STUDIES INTO THE FUNCTIONAL PROPERTIES OF THE PHARYNGEAL MUSCLE OF CAENORHABDITIS ELEGANS

By

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ABSTRACT

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STUDIES INTO THE FUNCTIONAL PROPERTIES OF THE PHARYNGEAL MUSCLE OF CAENORHABDITIS ELEGANS

Darrel John Pemberton

Caen*orhabditis elegans* is a well described nematode employed as a genetic model organism for studies into function, differentiation, development, and morphology of simple nervous and muscular systems. However, essential information regarding its physiology and pharmacology are lacking. To address this, electrophysiological recording techniques from pharyngeal muscle cells were developed to determine the ionic basis of the resting membrane potential and action potential. Resting membrane potential of pharyngeal muscle cells were relatively unaffected by changes in the extracellular concentrations of Cl^-, Na^+ or Ca^{++}. However, variations in extracellular concentrations of K^+, or exposure to ouabain, both elicited a depolarisation, although the depolarisations observed during elevations of extracellular K^+ were less than would be predicted if the membrane potential were completely dependent on K^+. It can be concluded therefore, that the resting membrane potential is largely determined by a K^+ permeability, and a ouabain-sensitive, electrogenic pump. Action potential height was reduced or increased in concentration-dependent manner following exposure to low or high extracellular Ca^{++} concentrations respectively. Furthermore, the L-type Ca^{++} channel blockers, verapamil and nifedipine, both reduced action potential amplitude and duration. This suggests a role for an L-type Ca^{++} channel in the action potential. However, action potentials persisted in Ca^{++} free saline. Action potential duration increased or decreased in a concentration-dependent manner following exposure to low or high Ca^{++} concentrations respectively. This suggests that the repolarisation phase is partly determined by a Ca^{++} activated K^+ channel. Possibly the most surprising finding was the absolute dependence of pharyngeal action potential generation on extracellular Na^+ concentration, especially as extensive searches of the *C. elegans* genome have failed to find any obvious candidate for a voltage-gated Na^+ channel.

Responses to a number of classical neurotransmitters/modulators on pharyngeal action potentials were determined. The pharyngeal muscle responded to all of the classical neurotransmitters/modulators examined. Dopamine, serotonin, muscarine, and the peptides AF1 and AF2 generated increases in action potential frequency whilst acetylcholine, nicotine and glutamate produced concentration-dependent membrane depolarisations. As receptors for most of these ligands have been shown to be expressed in the muscle, it is likely these effects are due to their direct action upon on the muscle although an indirect action *via* the pharyngeal nervous system cannot be discounted.

A detailed pharmacological study was then performed to characterise native pharyngeal glutamate-gated chloride channels (GluCl). These channels were chosen for study as they are unique to the invertebrate phyla and are the site of action of the potent anthelmintic ivermectin. The pharyngeal response to glutamate was a rapidly desensitising, reversible, chloride-dependent depolarisation (EC_{50} = 166μM), which was only weakly antagonised by picrotoxin. It was estimated that the equilibrium potential for chloride (E_{Cl}) was −40 mV, which is significantly more positive than calculated values for parasitic nematodes. The order of potency for agonists was ibotenate (EC_{50} = 17.8μM) > glutamate > kainate = quisqualate, which is typical of the profile for invertebrate GluCl channels. Ivermectin potently and irreversibly depolarised the pharynx (EC_{50}=2.7 nM). No further depolarisation was observed with coapplication of ivermectin and maximal glutamate indicating that both agonists are likely to depolarise the muscle by the same ionic mechanism. The potency of ivermectin was greater than that reported for any of the GluCl channels expressed in *Xenopus* oocytes. The selective glutamate uptake blocker PDC (trans-4-carboxy-L-proline/L-trans-pyrrolodine-2,4-dicarboxylic acid) depolarised the muscle suggesting the presence of tonic glutamate release which may be potentiating the ivermectin response.

