loop receptors like GluCls (Unwin, 2005; Wolstenholme, 2011). Once the drug binds the channel in the open state, it prevents the pore region from returning back to closed state resulting in a long-lasting channel-opening and a hyperpolarization of the cell which is no longer excitable. The other effects of the drug in parasite nematodes include long-lasting sterilization of *Onchocerca volvulus* adults *in vivo* (Awadzi *et al*., 1985; Wolstenholme, 2011) and lethal effects on *Brugia malayi* microfilariae (Moreno *et al*., 2010).

Table 2. A and **B** taken from Wolstenholme and Rogers (2005b). **A.** Shows the subunit genes of glutamate gated chloride channels in *C. elegans* and their properties. **B**. shows the glutamate gated chloride channels studied in parasitic nematodes and their properties.

A

Gene	Subunits Encoded	Properties
avr-14	GluClα3A GluClα3B	*avr-14* mutations cause moderate avermectin resistance in combination with *avr-15*, and very high level resistance as a triple mutant with *avr-15* and *glc-1*. GluClα3B forms glutamate- and ivermectin-gated channels: no channels have been reported for GluClα3A.
avr-15	GluClα2A GluClα2B	In *avr-15* mutants pharyngeal pumping is insensitive to ivermectin. GluClα2 subunits form glutamate- and ivermectin-gated channels and can co-assemble with β subunits.
glc-1	GluClα1	GluClα1 forms ivermectin-gated channels: glutamate binds but does not open the channel. When expressed with GluClβ, ivermectin-potentiated, glutamate-gated channels are formed.
glc-2	GluClβ	GluClβ forms glutamate-gated channels.
glc-3	GluClα4	GluClα4 forms glutamate- and ivermectin-gated channels.
glc-4	?	?

B

Subunit	Species in which found	Properties
GluClα/GluCla	*Haemonchus contortus*	Forms glutamate- and ivermectin-gated channels. Forms a high affinity ivermectin binding site.
GluClα3A GluClα3B	*Ascaris suum*•, *Cooperia oncophora*•, *Dirofilaria immitis*, *Haemonchus contortus*, *H. placei*, *Onchocerca volvulus*•	GluClα3A does not express in oocytes or mammalian cell lines. GluClα3B forms glutamate- and ivermectin-gated channels and a high affinity ivermectin binding site.
GluClβ	*Cooperia oncophora*, *Haemonchus contortus*	Forms glutamate-gated channels. Can be co-expressed with GluClα3B. Does not form a high-affinity ivermectin binding site.

• Only a single GluClα3 subunit has been identified in these species.

2.14.6 Biogenic amine neurotransmitters or neuromodulators

In nematodes there are four biogenic amines namely serotonin (5-hydroxytryptamine, 5HT), dopamine, octopamine and tyramine that modulate worm behavior in response to the surrounding environment. Most of these biogenic amines act on GPCRs to elicit their function. Serotonin is an exception that, it has receptors that are GPCRs (5HT1-2 and 3-7) as well as ion channels (5HT3). The main effects of the biogenic amines include activation or modulation of neurons to influence pharyngeal pumping, locomotion, learning, foraging, egg laying and defecation. The roles of dopamine and serotonin have established in mammalian nervous system. An abnormality in dopamine and serotonin signaling is implicated in diseases like Parkinson's disease, schizophrenia and depression. The precursors for 5HT is tryptophan which is converted to 5-hydroxytryptophan by tryptophan hydroxylase and later into serotonin (5HT) by aromatic amino acid decarboxylase. In the synthesis of other biogenic amines tyrosine is a precursor, acted upon by tryptophan hydroxylase, aromatic amino acid decarboxylase to form dopamine. Similarly, tyrosine is acted upon by tyrosine decarboxylase to from tyramine which is further catalyzed by tyramine-β-hydroxylase to form octopamine. Exogenous application of octopamine in *C.elegans* has been shown to stimulate worm movement but inhibit pharyngeal pumping and inhibition of egg laying. Octopamine has been shown to bind to tyramine receptors expressed in eukaryotic cells resulting in an increase in intracellular cAMP. Tyramine is secreted in low quantities from the same cells that synthesize octopamine. This implies tyramine may just be

an intermediate in the synthesis of octopamine. Exogenous tyramine in *C.elegans* was shown to inhibit egg laying and also inhibit serotonin-induced pharyngeal pumping (Komuniecki *et al*., 2004; Rex *et al*., 2004b; Martinez-Torres and Miledi, 2006; Chase and Koelle, 2007; Wragg *et al*., 2007).

Dopamine in mammalian brain acts on GPCRs characterized as D1-D5. According to activation of second messengers they are grouped as; D1-like (Gsα, increase cAMP) which includes D1 and D5; D2-like (Giα, decrease cAMP). The dopamine receptor is also linked to calcium and potassium channel activity. A similar characterization has been done in the dopamine receptors of *C.elegans* namely D1-like called DOP-1 and D2-like called DOP-2 (Suo *et al*., 2002, 2003; Sugiura *et al*., 2005). Dopamine signaling is part of mechanosensory neurons in *C.elegans* that modulate locomotion, behavior and learning. Dopamine also plays a role in the search for new food sources (Sawin *et al*., 2000; Chase and Koelle, 2007).

In nematodes, serotonin (5HT) plays a role in feeding, locomotion, egg-laying and metabolic regulation of nematodes. There are at least 7 subtypes of serotonin receptors in mammals of which 6 of them are GPCRs and one is an ion channel (namely 5HT3s). 5HT (EC50- 44 μM) causes an excitation of the pharynx and maintenance of pumping in an isolated *Ascaris* pharynx. 5HT causes an increase in cAMP resulting in glycogenolysis in *Ascaris* (Trim *et al*., 2001). In *C.elegans*, Serotonin is synthesized by the NSM (neurosecretory motor) neurons in *C.elegans* which helps in sensing food in the surroundings. Serotonin is also secreted by HSN (hermaphrodite-specific neurons) which helps to stimulate egg laying (Chase and

Koelle, 2007). 5HT activates 3 GPCRs (SER-1, SER-4 and SER-7) and an ion channel permeable to chloride (MOD-1). All these 5HT receptors are expressed in pharyngeal neurons and muscles of *C.elegans* (Hamdan *et al*., 1999). In *Ascaris*, two bilaterally symmetrical neurons in the pharynx have been shown to synthesize 5HT in immunoreactivity studies (Trim *et al*., 2001).

2.15 Anthelmintics and emergence of Anthelmintic resistance

Anthelmintic drugs control helminth infections by selectively disrupting worm physiology. Worms reside in the host gut, feed on the ingesta and maintain their site of predilection by constantly moving against the bowel movements. Cholinergic anthelmintics paralyze the worms by acting on the nAChRs found on the somatic muscle of the worm. Once paralyzed, worms fail to move and get expelled from the host gut. The cholinergic anthelmintics include: the imidazothiazoles (levamisole, tetramisole); tetrahydropyrimidines (pyrantel, morantel & oxantel); quaternary/tertiary amines (bephenium, thenium and tribendimidine); pyridines (methyridine) and AADs (monepantel) (Martin and Robertson, 2007). Antagonists of parasite muscle nAChRs include derquantel & phenothiazine (Robertson *et al*., 2002; Zinser *et al*., 2002). The anthelmintics which act as agonists of nAChRs produce spastic paralysis, while the antagonists produce flaccid paralysis of the worms. Piperazine mimics the natural ligand GABA and activates GABA gated chloride channels on the somatic muscle to cause hyperpolarization. The hyperpolarization of the somatic muscle of the parasite results in flaccid paralysis.

On the pharynx of the worms, glutamate gated chloride channels (GluCls) are present. Glutamate is the endogenous ligand for GluCls. The activation of GluCls produce hyperpolarization and inhibit the pharyngeal pumping. Pharyngeal pumping in worms is important for the ingestion of food from the host gut. Avermectins (ivermectin, doramectin, abamectin) and milbemycins (moxidectin, milbemycin) selectively modulate GluCls to potentiate glutamate responses causing inhibition of pumping in the pharynx. The inhibition of pharyngeal pumping impairs feeding, resulting in starvation of the worms (Wolstenholme and Rogers, 2005b). The starved slow moving worms are eliminated from the host gut during peristalsis.

At therapeutic doses, anthelmintics are selective on the parasite over the host and effective in controlling worm infections. Treatment with anthelmintics against parasitic worms fails when the worms develop resistance to drugs. “Anthelmintic resistance” refers to the ability of parasites to survive treatments that are generally effective at the recommended therapeutic doses. Intensive use of an anthelmintic agent to control worm infections in herds has led to the selection of genetically resistant parasites (Prichard et al., 1980; Prichard, 1990; Prichard, 1994; Waller, 1997; Silvestre et al., 2002; Kaminsky, 2003). In Australia (Edwards et al., 1986a; Jackson and Coop, 2000), Paraguay (Maciel *et al*., 1996; Waller *et al*., 1996) and S. Africa (van Wyk *et al*., 1997), anthelmintic resistance threatens the economics of the entire sheep industry. In humans, schistosomes and GI nematodes have shown resistance to anthelmintics following mass drug therapy programs in endemic regions (Ismail *et al*., 1996; De Clercq *et al*., 1997; Reynoldson *et al*., 1997; Ismail *et al*., 1999). After the introduction of ivermectin (in mid-1980’s) we did not see any

new anthelmintic come into the market for more than two decades (Geary, 2005). The limited number of anthelmintics available for the therapy, coupled with the onset of resistance in parasites poses a serious threat to livestock and human health (Kaplan, 2004b; Jones and George, 2005). Resistance has reduced the efficacy of currently used anthelmintics, limiting drug options to treat worm infections (James *et al*., 2009). Anthelmintic resistance is a global concern due to high prevalence of infections in both humans and livestock (Geerts and Gryseels, 2000; Kaplan, 2004b; Jones and George, 2005).

2.15.1 Anthelmintic resistance reports from United States

Anthelmintic resistance is on the rise in parasitic nematodes of domestic animals in the United States. Anthelmintic resistance reports have been recorded from beef cattle, sheep and goat farms. A commonly used approach to detect resistance is fecal egg reduction test (FECRT) carried out in herds. This test is carried out in growing animals which are not exposed to any dewormers preferably of even age and body weight. The fecal egg counts are made in controls and two weeks after the anthelmintic drug treatment. An effective anthelmintic drug reduces 90-95% of the egg counts in fecal samples collected two weeks after the treatment. Any reduction in egg counts less than 90-95% indicates a reduction in the efficacy of the anthelmintic and development of anthelmintic resistance in the parasites. A study conducted in beef cattle farms from 19 states across the United States involving 119 FECRTs from 4765 samples showed a reduction in efficacy of commonly used anthelmintic drugs. The efficacies of Ivermectin;40-76.2%, doramectin;89.9 %. However, moxidectin still remained effective, 98.1% in these studies (Bliss *et al*., 2008). *Cooperia species, Haemonchus species* infecting cattle have been observed to develop resistance to macrocyclic lactones namely, ivermectin and doramectin (Gasbarre *et al*., 2009). In sheep and goat farms, *H. contortus* was observed as the most common parasite in 46 farms across the South-eastern United States. In these farms, parasites resistant to benzimidazoles, levamisole, ivermectin and moxidectin have been recorded (Howell *et al*., 2008). Helminth infections namely toxocariasis, ascariasis, strongyloidiasis and cysticercosis have been found to affect

impoverished human populations distributed across the Mississippi Delta, Appalachian belts, US-Mexico borderlands and a few Southern United States. These infections have been referred as “neglected infections of poverty” and remain a growing concern to socioeconomically disadvantaged populations (Hotez, 2008).

2.15.2 Anthelmintic resistance reports from India

Benzimidazole resistance in small ruminants infected with *H. contortus* has been reported in Uttaranchal region of India (Varsheny, 1976; Dubey, 2010). Various tests used to identify anthelmintic resistance included fecal egg count reduction tests, egg hatch assays and larval migration inhibition assays (Easwaran, 2009). However, ivermectin still possess efficacy in sheep infected with *H. contortus* (Garg *et al*., 2007). Resistance was observed in *H.contortus, Telodorsagia species* to multiple anthelmintics namely levamisole, thiabendazole, has been encountered in sheep farms in Tamil Nadu, Southern India.

2.15.3 Electrophysiological studies to understand anthelmintic resistance

Electrophysiological studies in *A. suum* demonstrate that anthelmintics targeting the nAChRs of the somatic muscle selectively activate nAChR subtypes. There are 3 subtypes somatic muscle nAChRs: the N-subtype is preferentially activated by nicotine, oxantel and methyridine; the L-subtype by levamisole and pyrantel; and the B-subtype by bephenium (Martin and Robertson, 2000; Qian *et al*., 2006; Qian *et al*., 2008). The selectivity of derquantel on the B and L-subtype has allowed us to

discriminate between the N-subtype of receptors (Robertson *et al*, 2002). Martin and Robertson (2007) proposed that resistance to anthelmintics which target the nAChRs may be produced by four general mechanisms:

(1) changes in drug translocation (e.g. increased metabolism or excretion of the drug); (2) changes in receptor numbers (e.g. loss of a subpopulation of receptors) ; (3) changes in the drug binding sites on the receptors (e.g. amino acid substitution); or (4) post-receptor modification (e.g. changes in the downstream pathways after receptor activation). All of these mechanisms could play a role to result in anthelmintic resistance emphasizing its potential polygenic nature (Sangster *et al*., 1985; Sangster, 2003).

2.15.4 Using drug combinations to counter anthelmintic resistance

Traditional treatment against parasitic worms include: "*suppressive treatment"* with anthelmintics at 6–8 week intervals, "*strategic treatment"* aimed to remove parasites from hosts when pasture stages are minimal and "*targetedlcurative treatment*" targeted on those animals with a certain level of infection / suffering body condition below a threshold (Sangster, 1996; Sangster and Gill, 1999; Sangster, 2003). Other methods of treatment are to combine anthelmintics targeting different sites on the worm. Studies in Australia show that, resistance to both broad- and narrow-spectrum anthelmintics is widespread (Besier, 2003; Besier, 2007). The basis for combining anthelmintic drugs is to increase the spectrum for therapy, to achieve potentiation/synergism and to delay the onset of resistance. This strategy becomes

effective when the resistance genes are rare. Even though a parasite may carry one resistance gene, it would be less likely to carry the resistance genes to all the anthelmintics present in the combination. Using just one drug class is not ideal as strong selection against this class can occur because the parasite has to evolve in one direction only (Sangster, 2003). Le Jambre *et al* (2010) showed that even though the field-selected strains of *T. circumcincta* had resistance to several classes of anthelmintics when used as single drugs interestingly enough the combinations containing the same anthelmintic classes had improved efficacy. As the resistance developed by parasites towards anthelmintic drugs is evolving, anthelmintic combination therapy ensures removal of nematodes carrying one or two resistant genes. There are some limitations in combination therapy as it is expensive and also prevents sparing usage of a therapeutic agent. However, in countries like Australia and New Zealand, it is becoming clear that the combinations should be used before resistance levels climb too high. In the midst of anthelmintic resistance, our interest needs to be focused on understanding the physiology of the parasites in order to counter the resistance. Further, chemotherapy well supported by good management practices ensures greater wellbeing and productivity of both animals and humans.

CHAPTER 3. LEVAMISOLE AND RYANODINE RECEPTORS: AN ELECTROPHYSIOLOGICAL STUDY IN *ASCARIS SUUM*

[1]Modified from a paper published in *Mol Biochem Parasitol.* (2010)*; 171(1): 8–16.*

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3.1 Abstract

Resistance to antinematodal drugs like levamisole has increased and there is a need to understand what factors affect the responses to these anthelmintics. In our previous study, we examined the role of ryanodine receptors in muscle contraction pathways. Here we have examined interactions of levamisole receptors, ryanodine receptors (RYRs), the excitatory neuropeptide AF2, and coupling to electrophysiological responses. We examined the effects of a brief application of levamisole on *Ascaris suum* body muscle under current-clamp. The levamisole responses were characterized as an initial primary depolarization, followed by a slow secondary depolarizing response. We examined the effects of AF2 (KHEYLRF-amide), 1 μM applied for 2 min. We found that AF2 potentiated the secondary response to levamisole and had no significant effect on the primary depolarization (Trailovic *et al*., 2005) . Further, the reversal potentials observed during the secondary response suggested that more than one ion was involved in producing this potential. AF2 potentiated the secondary response in the presence of 30 μM mecamylamine suggesting the effect was independent of levamisole sensitive acetylcholine receptors. The secondary response, potentiated by AF2,

appeared to be dependent on cytoplasmic events triggered by the primary depolarization. Ion-substitution experiments showed that the AF2 potentiated secondary response was dependent on extracellular calcium and chloride suggesting a role for the calcium-activated anion channel. Caffeine mimicked the AF2 secondary response and 0.1 μM ryanodine inhibited it. 1.0 μM ryanodine increased spiking showing that it affected membrane excitability. A model is proposed showing ryanodine receptors mediating effects of AF2 on levamisole responses.

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3.2 Introduction

Nematode parasites are a severe burden on the productive lives of humans and animals (Jackson, 1993; Albonico *et al*., 2004; Albonico *et al*., 2005)[2-4]. Treatment of these conditions with anthelmintics is limited to three main classes of drugs, and drug resistance has emerged in humans (Albonico *et al*., 2004; Albonico *et al*., 2005) as well as in animals (Jackson, 1993; Kaplan, 2004b) against each of the three classes of anthelmintic. The appearance of multidrug-resistance in nematode

parasites (Jones and George, 2005) is a worrying development. These concerns emphasize the requirement for understanding the mode of action of these compounds and mechanisms of resistance.

Our laboratory has studied levamisole, pyrantel, oxantel and morantel which belong to an important group of nicotinic anthelmintic drugs (Robertson and Martin, 1993; Dale and Martin, 1995; Evans and Martin, 1996; Robertson *et al*., 2000; Martin *et al*., 2004; Levandoski *et al*., 2005) that are used for treatment of *ascariasis*, *Trichuris* sp. and hookworm infections (Hotez *et al*., 2005). The target sites of these drugs include the pharmacologically distinctive ion-channels that are nicotinic acetylcholine receptors (nAChRs) found on the body muscles of nematodes (Robertson et al., 1994; Robertson et al., 1999a; Qian et al., 2006). These drugs produce spastic paralysis of the parasitic nematode and have an advantage of acting rapidly on the parasite, effecting cures within 4 hours.

We have seen in the previous paper that ryanodine receptors (RYRs) of *A. suum* modulate the amplitude of the levamisole contraction by affecting gmax but not EC50. The cellular mechanisms that modulate responses to anthelmintics are important to recognize and describe because they may be modified in anthelmintic resistance. In this paper we extend our previous observations on the role of RYRs in muscle contraction in *A. suum*, using the current-clamp technique. We demonstrate that the RYRs and the entry of calcium play a role in modulating the secondary responses to levamisole and its potentiation by AF2. These observations demonstrate the role of RYRs in modulating the electrophysiological response, and

hence, affect the contractile response to levamisole. It is possible that RYRs are modified with the development of resistance to levamisole in parasitic nematodes.

3.3 Materials and Methods

3.3.1 Muscle flap preparation for electrophysiology

Adult *A. suum* were collected weekly from the Tyson's pork packing plant at Storm Lake, Iowa. Worms were maintained in Locke's solution [Composition (mM): NaCl 155, KCl 5, CaCl2 2, NaHCO3 1.5 and glucose 5] at a temperature of 32°C. The Locke's solution was changed daily and each batch of worms was used within 4 days of collection. We prepared 1 cm muscle tissue flaps by dissecting the anterior part of the worm, 2-3 cm caudal to the head. A body muscle flap preparation was then pinned onto a Sylgard™-lined double jacketed bath chamber maintained at 35°C by inner circulation of warm water (Fisher scientific Isotemp 3016H, PA, USA). The intestines were removed to expose the muscle cells (Trailovic *et al*., 2005). The preparation was continuously perfused, unless otherwise stated, with *Ascaris* Perienteric Fluid-Ringer (APF-Ringer) composition (mM): NaCl 23, Na-acetate 110, KCl 24, CaCl2 6, MgCl2 5, glucose 11, and HEPES 5; NaOH or acetic acid was used to adjust the pH to 7.6. The incoming perfusate was pre-warmed to 35°C with an inline heating system (SH 27B Warner instruments, CT, USA) before application. The rate of perfusion was 3.5 - 4 ml.min-1 through a 20 gauge needle placed directly above the muscle bag recorded from. The calcium substitution experiments were done using cobalt APF-Ringer, composition (mM): NaCl 23, Na-acetate 110,

KCl 24, CoCl2 6, MgCl2 5, glucose 11, and HEPES 5mM; pH 7.6. Chloride was substituted by acetate in chloride free APF-Ringer. The experimental compounds were dissolved in APF-Ringer, cobalt APF-Ringer or chloride free APF-Ringer as described in the results. 1 μM levamisole was applied for a period of 10-20 seconds as described in the results. AF2 (1 μM) was applied for 2 minutes and followed by a minute wash prior to applications of levamisole.

3.3.2 Electrophysiology

A two-microelectrode current-clamp technique was employed to examine the electrophysiological effects in the bag region of *A. suum* muscle (Fig.1A). Borosilicate capillary glass (Harvard Apparatus, Holliston, MA, USA, ID-0.86mm, OD- 1.5mm) microelectrodes were pulled on a P-97 Flaming Brown Micropipette puller (Sutter Instrument Co., CA, USA). We used 3M potassium acetate in the micropipettes which had resistances of 20-30 MΩ. The recordings were obtained by impaling the bag region of *A. suum* muscle with 2 microelectrodes, namely current injecting (I) and voltage recording electrodes (V). All experiments were performed using an Axoclamp 2A amplifier, a 1320A Digidata interface and Clampex 9 software (Molecular Devices, CA, USA). All data were displayed and analyzed on a PC based desktop computer. The current injecting electrode injected hyperpolarizing ramp or step currents, while the voltage recording electrode recorded the change in membrane potential in response to the injected currents.

Our ramp current was a hyperpolarizing step of -40 nA changing linearly with time to a depolarizing current of 10 nA over a duration of 3 s at 0.2 Hz. The step current was -40 nA for 500 ms at 0.3 Hz. Each set of experiments were repeated on preparations from separate batches of worms. Cells with constant membrane potentials more negative than -20 mV for 20 minutes and a stable input conductance of < 3.5 μS were selected for the recordings.

3.3.3 Drugs

AF2 (H - Lys - His - Glu - Tyr - Leu - Arg - Phe - NH2) [Sigma-Genosys, The Woodlands, TX, USA] 1 mM stock solutions were prepared in double distilled water every week and kept in aliquots at -20°C. AF2, stock solutions were thawed just before use. All other chemicals were obtained from Sigma-Aldrich (MO, USA) and Acros-Organics (NJ, USA).

3.3.4 Analysis

The peak change in membrane potential (δV) and conductance (δG) was determined in response to drug applications. The duration of the secondary depolarizing response (secondary response) to levamisole was measured as the time taken (min) for the peak primary depolarization to decline by 80% (T_{80}). We estimated reversal potentials by extrapolating from the membrane I-V plots using linear regression. We used the intracellular ionic concentration values of *Ascaris*

estimated by Brading and Caldwell (1971a) to calculate ion-reversal potentials. For a single ion species, the reversal potential was calculated from the Nernst equation using estimates of the intracellular and extracellular concentrations of that ion. When ion-channels selectively permeable to one species of ion open, then the membrane potential will move towards the reversal potential for that ion. We estimated the reversal potential from linear regression of the relationships between injected current and the membrane potential responses (the I-V plots) before and during conductance changes. The potential at which these plots cross, is the reversal potential of the ion-channel that has opened and is determined by ions that flow through the ion-channel. For example, a channel conducting only chloride ions will have a measured reversal potential that matches the chloride Nernst potential. If the measured reversal potential does not match the Nernst potential of a single ion, it implies that more than one ion is involved in generating the potential.

We defined spikes in *A. suum* as brief repeating action potentials with amplitude greater than 5 mV appearing as a single spike for duration up to 500ms. We measured the spike frequency (min^{-1}), amplitude (mV) and spike gradient ($mV.s^{-1}$). We tested the effects of ryanodine on spike parameters during before levamisole application and or during the rising phase of the levamisole depolarization. The spike gradient was measured on the rising phase and falling phase.

3.3.5 Statistics

All statistical analysis was done using Graph Pad Prism software (version 4.0/5.0, San Diego, CA, USA). Continuous recordings which had initial control application/s followed by test application/s were compared using paired t-tests. Control and test recordings made from separate preparations were compared using unpaired t-tests. Chi-squared tests were used to evaluate effects on muscle spiking.

3.4 Results

3.4.1 The levamisole response has two components: an initial primary depolarization followed by a slower secondary depolarizing response

Fig.1A shows a diagram of the technique used to observe the current-clamp responses of a somatic muscle cell; Fig 1B shows a representative current-clamp recording in APF-Ringer solution. The resting membrane potential of this cell, represented by the dark line in the trace, was -33.2 mV and its input conductance, determined from the amplitude of the downward voltage transients to injected current, was 1.7 μS. A brief application of levamisole (10 s) produced a primary depolarization of 9.3 mV and a change in conductance of 0.06 μS at its peak. After the primary depolarization, a slower secondary depolarizing response (the secondary response) followed which lasted more than 5 min. We quantified the duration of the secondary response as T80 (the time taken for the peak primary depolarization to decay by 80%) which was 4.2 min for the experiment in Fig 1B.

During the secondary response, we observed a secondary peak depolarization of 3.8 mV associated with a conductance increase of 0.07 µS. Similar secondary components were observed in more than 20 muscle cell preparations with 1 µM levamisole. The primary response is presumably initiated by the opening of N-, L- and B- subtypes of nAChRs present on the muscle bag membrane (Martin *et al*., 2005; Qian *et al*., 2006) during the application of levamisole and supported by voltage-activated channels (Verma *et al*., 2007). However, the cause for the slow secondary response when levamisole is being continuously washed off by APF-Ringer is an interesting, but as yet, unexplained effect of levamisole.

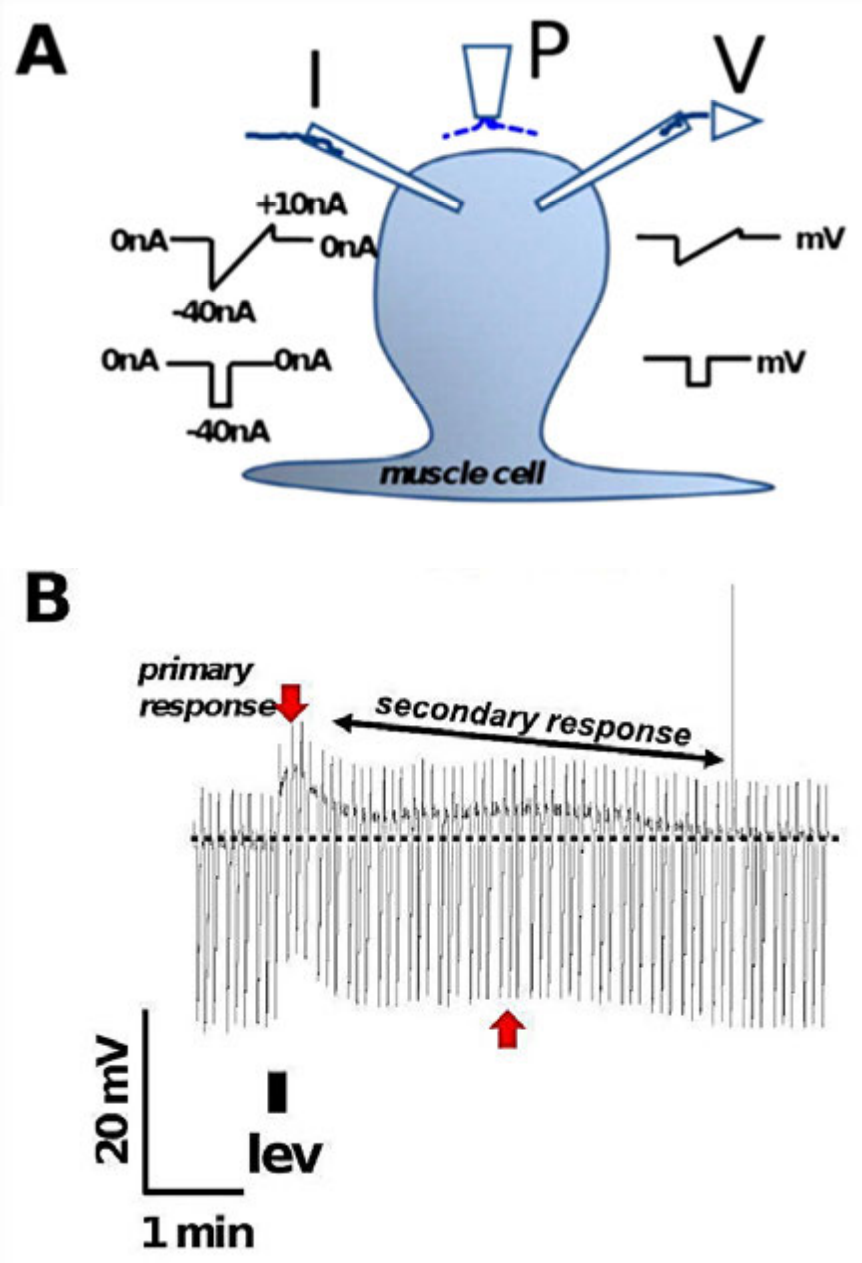

Fig. 1. A. Diagram showing the placement of the two micropipettes used for current-clamp and position of the micro perfusion system for continuous perfusion and application of drugs. P: microperfusion pipette. I: current-injecting electrode, injects

ramp currents or step currents. V: voltage-recording electrode. **B:** Representative trace showing the levamisole response and its 2 components in APF-Ringer namely, a primary depolarization and a secondary depolarizing response (secondary response). The darkest line of the recording is the membrane potential and the downward transients are the responses to injected current. The rapid primary depolarization (downward red arrow) is followed by a slow secondary response (red vertical arrow and oblique black double arrow). 1 μM levamisole was applied for 10 s as indicated by the filled rectangle below the trace. The discontinuous horizontal line indicates the original position of the resting membrane potential. The width of the trace is a reflection of membrane conductance; it gets narrower as membrane-ion channels open. The duration of the secondary response (T80) was measured as the time taken (min) for the peak primary depolarization to decline by 80%.

3.4.2 AF2 potentiates the secondary response to levamisole

Fig 2A shows a representative current-clamp trace where AF2 potentiated the secondary response to levamisole. There are two control levamisole applications (1 μM, 10 s) followed by AF2 treatment (1 μM, 2 min) with a brief wash (1 min) then two test applications of levamisole. The duration of the secondary response, T80, was significantly increased from 4.0 ± 1.4 to 16.1 ± 1.8 min after AF2 treatment ($n = 4$, $p < 0.05$ paired t-test, Fig 2B). Before and after the AF2 application the mean

primary depolarization responses were 6.9 ± 1.3 and 8.6 ± 2.1 mV respectively (n = 4, $p > 0.05$ paired t-test).

Fig 2A shows effects of AF2 treatment where we observed waves of conductance change during the secondary response. We examined the ionic basis of the secondary response by observing the current-voltage relationships and estimating the reversal potential. Fig 2C shows the I-V plots (fitted by linear regressions) at the two positions in Fig 2 B, after AF2 treatment. These positions were before the test application of levamisole as shown by black arrow and during the secondary peak as shown by the blue arrow. The estimated reversal potential was -20mV; this value did not match that of only one ion, and suggested that more than one ion was involved in its generation. The predicted Nernst potentials for K^+, Na^+, Cl^- and Ca^{2+} were E_K = -37.7, E_{Na} = +26.7, E_{Cl} = -42.9 and E_{Ca} = +45 mV (Brading and Caldwell, 1971a; Martin et al., 1992).

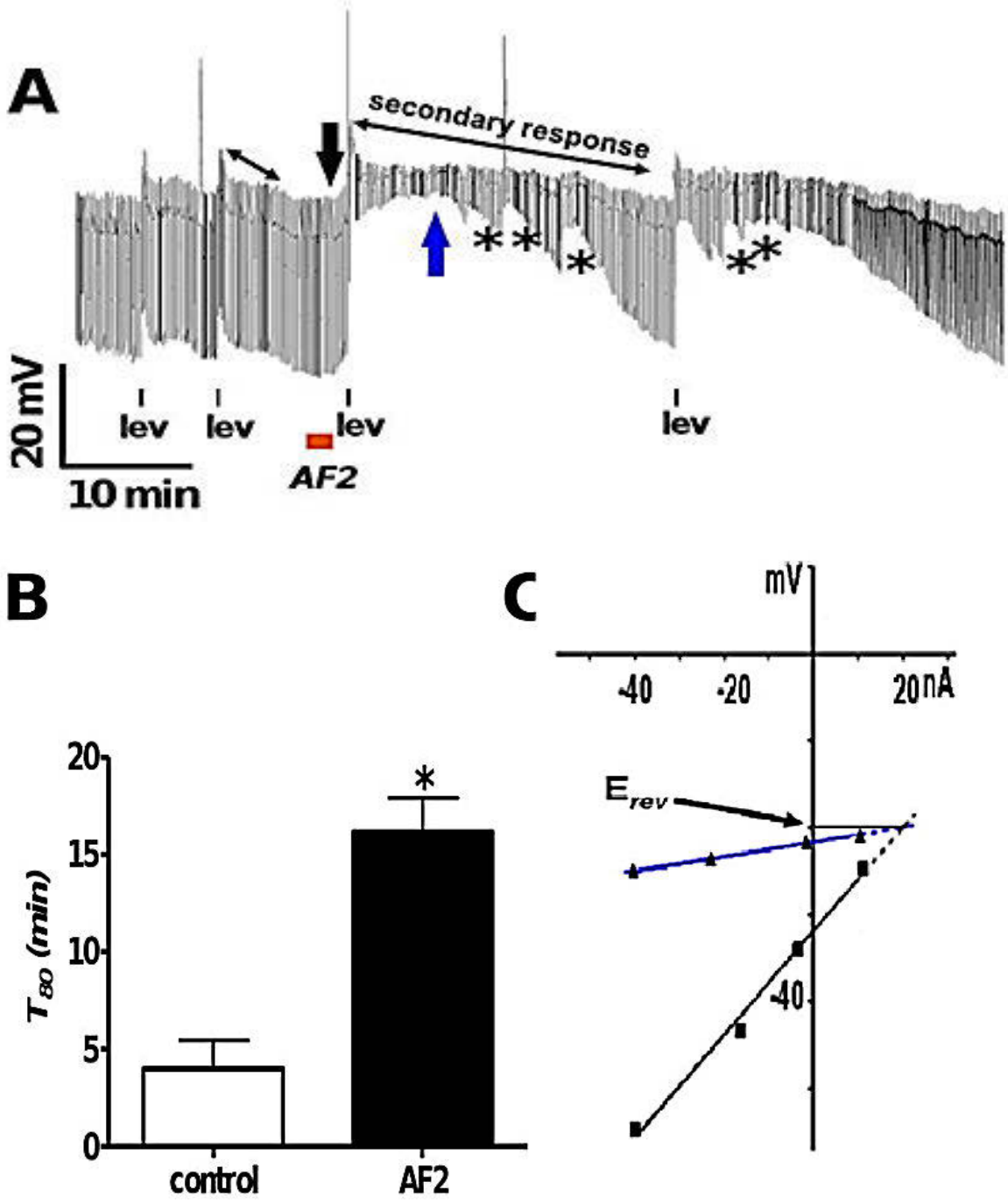

Fig. 2. A. Representative current-clamp trace showing AF2 potentiating the secondary response to levamisole. There are two applications of levamisole (1 μM) before and after AF2 treatment. The control levamisole applications are followed by

a 2 min application of AF2 (1 μM) with a brief wash (1 min), subsequently; there are two test levamisole applications. The double headed black arrows represent the secondary response before and after AF2 treatment. * represents waves of conductance change during the secondary response. **B.** Bar graph comparing T80 (min) before and after AF2 treatment in APF-Ringer. The secondary response to levamisole application was significantly increased after AF2 treatment as indicated by the increase in T80 (Fig 2B, $n = 4$, $p < 0.05$, paired t-test). **C.** The black line and downward arrow show the control current-voltage plot before the test levamisole application and the blue upward arrow and line show the current-voltage plot during the secondary peak from Fig. 2A. Plots were fitted by linear regression. The reversal potential, estimated by extrapolating the two current-voltage plots are shown. The reversal potential, E_{rev}, was -20 mV.

3.4.3 Potentiation continues after the initial effect of levamisole on nAChRs

We explored the properties of the AF2 potentiated secondary response. Levamisole, a membrane permeable drug (Robertson and Martin, 1993), adheres to the membrane and may maintain its effect, even after application has stopped. We used a high concentration of mecamylamine (30 μM) during the secondary response to inhibit any residual levamisole from affecting the secondary depolarization, thus isolating the nAChR independent component (Fig 3A). Although, as expected, mecamylamine reduced the duration of secondary response, we observed that AF2 still increased the duration of the secondary

response (Fig 3 B). The duration of the secondary response, T_{80} increased significantly from 0.4 ± 0.1 min (n = 4) to 0.8 ± 0.1 min (n = 5) after AF2 pretreatment ($p<0.05$, unpaired t-test). As an additional test we also measured the area under the response curve (RAUC) from the start of mecamylamine application to the return of the membrane potential to the resting level in control experiments, and in test experiments after AF2. The RAUC increased significantly from 84.6 ± 13.1 mV.s (n = 4) to 157.2 ± 21.5 mV.s (n = 5) after AF2 pretreatment ($p<0.05$, unpaired t-test). Thus, AF2 maintained its potentiating effect on the secondary response after blockade of the levamisole site of action. This indicated the activation of downstream pathways which are initiated during the primary levamisole response and give rise to the secondary response. We proceeded further to identify the ionic basis of the secondary response and its potentiation by AF2.

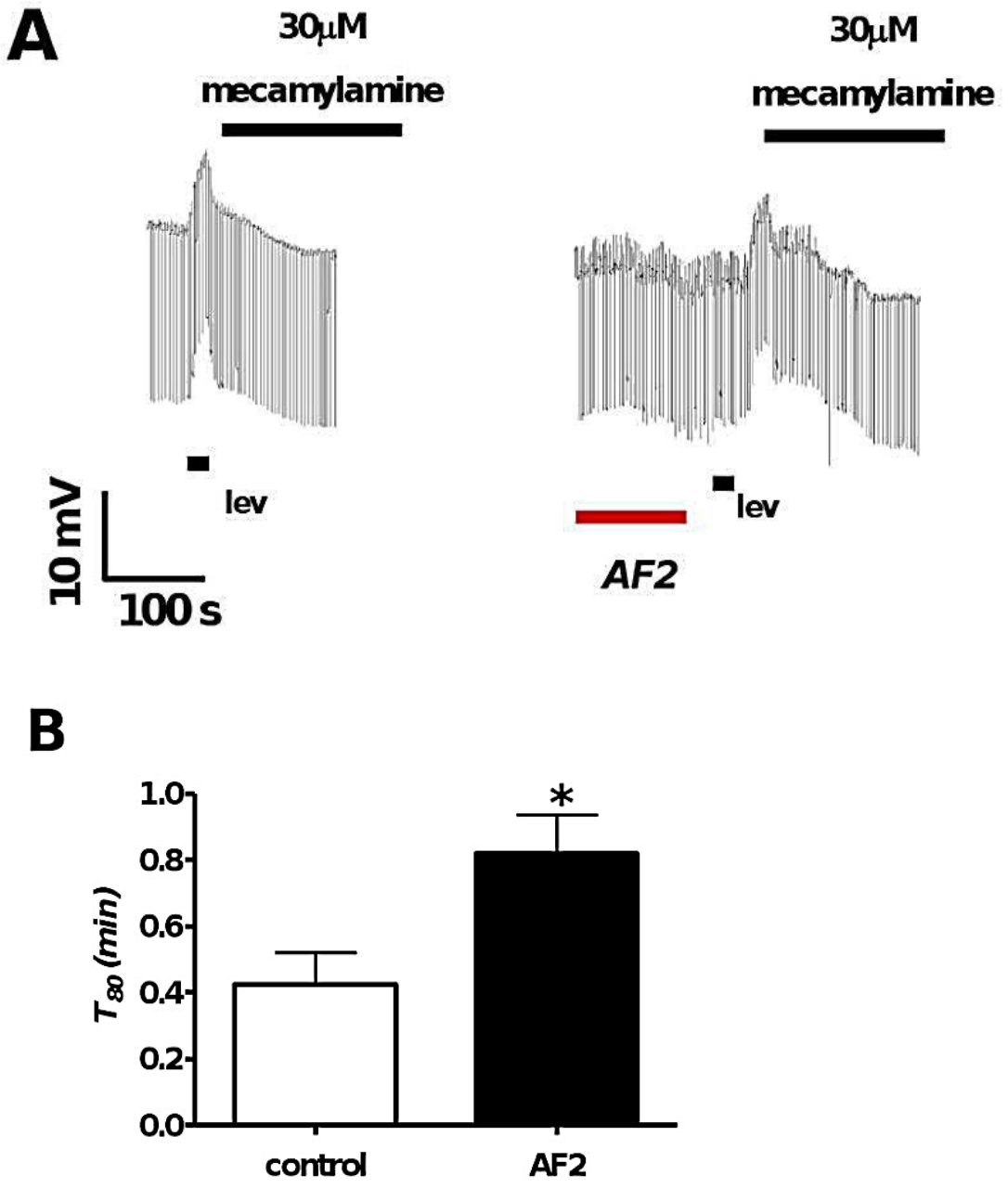

Fig. 3 A. Representative current-clamp traces where mecamylamine (30 μM), a nAChR antagonist, was applied immediately after the end of levamisole application in the control (n = 4) and the test (n = 5) recordings. The first trace shows a control and the second trace shows the test response after AF2. **B.** Bar graph comparing T_{80} controls and AF2 test pre-treatments in the presence of mecamylamine. AF2

potentiated the duration of levamisole secondary response T_{80}, in the presence of mecamylamine ($p < 0.05$, unpaired *t*-test).

3.4.4 Potentiation requires extracellular calcium

We investigated the ionic basis of the AF2 potentiated levamisole secondary response by substituting calcium with cobalt, a calcium channel blocker. We applied cobalt APF-Ringer (Ca^{2+} free) during the AF2 potentiated secondary response, Fig 4A. Calcium substitution caused a significant reduction in the duration of the secondary response, T80, which was reduced from 16.1 ± 1.8 to 2.6 ± 1.2 min ($p < 0.001$, n = 4 unpaired t-test Fig 4B). This indicated that external calcium is required for the AF2 potentiation of the secondary response. Calcium entry can initiate calcium induced calcium release mediated by RYRs and other calcium dependent events. Thorn and Martin (1987) demonstrated the presence of a high-conductance calcium-dependent chloride channel in the body muscle membrane of *A. suum* (Thorn and Martin, 1987). This raised the possibility that incoming calcium during the primary response leads to activation of these chloride channels.

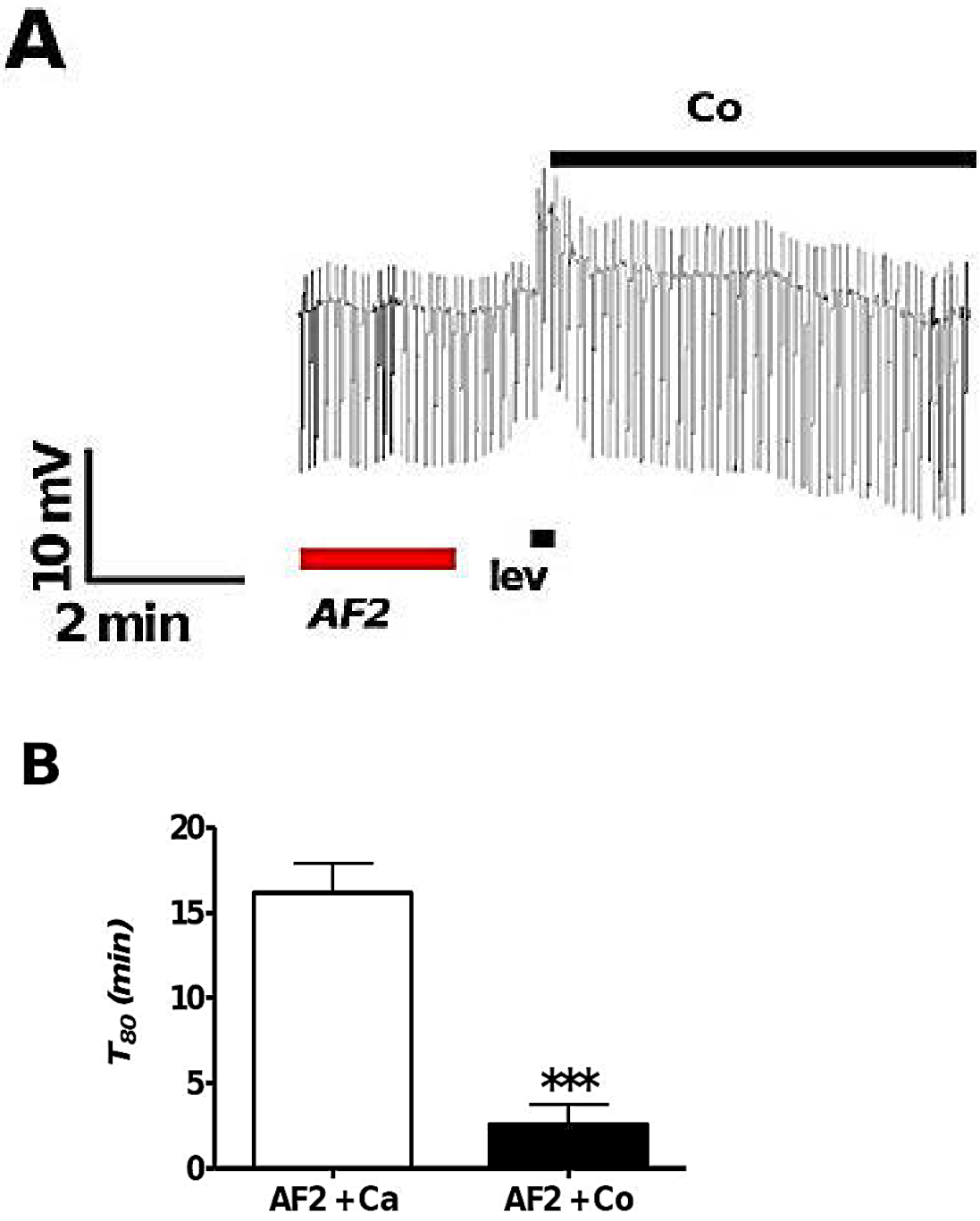

Fig. 4. A. Representative current-clamp trace showing the lack of an AF2 potentiated secondary response following replacement of calcium with cobalt APF-Ringer following the end of levamisole application (1 μM). **B.** Bar graph comparing

mean durations of secondary depolarizations, T80, from different preparations recorded after AF2 treatment in the presence and absence of calcium (calcium replaced using cobalt APF-Ringer). Calcium substitution caused a significant reduction in the duration of the secondary response ($p < 0.001$, $n = 4$, unpaired t-test).

3.4.5 Potentiation is sensitive to extracellular chloride

We removed chloride replacing it with acetate in our chloride free APF-Ringer solution. AF2 did not potentiate the levamisole secondary response in the absence of extracellular chloride as shown in Fig 5A. There was no significant difference in T_{80} ($p > 0.05$, paired t-test) (Fig 5B). The duration of the control secondary response (T_{80}) was 1.6 ± 0.4 min, while after AF2 treatment it was 2.9 ± 1.2 min ($n = 5$). Fig 5 A also shows that there was no increase in membrane conductance during the secondary responses following AF2; we found in all five experiments in chloride free APF-Ringer, that there was no increase in membrane conductance. These experiments showed that in addition to extracellular calcium, the AF2 potentiation required extracellular chloride.

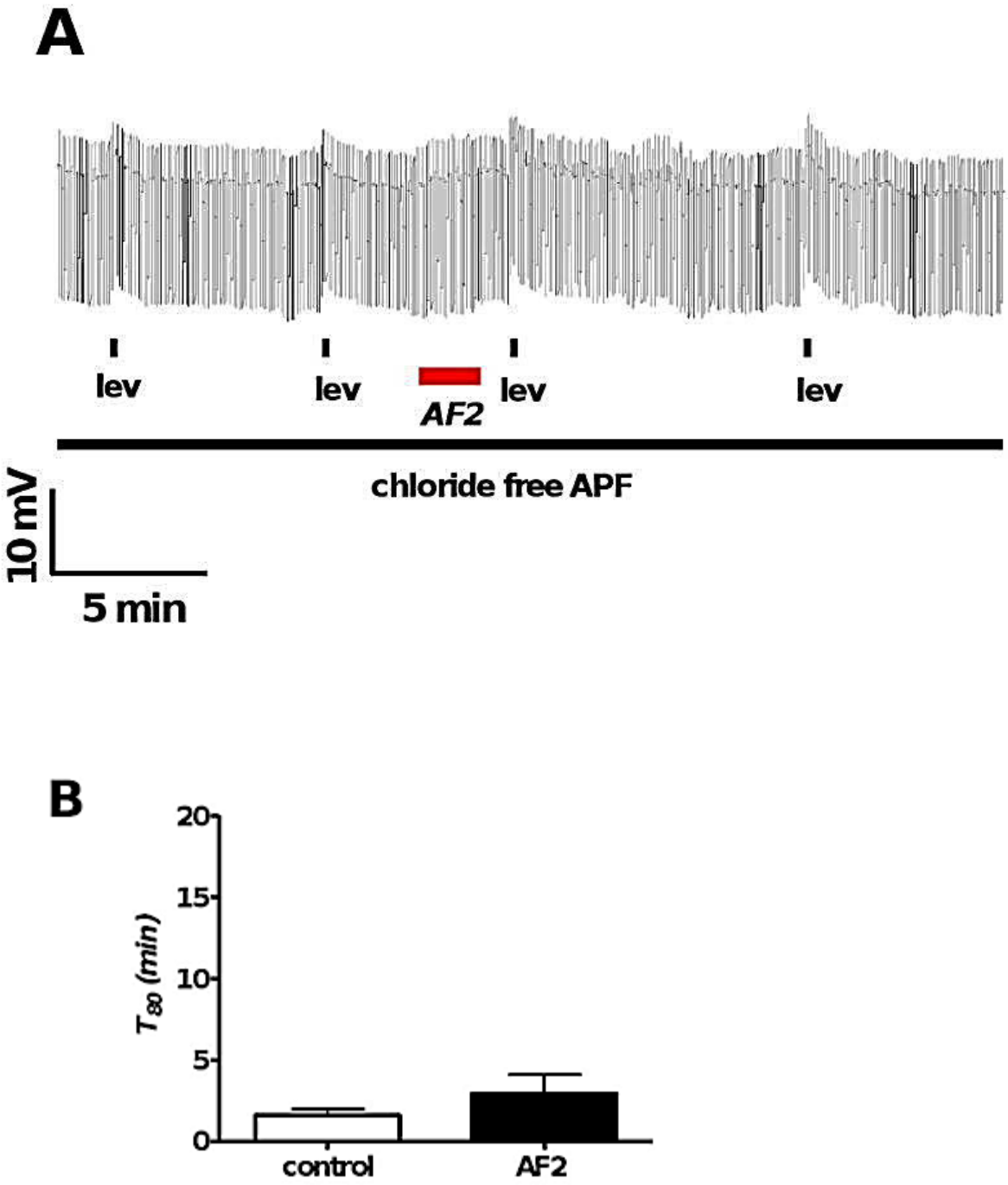

Fig. 5. A: Representative current-clamp trace showing levamisole (1 μM) applications before and after AF2 treatment in the chloride free in APF-Ringer. **B.** Bar graph comparing T_{80} before and after AF2 treatment. In the absence of extracellular chloride, T_{80} in control and test responses were not significantly

different ($p > 0.05$, $n = 5$, paired *t*-test). AF2 did not potentiate the levamisole secondary response in the absence of chloride as indicated by T_{80} measurements.

3.4.6 Caffeine mimics the AF2 potentiation

Recognizing the importance of extracellular calcium for AF2 potentiation of the levamisole secondary response, we explored the possibility of calcium entry causing calcium induced calcium release via ryanodine receptors (RYRs). The waves of changes in input conductances observed during AF2 potentiation, Fig 2A, suggested that release of intracellular calcium mediated by RYRs was involved. We used caffeine, an agonist of RYRs, to release calcium from the sarcoplasmic stores (Aoki and Ito, 1988; Ito *et al*., 1989; Sitsapesan *et al*., 1995). Fig 6A shows a representative trace of the effects of 30 mM caffeine on the membrane potential and conductance of the muscle cell. Caffeine (30 mM, 4 min) produced a slow depolarization of 3.5 mV associated with an increase in conductance of the cell membrane (0.9 μS). We compared the current-voltage relationship before caffeine application and at the peak of the caffeine response to determine the reversal potential. Similar to the AF2 potentiated secondary response Fig 2C, we observed a reversal potential of -12 mV during caffeine application, Fig 6B. The estimated reversal potential did not match that of one individual ion, suggesting that more than one ion was involved. The slow depolarization with a conductance increase had a similar time course to the AF2 potentiated levamisole secondary response. Based on these observations, we hypothesized that AF2 potentiated the secondary

response to levamisole by causing calcium release from the sarcoplasmic stores mediated by RYRs. We tested our hypothesis using ryanodine, a RYRs antagonist.

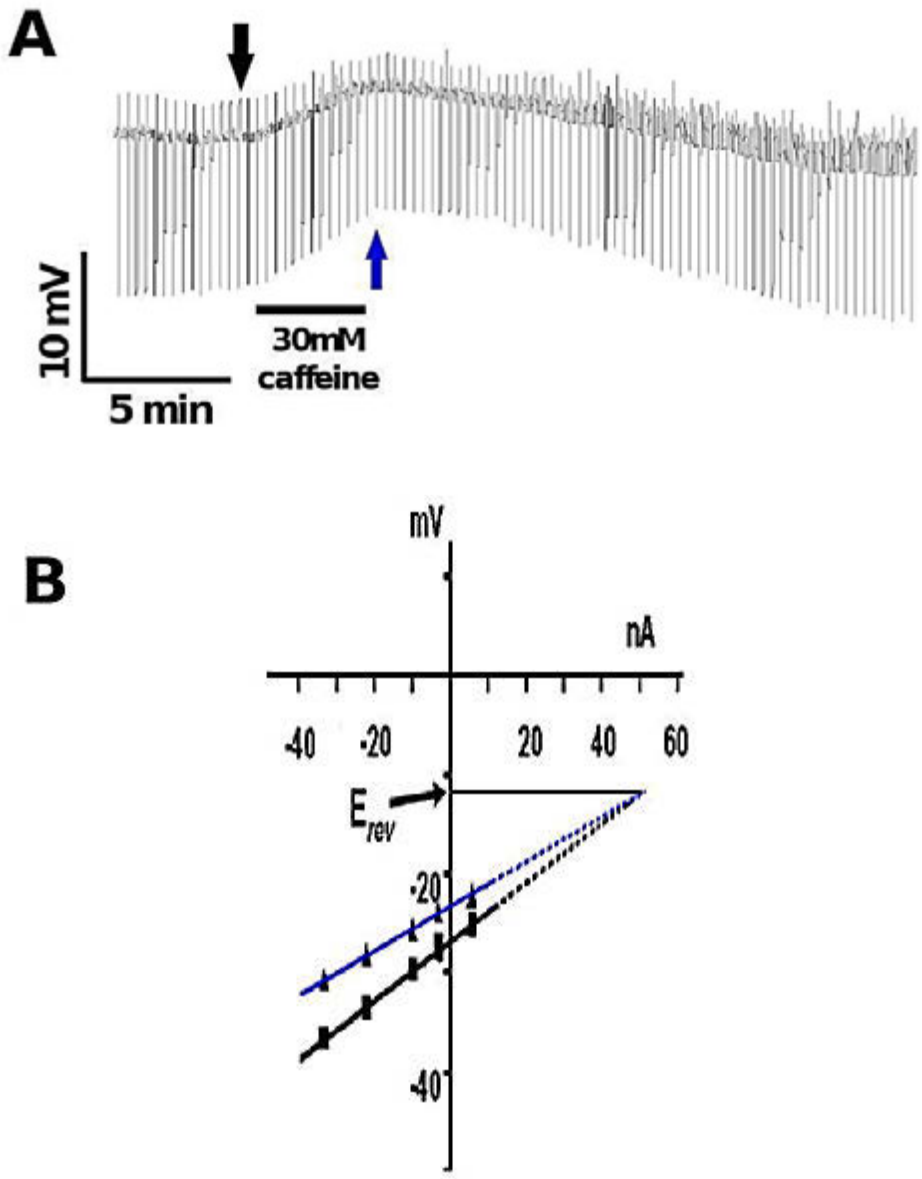

Fig. 6. A. Current-clamp trace showing the effect of caffeine (30 mM) on the membrane potential and conductance. Note that the application of caffeine produced a slow depolarization associated with an increase in conductance. **B.** The membrane potential responses to the injected ramp currents fitted with linear

regression before application of caffeine (black arrow Fig. 6 A) and at the peak depolarization (blue arrow Fig 6A) in the IV plot. The reversal potential, Erev, estimated after extrapolating the membrane potential responses was -12 mV.

3.4.7 Ryanodine antagonizes the AF2 potentiated secondary response

We hypothesized that AF2 potentiation involves calcium induced calcium release mediated by RYRs. Ryanodine is a plant alkaloid that inhibited calcium induced calcium release mediated by RYRs in muscle preparations (Kim *et al*., 1992; Sakube *et al*., 1993; Maryon *et al*., 1996). We tested the sensitivity of AF2 induced potentiation to application of ryanodine (a RYR antagonist). We applied 0.1 μM ryanodine continuously and tested the effects of AF2 on the levamisole responses (Fig 7A). We bathed the preparation for 20 min before control and test levamisole applications (1 μM, 10 s). We observed that AF2 no longer produced significant potentiation of the levamisole secondary response, Fig 7B. The duration of the control levamisole secondary response (T_{80}) was 1.7 ± 0.7 min and after AF2 treatment, it was 3.8 ± 1.6 min ($P>0.05$, paired t-test, n = 4). This demonstrated the involvement of RYRs during the AF2 potentiation of the levamisole secondary response. Interestingly, we also observed that prolonged ryanodine exposure produced large spikes (Fig 7A, 8A and 8B). We explored some of the properties of these spikes induced by ryanodine in our next experiments.