To determine which genes contribute to the function of the native GluCl channel, recordings were made from strains with loss-of-function mutations in specific GluCl channel subunits The effect of ivermectin was abolished in the mutant *avr-15*, which lacks a functional GluCl-α2 subunit. However, a chloride-dependent, non-desensitising glutamate and ibotenate response persisted although at a significantly reduced potency to that seen in wild-type. Therefore, the GluCl-α2 subunit confers ivermectin sensitivity and a high affinity response on the native muscle GluCl receptor. The potency of glutamate was also significantly reduced in the *avr-14* strain, which lacks a functional GluCl-α3 subunit. As this subunit has been reported to only be expressed outside the pharyngeal nervous system, immunocytochemistry was employed to check this. This indicated that GluCl-α3 is present in pharyngeal neurones, consistent with an indirect role for this subunit in mediating the effects of glutamate on the pharynx.

Overall, these studies have extended the utility of *C.elegans* as a model organism for investigating gene function and have delineated the role of GluCl channels in the action of the important anthelmintic ivermectin.

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Contents

Figures

Plates

Tables

Abbreviations

ACh	Acetylcholine
AChe	Acetylcholinesterase
AMPA	α-amino-3-hydroxl-5-methyl-4-isoxalone propionic acid
4-AP	4-Aminopyridine
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine Tetra-acetic Acid
GABA	γ-Aminobutyric Acid
GluCl	Glutamate-Gated Chloride Channel
5HT	5-Hydroxytrptamine
mRNA	Messenger Ribonucleic Acid
nAChR	Nicotinic Acetylcholine Receptor
NMDA	N-methyl-D-aspartate
RNA	Ribonucleic Acid

Chapter 1: Introduction

1. Introduction

1.1. Nematodes: an overview

"**Nem-a-tode\`nem-a-tōd\n**": A phylum of invertebrates comprising the roundworms. They are characterised by a smooth, narrow, unsegmented body, tapered at both ends. The microscopic free-living forms are found in all parts of the world, where they play an important role in the destruction and recycling of organic matter. The many parasitic nematodes are much larger, and include the Filaria *(Wuchereria)* and Guinea worm *(Dracunculus)*, which can cause serious diseases in man. ***Excerpted from A Concise Dictionary of Biology*** *(1990), Oxford University Press.*

Nematodes are the most abundant and widely dispersed group of metazoans that inhabit this planet. Although they exist generally unnoticed to the casual observer, they account for approximately 80% of the earth's organisms. Whilst estimates of the actual number of nematode species range from forty thousand to a staggering ten million (Platt et al. 1984); (Platt 1994); (Boucher and Lambshead 1994), the number of described species at present is only around twenty thousand (Platt 1994). Recent surveys of nematode habitats have led to some remarkable population discoveries; for instance, in one square metre of the sea floor close to the Dutch coast, 4,420,000 nematodes were counted and one acre of farmland in Northern China was estimated to contain up to five billion individual nematodes of varying species (Barnes 1980).

Nematodes are such a successful and adaptable group of organisms that they have been described in almost every possible location. As free-living organisms, they are found in soil, fresh water and seawater and as parasites, they infect animals, insects and plants. They have been isolated from sub-zero locations in the Andean mountains and from hot springs in Iceland (Croll and Mathews 1977). Some species have been recovered from samples taken at 30,000 feet, whilst other marine forms are extremely abundant in Arctic and Antarctic oceans at depths of 500 or more metres. Marine dwelling species account for approximately 50% of all nematodes and are very abundant in all oceans from inter-tidal zones to several hundred meters depth. Around 15% of nematodes are parasites of most animals, both invertebrate and vertebrate, and include man, domesticated pets and livestock. The plant parasitic and free-living

nematodes account for 10% and 25% of nematodes respectively (Croll and Mathews 1977).