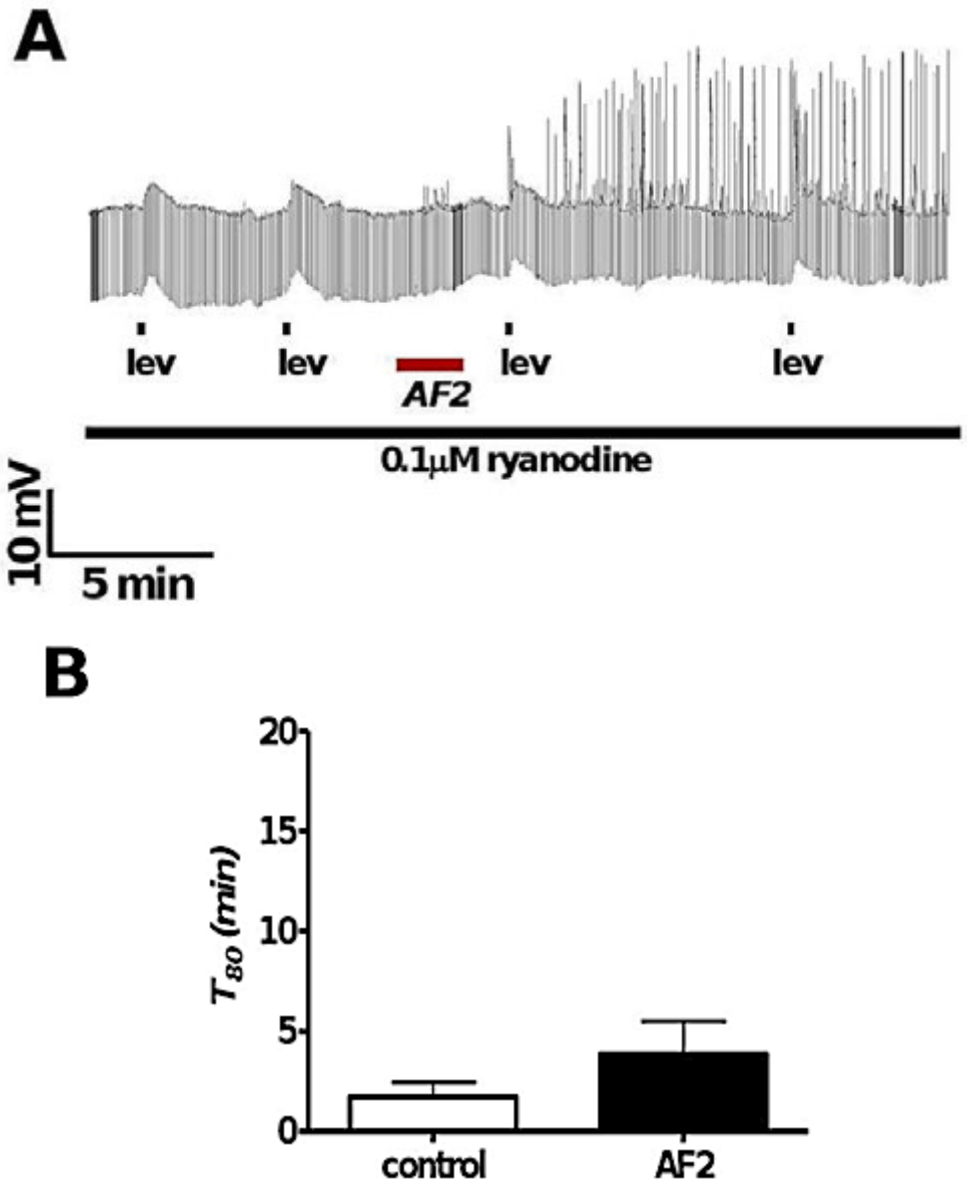

Fig. 7. A. Representative current-clamp trace showing levamisole (1 μM) applications before and after AF2 treatment in the presence of 0.1 μM ryanodine. **B.** Bar graph depicting T_{80} (min) in control and test applications. In the presence of 0.1 μM ryanodine, T_{80} in control and test responses were not significantly different ($p > 0.05$, $n = 4$, paired *t*-test). AF2 did not significantly potentiate the levamisole

secondary response in the presence of ryanodine, as indicated by T_{80} measurements.

3.4.8 Ryanodine increases the frequency of spikes

Under normal recording conditions (APF-Ringer) spontaneous membrane spikes were rare: 1 out of 10 experiments in this data set (example shown in Fig 8A). Spikes occurred more frequently during the rising phase of the primary levamisole depolarization; 3 out of 10 experiments in this data set. We found that ryanodine (0.1 or 1 μM) treatment increased the likelihood of observing spikes both at rest; 6 out of 10 experiments, and during levamisole application; 10 out of 10 experiments (Fig 8A & B). In both cases the difference was statistically significant ($p < 0.05$, Chi-square test, two sided) suggesting that ryanodine increases spiking. Next, we calculated spike frequency (min^{-1}) for recordings before and after ryanodine treatment on the rising phase of the levamisole depolarization. Treatment with 0.1 μM ryanodine significantly increased spiking from 0.8 ± 0.8 min^{-1} to 6 ± 2.1 min^{-1} ($n = 4$, $p < 0.05$, paired t-test). Treatment with 1 μM ryanodine significantly increased spiking from 7.2 ± 4.6 min^{-1} to 28.9 ± 4.8 min^{-1} ($n = 6$, $p < 0.05$, paired t-test). Spiking frequencies at 1 μM were also significantly greater than at 0.1 μM ($p<0.01$, unpaired t-test) demonstrating that the effect of ryanodine on spiking was concentration dependent (Fig 8C). We further characterized spike properties by measuring amplitude, rate of rise and rate of decay. The lack of spikes prior to ryanodine application rendered statistical analysis problematic. However, for the experiment shown in Fig 8A, 1 μM ryanodine caused a significant increase in spike

amplitude: at rest the amplitude was 6.5 ± 0.3 mV (n = 9) before and 40.4 ± 3.4 mV (n = 11) after 1 μM ryanodine ($p < 0.0001$, unpaired t-test). Similarly, 1 μM ryanodine increased both the rate of rise and rate of decay of the spikes. Prior to application of ryanodine, the rise rate was 0.7 ± 0.1 $mV.ms^{-1}$ and the decay rate was 0.2 ± 0.02 mV.ms-1 (n = 7); in the presence of 1 μM ryanodine, the rate of rise increased to 9.9 ± 0.7 mV.ms-1, and the rate of decay rate to 11.1 ± 0.06 mV.ms-1 (n = 11). The rates of rise before and after ryanodine application were significantly different ($p < 0.0001$, unpaired t-test), as were the rates of decay ($p < 0.0001$, unpaired t-test).

Spike action potentials appear to be a combination of voltage activated calcium currents, voltage-activated potassium currents (Martin *et al*., 1992) and calcium-activated chloride currents (Thorn and Martin, 1987) as modeled by Turner (2001). The spike gradient and spike amplitude observations suggest that ryanodine causes an increase in the inward voltage-gated calcium current and the outward voltage-gated potassium current.

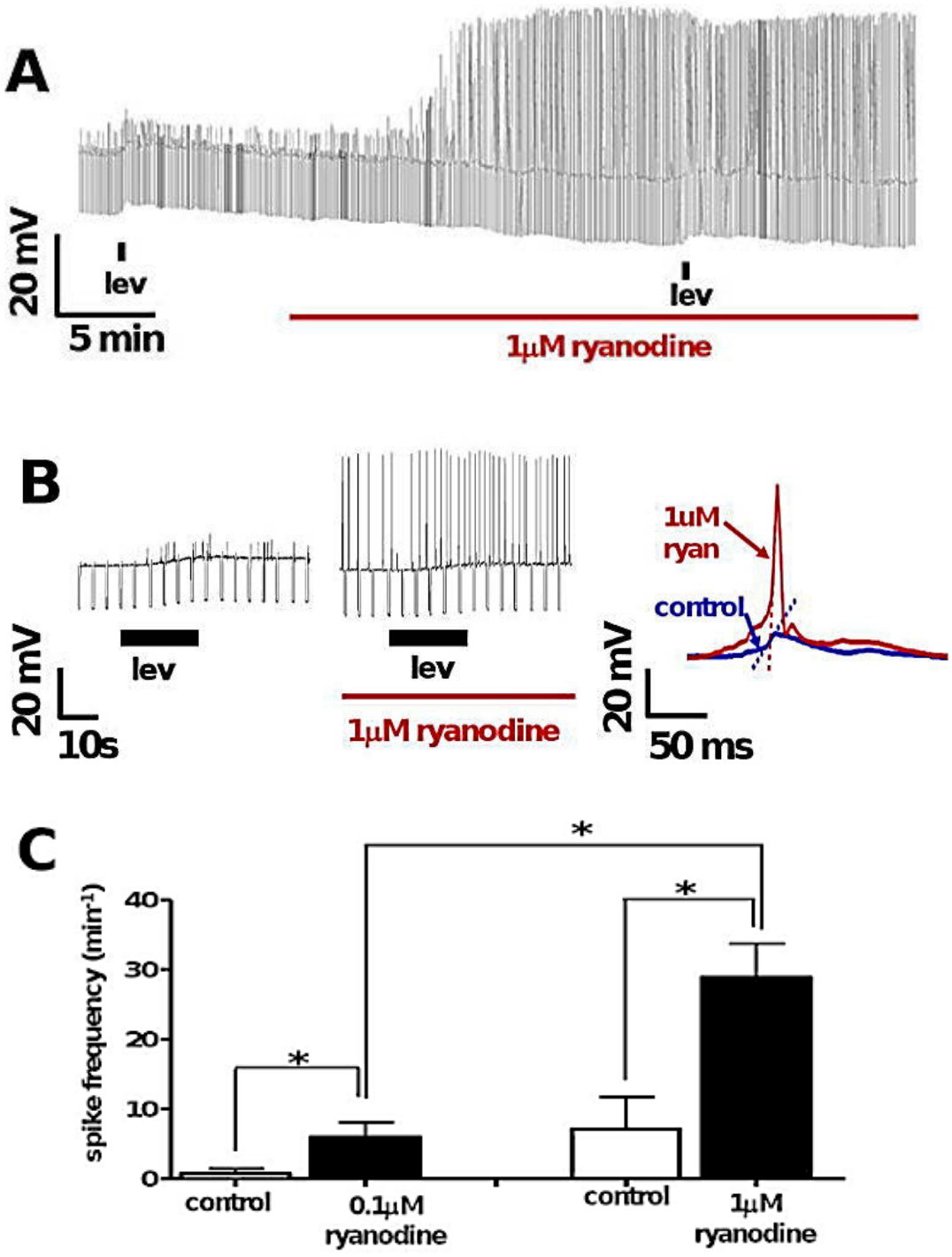

Fig. 8. A. Representative current-clamp trace showing levamisole (1 μM) application before and during ryanodine treatment (1 μM). **B.** Representative trace

of spikes seen at higher time resolution during levamisole application before and in the presence of 1 μM ryanodine. The recordings show an increase the spike amplitudes (left) and an increase in the gradient of the rising phase of the spikes (blue increased in red: right) in the presence of ryanodine. **C.** Bar graphs showing the mean ± S.E. spike frequency during the depolarizing phase of the response to levamisole before and in the presence of 0.1 μM ryanodine and in separate experiments 1 μM ryanodine. Treatment with 0.1 μM ryanodine significantly increased spiking from 0.8 ± 0.8 min-1 to 6 ± 2.1 min-1 ($n = 4$, $p < 0.05$, paired t-test). Treatment with 1 μM ryanodine significantly increased spiking from 7.2 ± 4.6 min-1 to 28.9 ± 4.8 min-1 ($n = 6$, $p < 0.05$, paired t-test). Spiking frequencies at 1 μM were also significantly greater than at 0.1 μM ($p<0.01$, unpaired t-test) demonstrating that the effect of ryanodine on spiking was concentration dependent.

3.5 Discussion

3.5.1 A model for the electrophysiological effects of AF2 and ryanodine on levamisole responses

Trailovic *et al* (Trailovic *et al*., 2005) showed that brief application of the neuropeptide, AF2, produces long-lasting potentiation of membrane potential and contraction responses to acetylcholine along with increased action potential generation. The effect on the levamisole response was to extend the duration of the depolarization. To explain these observations Trailovic *et al* (2005), proposed that AF2 increases cytosolic calcium by opening of voltage-gated calcium channels and stimulating release of calcium from sarcoplasmic stores. Subsequently Verma *et al*

(Verma *et al*., 2007) tested the effects of AF2 using voltage-clamp in *A. suum* muscle and found that AF2 potentiates the calcium currents. In this paper, we have extended these observations and described the more complex response to levamisole. The levamisole response consists of a primary depolarization followed by a slower secondary response and we have observed that AF2 potentiates the secondary response. We found that the AF2 potentiation was inhibited by calcium substitution with cobalt, inhibited by ryanodine, and required the presence of extracellular chloride. Application of caffeine produced a slow depolarization and an increase in membrane conductance like the secondary response. The reversal potentials of the levamisole secondary response when potentiated by AF2 were similar to the potentials produced by caffeine. We also observed that ryanodine alone produced an increase in the frequency of spiking. We tested for the persistence of levamisole following application as an explanation for the secondary response and found that application of a high concentration of mecamylamine did not abolish the secondary response. Importantly, the mecamylamine insensitive component of the secondary response to levamisole was potentiated by AF2. Fig 9 shows the previously proposed (Trailovic *et al*., 2005) sites of action of AF2 and in addition a currently proposed model that explains observations on the actions of ryanodine and AF2. The model proposes that AF2 acts via one or more G-protein receptors (Kubiak *et al*., 2003) to shift the opening of voltage-sensitive calcium channels to more hyperpolarized potentials so that they open more readily (Verma *et al*., 2007); the model also proposes that there is sensitization of the RYRs by AF2. The model proposes that the primary depolarization is initiated by opening of

the nAChRs and the flow of inward current; this depolarization will then secondarily activate voltage-activated channels. Calcium will enter the cytoplasm via the nAChRs and the voltage-activated channels producing an increase in cytoplasmic calcium that activates RYRs and further increase in cytoplasmic calcium. Activation of the calcium-activated anion channels (Thorn and Martin, 1987) follows the high rise in cytosolic calcium.

The AF2 potentiated secondary response to levamisole, in APF-Ringer, was sometimes associated with a large conductance change which had an oscillating pattern suggestive of intracellular calcium waves. The reversal potential, ~ -20 mV, was more depolarized than the predicted chloride reversal potential of -43 mV. We found that replacement of the chloride in the APF-Ringer with acetate inhibited the increase in conductance of the secondary depolarization, indicating the supporting role of the Ca-activated anion channel (Thorn and Martin, 1987). The actual reversal potential of the calcium-activated anion channel is likely to be more positive than the chloride reversal potential because of the presence of significant amounts of intracellular carboxylic acids from anaerobic respiration (Komuniecki *et al*., 1987) that permeates this anion channel (Valkanov and Martin, 1995). A rise in cytosolic calcium will, in addition, initiate homeostatic mechanisms that then lead to reduction of the cytosolic calcium, returning it to control levels; these mechanisms are expected to include the high capacity, low affinity Na-Ca exchanger and the low capacity high affinity calcium-ATPase systems that remove cytosolic calcium (DiPolo and Beauge, 2007).

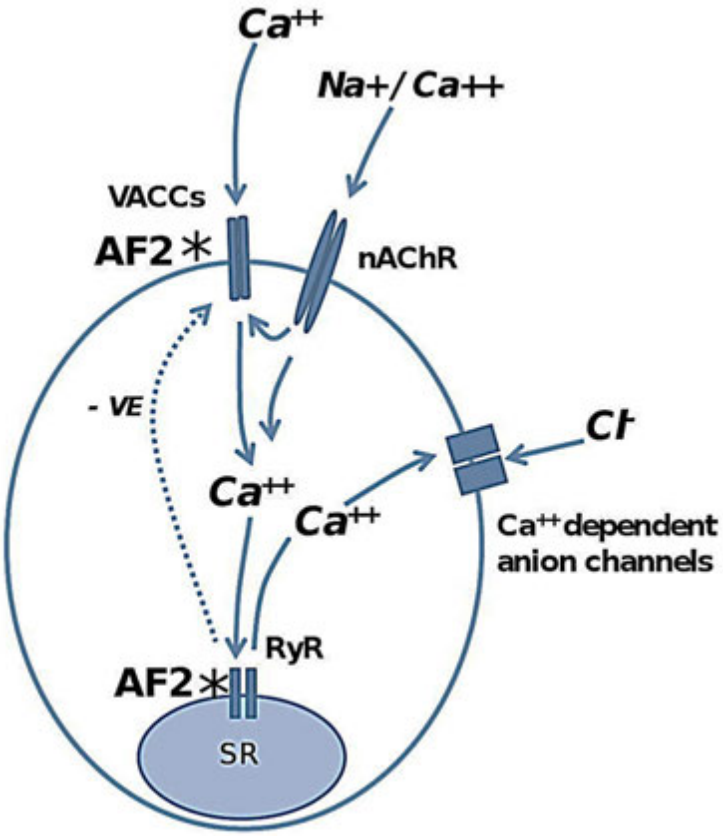

Fig 9. Proposed model and sites of action whereby AF2 modulates the responses to levamisole [1]. The primary depolarization follows levamisole binding to nAChRs and their opening to allow Ca++ and Na+ to enter the cell. The levamisole secondary response is initiated by the primary depolarization and involves activation of voltage-gated calcium channels (VACCs), ryanodine receptors (RyRs) and calcium-activated anion channels. VACCs are activated by the primary depolarization and allow more calcium to enter the cell. Increased intracellular calcium triggers calcium induced calcium release (CICR) from the sarcoplasmic reticulum (SR) and are gated by the ryanodine channels (RyRs). The calcium-activated anion channels are also activated during the cytoplasmic rise in calcium concentration. The calcium induced calcium release can inhibit the voltage-

activated calcium channels (VACCs) as a negative feedback (- ve). *: AF2 potentiates the levamisole secondary responses by increasing voltage-activated calcium entry through VACCs [19] and; * by sensitizing the RyRs to release more calcium from the sarcoplasmic reticulum in response to the calcium entry through the nAChRs and VACCs.

3.5.2 Ryanodine receptors and effects of AF2

In the following discussion, we suggest that AF2 increases cAMP in muscle cytoplasm (Reinitz *et al*., 2000) and that the raised camp leads to sensitization of RYRs and to the increases in cytoplasmic calcium. Nematode RYRs, like those of other eukaryotes are homo-tetramer calcium release channels found in the membrane of sarcoplasmic reticulum and that bind the alkaloid ryanodine (Bennett *et al*., 1996). In *C. elegans*, a single gene, unc-68, encodes for RYRs. *C. elegans* RYRs are located near the muscle surface membrane in vesicles that resemble junctional sarcoplasmic reticulum of the vertebrate striated muscle (Maryon *et al*., 1996) and the RYRs of *C. elegans* show 42% homology to mammalian RYRs (Sakube *et al*., 1993). RYRs channels in *C. elegans* have two conductance states, a 215 pS state and a 78 pS state; ryanodine at a concentration of 4 μM locks the RYRs channel in the 78 pS (sub conductance state) preventing transition between the states (Kim *et al*., 1992). The locking of the channel in the sub conductance state can lead to emptying of the calcium stores in the SR so that the effect of application of ryanodine on *C. elegans* is to produce an incomplete hyper

contraction and overall paralysis (Kim *et al*., 1992). In our muscle strips of *A. suum* however, ryanodine did not produce hyper contraction but reduced the maximum force of levamisole-induced contractions. The lack of contraction may be explained if there is slow emptying of the SR in *A. suum* which could be accommodated by homeostatic mechanisms like the sodium-calcium exchanger (Robertson *et al*., 2010).

RYRs release calcium from sarcoplasmic reticulum in vertebrate smooth muscle. Triggers for this release include an increase in cytosolic calcium and other ligands like caffeine (Fabiato and Fabiato, 1977; Kimball *et al*., 1996). In regular vertebrate skeletal muscle, RYRs release calcium in response to each action potential as a result of being coupled directly to the T-tubule system (Rios *et al*., 1991; Yano *et al*., 1995). In *Ascaris suum* however, we know from the work of Weisblat *et al*., (1976) that contraction of body muscle is not coupled to each spike, rather contraction is coupled to slower depolarizations referred to as modulation waves. In *C. elegans* RYRs play an important, but non-essential role in excitation-contraction coupling: calcium entry through plasma membrane voltage-activated channels can initiate contraction in the absence of RYRs (Kimball *et al*., 1996; Maryon *et al*., 1996). Thus RYRs enhance contraction in nematode body muscle by amplifying the calcium signal initiated by opening of nAChRs and voltage-activated calcium channels in the plasma membrane (Maryon *et al*., 1996). AF2 has been shown to produce a long-lasting increase in muscle cytoplasmic cAMP in *A. suum* (Reinitz *et al*., 2000). Activation of cAMP-dependent protein kinase-A can phosphorylate RYRs and

reduce the effects of the RYRs inhibitor proteins (FKBPs) (Petrovic *et al*., 2008). By this mechanism, AF2 could increase the probability that RYRs open in response to cytoplasmic calcium (Petrovic *et al*., 2008) and in turn increase contraction in response to a nicotinic anthelmintic like levamisole.

3.5.3 Ryanodine effects on spiking

We have observed that ryanodine increased the frequency of spiking and in two preparations, where spikes were present before the application of ryanodine; ryanodine increased the gradient of the rising phase of the spikes. Spikes in *A. suum* are produced by inward calcium currents and not sodium currents (Weisblat, 1976; Verma *et al*., 2007). Ryanodine increased the gradient of the rising phase of the spike (dV/dt) which is taken to be proportional to the inward calcium current (Martin *et al*., 1992; Turner, 2001) indicating that ryanodine increased the inward calcium current. It is of interest to consider what the mechanism for the spiking and increased calcium current could be. Voltage-activated calcium channels are subject to calcium-induced inactivation mediated by calmodulin (Catterall, 2000; Dunlap, 2007). Since ryanodine inhibits the calcium-induced calcium release (we believe by emptying the calcium SR store), ryanodine could lead to the removal of inhibition of calcium channels by calmodulin. Other less likely mechanisms include ryanodine modulation of presynaptic transmitter release or inhibition of potassium channels in muscle. Modulation of presynaptic transmitter release does not seem likely because

ryanodine (100 μM) has been shown to reduce excitatory neurotransmitter release in *C. elegans* (Liu *et al*., 2005); this would be seen as an inhibitory effect not an excitatory effect. With regard to an effect on potassium channels, we found that ryanodine did not affect the resting membrane potential of *A. suum* muscle nor was it observed to have an effect on the duration of the *A. suum* spikes. A closing of potassium channels would be expected to depolarize the cell membrane, to decrease the rate of spike decay and increase spike duration; it did not. To our knowledge, there are no reports of direct effects of ryanodine on *C. elegans* potassium channels. However, we cannot rule out an indirect effect via changes in cytosolic calcium having effects on calcium-activated potassium channels (Salkoff *et al*., 2005). Taken together, these observations suggest that ryanodine affects spiking via an action on calcium channels. Despite this increase in spiking, we have observed that ryanodine does not increase the force of muscle contraction [Robertson *et al*, this issue] demonstrating the physiological separation of spikes and contraction in *A. suum* muscle.

3.5.4 Nematode ryanodine receptors and levamisole resistance

We have seen that the force of levamisole induced contractions in *A. suum* is sensitive to ryanodine as is the secondary response of levamisole following AF2 treatment. The genetic basis of resistance to levamisole has been studied in *C. elegans* where it has been found that null-mutants of components of the levamisole-signaling excitation-contraction cascade cause resistance (Dick *et al*., 2008).

Components of the levamisole-signaling pathway include the subunits of the levamisole receptor: UNC-38, UNC-63, UNC-29, LEV-1 and LEV-8. They also include the RYRs encoded by the unc-68 gene. unc-68 *C. elegans* null-mutants show reduced sensitivity to levamisole (Robertson and Martin, 2007). It is likely that null-mutants of unc-68 of parasitic nematodes would also show reduced response to levamisole because the reduction in gmax (Robertson *et al*., 2010). Mutations of RYRs in parasitic nematodes are predicted to reduce the response to treatment with nicotinic anthelmintics like levamisole and pyrantel and to be associated with resistance

3.6 Acknowledgements

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3.6 Footnotes

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CHAPTER 4. DERQUANTEL AND ABAMECTIN: EFFECTS AND INTERACTIONS ON ISOLATED TISSUES OF *ASCARIS SUUM*

A paper to be submitted to *European Journal of Pharmacology, 2012*

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4.1 Abstract

Startect[R] is a novel anthelmintic combination of derquantel and abamectin, Fig 1, which selectively paralyses parasitic nematodes. It is hypothesized that derquantel and abamectin interact pharmacologically. We investigated the effects of derquantel, abamectin and their combination on somatic muscle nicotinic acetylcholine receptors (nAChRs) and pharyngeal muscle glutamate gated chloride receptor channels (GluCls) of *Ascaris suum.* We used muscle-strips to test the effects of 0.3 µM abamectin, 1 µM derquantel, and 0.3 µM abamectin + 1 µM derquantel together on the contraction responses to different concentrations of acetylcholine. We found that abamectin reduced the response to acetylcholine, as did the derquantel. When abamectin was added along with derquantel, the inhibition of the acetylcholine response was increased. A two-micropipette current-clamp technique was then used to study the electrophysiological effects of the anthelmintics on: 1) acetylcholine responses in somatic muscle flaps and; 2) L-glutamate responses in pharyngeal preparations. On somatic muscle, application of

derquantel (0.1 - 30 μM) produced a potent (IC_{50} = 200 nM) reversible antagonism of acetylcholine depolarizations. Application of abamectin (0.3 μM) produced inhibition of acetylcholine depolarizations that were slow in onset. To examine the electrophysiological interactions of abamectin and derquantel, we compared effects of these drugs on muscle preparations pretreated for 30 minutes with these drugs. The effect of the combination was greater than either drug alone and suggested synergism. On the pharynx, application of derquantel produced no significant effect by itself or on responses to L-glutamate. Abamectin increased the input conductance of the pharynx in a concentration-dependent manner (EC_{50}: 400 nM). The effects of abamectin and L-glutamate on conductance were additive when applied together. Our study shows that derquantel and abamectin do not interact at L-glutamate receptors on the pharynx but that they do interact on acetylcholine receptors of the somatic muscle.

Key words: abamectin, derquantel, combination, interaction, nAChRs and GluCls.

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4.2. Introduction

Nematode parasites cause severe problems for humans and animals. Globally, more than a billion people are infected with ascariasis, hook worms, and whipworms which are soil transmitted gastro-intestinal (GI) nematodes. These nematode infections are endemic to a majority of tropical countries (Savioli and Albonico, 2004; Hotez et al., 2007a). Similar GI nematodes are also present in most domestic animals. The high global prevalence of nematode infections in both humans and livestock results in debility, reduced productivity, severe economic losses and contributes to poverty (de Silva et al., 2003; Kaplan, 2004b).

In the absence of effective vaccines and sanitation, anthelmintics are used for treatment and prophylaxis. Unfortunately, the regular use of anthelmintic drugs has resulted in the appearance of anthelmintic resistance in domestic animals and recently in humans. In Australia (Edwards et al., 1986a, b; Jackson and Coop, 2000), Paraguay (Maciel *et al*., 1996; Waller *et al*., 1996) and S. Africa (van Wyk *et al*., 1997), anthelmintic resistance threatens the economics of the entire sheep industry. In humans, GI nematodes have shown resistance to anthelmintics following mass drug administration (MDA) programs in endemic regions (Ismail *et al*., 1996; De Clercq *et al*., 1997; Reynoldson *et al*., 1997; Ismail *et al*., 1999). The limited number of anthelmintics available for therapy, coupled with the onset of resistance in parasites poses a serious threat to human health and livestock (Kaplan, 2004b; Jones and George, 2005).

A majority of anthelmintics exert their effect selectively on membrane ion-channels of nematode parasites, disrupting normal opening. Nicotinic acetylcholine receptors

(nAChRs) are present on nematode somatic muscles and nerves. As a result, anthelmintics that act as selective agonists at muscle nAChRs produce spastic paralysis of the worms, while selective antagonists produce flaccid paralysis. The nAChR agonists include: the imidazothiazoles (levamisole); the tetrahydropyrimidines (pyrantel, morantel & oxantel) (Martin and Robertson, 2007) and; the amino-acetonitrile derivatives (monepantel) (Kaminsky *et al*., 2008). The nAChR antagonists include the spiroindoles (derquantel, Fig. 1) (Robertson *et al*., 2002). Inhibitory glutamate gated chloride channels (GluCls) are present on nematode pharyngeal muscle (Martin, 1996) and the avermectins (ivermectin, doramectin, abamectin) and milbemycins (moxidectin, milbemycin) increase opening of the GluCls (Martin, 1996; Wolstenholme and Rogers, 2005b), inhibiting pharyngeal pumping and feeding (Sheriff *et al*., 2002).

If control of parasitic nematodes relies on only a single class of anthelmintic drug, the selection pressure for resistance is strong (Sangster, 2003). However, if a combination of anthelmintic drugs is used from different drug classes, the development of resistance is predicted to be slower because simultaneous development of resistance for two classes of anthelmintic is required (Smith, 1990; Barnes et al., 1995; Albonico et al., 2003; Coles, 2005; Stepek et al., 2006). The combination of two or more anthelmintics also has the potential of producing additive or synergistic effects, increasing the efficacy of the combination.

In this study, we have used isolated tissues from *Ascaris suum* to study effects and examine the interactions of derquantel and abamectin. We used somatic muscle flaps for contraction assays. We used somatic muscle flaps and pharyngeal

muscles for electrophysiological assays. We studied the effects of derquantel alone and abamectin alone and both in combination; we found that the effects of derquantel and abamectin were additive and suggested synergism on the nAChRs of the muscle but derquantel did not affect the pharynx and effects were not additive on the GluCls of the pharynx.

4.3. Materials and methods

Adult *A .suum* were collected weekly from the JBS packing plant at Marshalltown, Iowa. Worms were maintained in Locke's solution [composition (mM): NaCl 155, KCl 5, $CaCl_2$ 2, $NaHCO_3$ 1.5 and glucose 5] at a temperature of 32 °C. The Locke's solution was changed twice daily and each batch of worms was used within 4 days of collection.

4.3.1 Muscle-flap for contraction

We prepared 1 cm muscle body flaps by dissecting the anterior part of the worm, 2–3 cm caudal to the head. Each flap was monitored isometrically by attaching a force transducer in an experimental bath maintained at 37^{O}C containing 10 ml *Ascaris* Perienteric Fluid Ringer/APF Ringer (mM): NaCl, 23; Na-acetate, 110; KCl, 24; $CaCl_2$, 6; $MgCl_2$ 5; glucose, 11; HEPES, 5; pH 7.6 with NaOH and 0.1% DMSO and bubbled with nitrogen. After dissection, the preparations were allowed to equilibrate for 15 min under an initial tension of 0.5 g. Different concentrations of acetylcholine were then added to the preparation and the maximum contraction observed before

washing and subsequent application of the next concentration of acetylcholine. The responses for each concentration were expressed as a % of the maximum tension produced by each individual flap preparation. The effects of abamectin, and derquantel on control acetylcholine dose-response plots were determined. Contraction was monitored on a PC using a MacLab interface. The system allows for recording, displaying and analysis of experimental data. Sigmoid dose-response curves for each individual flap preparation at each concentration of antagonist were described by the Hill equation.

4.3.1a Muscle flap for current-clamp recording

We also prepared the 1 cm muscle body flaps for electrophysiology by dissecting the anterior part of the worm, 2–3 cm caudal to the head which were then pinned onto a Sylgard™ contained in lined double jacketed bath chamber maintained at 35 °C by an inner circulation of warm water (Fisher scientific Isotemp 3016H, PA, USA). The preparation was continuously perfused, with APF-Ringer, composition (mM): NaCl 23, Na-acetate 110, KCl 24, $CaCl_2$ 6, $MgCl_2$ 5, glucose 11, and HEPES 5; NaOH or acetic acid was used to adjust the pH to 7.6; 0.1 % DMSO was also added to dissolve the abamectin and derquantel. The incoming perfusate was pre-warmed to 35 °C with an in-line heating system (SH 27B Warner instruments, CT, USA) before application. The rate of perfusion was 3.5–4 ml min^{-1} through a 20 gauge needle placed directly above the muscle bag recorded from. Test compounds were dissolved in APF-Ringer and applied as described in the results. A two-microelectrode current-clamp technique was employed to examine the

electrophysiological effects in the bag region of somatic muscle. We used 3 M potassium acetate in the micropipettes which had resistances of 20–30 MΩ. The recordings were made by impaling the bag region of somatic muscle with two microelectrodes, namely current-injecting (I) and voltage-recording electrodes (V). The step current was -40 nA was injected for 500 ms at 0.3 Hz. All experiments were performed using an Axoclamp 2A amplifier, a 1320A Digidata interface and Clampex 9 software (Molecular Devices, CA, USA). All data were displayed and analyzed on a PC based desktop computer. Our somatic muscle preparations had resting membrane potentials greater than -25 mV and the resting input conductances less than 4 μS.

4.3.2 The pharyngeal muscle preparation

The pharynx of *Ascaris* is a large muscular tube amenable to electrophysiological study. The cuticle and the muscle in the head region were dissected out to expose the pharynx. The beginning of the intestine was pinned to the muscle and the cuticle to secure the pharynx for recording. We increased the stability of the preparation by using calcium-free APF Ringer to limit contraction and by lowering the temperature of the incoming perfusate to 28°C. A microperfusion needle with a flow rate of 3.5–4 ml min^{-1} was used for perfusion of the pharynx. The recordings were made by impaling the pharynx at the posterior region of pharynx with two microelectrodes, namely current-injecting (I) and voltage-recording electrodes (V). The step current was -1000 nA was injected for 500 ms at 0.3 Hz. Our pharyngeal muscle

preparations had resting membrane potentials greater than -15 mV and the resting conductances less than 250 μS. Test compounds were dissolved in the calcium free APF-Ringer and applied as described in the results.

4.3.3 Drugs

Derquantel (Der) and abamectin (Aba) were provided by Pfizer Animal Health (Pharmacia and Upjohn Co., Kalamazoo, MI). Acetylcholine and L-glutamic acid as the monosodium salt hydrate obtained from Sigma-Aldrich (MO, USA). GABA was obtained from Calbiochem (EMD Serono, Inc, Rockland, MA, USA).

4.3.4 Analysis

In contraction assays, sigmoid concentration response curves for each were described by the Hill equation:

$$\% \text{ response} = 1/ (1+ [EC_{50}/X_a]^{nH}), \qquad \textit{equation 1},$$

where EC_{50} is the concentration of agonist (X_a) producing 50% of the maximum response and *nH* is the Hill coefficient (slope). Prism 4.0/5.0 (GraphPad Software, San Diego, CA.) was used to estimate the constants EC_{50} and *nH* in equation 1, by non-linear regression for each preparation.

In electrophysiology, we used parameters which were consistent and measurable across different batches of worms in order to describe and study the responses to the drugs. In somatic muscle preparations we determined changes in resting

membrane potential and in pharyngeal preparations we determined changes in the resting conductance. Our experiments were spread across different batches of worms to minimize the batch variations.

We calculated fractional inhibition of the acetylcholine induced depolarizations to determine the different interactions (additive, antagonistic or synergistic) of drugs on the somatic muscle nAChRs (Greco *et al*., 1996). We calculated: the mean of the depolarizations to each concentration of acetylcholine (control dose-response); the mean of the depolarizations (±S.E., standard error) to each of the concentrations of acetylcholine in the presence of derquantel (derquantel effect); the mean (±S.E.) of the depolarizations to each concentration of acetylcholine in presence of abamectin (abamectin effect).

We determined the fractional inhibition produced by derquantel alone (the reduction in membrane potential response ÷ control membrane potential response) and abamectin alone at each concentration of acetylcholine. The fractional inhibition produced by derquantel alone was denoted as *Fd*. Similarly, the fractional inhibition produced by abamectin alone was denoted *Fa*. The fractional inhibition produced by abamectin when derquantel is already present was calculated from multiplying *Fa*, by the remaining possible response, 1-*Fd* as Fa*(1-*Fd*). The calculated total fractional additive inhibition produced by the mixture of the two drugs is then:

*Fd+Fa (*1-*Fd)* *equation 2.*

This equation calculates the effects of two drugs that are behaving additively (Greco *et al*., 1996). We compared this calculated fractional additive inhibition with the observed fractional inhibition to detect synergism.

4.3.5 Statistics

All statistical analysis was done using Graph Pad Prism software. Mean values ± S.E. values are quoted throughout. Paired *t*-tests were used to test control recordings that were followed by test recordings from the same cell. Unpaired *t*-tests were used to compare control and test responses recorded from separate cells or preparations.

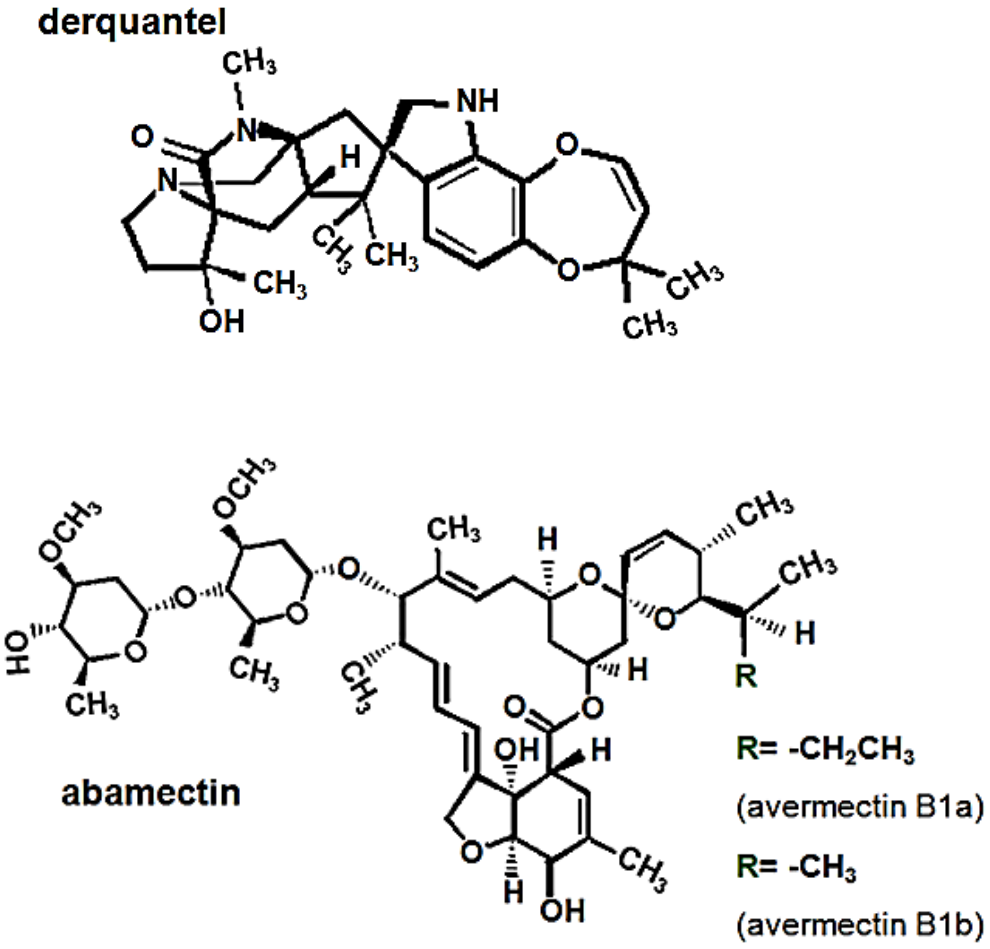

Fig 1. Structures of derquantel and abamectin. **A**. Structure of derquantel. **B.** Structure of abamectin. Abamectin is a mixture containing more than 80 % avermectin B1a and less than 20 % avermectin B1b. Avermectin B1a differs from avermectin B1b by a functional group at the 'R' position.

4.4. Results

4.4.1. Contraction: Inhibitory effects of derquantel and abamectin

Fig 2A shows a representative trace of the effects of adding increasing concentrations of acetylcholine and washing on isometric contractions of an *Ascaris* muscle flap preparation. When 1 μM derquantel was added there was little or no change in the resting contraction but the responses to the concentrations of acetylcholine were inhibited. When 0.3 μM abamectin was added in addition to derquantel, the response to the concentrations of acetylcholine were further inhibited. Washing reversed the inhibition but did not completely return the contractions to the control levels even after 10 minutes.

Fig 2B shows the concentration-response plots (mean ± S.E.) from 11 similar experiments. The control EC_{50} for acetylcholine was 7 μM with a g_{max} of 3.7 g and the inhibitory effect of 1 μM derquantel was to increase the EC_{50} to 52 μM with little change in the g_{max} to 3.4 g. When 0.3 μM abamectin was added there was further inhibition but with only a small change in the EC_{50} to 68 μM but there was a bigger reduction in g_{max} to 2.4 g. Washing the preparation partially reversed the inhibition so that the EC_{50} became 14 μM and the g_{max} increased to 3.2 g. Thus the main effect of derquantel was to increase EC_{50}, like a competitive antagonist, while the main effect of abamectin was to reduce g_{max}, like a non-competitive antagonist.

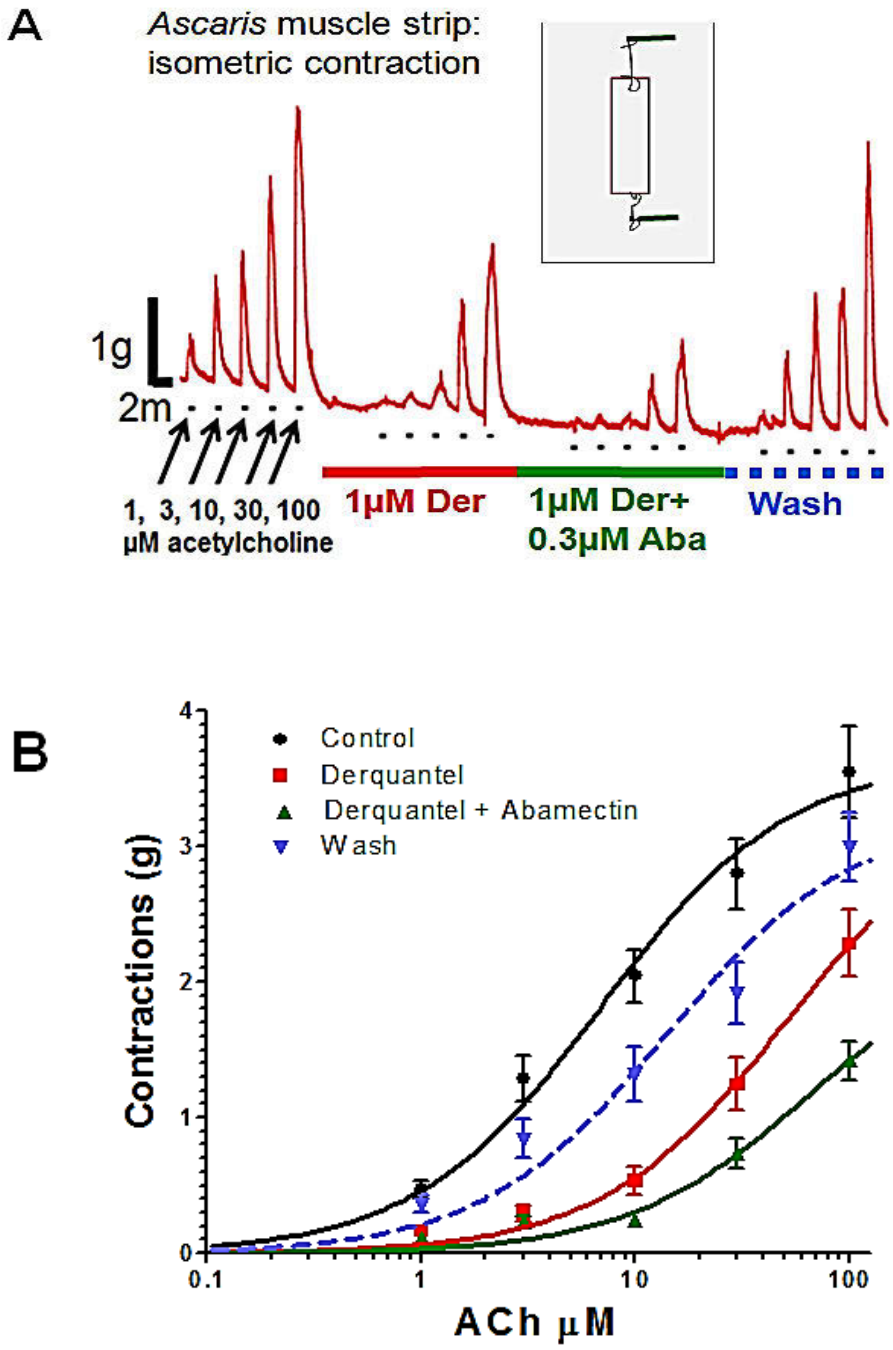

Fig. 2. A. Isometric contraction of *Ascaris suum* muscle strips produced by application of increasing concentrations of acetylcholine and antagonism by 1µM derquantel (red bar), 1µM derquantel+0.3µM abamectin (green bar) and wash (blue

bar). Note that derquantel decreases the responses to acetylcholine and that the addition of abamectin increases the inhibition. **B.** The concentration-depolarizing-response plot of acetylcholine showing mean ± S.E. bars (n=11). Control (black); in the presence of 1μM derquantel (red); 1μM derquantel+0.3μM abamectin (green) and wash (blue). Note that abamectin increases the inhibition produced by derquantel.

4.4.2. Electrophysiology: derquantel inhibits muscle nAChRs

Derquantel (0.1 - 30 μM) by itself produced little or no significant change in the muscle membrane potential or conductance. However, derquantel had a rapid (within 4 min) reversible inhibitory effect on acetylcholine depolarizations under current-clamp. Fig. 3A shows a representative trace of the inhibition produced by short application of 0.1 μM derquantel: the mean (n=9) depolarizations were decreased from 7.2 ± 1.0 mV to 4.6 ± 0.5 mV by derquantel and recovered to 7.0 ± 1.1 mV on wash. The bar chart in Fig. 3B summarizes results from 9 separate preparations: derquantel produced a significant inhibition ($p = 0.001$, paired *t-test*) which was reversible on washing ($p = 0.004$, paired *t-test*). Fig 3C illustrates the concentration-dependent inhibition of these acetylcholine depolarizations by derquantel and shows that the IC_{50}, was 200 nM (n = 18).

4.4.2a Effects of derquantel on the ACh concentration response

Fig 4A is representative trace showing the concentration-dependent effects of acetylcholine (0.3 to 30 μM, each applied for 15s) in a control preparation. Fig 4B shows representative trace of responses to acetylcholine applications in the presence of derquantel (1 μM). The control EC_{50} was 4.5 μM and the maximal response (R_{max}) was 21 mV (n = 11), Fig 5A. With derquantel the EC_{50} was 10.2 μM and the R_{max} of 14.5 mV (n = 8). The major effect of derquantel was to produce a shift to the right of the EC_{50} like a competitive antagonist.

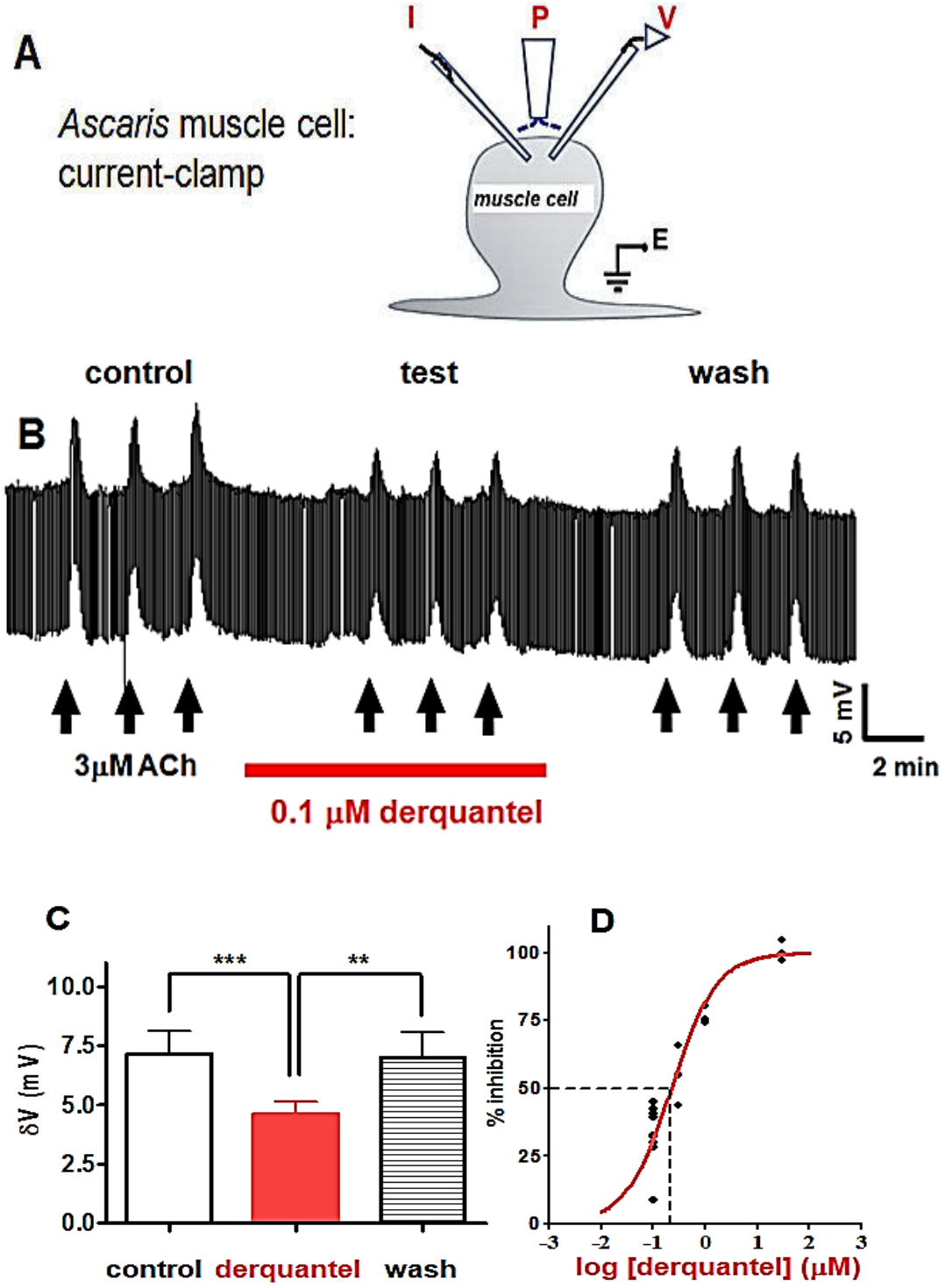

Fig 3. Current-clamp setup for making recordings from the somatic muscle and the pharynx in *A .suum.* **A.** A diagram the position of the positioning of I; current

injecting electrode and **V**; voltage recording electrode for making current clamp recording from the bag region of *A .suum* muscle cell. **P**; perfusion needle over the muscle bag for a localized perfusion of APF Ringer and or drugs, **E**; the earth electrode to complete the circuit. **B.** Representative trace shows depolarizations to three control applications of 3 μM acetylcholine (15s) vertical arrows. The applications of acetylcholine were repeated in the presence 0.1 μM derquantel after exposure (4 min) of the preparations to derquantel. **C.** Bar chart summarizing the results show a significant reduction in amplitude of control acetylcholine depolarizations in the presence of derquantel (0.1 μM) ($p < 0.001$, paired t-test, n = 9). Note that during the wash period the acetylcholine depolarizations recovered when compared to the test ($p = 0.004$, paired t-test, n = 9).

D. Concentration-inhibition plot for derquantel to inhibit 3 μM acetylcholine induced depolarizations. Concentration-inhibition responses were fitted with non-linear regression to determine the IC_{50}. The IC_{50} determined from this study was 0.2 μM (n = 18).

4.4.2b Effects of abamectin on the ACh concentration response

Abamectin application by itself produced no significant change in membrane potential ($p > 0.05$, one sample t-test, n=9). Fig 4C shows a representative trace of the effects of abamectin (0.3 μM) on the ACh concentration responses. In the presence of abamectin, the EC_{50} was 4 μM and the R_{max} of 12 mV (n = 9). Comparison with the control, Fig. 5A, shows that the major effect of abamectin was

to produce a reduction in the R_{max} without changing the EC_{50} in the manner of a non-competitive antagonist (negative allosteric modulator).

4.4.2c Combination can produce greater than additive inhibition

When we tested the effects of 0.3 μM abamectin with + 1 μM derquantel on the acetylcholine responses, we found that the inhibition was greater than produced by either drug alone, Fig 4D & Fig 5A. The EC_{50} was 10.1 μM and the R_{max} was 7.5 mV in the presence of the combinations (n = 7). The effect of the combination was to produce both a shift to the right of the EC_{50} and a reduction in $R_{max.}$

We determined the fractional inhibition (equation 2) of the acetylcholine responses produced by derquantel, abamectin and the combination. Fig 5B shows a plot of 1-fractional inhibition plotted against the concentration of acetylcholine. The dashed line in Fig 5B indicates the predicted line of additive inhibition for derquantel and abamectin (Greco *et al*., 1996). Note that the combination of derquantel and abamectin Fig 5B actually produces a greater than additive effect at higher concentrations of acetylcholine. This effect is predicted if the two drugs act at separate sites on the acetylcholine receptor: derquantel acting mostly as a competitive antagonist and abamectin acting as a non-competitive antagonist.

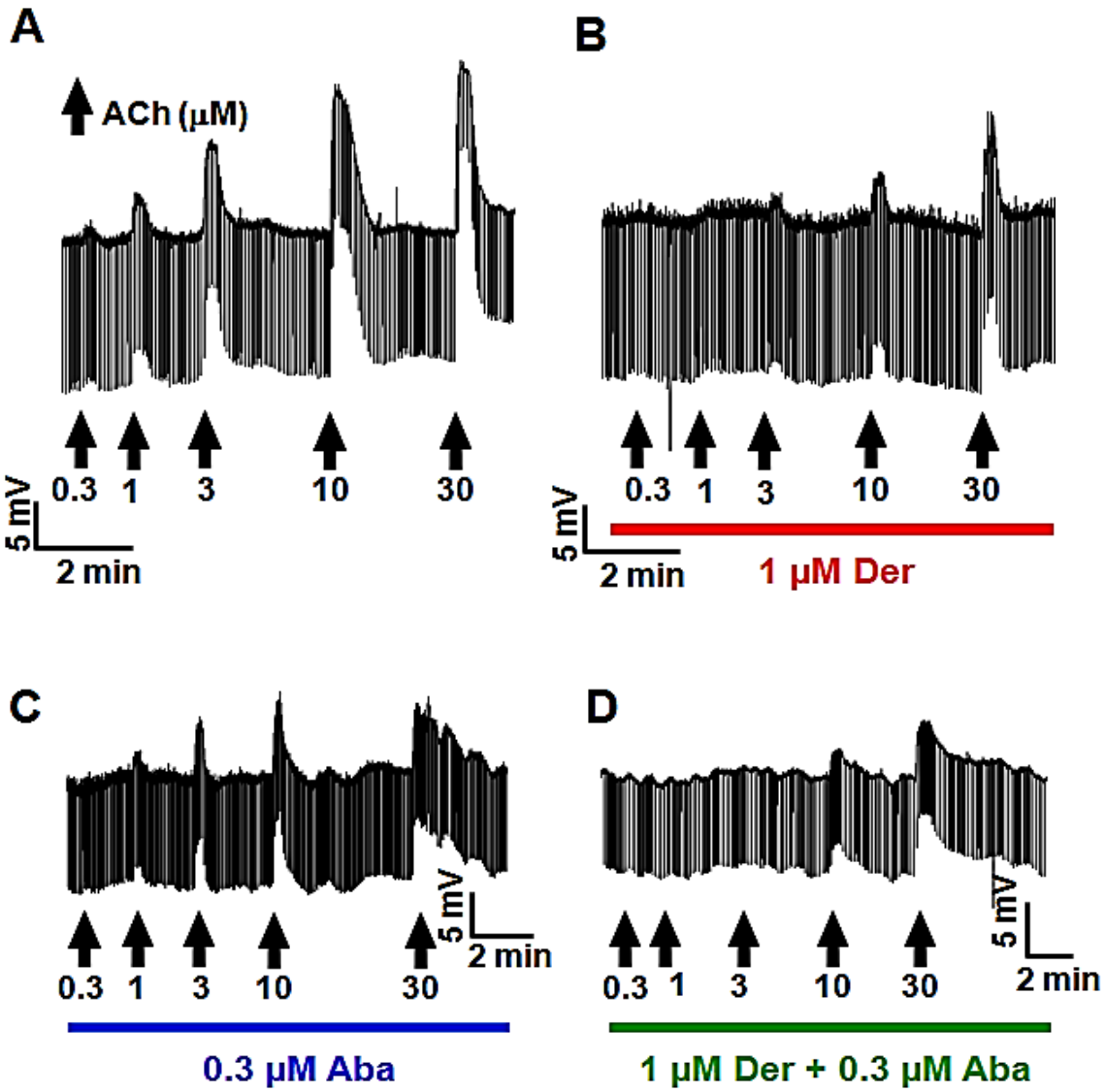

Fig 4. Representative traces showing acetylcholine concentration responses of controls and in the presence of 1 μM derquantel, 0.3 μM abamectin and the combination. Increasing concentrations of acetylcholine (0.3 - 30 μM) shown in black arrows were applied for 15s in controls. In test, the acetylcholine applications

followed 30 min pretreatment of the preparations with the drugs. **A**. Representative trace, one from 11 such experiments, shows a concentration dependent increase in the depolarizations to the acetylcholine applications in the controls. **B.** Representative, one from 8 such experiments trace shows, a reduction in acetylcholine induced depolarizations in the presence of derquantel. **C.** Representative trace, one from 9 such experiments shows a reduction in the acetylcholine induced depolarization in the presence of abamectin. **D.** Representative trace, one from 7 such experiments shows a reduction in acetylcholine induced depolarizations in the presence of the combination.