Whilst some nematodes have a worldwide coverage the distribution of others is restricted by geographical or environmental conditions. A good example of the latter is a species of nematode that has only been described living upon felt beer coasters within a few towns in northern Germany (Viglierchio 1991). Most plants and animals have at least one parasitic nematode, which is uniquely adapted to exploit the concentration of food and resources that the host represents. It is not uncommon however, for several species of nematodes to be found in the same host or habitat (Croll and Mathews 1977). Although nematodes are generally concealed, either due to their microscopic size or containment in their host, descriptions can still be found within historical literature. Written references to nematodes have been recovered from literature of the civilizations of the Mediterranean, Middle East, and Orient (Maggenti 1981). The oldest reference to parasitic nematodes was found in Huang Ti Nei Ching or The Yellow Emperor's Classic of Internal Medicine from China circa 2700 B.C. This account is quite detailed in that it indicates food to be avoided as well as symptomatology and treatment for problems caused by human parasites (Maggenti 1981).

A proportion of the early references to nematodes can be found within the Bible. For example, some interpret the passages of Moses relating to Hebrew laws of sanitation and hygiene as emanating from early information from Egyptian physicians about parasites. It has been proposed that Moses 'fiery serpent' is in fact a reference to the Guinea worm and the technique to remove this pest from tissues by winding it around a stick is still practiced today in many parts of North Africa and the Middle East. It is notable that Hypocrates, circa 430 BC was aware of nematodes as parasites and was probably the first to record knowledge of the pinworm. He mentioned the presence of pinworms in the vagina of women and of similar organisms in horses, the latter being the first observation in domestic animals. Aristotle also knew of nematodes as parasites, especially *Ascaris*. He stated, "these intestinal worms do not propagate their kind". This supported the theory of "spontaneous generation" which had such a disastrous influence on science that it was in a dark age for almost 2000 years. However, a few of the isolated advances within nematology during this period were: Celsus (53 B.C.-7 A.D.) distinguished roundworms from flat worms; Columella, circa. 100 A.D., mentioned an

Ascarid from a calf; Galen (130-200 A.D.) recorded nematodes in fish and Albertus Magnus (1200-1280) A.D. recorded nematodes from birds, namely, falcons (Maggenti 1981).

In terms of size nematodes range from the exceptional *Placentonema gigantissima*, a nine metre giant parasite that inhabits the placenta of the sperm whale, to the smallest free-living species, only 250 μm in length (Barnes et al. 1988). Free-living nematodes play an extensive role in the decomposition of organic matter, humus production, recycling of nutrients and energy, soil metabolism, and the production of compounds that cause soil aggregates to form. Moreover, many are in symbiotic relationships with plants and animals serving as nitrogen fixers and gut microbes. They therefore function as a substantial part of the food web. Parasitic nematodes on the other hand can affect humans directly or indirectly through their domesticated animals. The parasitic nematodes include the common roundworms, which infect more than half of the world's human population. Pinworms, another extremely common parasite, even in developed countries such as the United States, and filarial worms, primarily tropical parasites that cause diseases such as filariasis (elephantiasis) and onchocerciasis (river blindness).

A major group of parasitic nematodes are the intestinal parasites. Intestinal parasitic and protozoan infections are amongst the most common infections worldwide. It is estimated that some 3.5 billion people are affected, and that 450 million are ill as a result of these infections, the majority being children. Each year, some 65,000 deaths are directly attributable to hookworm infections, and another 60,000 to *Ascaris lumbricoides*. *Entoameoba histolytica* that causes amoebiasis is estimated to cause severe disease in 48 million people, killing 70,000 each year. Multiple infections with several different parasites (e.g., hookworms, roundworms and amoebae) are common, and their harmful effects are often aggravated by co-existent malnutrition or micronutrient deficiencies. Furthermore, approximately 44 million pregnant women have hookworm infections that cause chronic blood loss from the intestine and predisposes to the development of iron deficiency anaemia, sometimes of great severity, constituting a major public health problem (World Heath Organsization 2001). The numbers of infected people are steadily increasing in WHO monitored areas and by 2025 more than half of the population in developing countries will be urbanized. As a

direct consequence of urbanization, a large number of people will live in shanty towns where *E. histolytica, Giardia intestinalis, Ascaris lumbricoides* and *Trichuris trichiura* will find a favourable ground for transmission (World Heath Organsization 2001). There is therefore considerable economic and moral incentive in developing and implementing effective means of parasite control.