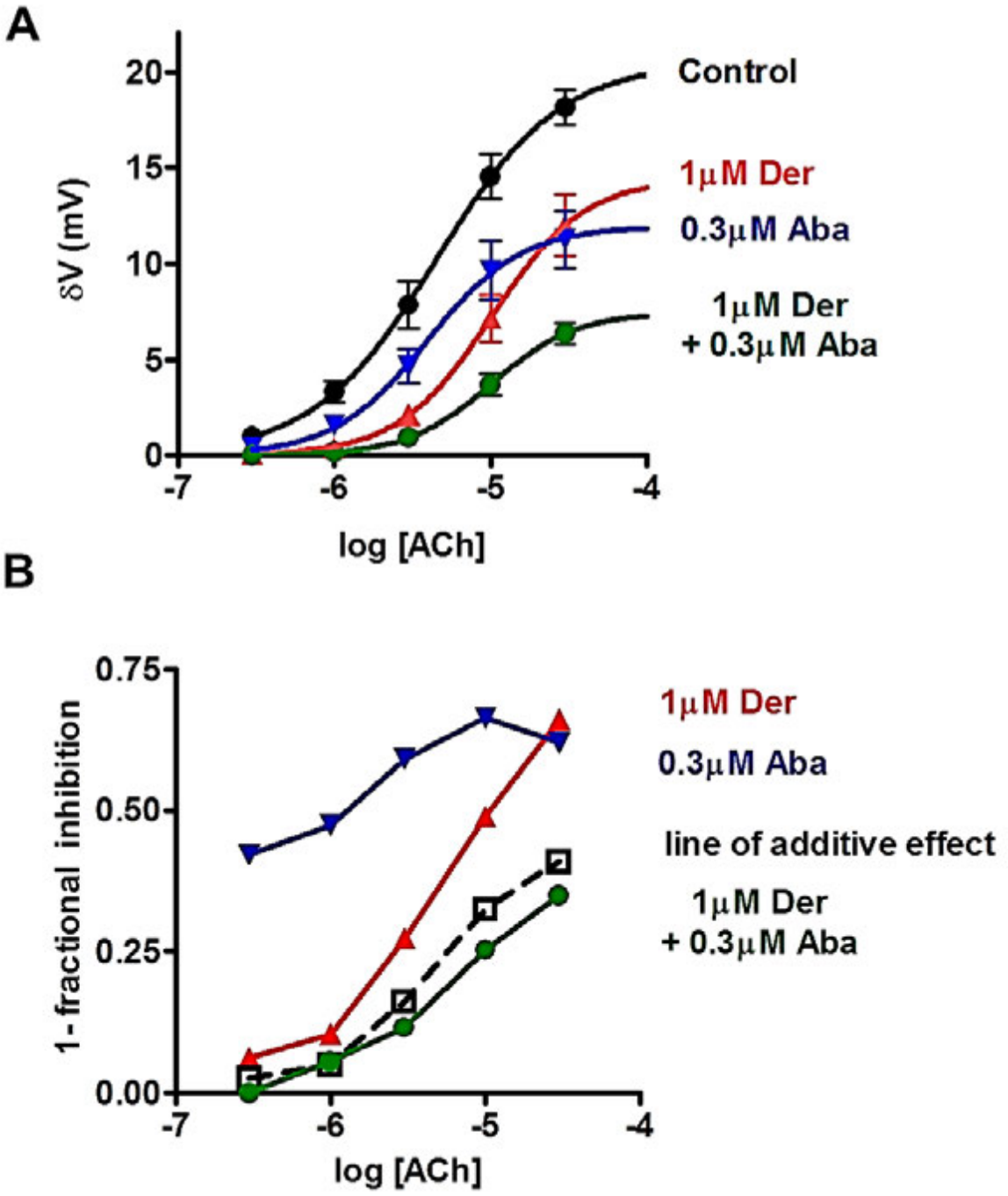

Fig 5. Acetylcholine concentration-depolarization and fractional inhibition of acetylcholine depolarization plots in the presence of derquantel, abamectin and the combination. **A.** Concentration-depolarization responses for acetylcholine (0.3 - 30

μM, 15 s) in the controls fitted with non-linear regression ●. The test consists of increasing concentrations of acetylcholine were applied after a 30 min pretreatment of the preparations with 1 μM derquantel ▲, 0.3 μM abamectin ▼ and the combination (1 μM derquantel + 0.3 μM abamectin) ●. The EC_{50} and R_{max} of acetylcholine in the controls was 4.5 μM and 20.6 mV (n = 11). The EC_{50} and R_{max} of acetylcholine in the presence of derquantel was 10.2 μM and 14.5 ± 3.3 mV (n = 8). The EC_{50} and R_{max} of acetylcholine in the presence of abamectin was 4 μM and 12 ± 1.7 mV (n = 9). The EC_{50} and R_{max} of acetylcholine in the presence of the combination was 10.1 μM and 7.5 ± 1.2 mV (n = 7). **B.** The fractional inhibition of acetylcholine depolarization in the presence of derquantel ▲, abamectin ▼and the combination ●. The dashed black line shows the predicted line for the additive effect of derquantel and abamectin in inhibiting acetylcholine depolarizations. The fractional inhibition of acetylcholine response in the presence of the combination is greater than the predicted line of additive effect.

4.4.3 No interaction at GABA receptors on somatic muscle

GABA is an inhibitory neurotransmitter that opens GABA gated chloride channels on the somatic muscle resulting in hyperpolarization. We tested the effects of derquantel, abamectin and the combination, applied for 4 min, on the hyperpolarizing response to GABA (10 μM, applied for 15s). We did not observe a significant ($p > 0.05$, paired t-test) effect of derquantel (1 μM, n = 4), abamectin (0.3

μM, n = 5) or the combination (1 μM derquantel+ 0.3 μM abamectin, n = 4) on the GABA responses. Our study suggests that the combination does not interact on GABA gated chloride receptor channels on the somatic muscle of *Ascaris*.

4.4.4 Abamectin activates GluCls of the pharynx

The *Ascaris* pharynx contains inhibitory glutamate-activated chloride channels (GluCls, Martin 1996). Abamectin activated these channels when we applied abamectin for 2 min in cumulative doses in concentrations ranging from 0.01 to 1 μM. It produced hyperpolarization and a concentration-dependent increase in membrane conductance of the pharyngeal muscle, Fig 6B &D. The hyperpolarization varied between preparations and depended on the initial resting membrane potential. The resting input conductance of these preparations was 186.5 ± 18 μS and the change in conductance at the end of 1 μM abamectin (the highest concentration) was 282.6 ± 62.1 μS (n = 4). The EC_{50} for the concentration-conductance response for abamectin was 0.4 μM.

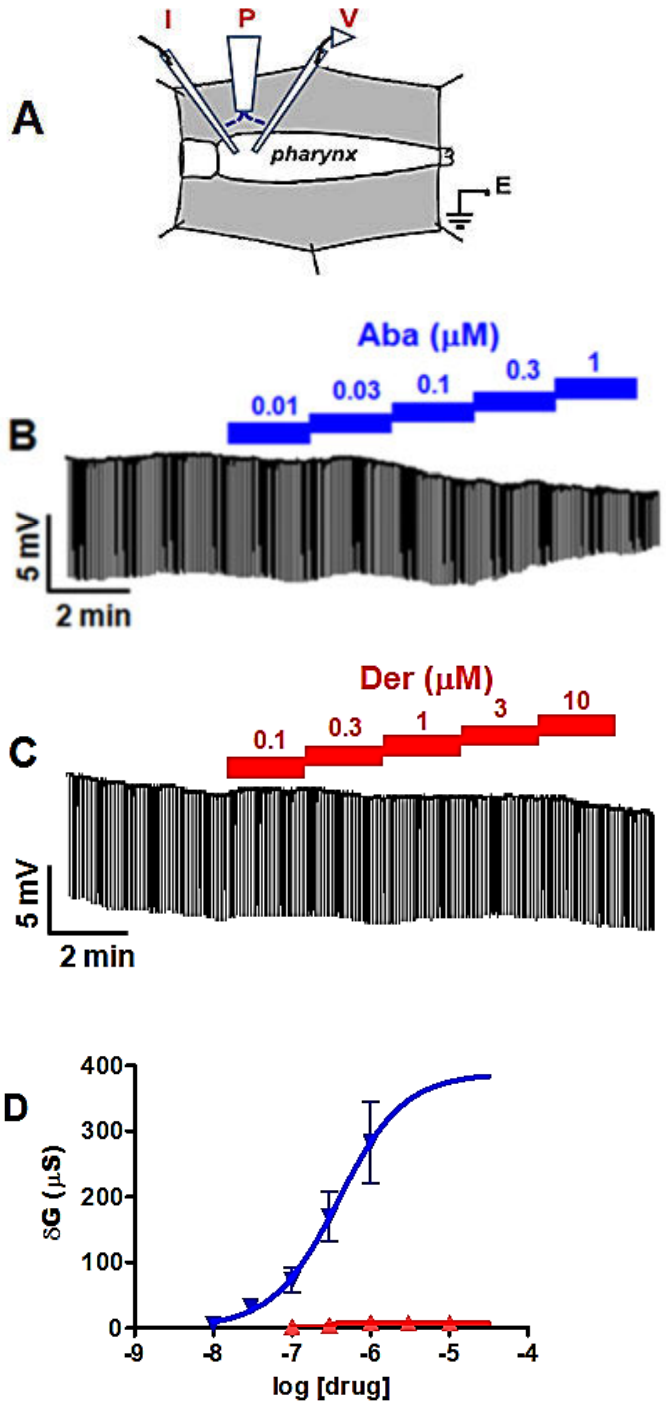

Fig 6. Recording technique used for the pharynx to record the conductance changes produced in response to cumulative applications of abamectin and

derquantel in pharyngeal preparations. **A.** Diagram of the technique. Positioning of: **I,** current injecting electrode and; **V,** voltage recording electrode in the terminal bulb of pharynx. **P**, perfusion needle above the pharynx for a localized perfusion of calcium free APF Ringer and or drugs **E**, the earth electrode to complete the circuit. The cumulative applications of abamectin (0.01 to 1 µM) and derquantel (0.1 to 10 µM).

B. Representative trace showing the conductance changes produced in response to cumulative applications of abamectin. **C.** Representative trace showing the conductance changes produced by the cumulative applications of derquantel. **D.** The concentration conductance plots fitted with non-linear regression for abamectin ▼ and derquantel ▲. The EC_{50} for abamectin was 400 nM (n = 4). It was not possible to fit non-linear regression for derquantel due to a little or no conductance change produced by derquantel (one sample t-test, n = 4).

4.4.5. Derquantel does not affect GluCls of the pharynx

Derquantel was applied for 2 min in cumulative doses starting at a concentration of 0.1 and increasing up to10 µM. Fig 6C & D shows that derquantel produces no significant effect on the membrane potential or input conductance of the pharyngeal muscle (n = 4). We also tested the effects of cumulative-concentration application of abamectin in the presence of 0.1 and 1 µM derquantel, each set of 4 preparations. In the presence of 0.1 µM, the EC_{50} of abamectin was 0.3 µM; in the presence of 1 µM derquantel the EC_{50}, was also 0.3 µM. Derquantel did not change

Log EC_{50} values for abamectin ($p > 0.05$, F-test) suggesting that derquantel does not interact with abamectin at the GluCls of the pharynx.

We tested the effect of derquantel on the conductance changes produced by applications of 10 μM and 100 μM L-glutamate for 15s. Fig 7 A shows a representative trace of the L-glutamate control responses and L-glutamate responses in the presence of 1 μM derquantel. The input conductance response to 10 μM L-glutamate was 32 ± 8 μS (n = 4) and to 100 μM L-glutamate it was 126 ± 43 μS (n = 4). In the presence of 1 μM derquantel, the input conductance response to 10 μM L-glutamate was 51 ± 18 μS (n = 4) and the response to 100 μM L-glutamate was 116 ± 37 μS (n = 4). The presence derquantel did not significantly change the L-glutamate (10 or 100 μM) responses ($p > 0.1$, paired t-test). These observations suggest that derquantel does not interact with L-glutamate at the GluCls of the pharynx.

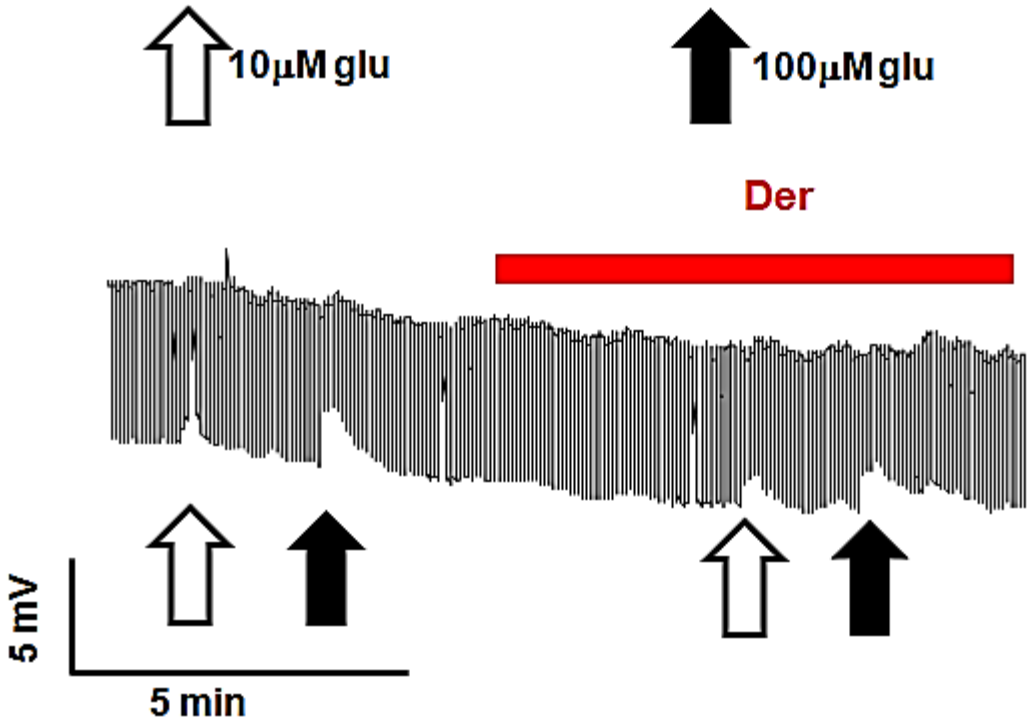

Fig 7. L-glutamate responses in the presence of derquantel in pharynx. Unfilled and filled arrows represents 15s applications of 10 μM L-glutamate and 100 μM L-glutamate respectively. The applications of L-glutamate followed after 4 min of 1 μM derquantel treatment. Representative trace shows control L-glutamate responses and those in the presence of derquantel (1 μM). Note that there is no effect of derquantel on the L-glutamate responses.

4.5 Discussion

4.5.1 Derquantel is a potent competitive nematode nAChR antagonist

Robertson *et al*. (2002) used an *Ascaris* muscle strip contraction assay to describe the antagonistic effects of derquantel against a panel of cholinergic anthelmintics.

Derquantel behaved like a competitive antagonist. The pA_2 values for a panel of nicotinic agonists and cholinergic anthelmintics (Martin *et al*., 2003; Martin *et al*., 2004) were: methyridine (5.3), oxantel (5.4), thenium (5.5), levamisole (5.7), pyrantel (5.9), nicotine (6.3) and bephenium (6.5). Analyses of these observations lead to the conclusion that there are 3 subtypes of nAChR present in *Ascaris* muscle strips. There is the N-subtype preferentially activated by nicotine and oxantel; there is the L-subtype preferentially activated by levamisole and; the B-subtypes preferentially activated by bephenium. In our present contraction studies with acetylcholine, we found that 1 μM derquantel shifted the acetylcholine EC_{50} from 7 μM to 52 μM giving a dose-ratio of 7.4 and pA_2 of 6.8 (Schild equation). Interestingly, the pA_2 is closest to bephenium suggesting that acetylcholine, a quaternary ammonium like bephenium, may stimulate the B-subtypes of nAChR during the contraction studies (Martin *et al*., 2004). The potent inhibitory effects of derquantel in our *Ascaris* (Clade III nematode) contraction assays, is comparable to the potent inhibitory effects of derquantel seen in Clade V nematode parasites. For example, in *T. colubriformis* motility is inhibited by 100 nM derquantel (Zinser *et al*., 2002) and in *H. contortus*, the EC_{50} for inhibition in motility assays is 200 nM (Johnson *et al*., 2004).

In our electrophysiological studies we found, like Zinser *et al* (2002), that derquantel has a rapid onset of action and had no effect on membrane potential or conductance of the *Ascaris* somatic muscle and that derquantel is a potent acetylcholine antagonist. In the electrophysiological studies, 1 μM derquantel shifted the acetylcholine concentration-depolarization plots to the right: the EC_{50}

values were shifted from 4.5 μM to 10.2 μM giving a calculated pA_2 of 6.1. The pA_2 value of 6.1 is less than we saw in the contraction studies and may be explained if the mixture of subtypes of nAChR associated with contraction contains more B-subtypes than the nAChRs on the bag region of the *Ascaris* muscle which contain more L-subtypes (Qian *et al*., 2006). The speed of action of derquantel and competitive mode of action is consistent with a site of action in the extracellular aqueous phase of the nAChR ion-channel.

In contrast to the effects on the somatic muscle, we found that derquantel had little or no effect on the GABA induced hyperpolarizations of somatic muscle or on effects of glutamate or abamectin of the pharynx. The potent effects on muscle nAChRs and the lack of effect on GABA receptor and GluCls suggest that the somatic muscle nAChRs are a major target site for the therapeutic action of derquantel.

4.5.2 Effects of abamectin on the somatic muscle

Application of abamectin produced a slowly developing non-competitive antagonism of acetylcholine depolarizations and muscle contraction; the effect was to produce a reduction in the maximum response without a shift in the EC_{50}. This inhibitory effect of abamectin, initially, was unexpected since a potentiating (positive allosteric effect) of the ivermectin has been described on nAChRs (Krause *et al*., 1998; Bertrand and Gopalakrishnan, 2007). The mode of action of ivermectin, a macrocyclic lactone like abamectin, as positive allosteric modulator on nAChRs involves the transmembrane domains (TM1, TM2 and TM3) of the ion channel (Collins and Millar, 2010) as it does for the GluCls (Hibbs and Gouaux, 2011). Interestingly however, the positive allosteric effect of ivermectin on nAChRs can be converted to an antagonist by one of three mutations (S222M, M253L, and S276V located in TM1, TM2, and TM3 regions) (Collins and Millar, 2010). With these mutations, ivermectin behaves like a non-competitive antagonist of the nAChRs that had no effect on the EC_{50} but reduced the maximum response as we see here for abamectin. Thus abamectin may act like ivermectin in the outer lipid phase of the membrane to combine with a lipophilic region of the ion-channel (Martin and Kusel, 1992; Hibbs and Gouaux, 2011) and depending on the amino acids present in the transmembrane regions, acts as a non-competitive antagonist (negative allosteric modulator) as we see here. A site of action within the lipid phase of the membrane is consistent with a slow onset action. At the *Ascaris* somatic muscle nAChR, it behaved like a non-competitive antagonist.

4.5.3 Effects of the combination on the somatic muscle

We have seen that the effects of derquantel have a rapid onset and derquantel behaves in a competitive manner suggesting a site of action on the extracellular surface of the nAChR. Abamectin is slower in onset, non-competitive and may have a site of action within the lipid phase of in the transmembrane region of the nAChR. When the two inhibitors are applied together the action at the two different putative sites of action on the nAChR combine and the effects are greater than additive at higher (> 3 μM) concentrations of acetylcholine. At vertebrate neuro-muscular junctions, the released acetylcholine concentration can reach as high as 5 mM (Jin, 2010). Hence, the inhibition of acetylcholine response at the somatic muscle of the worm produced by the combination may be higher than we have observed here. The inhibitory effects of the combination on the somatic muscle may translate into a greater therapeutic effect than using either of the individual drugs alone for treatment.

4.5.4 Effects of abamectin on the pharynx

The *Ascaris* nematode pharynx contains glutamate gated chloride channels (GluCls) which are the targeted by avermectin anthelmintics (Martin, 1996). In *C. elegans,* glutamate mediates the actions of inhibitory motor neuron M3 on the pharyngeal muscle (Avery, 1993a). GluCl activation is implicated in reducing the locomotion mediated via command interneurons (Wolstenholme and Rogers, 2005b). Single channel studies from the pharyngeal muscle vesicles of *A .suum*

show that the GluCls are activated by glutamate and ivermectin and reversibly blocked by picrotoxin (Adelsberger et al., 1997b). The GluCl subunits in *C. elegans* are encoded by at least 6 genes: avr-14, avr-15, glc-1, glc-2, glc-3 and glc-4.

Previous studies on the GluCls show that they are sensitive low concentrations (nanomolar) of avermectin anthelmintics. The EC_{50} values for ivermectin in *C .elegans* GluCls were 0.1 to 0.2 μM (Cully *et al*., 1994) and *H .contortus* GluCls expressed in *Xenopus* oocytes had EC_{50} in the 0.1 to 1nM range (McCavera *et al*., 2009). In our pharyngeal preparations, abamectin produced hyperpolarization and a concentration dependent increase in the input conductance we attribute to the opening of GluCls of the pharynx. Abamectin had an EC_{50} of 0.4 μM showing that pharynx is sensitive to low concentrations of abamectin. The potency of avermectin anthelmintics on nematode worms is a combined effect of endogenous glutamate complimented by exogenous avermectins. The resultant effect is a massive increase in the chloride conductance of the pharyngeal muscle through irreversible opening of GluCls (Hejmadi *et al*., 2000; Pemberton *et al*., 2001; Wolstenholme and Rogers, 2005b).

4.5.5 Effects of derquantel on the pharynx

Derquantel had no effect on the resting membrane conductance or on the conductance change produced by glutamate or abamectin. These observations suggest that derquantel has no effect on their GluCls and that GluCls are not involved in the mode of action of derquantel.

4.5.6 Anthelmintic resistance and the use of combination drugs in therapy

Anthelmintic resistance is a concern in parasitic nematode of both humans and livestock (Geerts and Gryseels, 2000; Kaplan, 2004b; Jones and George, 2005). Resistance can limit the efficacy of current anthelmintics (James *et al*., 2009). Electrophysiological studies in *A. suum* somatic muscle suggest that cholinergic anthelmintics preferentially activate pharmacologically distinct subtypes of nAChRs. The N-subtype is preferentially activated by nicotine and oxantel ; the L-subtype by levamisole; and the B-subtype by bephenium. Further, derquantel preferentially antagonizes the B-subtype of nAChRs (Robertson *et al*., 2002). Our studies show that abamectin can potentiate the effects of derquantel in the somatic muscle of *A .suum*.

Studies in Australia show that, resistance to both broad- and narrow-spectrum anthelmintics is widespread (Besier, 2003). Suitable anthelmintic combinations favor elimination of those parasitic nematodes that carry resistant genes to only one of the anthelmintics. Despite the limitations of anthelmintic combinations being expensive, it is becoming clearer that the combinations should be used before resistance levels climb too high (Le Jambre, 2010). The use of combination therapy is also favored when the two anthelmintics have additive or synergistic effects. The combination of anthelmintics is less likely to be successful if the compounds have an inhibitory effect on each other.

4.6 Conclusion

We have studied the interaction of derquantel and abamectin a novel anthelmintic combination. Our study focused on two important anthelmintic drug target sites in the worm namely the somatic muscle nAChRs and the pharyngeal GluCls. Our experiments on worm somatic muscle demonstrate that the abamectin acts non-competitively at the nAChRs of somatic muscle and potentiates the competitive antagonism produced by derquantel. It is anticipated that the combination will allow a slower rate of development of resistance than either of the two drugs alone. The introduction of anthelmintic drugs with new mechanisms or new combinations will help us to move ahead in the battle against the evolving resistance appearing in nematode parasites.

CHAPTER 5. ELECTROPHYSIOLOGICAL CHARACTERIZATION OF THE NICOTINIC CHOLINERGIC RESPONSE IN *ASCARIS SUUM* PHARYNX

Sreekanth Puttachary[1], Richard J. Martin[2], and Alan P. Robertson [2,3]

5.1 Abstract

Soil transmitted nematode infections including round worms (*Ascaris lumbricoides)*, hook worms (*Ancylostoma duodenale & Necator americanus)*, and whipworms (*Trichuris trichura*), affect more than a billion people worldwide. Nematode infections of humans and livestock cause debility, reduced productivity and severe economic loss. Chemotherapy is widely used to control these infections. However, there are a limited number of effective compounds and therefore a clear need to develop new anthelmintic drugs.

Both the muscular pharynx and the body wall muscle are validated target tissues for current anthelmintic drugs. In the present study, we characterized a novel nicotinic acetylcholine receptor (nAChR) on the pharynx of the parasitic nematode *Ascaris suum* using electrophysiological techniques. Acetylcholine (ACh) application (100 μM) produced a large depolarization accompanied by an increase in membrane conductance. Selected muscarinic receptor agonists (arecoline, pilocarpine, oxotremorine, methylfurmethiodide, all 100 μM) produced a negligible change in membrane conductance (δG) indicating the response to ACh was not muscarinic in origin. Agonists of vertebrate nAChRs also produced depolarizations and increased

conductance in the pharynx, with a rank order potency series of: ACh > nicotine > cytisine > epibatidine > DMPP > choline.

Interestingly, existing anthelmintics that act on body muscle nAChRs in the nematode had no significant effect on pharyngeal membrane potential or input conductance. Further characterization of the nAChR on *Ascaris* pharynx has revealed pharmacological differences from both the nAChRs found in the vertebrate host and those found on nematode body muscle, indicating that this receptor may be a suitable target site for new anthelmintic compounds.

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5.2 Introduction

Gastrointestinal (GI) nematodes affect humans and livestock worldwide. The Global prevalence of human GI nematode infections is more than a billion and severe infections have resulted in deaths (Savioli and Albonico, 2004; Hotez et al., 2007a). GI nematodal infections affect livestock health and productivity resulting in substantial economic losses (Kaplan, 2004b). Chemotherapy is a widely used approach for controlling these worm infections. There are a limited number of anthelmintics available in the market. Repeated use of these anthelmintics to treat

worm infections has led to the emergence of resistance in the parasites (Kaplan, 2004b; Jones and George, 2005). Resistance has reduced the efficacy of currently used anthelmintics thus limiting treatment options (James *et al*., 2009). To achieve effective worm-control, research to identify new drug targets on worms has become indispensable.

For decades, ion channels on the worm have been exploited as anthelmintic targets. These ion channels include nicotinic acetylcholine receptors (nAChRs) on muscle and nerve, GABA gated chloride channels on the somatic muscle and glutamate-gated chloride channels on the pharynx. In the present study we propose that, the nAChRs of the worm pharynx are a novel pharmacological target. We discuss how pharynx nAChRs play a role in feeding and how this physiology can be exploited to control worm infections. The pharynx, a muscular pumping organ in worms, aids ingestion of food from the surroundings within the host gut. A rhythmic peristalsis of the pharynx permits ingestion while the secreted digestive enzymes within the pharyngeal lumen help digestion. Pharyngeal peristalsis in nematodes is well coordinated by rhythmic activations of excitatory (cholinergic) and inhibitory (glutamatergic) motor neurons innervating the pharyngeal muscles (Raizen *et al*., 1995; Brownlee *et al*., 1997). At the neuromuscular synapses, cholinergic motor neurons activate the pharyngeal muscles to initiate and maintain peristalsis. ACh released from the cholinergic motor nerve binds to the nAChRs on the pharyngeal muscles causing depolarization and contraction (Raizen *et al*., 1995). Glutamate released from the glutamatergic motor nerve endings binds to the glutamate gated

chloride channels on the pharyngeal muscles causing hyperpolarization and relaxation (Martin, 1996; Brownlee *et al*., 1997). A loss of peristaltic rhythm of the pharynx leads to failure of ingestion and starvation of worms (Wolstenholme and Rogers, 2005b). Starved and therefore slow moving worms within the host gut are expelled.

The importance of pharyngeal peristalsis for worm survival suggests that the receptors involved are attractive targets for pharmacological control of worm infections. We propose that, like worm somatic muscle nAChRs (Barragry, 1984; Qian *et al*., 2006), excessive activation of the pharyngeal nAChRs will cause paralysis of the worm pharynx. The current literature on the nematode pharynx nAChRs is limited.

5.3. Materials and methods

Adult *A .suum* worms were collected weekly from the JBS packing plant at Marshalltown, Iowa. Locke's solution [composition (mM): NaCl 155, KCl 5, $CaCl_2$ 2, $NaHCO_3$ 1.5 and glucose 5] at a temperature of 35 °C was used to store the worms. Locke's solution was changed twice daily and worms from each batch were used for experiments within 4 days of collection.

5.3.1. Current-clamp recordings from the pharynx

The pharyngeal muscle preparation

The pharynx of *Ascaris* is a large muscular cylindrical tube amenable to electrophysiological study. About 1.5-2 cm of worm from the head region containing the pharynx was dissected out on a Sylgard™-lined double jacketed bath chamber. The cuticle and the muscle were carefully removed by an incision along one of the lateral line to expose pharynx. The muscle layer surrounding the anterior 3rd of the pharynx was preserved for anchoring. The intestine attached to the posterior end of the pharynx was used for stretching and pinning down. The temperature of the bath chamber was maintained at 28 °C by inner circulation of warm water (Haake FJ, Berlin, Germany). The preparation was continuously perfused with calcium free *Ascaris* Perienteric Fluid-Ringer (calcium free APF-Ringer) composition (mM): NaCl 23, Na-acetate 110, KCl 24, $MgCl_2$ 11, glucose 11, and HEPES 5; NaOH or acetic acid was used to adjust the pH to 7.6. The experimental compounds were dissolved in calcium free APF-Ringer and applied as described in the results. An in-line heating system (SH 27B Warner instruments, CT, USA) was used to pre-warm the incoming perfusate to 28 °C before application. The rate of localized perfusion was 3.5–4 ml min^{-1} through a 20 gauge needle which was placed directly above the recording region of pharynx. The posterior region of pharynx 1-2mm ahead of the attachment of the intestine was used for making electrophysiological recordings. In order to prevent contraction artifacts affecting our pharyngeal recordings we destroyed most of the somatic muscular layer surrounding the pharynx. Pharyngeal

preparations with resting membrane potentials greater than -10 mV and the resting conductances less than 250 μS were selected for analysis. In individual recordings, the peak change in membrane conductance (δG) in response to each drug application was determined.