1.2. General nematode morphology

Phylogenetically nematodes represent one of the lowest metazoan groups and in comparison to other animal phyla, their anatomical plan is highly conserved. All nematodes are vermiform, bilaterally symmetrical, non-segmented pseudocoelomates (Barnes et al. 1988). The nematode body is covered with a thick cuticle secreted by a cellular or syncytial epidermis that is moulted during development. Beneath the epidermis lies a set of longitudinally orientated somatic muscle cells. This sheet of cells, which run the whole length of the worm, is primarily subdivided by the lateral lines into the dorsal and ventral fields. Each field is then further divided into two quadrants by the dorsal and ventral nerve cords respectively (Maggenti 1981). The nematode gut is complete with mouth at the anterior tip of the body and anus on the ventral surface near the posterior tip. The gut comprises of ectodermal foregut and hindgut and an endodermal midgut. The body cavity, or pseudocoel, is not lined with peritoneum and there are no muscles, connective tissue, or other mesodermal derivatives associated with the midgut.

In general, nematodes are diecious with the females usually being larger than the males. The female reproductive system opens to the exterior via the vulva on the ventral surface of the worm, which in many species is about half way along the length of the worm. Males have a cloaca, which is a common urogenital opening also located on the ventral surface at the posterior end of the worm and usually marks the start of the tail. In addition, they have accessory copulatory organs called spicules. In some species of nematodes females can be parthenogenic or hermaphroditic. Development is direct and includes four juvenile and one adult stage separated by molts (Barnes 1980). The nematode nervous system is very simple and consists of a central ganglion or circumpharyngeal nerve ring from which projects the dorsal and ventral nerve cords that run to the posterior of the worm. Arising from the same nerve ring and running

anteriorly are a variety of cephalic nerves, which innervate a number of sensory receptors located in the head region (Barnes et al. 1988). There may be unique chemosensory organs known as amphids as well as sensory bristles around the mouth. Most nematodes lack cilia or flagella, even in the spermatozoa. There are, however, ciliary derivatives in the amphids. (Barnes et al. 1988).

1.3. *Caenorhabditis elegans.*

C. elegans is a small, rapidly growing member of the rhabditid nematode family. It is non-parasitic and can be found in most regions of the world where it inhabits the soil and feeds on bacteria. There are two sexual forms: a self-fertilising hermaphrodite, typically 1 mm in length, and males which are slightly smaller (Figure 1.1). In June 1963, *C. elegans* was proposed as a potential genetic model by the molecular biologist Sydney Brenner who wanted to extend current molecular biological techniques into other fields of biology. In particular, Brenner was interested in discovering how a simple nervous system becomes correctly connected during development (Brenner 1988). In considerably more complicated organisms such as mice, rabbits or humans this problem appeared an impossible task, but because *C. elegans* is both small (959 cells) and transparent (developing cells can be seen and tracked) it turned this question into a potentially solvable problem.

The other attraction of studying *C. elegans* for Brenner was its advantages as a genetic system (Brenner 1974). All of the outstanding successes seen in molecular genetics have been dependent on the use of very simple organisms that can be handled in large numbers: bacteria and bacterial viruses for example. Brenner therefore required a multicellular organism that has a short life cycle, can be easily cultivated, and is small enough to be handled in large numbers. *C. elegans* proved to be an ideal candidate in that it has the following properties: It is a self-fertilizing hermaphrodite; sexual propagation is therefore independent of population size. Males are also found (0.1%), which can fertilize the hermaphrodites, allowing stocks to be constructed by genetic crosses.