Current-clamp technique

We used two microelectrode current-clamp technique to examine the electrophysiological responses in the *A. suum* pharyngeal muscle preparation (Fig1A). We used the borosilicate capillary glass (Harvard Apparatus, MA, USA, ID-0.86 mm, OD-1.5 mm) to prepare voltage recording microelectrodes (V in the Fig 1A) by pulling on a P-97 Flaming Brown Micropipette puller (Sutter Instrument Co., CA, USA). We used thin walled capillary patch glass (Warner instruments, CT, USA, OD-1.5 mm, ID-1.16 mm) to prepare the current injecting microelectrode (I in Fig.1A) by pulling on a dual stage glass micropipette puller (PC-10,Narishige Co, Tokyo, Japan). We used 3 M potassium acetate in our micropipettes to get the final resistances of 4-7 MΩ for the voltage recording and 0.5-1 MΩ for the current injecting electrode. The recordings were made by impaling the posterior region of the *A. suum* pharyngeal muscle with two microelectrodes, namely current-injecting (I) and voltage-recording electrodes (V). We used an Axoclamp 2A amplifier, 1320A Digidata interface with Clampex 9 software (Molecular Devices, CA, USA) to record and analyze data on a PC based desktop computer.

The current-injecting electrode injected hyperpolarizing step currents of −1000 nA for 500 ms at 0.3 Hz. The voltage-recording electrode recorded the change in

membrane potential in response to the injected currents. Pharyngeal preparations with constant resting membrane potentials more negative than −10 mV for 20 min and a stable input conductance of <250 µS were selected for the recordings.

5.3.2 Drugs

Acetylcholine, nicotine, epibatidine, cytisine, pyrantel, oxantel and bephenium were obtained from Sigma-Aldrich chemicals (MO, USA). Levamisole was obtained from Acros-Organics (NJ, USA). Derquantel (der) was provided by Pfizer Animal Health (Pharmacia and Upjohn Co., Kalamazo, MI). 100 mM stock solutions were prepared in double distilled water every week and frozen in aliquots at −20 °C. Stocks of epibatidine, nicotine, oxantel, bephenium were prepared in DMSO, with the final concentration of DMSO in calcium free APF not exceeding 0.1%. Stock solutions were thawed just before use. ACh stock was always prepared fresh before the start of experiments.

5.3.3 Analysis

In our preliminary studies, we observed a depolarization with an increased membrane conductance in response to the application of ACh. The change in membrane potential (δV) and the change in membrane conductance (δG) were the two parameters used to measure the responses to the applied drugs. We observed

the size of the depolarizations (δV) in response to drugs was dependent on the resting membrane potential (RMP) of the preparation. The RMPs recorded from preparations across different batches of worms showed variability. The change in membrane conductance (δG) in response to a drug across batches of worms was more consistent. Hence, we chose change in membrane conductance (δG) as the parameter to compare responses to different drug applications. To further limit the variations across different batches of worms all our preparations had control application/s of 100 μM ACh (applied for 10s). The δG produced by 100 μM ACh was set as 100% within each preparation containing other drug applications. The δG responses to different drug applications within the preparations were normalized to the 100 μM ACh response for comparison.

5.3.4 Statistics

The peak changes in membrane conductance (δGmax) in response to drug applications were normalized to δG response to acetylcholine application (100 μM ACh, applied for 10s) within each preparation. The δG responses to other drugs were normalized to δG response to acetylcholine to observe their rank order potency. We constructed sigmoidal concentration response plots by fitting the data by nonlinear regression (GraphPad Prizm V5, San Diego, Ca) to determine the EC_{50} and the maximal response (R_{max}). The logEC_{50}, slope and maximal response

of control and test concentration response plots were compared using extra sum of squares F-test. The significance levels was set to $p < 0.05$.

5.4 Results

5.4.1 Nicotinic receptor activation contributes to a majority of ACh response in pharynx

ACh is a known agonist at nicotinic and muscarinic receptors in vertebrates. Apart from the nAChRs, the *Ascaris* pharynx is also known to contain G-protein coupled acetylcholine receptors (GARs) with a high structural homology to the *C. elegans* GAR-1 receptor. The rank order potency of muscarinic agonists (all used at 100μM) observed in yeast functional expression studies was ACh (100%) > carbachol (80%) ≅ arecoline (76%) > oxotremorine (61%) ≅ bethanechol (58%) > pilocarpine (14%) (Kimber *et al*., 2009). Here we tested the hypothesis that the observed change in membrane conductance response (δG) to ACh on the pharynx is produced by activation of nAChRs and mAChRs (GARs).

Our pharyngeal preparations in this group had a resting membrane potential (RMP) of -17.6 ± 1.3 mV and a resting conductance of 147.8 ± 13.1 μS (n=8) (mean ± SE). We tested responses to the muscarinic agonists ACh, 5-methylfurmethiodide (MFI), oxotremorine, arecoline, pilocarpine (all 100μM, 10s application). In applications containing arecoline and pilocarpine, we used mecamylamine, a nAChR antagonist (30μM) to inhibit nAChRs and to allow only mAChR activation. A control application

of ACh (100 μM, 10s) in the presence of mecamylamine was used for comparison of response to nAChR activation. We normalized the conductance change (δG) produced by the control ACh application (100 μM, 10s) to 100% in order compare the relative responses to other muscarinic agonists.

The normalized responses to muscarinic agonists (mean ± SE, %) were MFI [4.0 ± 0.6 % (n=4)], oxotremorine [1.8 ± 0.8 % (n=4)], arecoline [2.6 ± 2.0 % (n=4)], pilocarpine [0.7 ± 0.7 % (n=4)]. Mecamylamine (30 μM) inhibited 92% of the ACh δG response suggesting nicotinic receptor activation rather than the muscarinic receptor activation was responsible for the observed changes in conductance and membrane potential (Fig 1.B). Earlier studies have described the pharmacological properties of mAChRs in *A. suum.* Our results demonstrate their contribution to the acetylcholine induced conductance changes is negligible. Therefore it was unnecessary to further use muscarinic receptor antagonists in our experiments to characterize the nAChRs. Other work has documented the phenomenon of concentration dependent reversible channel block produced by atropine (IC_{50} – 4-10μM) on $\alpha_3\beta_4$, $\alpha_3\beta_2$, $\alpha_4\beta_4$ and $\alpha_4\beta_2$ vertebrate nAChRs (Parker *et al.*, 2003).

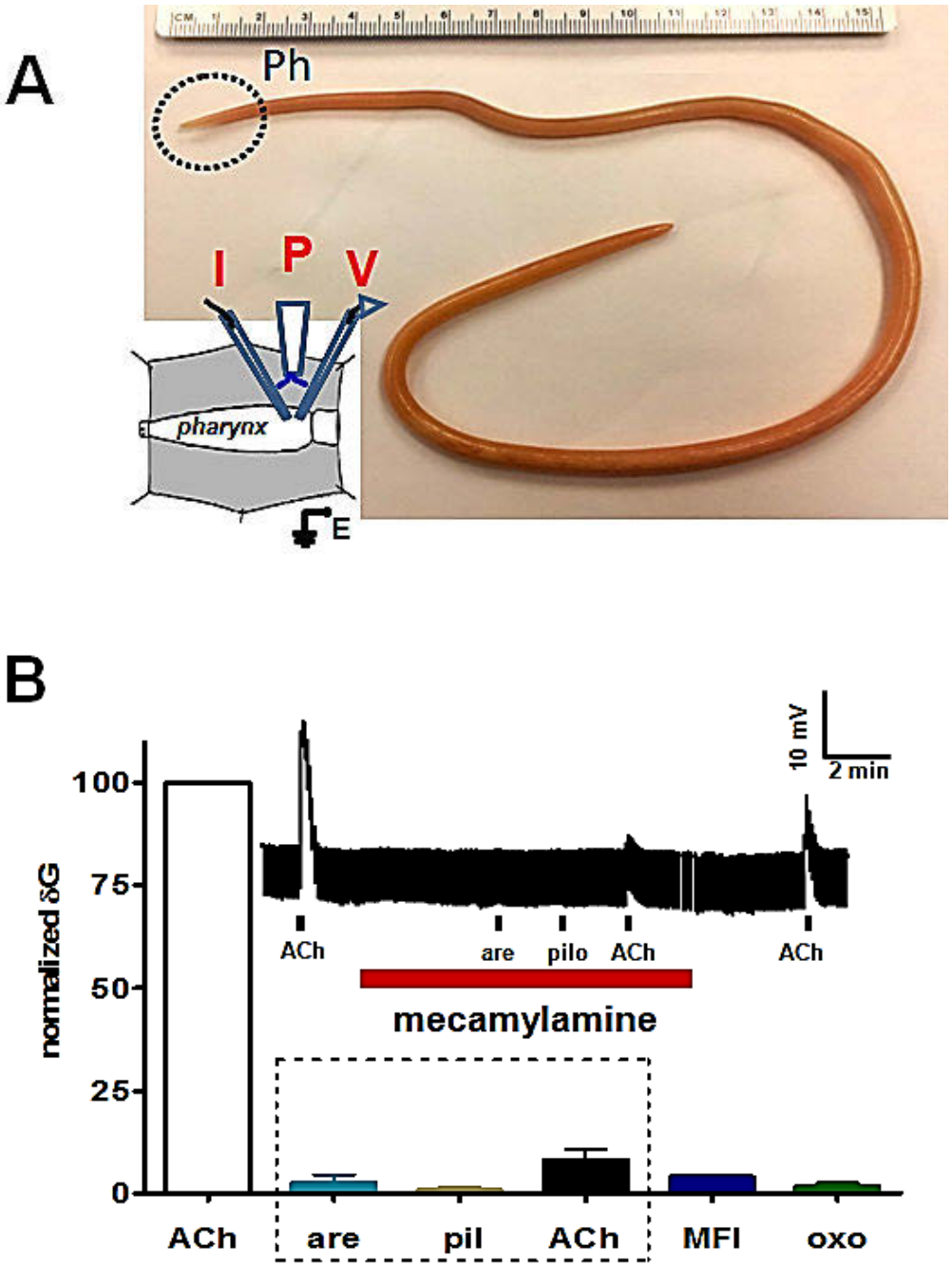

Figure 1. A. A photograph of female *A .suum* showing the head region, where the pharynx (Ph) is located. A diagram showing current clamp setup for making recordings from the posterior region of pharynx **I**; current injecting electrode **V**;

voltage recording electrode, **P**; perfusion needle for localized perfusion of Ca^{2+} free APF Ringer or drugs **E**; earth electrode. **B.** Representative current clamp trace as an inset to the bar graph showing the normalized δG response to ACh and muscarinic agonists. Arecoline, pilocarpine and ACh applications in the presence of mecamylamine to inhibit nAChR responses and to observe only muscarinic receptor responses.

5.4.2 Pharyngeal nAChRs are not activated by existing cholinomimetic anthelmintics

Fig 2 shows the rank potency series of cholinergic anthelmintics on the *A. suum* pharynx. Our pharyngeal preparations in these experiments had a resting membrane potential of -19.3 ± 1.1 mV and a resting conductance of 150.5 ± 11.9 μS (n=21) (mean ± SE). We used selected cholinergic anthelmintics and ACh at 100 μM for 10s. Tribendimidine was used at 30 μM due to solubility limitations. The normalized δG responses (mean ± SE, %) were bephenium [7.2 ± 3.5 (n=5)], thenium [6.1 ± 1.6 (n=4)], levamisole [1.8 ± 0.6(n=4)], morantel [0.3 ± 0.3 (n=4)], pyrantel [0 ± 0 (n=4)], oxantel [0 ± 0 (n=4)], methyridine [0 ± 0 (n=4)] and tribendimidine [0 ± 0 (n=4)] (Fig 2).

The rank order potency series for cholinergic anthelmintics on the *A. suum* pharynx is ACh > bephenium > thenium > levamisole ~ morantel ~ pyrantel ~ oxantel ~ methyridine ~ tribendimidine (Fig 2). Pharyngeal nAChRs differ pharmacologically

from the somatic muscle nAChRs of the worm. The rank order potency series of cholinergic anthelmintics on pharynx, and the somatic muscle nAChRs of the worm are shown in inset table (Martin *et al*., 2004; Trailovic *et al*., 2008).

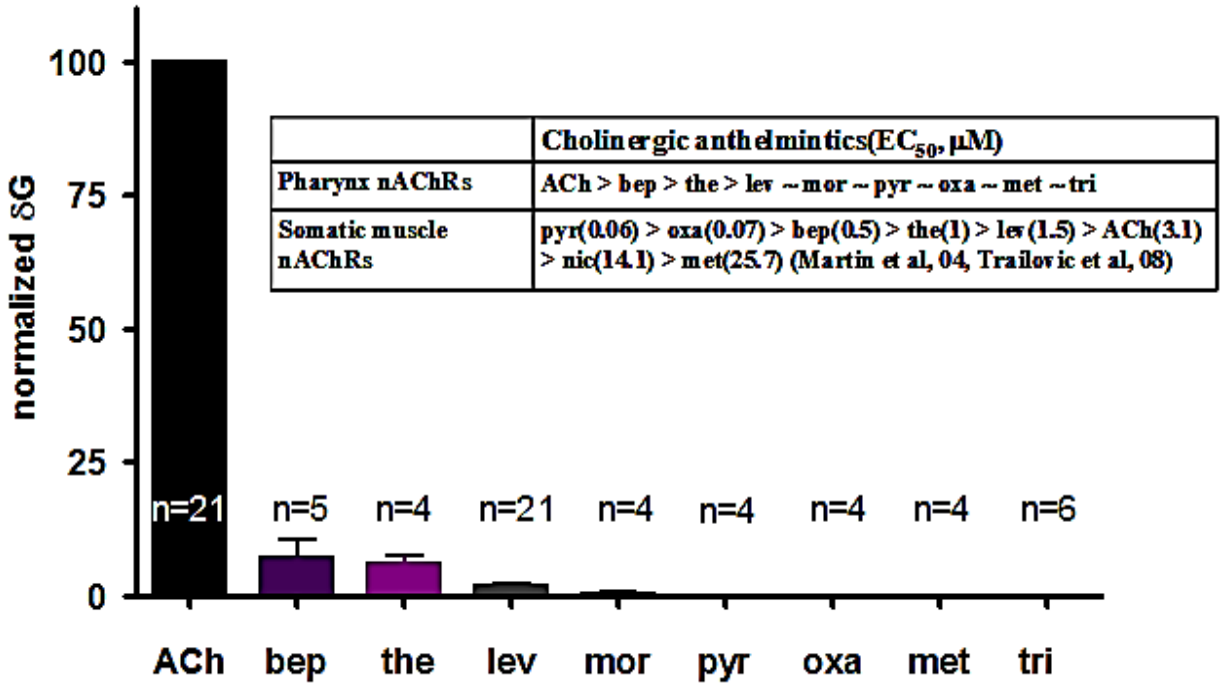

Figure 2. Bar graph showing the rank order potency series of selected cholinergic anthelmintics producing % change in membrane conductance on *A. suum* pharynx. **Table (inset**) comparing the rank order potency of cholinergic anthelmintics on pharynx and somatic muscle of *A. suum.* The pharyngeal nAChRs differ pharmacologically from the nAChRs of somatic muscle of the worm.

5.4.3 Rank order potency series for selected vertebrate nicotinic agonists show pharyngeal nAChRs are pharmacologically distinct

Three subtypes of nAChRs (N-, L- and B-) have been characterized and studied at the single channel level on the somatic muscle of *A. suum* (Qian *et al*., 2006). The N-subtype was preferentially activated by nicotine, oxantel and methyridine. Similarly the L-subtype was activated by levamisole and pyrantel, while the B-subtype was activated by bephenium. The N-subtype agonist sensitivity was distinguished from L or B subtype by the antagonists paraherquamide and derquantel (Martin *et al*., 2004; Qian *et al*., 2006).

In this study we pharmacologically characterized the nAChRs of the *A. suum* pharynx. We determined whether the pharyngeal nAChRs are pharmacologically distinct from the somatic muscle nAChRs of the worm. Our pharyngeal preparations in this group had a resting membrane potential of -21.3 ± 1.3 mV and a resting conductance of 136.4 ± 14.9 μS (n=13) (mean ± SE). We applied ACh and selected nicotinic agonists at 100μM for 10s. The δG responses to test applications of selected nicotinic agonists were normalized to the ACh δG. The normalized δG responses (mean ± SE, %) produced to selected nicotinic agonists were: nicotine [91.9 ± 6.2 (n=9)], cytisine [71.2 ± 5.0 (n=4)], epibatidine [30.5 ± 3 (n=7)], DMPP [11.8 ± 2.9 (n=9)] and choline [0 ± 0 (n=4)] (Fig 3). From these studies, the rank order potency series for vertebrate nicotinic agonists on the *A. suum* pharynx nAChRs was, ACh > nicotine > cytisine > epibatidine > DMPP > choline (Fig 3). The

rank order potency series of selected nicotinic agonists on the pharynx differs from that of somatic muscle nAChRs and that of the vertebrate host nAChRs (Table 1).

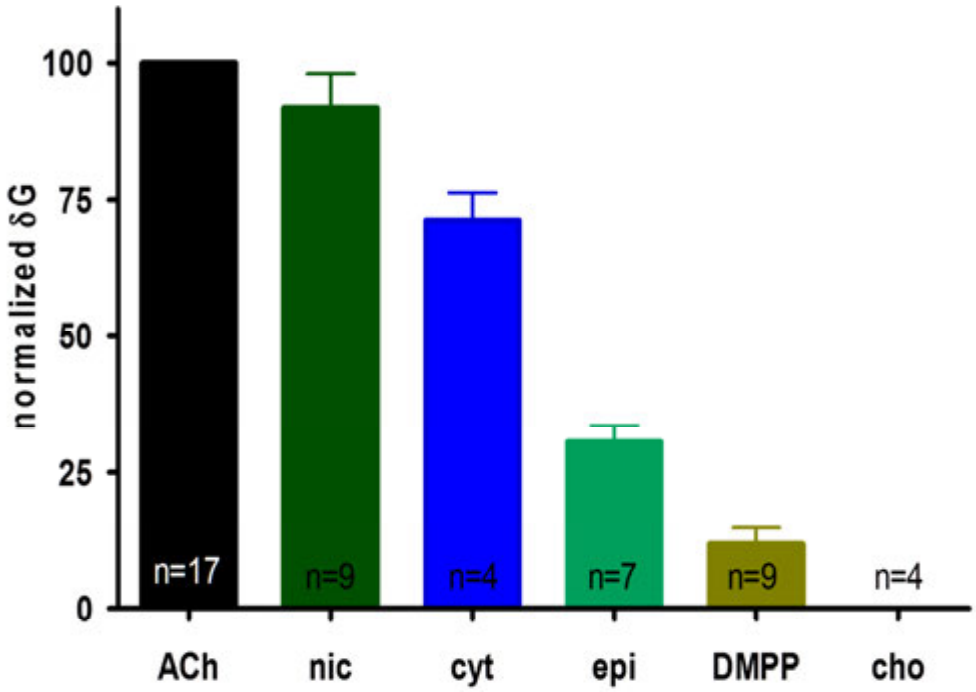

Figure 3. Bar graph showing the rank order potency of selected vertebrate nAChR agonists producing % change in membrane conductance in pharynx. The responses to agonists were normalized to δG response to control ACh application.

5.4.4 Rank order potency series for selected vertebrate nicotinic antagonists confirms pharyngeal nAChRs are pharmacologically distinct

Our pharyngeal preparations in this group had a resting membrane potential of -20.2 ± 1.1 mV and a resting conductance of 129.2 ± 6.8 μS (mean ± SE, n=34). We

used selected nicotinic antagonists (all at 30μM) to inhibit 100μM ACh responses (10s ACh applications). The δG produced by a control application of ACh (100μM) was set as 100%. We calculated the % inhibition of the δG response to ACh by nicotinic antagonists to determine a rank order potency series. The % inhibition of the ACh (100μM) δG response by selected nicotinic antagonists were (mean ± SE, %): d-tubocurarine [94.6 ± 0.2 (n=4)], mecamylamine [92.2 ± 1.9 (n=9)], methyllycaconitine [62.6 ± 3.7 (n=5)], paraharquamide [37.2 ± 8.7 (n=4)], derquantel (derq) [30.6 ± 7.0 (n=4)], hexamethonium (hexa) [26.8 ± 1.9 (n=4)] and dihydro-β-erythroidine (DHβE) [17.9 ± 5.0 (n=4)].

The rank order potency series for nicotinic antagonists on the *A. suum* pharynx nAChRs was, d-tc > mec > MLA > para > derq > hex > DHβE (Fig 4). The rank order potency series of nicotinic receptor antagonists on the pharynx differs from that of vertebrate nAChRs (Table 1).

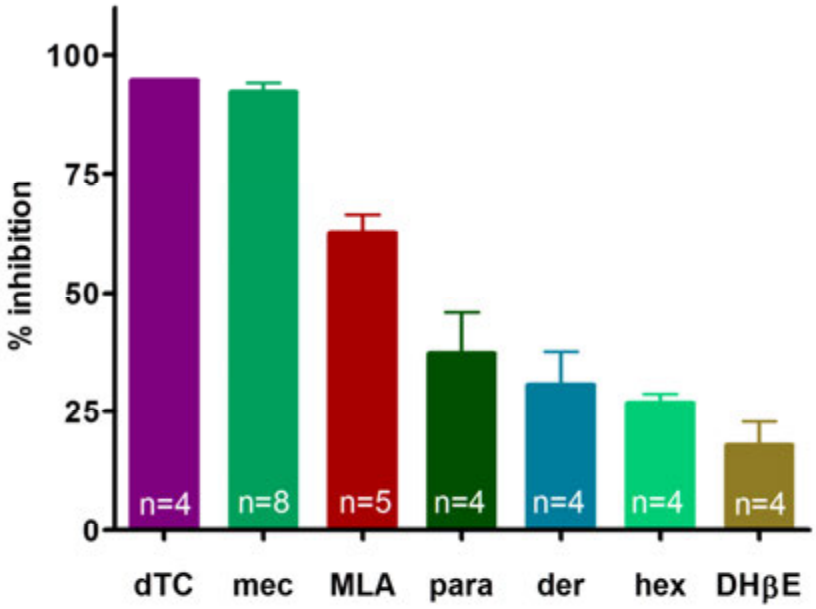

Figure 4. Bar graph showing the rank order potency of selected vertebrate nAChR antagonists producing % inhibition of ACh membrane conductance in pharynx.

Table 1. Rank order potencies of nAChR agonists and antagonists in *Ascaris* pharyngeal nAChRs observed from our study, *Ascaris* somatic muscle nAChRs and nAChRs of the vertebrate hosts (Harrow and Gration, 1985; Colquhoun *et al*., 1991; Buisson *et al*., 1996; Virginio *et al*., 2002; Wannacott, 2007; Trailovic *et al*., 2008).

			Nicotinic agonists	Nicotinic antagonists
Ascaris	**Pharynx nAChRs**		**ACh > nic > cyti > epibat > DMPP > chol** **ACh > beph > then > lev ≅ mora ≅ pyr ≅ oxa ≅ methy ≅ trib**	**d-tc > mec > MLA >para >derq> hexa > DHβE**
	Somatic muscle nAChRs		DMPP > ACh > nic (Colquhoun et al, 91) mora = pyrantel > levamisole > ACh (Harrow and Gration, 85) pyr >oxa >beph >then >lev >ACh> nic >methy (Martin et al, 04, Trailovic et al, 08)	mec > d-tc > hexa > DHβE (Colquhoun et al, 91)
Vertebrate	**Skeletal muscle nAChRs**	**Adult**	cyti > DMPP > nic (Yost and Winegar, 97)	
	CNS /neuronal nAChRs	**α4β2**	epibat > cyti > DMPP > nic > ACh (Wannacot, 07)	DHβE > MLA (Buisson et al, 96)
		α3β4	epibat > cyti > DMPP > nic > ACh (Wannacot, 07)	mec > d-tc > DHβE>hexa (Wannacot, 07)
		α7	cyti >nic>ACh>choline (Virginio et al, 02)	MLA > mec > DHβE (Virginio et al, 02)

5.4.5. Effects of nAChR antagonists on concentration response curves of acetylcholine and nicotine.

We determined the concentration response curves by plotting the concentration of agonists (1-1000μM, applied for 10s) against the response (δG) produced. The response (δG) produced during the increasing concentations of agonist was normalized to the response (δG) produced by 100 μM ACh (applied for 10s) within each experiment. Fig 5 A shows the concentration response curves for ACh and nicotine. The EC_{50} of ACh and nicotine were 9.5 μM (n=6)and 11.6 μM (n=8). The maximal response of ACh was 103.7 ± 2.4 μS and nicotine was 79 ± 3.9 μS. Nicotine reached about 80% maximal response of ACh.

We determined concentration response of ACh in the presence of nicotinic receptor antagonists. The nicotinic receptor antagonists chosen from the rank order potency series (Fig 4) were d-tubocurarine (d-tc) (10μM), methyllycaconitine (MLA) (10μM), paraherquamide (para) (10μM) and dihydro-β-erythroidine (DHβE) (30μM). The response (δG) produced during the application of agonists alone or agonist in the presence of antagonist were normalized to the response (δG) produced by 100 μM ACh (applied for 10s) within each experiment.

Fig 5 B shows the panel of nAChR antagonists d-tc, MLA and para on ACh concentration response. The EC_{50} of ACh concentration response was 9.5 μM (n=6). The EC_{50} values of ACh were 9.8 μM (n=8) in the presence of MLA (10 μM), 12.9 μM (n=3) in the presence of d-tc (10 μM) and 8.3 μM (n=3) in the presence of

paraherquamide (10 μM). The $logEC_{50}$ of the ACh concentration response curve did not significantly differ in the presence of methyllycaconitine ($p>0.05$, ESS F-test), d-tubocurarine ($p>0.05$, ESS F-test) or paraherquamide. We found that the maximal response for ACh was inhibited. The maximal response (δG) (mean ± SE, μS) for the control ACh concentration response curve was 103.7 ± 2.4 μS. The maximum response (δG) was reduced to 57 ± 4.6 μS in the presence of MLA, 23 ± 3 μS in the presence of d-tc and 87.7 ± 5.7 μS in the presence of paraherquamide. For methyllycaconitine, d-tubocurarine and paraherquamide the unchanged EC_{50} and reduced maximum suggests these compounds acted as non-competitive antagonists on the ACh receptor.

Fig 5 C and D shows ACh and nicotine concentration responses in the presence of dihydro-β-erythroidine (DHβE). On ACh concentration responses, DHβE (at 30 μM) produced a right shift in the EC_{50} without changing the slope of the curve (Fig 5C). The EC_{50} for the ACh concentration response curve was 9.5 μM (n=6) and in the presence of DHβE was 16.5 μM (n=7). The $logEC_{50}$ for the ACh concentration response curve was significantly different in the presence of DHβE [$p=0.001$, extra sum of squares (ESS) F-test]. The change in EC_{50} produced by DHβE was suggestive of competitive antagonism.

Fig 5.D shows the effect of DHβE (30 μM) on the nicotine concentration responses Interestingly, DHβE (30 μM) had no significant effect on the $logEC_{50}$ ($p>0.05$, ESS F-test), the slope of the curve ($p>0.05$, ESS F-test) or the maximal response

($p>0.05$, ESS F-test). The EC_{50} value for the nicotine concentration response was 11.6 μM and in the presence of DHβE was 9.8 μM. The maximal response (δG) (mean± SE, μS) produced to nicotine was 79 ± 3.9 μS and in the presence of DHβE was 73.6 ± 2.9 μS.

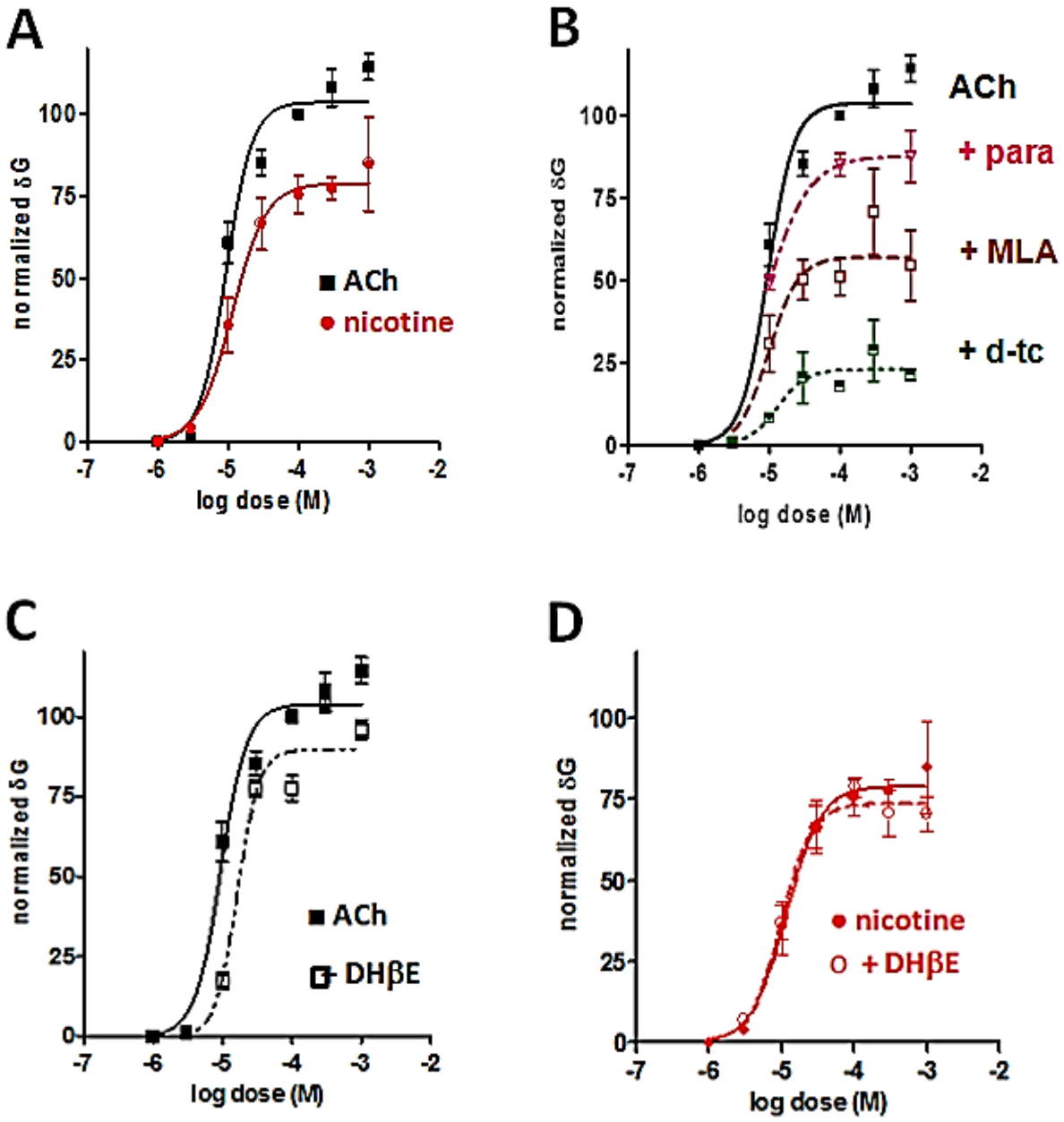

Figure 5. Concentration conductance plots for **A.** Concentration conductance plots for ACh and nicotine plotting % change in conductance vs log molar concentration of the drugs. **B.** Concentration conductance plots of ACh and ACh +nAChR antagonists; paraharquamide (par), methyllycaconitine (MLA) and d-tubocurarine

(d-tc). These antagonists behaved like non-competitive antagonists on ACh concentration response. **C**. ACh and ACh+30 μM DHβE **D.** nicotine and nicotine+30μM DHβE. The graphs clearly demonstrate that DHβE competitively antagonized the ACh concentration response but not the nicotine response, demonstrating the presence of more than one nAChR subtype.

5.4.6 Evidence suggesting multiple nAChR subtypes in *A. suum* pharynx

From our present study in *A. suum* pharynx, the maximal response (δG) to nicotine was about 80% of the maximal response (δG) produced by ACh. Suggesting that about 20% of the ACh response (δG) is from a nicotine insensitive component. In addition, dihydro-β-erythroidine (DHβE) produced a competitive antagonism of the ACh concentration response while, it did not antagonize the nicotine concentration response. These observations demonstrate the presence of more than one nAChR subtype in *A. suum* pharynx (Fig.6). These are 1) nicotine sensitive nAChRs and 2) nicotine insensitive nAChRs. Acetylcholine activates both the nicotine sensitive and nicotine insensitive subtypes of nAChR. DHβE antagonizes the nicotine insensitive subpopulation of nAChR but not the nicotine sensitive subtype of nAChRs.

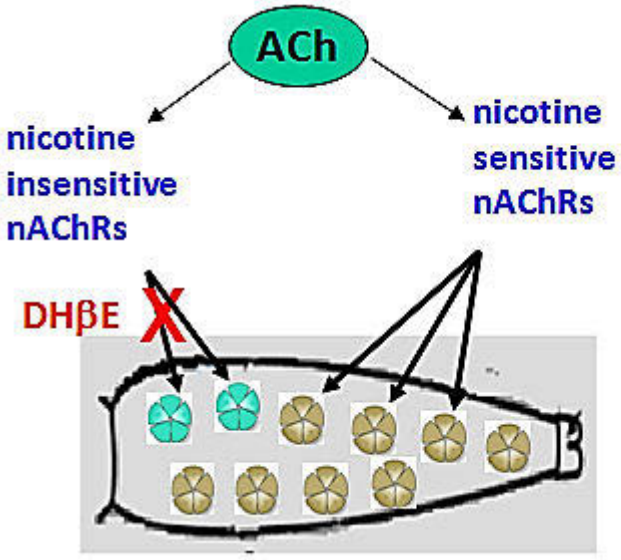

Fig. 6. Model describing the nAChR population on the pharynx. A majority includes nicotine sensitive nAChRs and a minor population includes nicotine insensitive nAChRs. The nicotine insensitve nAChRs are antagonized by dihydro-beta-erythroidine.

5.5 Discussion

5.5.1. Ion channels of the worms as anthelmintic target

Ion channels on parasitic worms have been attractive targets for selective targeting and development of anthelmintic drugs. Some of these ion channels exploited as drug targets are nAChRs, GABA gated chloride channels (on the somatic muscle), and glutamate gated chloride channels (on the pharynx). Unlike anthelmintics like benzimidazoles that produce a gradual effect by interfering with parasite physiology, drugs acting on membrane ion-channels of the nematode have a rapid onset (less

than 4 hours). The anthelmintics can act on the membrane ion-channels of the parasites as agonists (mimicking natural ligands) or antagonists (competing with the natural ligands) or allosteric modulators of ion-channels. On the parasite somatic muscle, nicotinic acetylcholine receptors (nAChRs) and GABA gated chloride channels are present. The endogenous neurotransmitters namely ACh and GABA produce excitation and inhibition of somatic muscle to facilitate locomotion of the worm. Cholinomimetic anthelmintics include imidazothiazoles (levamisole, tetramisole); tetrahydropyrimidines (pyrantel, morantel & oxantel); quaternary/tertiary amines (bephenium, thenium and tribendimidine) and pyridines (methyridine). Cholinomimetic anthelmintics mimic ACh when they act on nAChRs of the somatic muscle of the worm. Unlike ACh, these cholinomimetic anthelmintics are not acted upon by cholinesterase causing persistent activation of nAChRs resulting in spastic paralysis (Martin and Robertson, 2007). Derquantel acts as competitive antagonist of parasite somatic muscle nAChRs (Robertson *et al*., 2002; Zinser *et al*., 2002). The anthelmintics that act as antagonist of nAChRs produce flaccid paralysis of the worms. Piperazine mimics the natural ligand GABA and activates GABA gated chloride channels on the somatic muscle to cause hyperpolarization. The hyperpolarization of the somatic muscle of the parasite results in flaccid paralysis.

Glutamate gated chloride channels are present on the pharyngeal muscle of the nematodes. Glutamate is the endogenous ligand for activating glutamate gated chloride channels. The activation of glutamate gated chloride channels produce

hyperpolarization and inhibit the pharyngeal pumping. The macrocyclic lactones allosterically modulate glutamate gated chloride channels to potentiate endogenous glutamate responses causing paralysis of the pharynx. The macrocyclic lactones include ivermectin, doramectin, abamectin and moxidectin. The anthelmintic effect of macrocyclic lactones is due to inhibition of pharyngeal pumping resulting in starvation of the worms (Wolstenholme and Rogers, 2005b). Currently, there are reports of resistance to drugs belonging to all major groups of anthelmintics in major livestock producing areas (Kaplan, 2004b).

5.5.2. nAChRs of the worm pharynx: a novel anthelmintic target

In our present study, we describe the nematode pharynx nAChRs as a potential pharmacological target for developing new anthelmintic drugs. The pharyngeal muscles contain nAChRs at the neuromuscular synapses. The pharynx of the nematode is a muscular pumping organ necessary for feeding. Pharyngeal peristalsis is required for ingestion of food, while digestive enzymes secreted by the pharynx aid in digestion. Pharyngeal peristalsis is coordinated by excitatory (cholinergic) (Raizen *et al*., 1995) and inhibitory (glutamatergic) (Brownlee *et al*., 1997) motor neuronal inputs on to pharyngeal muscles. Drugs like the avermectins modulate glutamate gated chloride channels to inhibit pharyngeal function. These drugs potentiate the response to glutamate to produce hyperpolarization and inhibition of pharyngeal peristalsis in worms (Martin, 1996; Brownlee *et al*., 1997). Currently, resistance has been reported to avermectins in livestock parasites

(Kaplan, 2004b). An alternative approach to impair pharyngeal peristalsis in worms is by excessive activation of pharyngeal muscle nAChRs. A selective drug mimicking ACh will persistently activate pharyngeal nAChRs. An excessive activation of somatic muscle nAChRs by cholinergic anthelmintics is known to produce spastic paralysis in worms (Barragry, 1984). As pharyngeal peristalsis is a coordinated event of excitation and relaxation of pharyngeal muscles, an excessive activation of nAChRs will result in spastic paralysis of the pharynx. A paralyzed pharynx impairs feeding in worms resulting in starvation (Wolstenholme and Rogers, 2005b). The limited literature prompted us to investigate pharyngeal nAChRs of the nematodes. We used the pharynx of *A. suum* as a model and pharmacologically characterized these pharynx nAChRs. In this study we have shown that pharyngeal nAChRs are pharmacologically distinct from the nAChRs of somatic muscle of *Ascaris.* Further, we determined that pharynx nAChRs are pharmacologically distinct from that of nAChRs of the vertebrate host.

5.5.3. Electrophysiological studies on the nAChRs of the parasitic worms

Electrophysiological studies have helped us understand the mode of action of the cholinergic anthelmintics in parasitic worms. The studies have revealed that cholinergic anthelmintics act as agonists on distinct subtypes of nAChRs (N, L and B subtypes) in the somatic muscle of *A. suum* (Qian *et al*., 2006). The cholinergic anthelmintics like oxantel and methyridine are selective towards nicotine sensitive N-subtypes of nAChRs. Pyrantel preferentially activates levamisole sensitive

subtype (L-subtype) while, bephenium preferentially activate the B-subtype of nAChRs. Similar studies on the somatic muscle of *C. elegans* have confirmed presence of pharmacologically distinct subtypes (N- and L-subtype) of nAChRs (Richmond and Jorgensen, 1999a; Qian et al., 2008). Our studies on pharynx have revealed at least two subtypes of nAChRs. Nicotine sensitive nAChRs formed a major population while nicotine insensitive nAChRs formed a minor population. Dihydro-β-erythroidine distinguished these two nAChR subtypes on the pharynx. Dihydro-β-erythroidine had no effect on the nicotine sensitive nAChRs while competitively antagonized a minor population of nicotine insensitive nAChRs.

Some of the predicted mechanisms of resistance to cholinergic anthelmintics have been proposed. It is thought to involve a loss of drug sensitive nAChR subtypes on the somatic muscle of the parasite. In addition, other predicted mechanism of resistance also involve intracellular pathways downstream of nAChR activation meditated by ryanodine receptors (Puttachary *et al*.). Due to a limited number of therapeutic agents in the market (James *et al*., 2009) and existence of anthelmintic resistance worldwide, there is an urgency to drug development efforts and the need to explore new drug targets.

5.5.4. Novel drug targets to counter anthelmintic resistance in parasites

The nicotinic receptors of the worm are still attractive targets for developing anthelmintic drugs. This is in light of new anthelmintic drugs that have been

released in to the market namely derquantel and monepantel. Unlike derquantel which acts as antagonist of somatic muscle nAChRs of worms, monepantel acts as positive allosteric modulator of DEG3/DES2 subfamily of *C. elegans* nAChRs found on the mechanosensory neurons of the head region and interneurons of the ventral nerve cord (Treinin *et al*., 1998; Rufener *et al*., 2010). In the midst of wide spread reports of anthelmintic resistance in farm animals, after release of ivermectin (in mid 1980s), there were no new anthelmintics on the market for more than two decades (Geary, 2005). In addition to two new anthelmintic drugs in the market, exploring potential drug candidates to target nAChRs of the pharynx will prove beneficial to counter emerging anthelmintic resistance. Preliminary, semi-quantitative PCR studies suggest that the *Asu-acr-21* gene may contribute to the mature pharyngeal nAChR. Our current studies are focusing on identifying other genes involved in receptor formation and expression of the pharyngeal nAChR in *Xenopus* oocytes as a platform for drug screening.

CHAPTER 6. GENERAL DISCUSSION

My present research address different approaches employed to counter anthelmintic resistance. The research in chapter 3 suggests a mechanism to increase potency of existing cholinomimetic anthelmintics like levamisole. In chapter 4, I have tested a novel anthelmintic combination from Pfizer released to control parasites which are resistant to anthelmintic drugs. In chapter 5, I have explored a novel drug target, the pharyngeal nAChRs in the parasitic nematode *A. suum.* Pharyngeal nAChRs can be screened for developing novel anthelmintics that can be used to control resistant parasites.

The background work for Chapter 3 was extending the observations of Trailovic *et al* (2005) and Verma *et al* (2007) on the AF2 receptor responses in somatic muscle of *A. suum.* My research on the AF2 receptor activation suggests that similar to the potentiation of ACh responses we can also potentiate the responses of cholinergic anthelmintics like levamisole. AF2 receptor activation did not potentiate the amplitude of levamisole response as observed by Trailovic *et al* (2005) on the ACh response. Rather, the potentiation of the levamisole response was on the persistence of its action upon AF2 treatment. After AF2 treatment, the duration of the secondary response to levamisole significantly increased compared to the control. The potentiation of the levamisole response after AF2 treatment was dependent on extracellular a calcium and chloride suggesting the involvement of a calcium dependent chloride channel. The potentiation of levamisole response was also dependent on the elevation of cytosolic calcium mediated by ryanodine receptors. These observations suggest that the potency of cholinergic anthelmintics

can be increased by activating neuropeptide receptors. Further, developing new anthelmintic drugs which activate the AF2 receptor is expected to produce synergy with the actions of existing cholinergic anthelmintics. Future work involving, calcium imaging can help us to observe the elevation of cytosolic calcium after AF2 receptor activation. Second messengers (cAMP or calcium levels) assays are also important as they can reveal the mechanisms involved after AF2 receptor activation until the potentiation of nAChR responses.

In Chapter 4, I have tested the interactions of derquantel and abamectin released into the market as a novel anthelmintic combination Startect®. Anthelmintic combinations are generally useful to control parasites which have developed resistance to one or more groups of anthelmintic drugs. The inhibitory actions of Derquantel on the ACh concentration responses suggested a competitive antagonism. The slow inhibitory actions of abamectin on the ACh concentration responses suggested a non-competitive antagonism. The effects of the combination of derquantel and abamectin were greater than the predicted addictive effect on the ACh concentration responses. The combination of derquantel and abamectin is predicted to be greater than the responses of individual drugs on the somatic muscle of *A. suum.*

On the pharynx of *A. suum,* abamectin produced a profound increase in the membrane conductance but, derquantel produced no significant effects. The conductance response of the combination was not significantly different from the response of abamectin. We did not observe any significant interaction of the abamectin and derquantel on the pharynx of *A. suum.* We observed that the

combination of derquantel and abamectin was favorable due to their effects on two important targets on the parasitic worm. On the somatic muscle we observed a greater than additive effect of the combination and on pharynx, abamectin alone produced a profound response.

In chapter 5, I have pharmacologically characterized nAChRs of the pharynx that are unexploited drug targets. In addition, my studies infer that the pharyngeal nAChRs are pharmacologically distinct from the known nAChRs of the somatic muscle the worm or the vertebrate host. The currently available cholinergic anthelmintics do not act on these receptors implying these receptors are novel drug targets. The distinct pharmacology of pharyngeal nAChRs is an advantage for selective targeting of parasites with minimal side effects to the vertebrate host. Future studies for the identification of subunit genes responsible for forming the mature pharyngeal nAChRs are important. Identification of subunits genes helps us to express the pharyngeal nAChRs in *Xenopus* oocytes for high throughput screening for potential drug candidates. Expression studies can also help us to observe the pharyngeal nAChRs at the single channel level using patch clamp. Expression of nematode pharyngeal nAChRs in *Xenopus* can reveal the receptor combinations and pharmacology to make comparisons with the native receptors of the pharynx of the parasitic nematode *A. suum* and others.

APPENDIX 1. ELECTROPHYSIOLOGICAL RECORDING FROM PARASITIC NEMATODE MUSCLE

[1]Modified from a paper published in *Invertebrate Neuroscience* (2008) 8: 167-175.

Alan P. Robertson[2, 3], Sreekanth Puttachary[3], Samuel K Buxton[3], Richard J Martin[3]

1.1 Abstract

Infection of man and animals with parasitic nematodes is recognized as a significant global problem (McLeod, 1994; Hotez et al., 2007b). At present control of these infections relies primarily on chemotherapy. There are a limited number of classes of anthelmintic compounds and the majority of these acts on ion-channels of the parasite (Martin *et al*., 1996a). In this report, we describe electrophysiological recording techniques as applied to parasitic nematodes. The aim of this report is: (1) to promote the study of ion channels in nematodes to help further the understanding of antinematodal drug action; (2) to describe our recording equipment and experimental protocols; and (3) provide some examples of the information to be gleaned from this approach and how it can increase our understanding of these important pathogens.

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1.2. Introduction

Nematode infections are a significant problem in both human (Hotez *et al.,* 2007) and veterinary medicine (McLeod, 1994). Chemotherapy is widely used for treating these infections. The range of drugs available for treatment is limited and repeated large scale use has led to the development of drug resistance in numerous parasite species (Kaplan, 2004a). It is anticipated that the problem of drug resistance will get worse particularly since only one new class of anthelmintic has come to market recently (emodepside). A Consortium on Anthelmintic Resistance SNPS (CARS) has been set up to monitor drug resistance and advance molecular methods for detecting resistance (http:// consortium.mine.nu/cars/pmwiki.php/Main/HomePage).

The majority of anthelmintic compounds act on the neuromuscular system of the worm, for review see Robertson and Martin (2007). As with any excitable system, ion-channels are central to nematode neuromuscular signaling and function. Here we review the methods we have used to study ion-channels on nematode muscle that are either potential or actual target sites of new and existing compounds. The current anthelmintics that act on nematode ion-channels include: the avermectins/milbemycins which act on glutamate-gated chloride channels and/or GABA channels; the nicotinic anthelmintics (pyrantel, etc.) gate non-selective cation channels (nicotinic acetylcholine receptors). However, our understanding of the receptors activated during the therapeutic response is incomplete. In addition, there are many other ion-channels (peptide-gated, potassium and calcium selective channels) that may have critical roles for neuromuscular function in the nematode.

Here we describe the electrophysiological methods we have used to examine nematode ion channels. These techniques are widely used by biologists to study channels in almost every living system and are not specific to our approach. We give details of methods of how we use them to study parasitic nematode ion channels. Our aim is to encourage others to study this important but overlooked field. This report is not intended as an introduction to electrophysiology. It is intended to highlight the small alterations in methodology required to adapt these classical electrophysiological techniques to study currents and channels in parasitic nematodes.

1.3 Methods

1.3.1 Nematode tissue

Successful electrophysiological studies require a regular supply of live, viable parasite tissue (Fig. 1). This, in itself, is a common limiting experimental step: many parasitic species cannot easily be maintained for long periods *in vitro*. We have found *Ascaris suum* can be obtained from the local abattoir, although the ease of collection (related to the incidence of infection in the local swine population) appears to be somewhat seasonal. Adult worms remain viable for 4–7 days when kept at 30–35_C in Locke's solution (mM): NaCl 155; KCl 5; CaCl2 2; NaHCO3 1.5; D-glucose 5. It is possible, though significantly more labor and cost intensive, to maintain experimental infections of different parasite species; the

Oesophagostomum dentatum life-cycle can be successfully maintained by passage through pigs (the native host) and will also yield useful adult worms on euthanasia of the hog. An additional benefit of using laboratory infections is the possibility of maintaining specific isolates, e.g. drug resistant isolates that have less genetic diversity than sampling the wild population. Obtaining viable material from other parasite species (e.g. human pathogens) can be more problematic and may necessitate the studies to be carried out on non-adult life cycle stages or even expression of the ion channel of interest in a heterologous system, e.g. *Xenopus laevis* oocytes.

1.3.2 Dissection

Ascaris are large worms and the dissection needed to expose the muscle cells for recording is simple. A ~1 cm section of the worm is cut from the anterior region of the parasite. The resulting tube is then cut along one of the lateral lines and pinned onto a Sylgard lined recording chamber cuticle side down. The gut is easily removed using fine forceps to expose muscle bags. With smaller nematodes the same approach can be applied but this time using the whole length of the worm. For adult *O. dentatum*, the entire worm (~1 cm) is pinned into the chamber (head and tail only) and then cut along a lateral line using a scalpel. The gut and reproductive tissue can then be removed and the preparation pinned out further to reveal the somatic muscle cells. A similar approach has been developed for electrophysiological recording from the muscle cells in *Caenorhabditis elegans*

(Richmond and Jorgensen, 1999b). For *C. elegans*, the small size of the worms means that the pins have been replaced by cyanoacrylate glue, but the principles of sticking the worm down, cutting it open, removing the gut and reproductive tissue and producing a ‘‘flap’’ or ‘‘filleted worm’’ remain the same. Thus, electrophysiological techniques have been applied to worms from ~30 cm to less than 1 mm in size. It should be noted, however, that as the worm size decreases the technical difficulty of the dissection increases substantially.

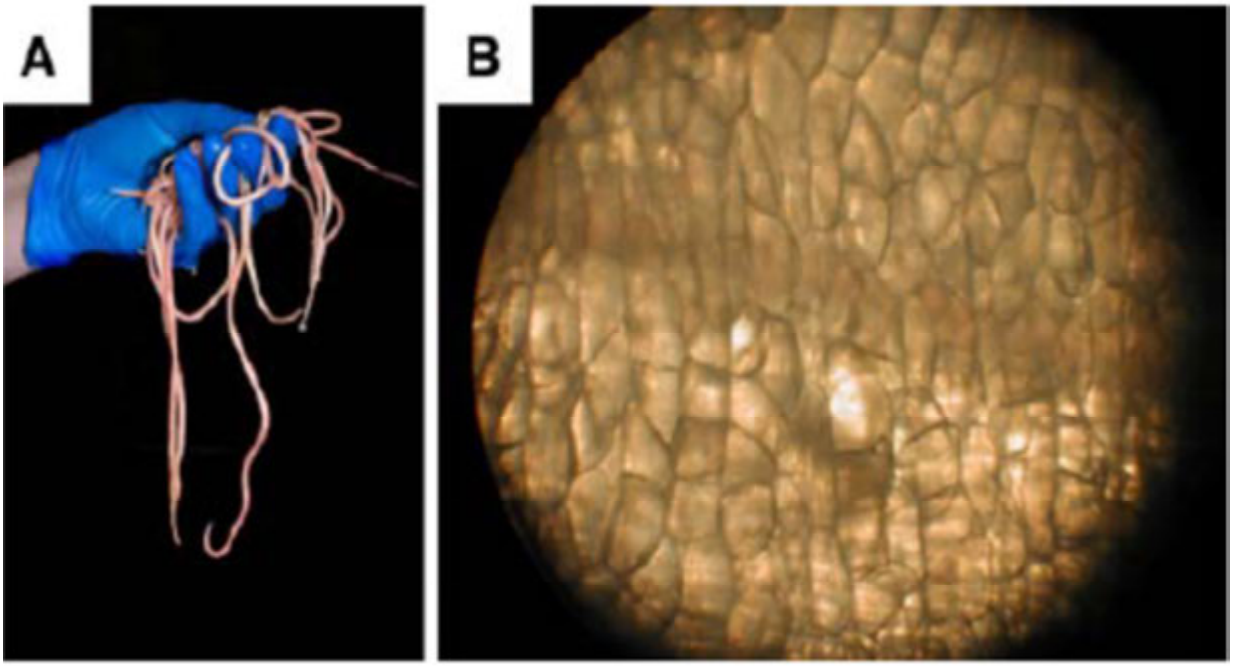

Figure 1. A. Photograph of adult *Ascaris suum*; **B.** photograph of muscle flap preparation showing muscle cell bags (~200 µlm diameter) suitable for two-electrode recording techniques. The faint horizontal line is the ventral nerve cord in this preparation.

1.3.3 Two electrode current-clamp

The large size of many nematode cells makes them amenable for study using classical two-electrode recording techniques. The electrophysiology ‘‘rig’’ used for both current-clamp and voltage-clamp experiments is essentially identical (Fig. 2). The electronic components are: a current/voltage amplifier (Axoclamp 2A or 2B); a digitizer to convert the amplified signals from analog to digital format (Digidata 1320A/1322A) and a computer for running the data acquisition software. The computer software (Clampex v8 or v9, Axon Instruments) not only acquires the data but can be used to control the perfusion system and command the amplifier to inject current or voltage through either electrode. The tissue is perfused by a system controlled by six valves, a computer and a Warner VC-6 valve controller. The incoming perfusate is warmed to the desired temperature by a Warner SH-27B inline heater controlled by a Warner TC-324B heater controller. The preparation is viewed using a Stereo zoom dissecting microscope (Bausch & Lomb) and a fiber optic light source. The tissue is mounted in a Sylgard lined Perspex chamber (custom made) surrounded by a water jacket to maintain temperature. The water jacket is perfused with warm water using a heated water pump (Isotemp 301b, Fisher Scientific). Microelectrodes are mounted on the amplifier head stages and maneuvered into position using a Leica micromanipulator.

For current-clamp experiments we pull microelectrodes using standard walled borosilicate glass with filament, o.d. 1.5 mm, i.d. 0.86 mm (G150F-6, Warner Instruments). Microelectrodes are fabricated using a Flaming/Brown horizontal

electrode puller (Model P-97, Sutter Instruments) and are typically pulled to a resistance of 20–30 MΩ. The filament allows easy backfilling of the electrodes with the relevant solution, typically for current-clamp this is 3 M potassium acetate. The recording chamber is mounted on a nitrogen supported anti-vibration table (TMC Corp.) to minimize mechanical noise. A Faraday cage (TMC Corp.) surrounds the recording chamber to reduce electrical noise. Microelectrodes are positioned directly over the cell to be recorded from. The muscle cell is carefully impaled with both electrodes. Typically resting membrane potentials are in the range -25 to -40 mV for somatic muscle cells in *Ascaris*. The current injecting protocol is then applied through one microelectrode (I_m, Fig. 2D); our standard protocol is 0.5 s pulses of -40 nA current at a frequency of 0.25 Hz. Another microelectrode (V_m, Fig. 2D) can then be used to monitor the membrane potential and also the input conductance of the cell (typically 1–3 μS). The signal is filtered at 0.3 kHz, digitized and stored on the computer hard drive for later analysis. The effect of perfused drugs can then be monitored. It is possible to record for ~1 h from a single cell in a healthy preparation. Our basic recording solution, *Ascaris* Perienteric Fluid (APF) consists of NaCl (23 mM), Na-acetate (110 mM), KCl (24 mM), CaCl2 (6 mM), MgCl2 (5 mM), glucose (11 mM), HEPES (5 mM), pH 7.6, adjusted with NaOH, and can be modified when necessary to determine the ionic basis of drug effects.

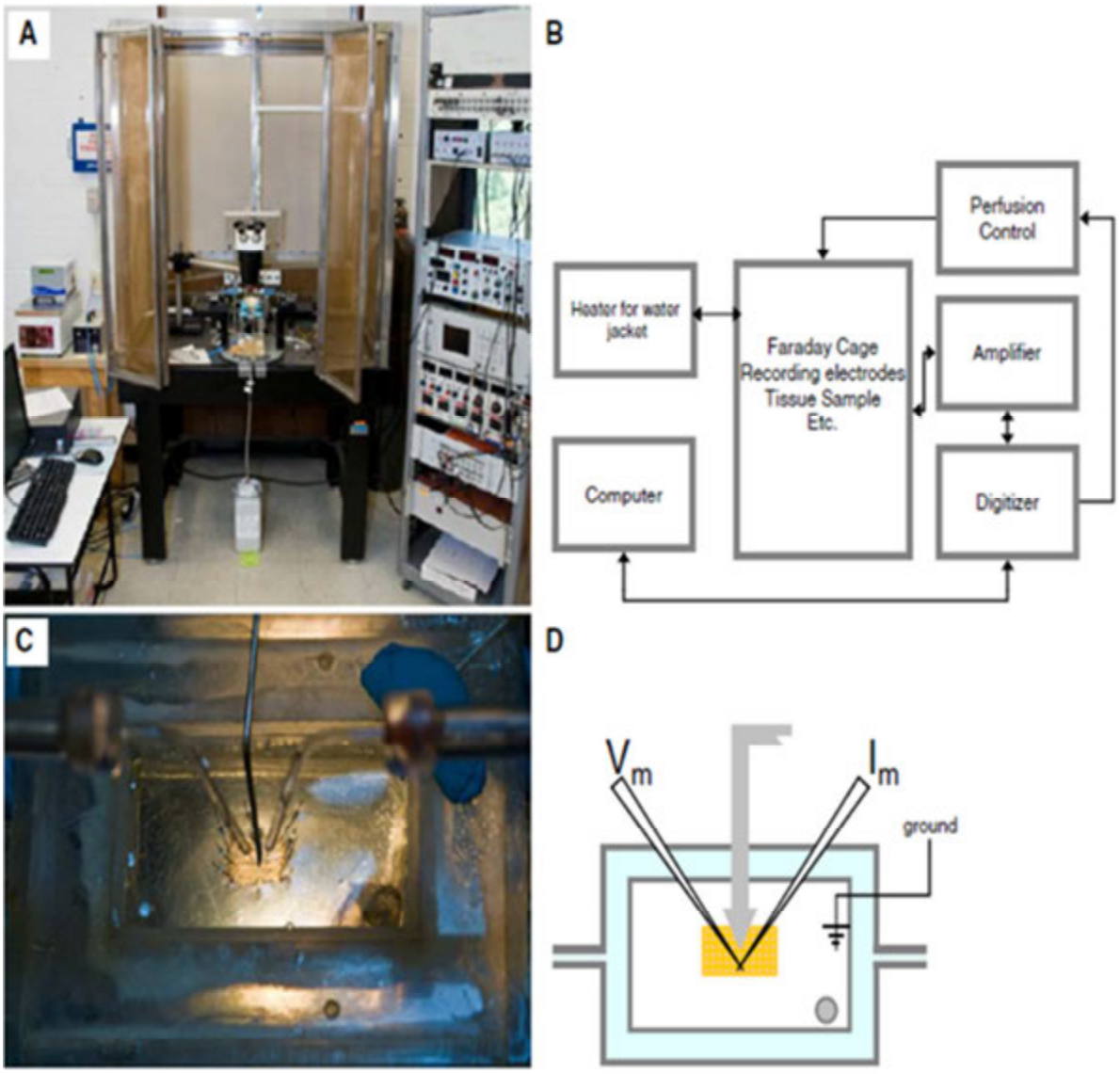

Figure 2. A. Photograph; **B.** diagram of two electrode current-clamp ‘‘rig’’; **C.** photograph and **D.** diagram of the recording chamber for current-clamp experiments. The muscle flap is clearly seen with both microelectrodes visible. The perfusate is applied via a 20-gauge needle (gray arrow in diagram) and excess removed by gravity through the outflow on the bottom right of the photograph and diagram (gray circle).

1.3.4 Two electrode voltage-clamp

The electrophysiology ‘‘rig’’ for two-electrode voltage clamp is identical to that used for current-clamp experiments. However, there are some small but significant changes required to perform successful voltage-clamp experiments. Firstly, in electrode manufacture, the large size of the *Ascaris* muscle cell means that space clamp is quite poor. The injection of the large currents required to effect the desired voltage change requires a lower resistance current injecting electrode. Typically for voltage-clamp experiments we use a current injecting electrode (I_m) with a resistance of 2–5 MX. This is easily achieved by carefully breaking the tip of a standard current-clamp electrode using a piece of tissue paper. The voltage sensing electrode (V_m) is a standard current-clamp electrode.

Secondly, in voltage-clamp experiments, it is desirable to investigate the current flow through specific ion channel types or currents carried by individual ion species, e.g. outward K currents or inward Ca currents. To this end it is desirable to eliminate, as much as possible, currents carried by other ions and channels. Traditionally, this is achieved by either elimination/substitution of ions (other than the ion of interest) from recording solutions or by pharmacological block of other channel types present. For example, to record voltage activated inward calcium currents we have added cesium to the pipette filling solution (intracellular Cs blocks potassium currents, electrode fill solution is 1.5 M Cesium acetate + 1.5 M potassium acetate) and 4-amino pyridine (4-AP) to the bathing solution (4-AP is a selective blocker of K channels). Conversely, we have found that voltage-activated

outward potassium currents are more easily studied when calcium is substituted for magnesium in the bathing medium, thus eliminating voltage activated inward calcium currents. It is also possible to isolate a current of interest by varying the voltage changes applied to the cell.

Isolating and optimizing the current to be studied is often the most demanding and time consuming aspect of these experiments. Unfortunately, parasitic nematodes are not the most widely studied group of organisms and drugs that affect ion channels in other preparations have been found to be inactive or significantly less active on *Ascaris* muscle cells. For example, the calcium channel blocker verapamil is frequently used to eliminate certain types of calcium current in vertebrate preparations, thus facilitating the study of other current types. Unfortunately, in *Ascaris* verapamil has no significant effect on voltage gated inward currents.

1.3.5 Single-channel patch-clamp

The majority of parasitic nematode cell types we have worked with are too large to render whole cell patch-clamp recording a viable option; so we use two-electrode techniques. Whole cell patch recording has been successfully developed for investigating the muscle cells of *C. elegans* (Richmond and Jorgensen, 1999a) and is not described further here. It should be noted, however, that this approach may be suitable for the study of smaller nematode cells where impalement with two sharp electrodes is not possible. It is possible to use the patch-clamp technique to

measure the properties of individual ion channel molecules. This ‘‘single-channel’’ patch recording technique is relatively straightforward using parasitic nematode muscle cells. The principle of this technique is the electrical isolation of a small ‘‘patch’’ of membrane containing one (or very few) ion channel molecules. Then conventional voltage protocols are applied to the membrane patch and the opening and closing of the single channel molecule can be measured.

The principles behind this technique are straightforward but in practice this is probably the most technically demanding compared to the other approaches outlined in this review. For single-channel recording from nematode muscle we use an anti-vibration table and Faraday cage (TMC Corp.) as in the current-clamp ‘‘rigs’’. The amplifier is an Axopatch 200B (Axon Instruments) connected to a PC (Dell) via a digitizer (Digidata 1320A/1322A) and controlled by Clampex (v8 or v9) data acquisition software (Axon Instruments). Nematode muscle cells or muscle cell derived vesicles are held in a recording chamber (Warner Instruments) and viewed through a Nikon TE2000 inverted light microscope at 9400 magnification. Vesicles are easily viewed under normal light but small *C. elegans* muscle cells are best viewed using DIC optics. The amplifier head stage and microelectrode are positioned using a Narishige (MHW-3, Narishige Inc.) hydraulic micromanipulator.

Microelectrodes for patch clamp studies are pulled from thin walled glass capillaries, o.d. 1.5 mm, i.d. 1.16 mm with no filament (G85150T-3, Warner Instruments) using a two stage vertical electrode puller (models PP-830 or PC-10, Narishige Inc.). Electrodes are coated close to the tip with Sylgard to improve frequency responses

and fire polished (MF-900 micro-forge, Narishige Instruments) to the desired resistance, typically 2–5 MX.

A major requirement for successful patch-clamp experiments is the formation of a high resistance seal (>1 GΩ, a giga seal) between the glass microelectrode and the cell membrane. Giga seal formation requires clean debris free membranes, which are reasonably common in cells in tissue culture but less so in intact tissue. *Ascaris* and other nematodes have a large amount of collagen overlying the muscle cell preventing giga seal formation. This must be removed by enzyme treatment using collagenase (type 1A, Sigma). Collagenase treatment removes the collagen matrix and allows access of the patch pipette to clean muscle cell membranes. One result of collagenase treatment is the ‘‘budding’’ off of clean membrane vesicles from the bag region of the muscle cells. By applying the patch clamp technique Martin *et al*. (1990) discovered that these membrane vesicles contain functional ion-channels. We have successfully applied this method to record ion channels from vesicles originating from *O. dentatum* muscle cells (Robertson et al., 1999b). Details of vesicle preparation and recording protocols are given below.

Ascaris were dissected and a muscle flap was prepared and pinned cuticle side down onto a plastic dish lined with Sylgard. The muscle flap preparation was washed with maintenance solution to remove fragments of the gut. Maintenance solution is (in mM): 35 NaCl, 105 sodium acetate, 2.0 KCl, 2.0 $MgCl_2$, 10 HEPES, 3.0 D-glucose, 2.0 ascorbic acid, 1.0 EGTA, pH 7.2 with NaOH. The maintenance solution was then replaced with collagenase solution. Collagenase solution is

maintenance solution without EGTA and with 1 mg/ml collagenase Type 1A added (Sigma). After collagenase treatment for 4–8 min at 37°C, the muscle preparation was washed (5–10 times) and incubated in maintenance solution at 37°C for 20–40 min. Small membranous vesicles, 10–50 lm in diameter, grew out from the membrane of the muscle cells. These membranous vesicles are transferred to a recording chamber using a glass Pasteur pipette. For *O. dentatum* the vesicle preparation protocol is unchanged, however, the yield of vesicles is significantly less due to the smaller size of the parasite. We have found that vesicle yield and quality can vary significantly between batches of worms and worms of different size. As a guide, smaller worms require less collagenase treatment than larger ones. Prolonged collagenase treatment yields an abundance of vesicles but they are more fragile and rapidly become unusable. Shorter collagenase treatment yields fewer vesicles but they are generally more robust. For worms as small as *C. elegans* the collagen matrix is significantly less of a problem and collagenase treatments of 0.5 mg/ml for 5–10 s are adequate to clean the muscle cell membrane and allow seal formation directly from the body wall muscle cells. Finally, we have found that collagenase from different suppliers or even different batches from the same supplier can affect the quality of vesicles produced.

Vesicles are placed in the recording chamber and patch experiments are carried out in the isolated inside out patch configuration. Achieving the outside-out patch configuration is considerably more difficult when using membrane vesicles as they tend to implode when rupturing the patch membrane. The recording conditions for

studying nAChR channels are given below. Voltage protocols and solution recipes can be altered depending on the ion-channel to be studied.

The pipette was filled with pipette solution containing (mM): CsCl, 140; $MgCl_2$, 2; HEPES, 10; EGTA, 1; pH 7.2 with CsOH. The pipette solution also contained the agonist (levamisole, acetylcholine, etc.) at the desired concentration. The bathing solution was (mM): CsCl, 35; Cs acetate, 105; $MgCl_2$, 2; HEPES, 10; EGTA, 1; pH 7.2 with CsOH. As in other voltage clamp experiments, it is desirable to isolate the specific ion-channel of interest. To this end the bathing solutions contained symmetrical Cs as it permeates the nAChR but blocks potassium channels. The chloride concentration was asymmetrical to identify contaminating chloride channels by their non-zero reversal potentials on later analysis. Calcium is absent from the solutions to prevent contamination of the recordings with Ca-dependent chloride channel openings. Typically for ligand-gated ion channels we record for approximately 1 min at several different holding potentials between -100 and +100 mV (normally, -100, -75, -50, +50, +75 and +100 mV). Membrane breakdown is common at both -100 and +100 mV. In some preparations, we have found that addition of 0.5 mM dithiothreitol helps to stabilize the membrane at more extreme potentials (Robertson *et al*. 1999). Recordings are viewed in real time by filtering at 2.5 kHz (8-pole Besel filter, custom made) and viewing on a digital storage oscilloscope (Hitachi VC-6025). The recordings are also filtered by the amplifier (5 KHz, Besel filter) digitized and stored on the PC for later analysis.

As with all recordings made using the above methods the data generated is suitable for analysis using standard methods. In the case of nAChR single-channel currents we normally calculate the single-channel conductance, mean open-time, mean closed-times and the probability of the channel being in the open state (P_{open}). Other more complex single-channel analysis is possible but beyond the scope of this manuscript.

1.4 Results

Examples of the type of data available from each of our experimental approaches are given below. The data in this section was obtained from *Ascaris* somatic muscle.

1.4.1 Illustrative results using two electrode current-clamp

Figure 3 is a current-clamp recording from *Ascaris* somatic muscle. Figure 3A is a low time resolution display covering approximately 30 min. The blue arrow (dark gray) indicates the resting membrane potential of the cell (-37 mV in this experiment). The red arrow (light gray) shows the voltage response to the -40 nA injected current pulses. The size of the voltage response is inversely related to the input conductance of the cell. The size of the response increases as the conductance decreases (when ion channels close) and vice versa. Figure 3A clearly demonstrates that levamisole application induces a rapid depolarization. When the

trace is examined in more detail (Fig. 3B, C) the effect on the cell's conductance also becomes apparent. In Fig. 3B, C, the red arrow (light gray) again represents the response to injected current and the blue arrow (dark gray) this time represents the depolarization induced by levamisole. In Fig. 3B the depolarization induced by levamisole (blue arrow, dark gray) is clearly seen. Levamisole is an agonist of the nicotinic acetylcholine receptor (nAChR) ion channel; application of the drug causes these channels to open and cations to enter the cell thus causing the depolarization. The opening of the ion channels causes an increase in input conductance during the depolarization. The red arrow highlights the voltage response to injected current and at the peak of the depolarization this response is reduced, reflecting the conductance increase due to nAChR opening. Figure 3B is the levamisole response after a 2 min application of the neuropeptide AF2 (1 μM). It is apparent that both the levamisole induced depolarization (blue arrow) and conductance change are substantially increased by treatment with this peptide. Figure 3A also demonstrates that AF2 treatment prolongs the recovery time after levamisole treatment.

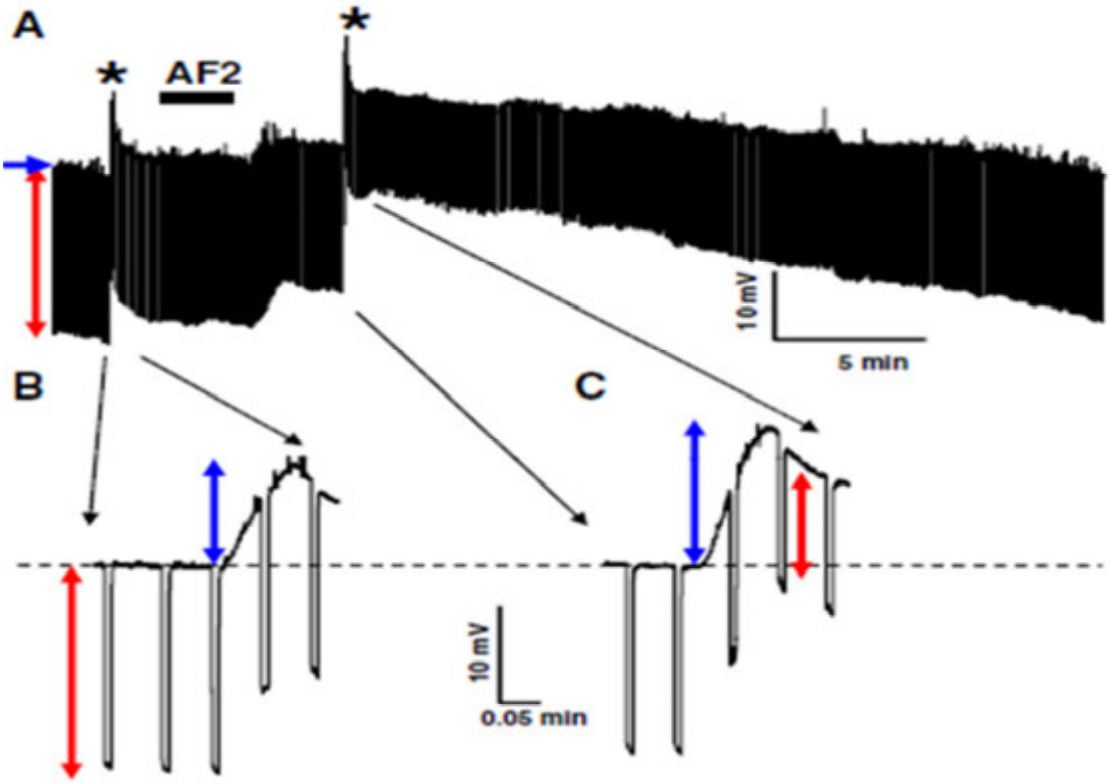

Figure 3. A. Low time resolution current-clamp trace illustrating the effects of 20 s applications (asterisk) of 1 μM levamisole (4 ml/min flow rate) before and after treatment of the muscle flap with 1 μM AF2 (a nematode FMRF-related neuropeptide). Blue arrow illustrates the resting membrane potential while the red arrow illustrates the size of the voltage response to -40 nA injected current. Levamisole induces an obvious depolarization of the cell; **B**, **C** higher time resolution view of sections of the recording in **A**. Red arrow (light gray) illustrates the voltage response to injected current and blue arrow (dark gray) illustrates the amplitude of levamisole induced depolarization. It can be clearly seen that both the depolarization and conductance change in response to levamisole are larger after AF2 treatment **C** than before **B**.

1.4.2 Illustrative results using two electrode voltage-clamp

A sample experiment using two electrode voltage-clamp recording on *Ascaris* muscle is shown in Fig. 4. In this experiment, we have isolated the voltage gated potassium currents and examined the effects of the potassium channel blocker 4-amino pyridine (4-AP). To study the potassium currents in isolation we have replaced calcium (a permeant ion) in our recording solutions with the same concentration of magnesium (an impermeant ion) to remove the voltage activated inward currents carried by calcium. Figure 4A are the outward currents carried by potassium in response to 40 ms step voltage changes in the holding potential of the cell. In this instance, the cell was held at -35 mV and stepped to -25, -20, -15, -10, -5, 0, 5, 10, 15, and 20 mV. The same voltage step protocol was applied in the presence of 5 mM 4-AP (Fig. 4B) which substantially reduced the amplitude of the outward potassium currents.

After a 30-min wash period the currents had partially recovered (Fig. 4C). The maximum current at each voltage step was plotted (Fig. 4D) and clearly shows the inhibitory effect of 4-AP and this effect was partially reversible.

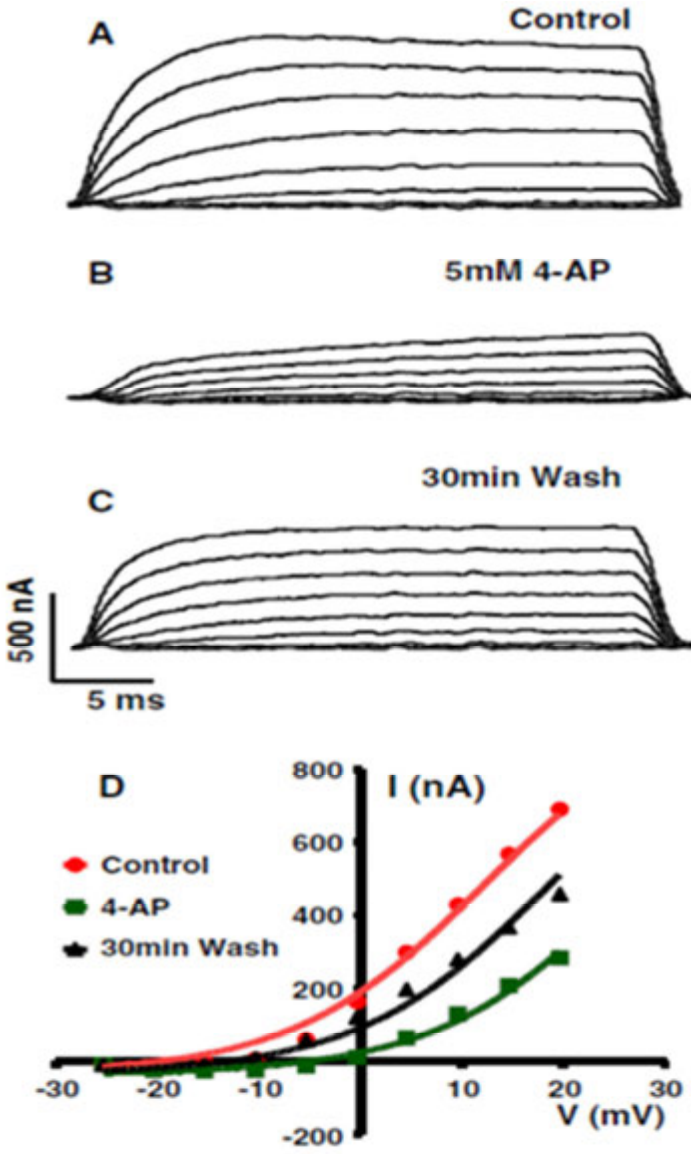

Figure 4 Voltage activated potassium currents from Ascaris muscle bags recorded under two electrode voltage-clamp; **A.** under control conditions (Ca free APF solution); **B.** during application of 5 mM 4-amino pyridine (4-AP); and **C,** after 30 min wash in calcium free APF solution; **D.** current–voltage relationship for the

recordings in A–C clearly showing the inhibitory effect of 4-AP and that it is partially reversed on washing.

1.4.3 Illustrative results using single-channel patch-clamp

A sample of a recording from an *Ascaris* muscle derived vesicle is shown in Fig. 5A. The isolated inside-out patch was held at +75 mV and the patch pipette contained 30 μM levamisole. Rectangular channel openings are clearly visible ranging from ~2 to 4 pA in size and ~0.3 to 10 ms in duration. In this experiment, there are openings to more than one level indicating the presence of multiple subtypes of nAChR present in this isolated patch of membrane. In Fig. 5B, we plotted an amplitude histogram of all openings in the recording and fitted with Gaussian distributions to calculate the mean amplitude for each of the three peaks. By using multiple agonists, concentrations and antagonists we have been able to characterize three subtypes of nAChR on *Ascaris* muscle cells that have different single-channel and pharmacological properties. Figure 6 is a summary diagram of these findings where N-type refers to nicotine preferring subtype of nAChR, L-type refers to a levamisole preferring subtype of nAChR and B-type refers to a bephenium preferring subtype of nAChR.

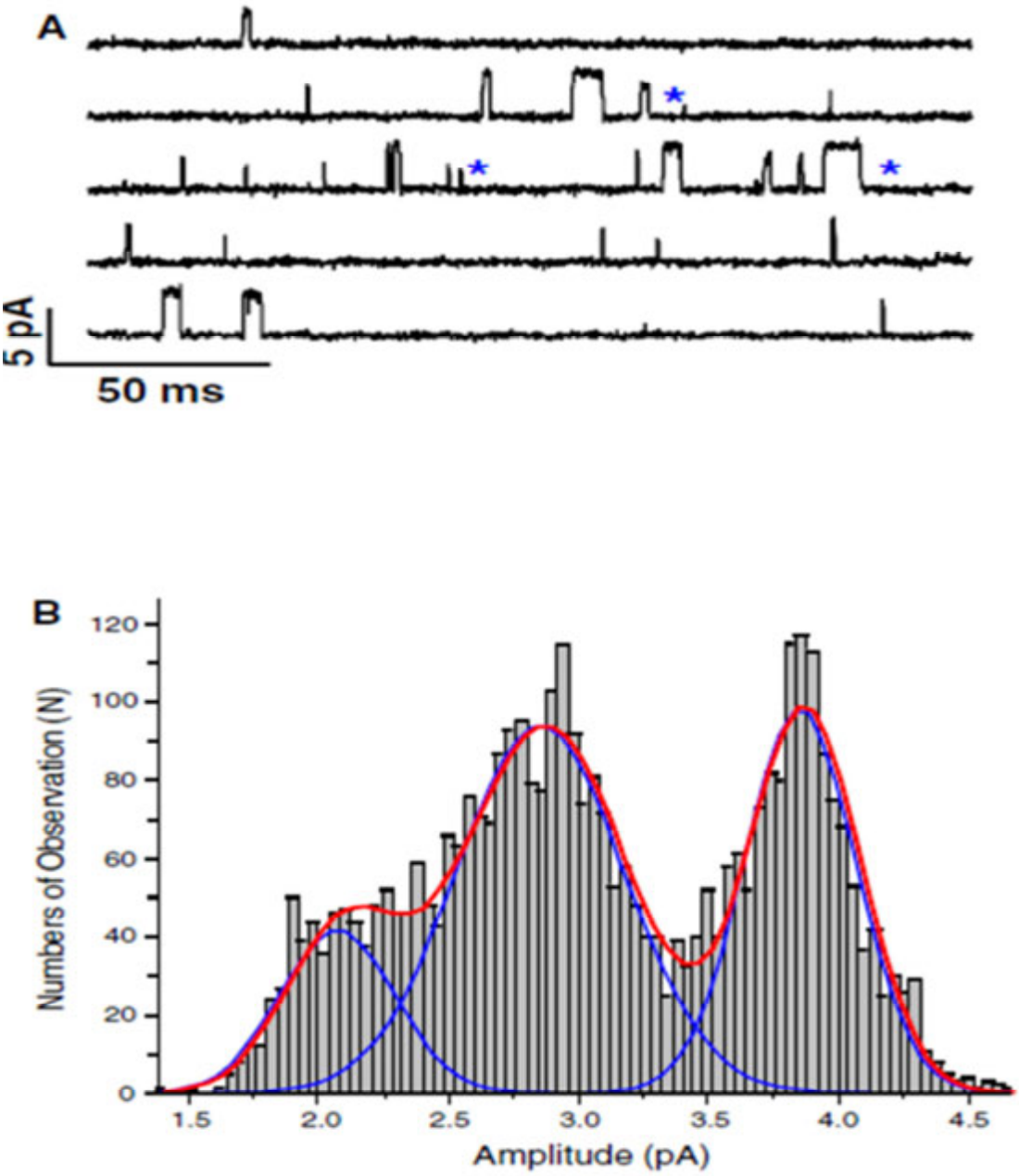

Figure 5 A. Sample of a single-channel recording from a membrane patch of Ascaris muscle vesicle held at +75 mV. Discrete single-channel openings are visible as rectangular current pulses of ~2 – 4 pA. Blue asterisk highlight the presence of three separable open levels and therefore three different ion channel molecules in this membrane patch; **B.** histogram of all channel openings from the recording

illustrated in **A**. Three separable peaks are obvious and have been fitted using Gaussian distributions to determine the amplitude of channel opening at +75 mV for each channel type.

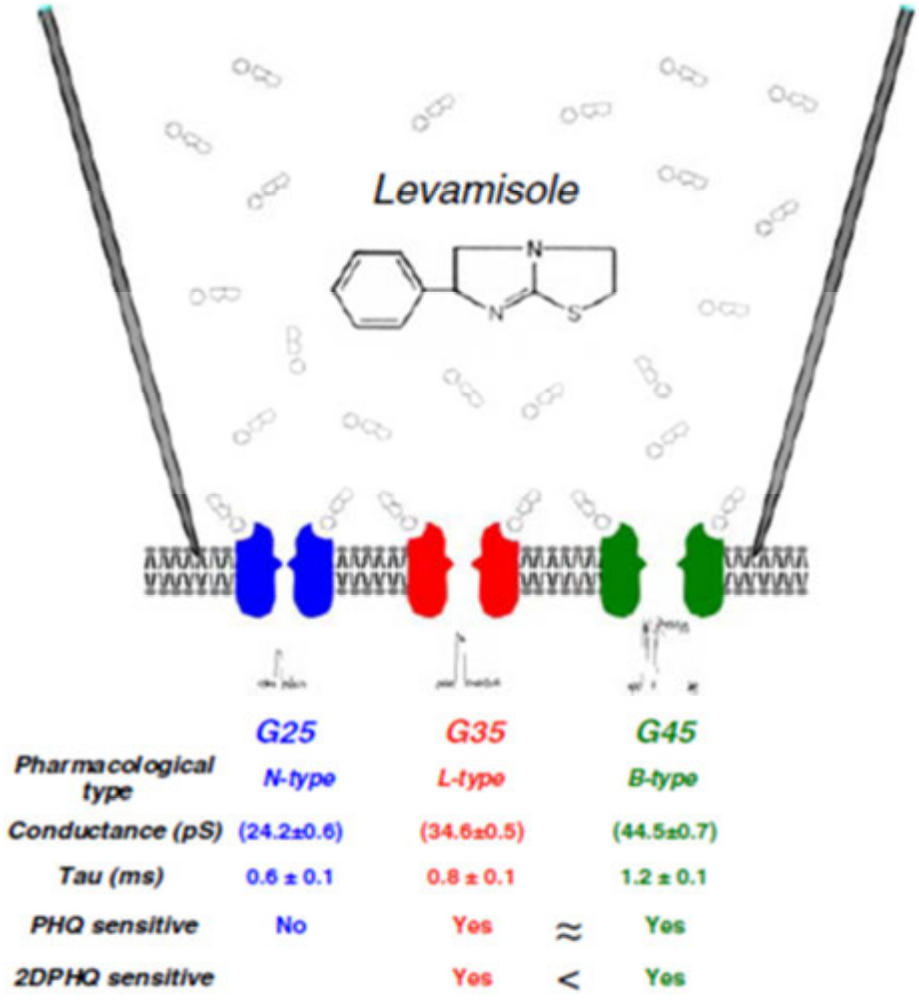

Figure 6 Summary diagram representing a membrane patch containing the three nAChR subtypes present on Ascaris muscle with some of their single-channel and pharmacological properties illustrated.

1.5 Discussion

The development of electrophysiological methods has taken ~50 years to mature. Early studies concentrated on large easily observable cells that were easy to impale, e.g. the squid giant axon. Interestingly, *Ascaris suum* muscle cells were investigated as early as the 1950s (Jarman, 1959). As the techniques were refined the need for large cells decreased. Additionally, Brading and Caldwell (1971b) found that *Ascaris* had different properties to other more typical cell types. These developments possibly led to the conclusion that *Ascaris* was not necessarily a good model for general electrophysiology studies of cells and have thus restricted the amount of research carried out on this and other parasitic nematodes using electrophysiological techniques.

We have described some of the electrophysiological methods that can be used to study ion-channels in *Ascaris* and other nematodes. Included in the methods section are additional details that we have found important for successful studies, details that are seldom discussed at length in other publications due to space constraints. The aim of this report is to provide detailed information to facilitate the study of ion channels in parasitic nematodes by any interested researchers.

The importance of studying these parasite ion-channels is readily apparent. There are a number of groups of anthelmintic compound that act on channels in parasites. These include: the cholinomimetics (pyrantel, etc.) that act as agonists of nAChRs on muscle (Harrow and Gration, 1985); the avermectins are allosteric activators of glutamate-gated chloride channels in the pharynx (Wolstenholme and Rogers,

2005a) and/or GABA-gated chloride channels on muscle; piperazine an agonist of GABA-gated chloride channels on muscle (Martin, 1982b); emodepside is proposed to have an effect on potassium currents (Guest *et al*., 2007); and recently the amino acetonitrile derivatives (AADs) are proposed to be nAChR antagonists (Kaminsky *et al*., 2008).

We have detailed our approaches on nematode muscle. Several other groups have successfully used electrophysiological techniques in a variety of preparations including the musculature (Holden-Dye and Walker, 1990) to examine ion-channel properties, drug action and more basic biological questions. The pharynx of *Ascaris* has been investigated using whole cell current-clamp (Martin, 1996) and voltage-clamp (Byerly and Masuda, 1979a), while Adelsberger *et al*. (1997a) successfully developed vesicle production from the pharynx to make patch recordings of glutamate-gated chloride channels. The electrophysiological properties of parasite nerve cells have also been investigated in detail (Davis and Stretton, 1996). While more recent work on *C. elegans* has developed techniques for recording whole cell currents from body wall muscle (Richmond and Jorgensen, 1999a), single-channel recording of nAChRs from body wall muscle (Qian *et al*., 2008) and even electrical recording of pharyngeal activity (Cook *et al*., 2006).

1.6 Acknowledgments

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