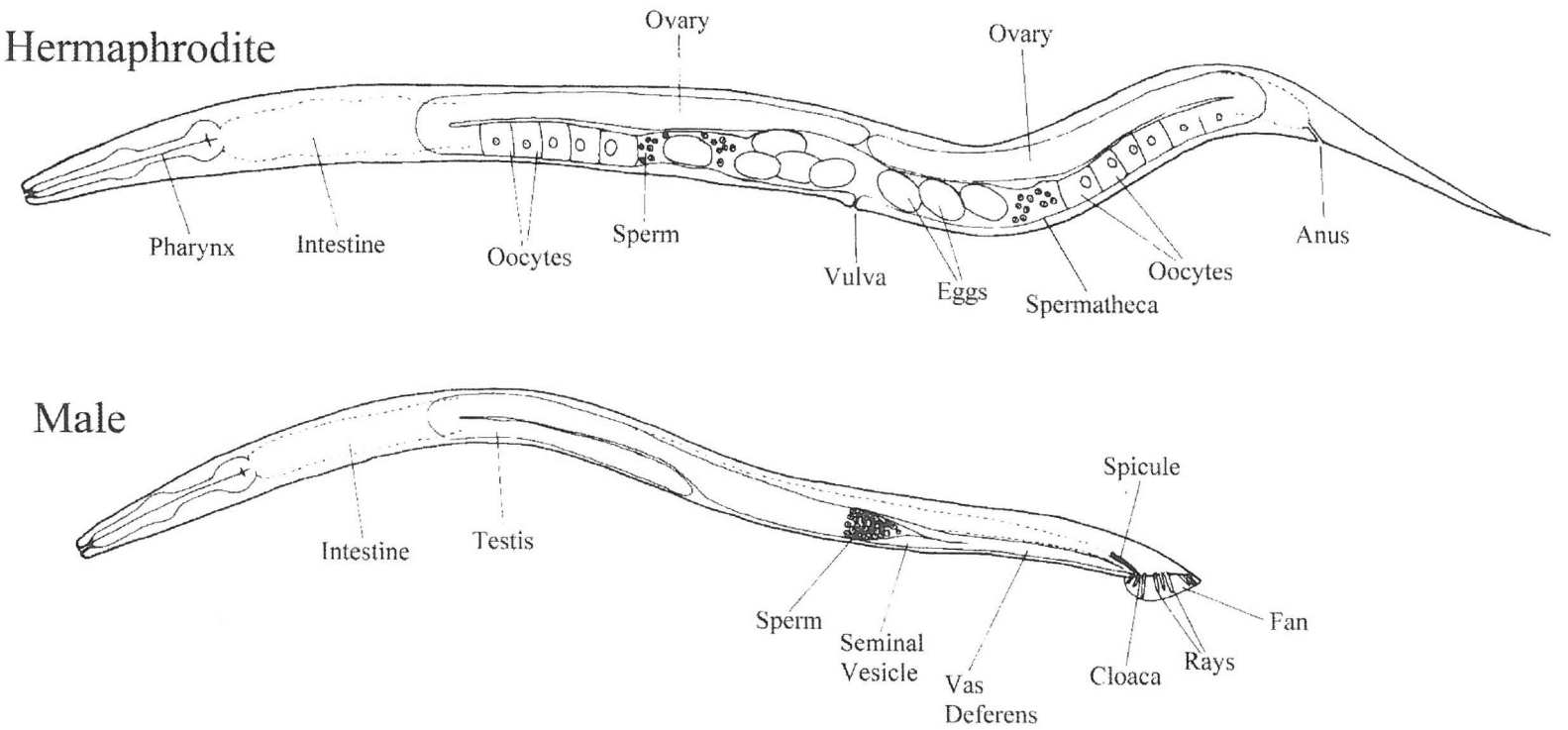


Figure 1.1. Anatomical plan of *Caenorhabditis elegans*. With few exceptions nematodes possess an anatomical organisation that is both relatively simple and highly conserved across the phylum. Identical to all nematodes, *C. elegans* essentially comprises of a cylinder, the exterior cuticle, containing two smaller cylinders, the pharynx and intestine, and the reproductive system. Figure adapted from Wood, 1987.

Each worm can lay up to 200 eggs that hatch within twelve hours, producing larvae approximately 80 microns in length. These larvae grow to a maximum length of 1 mm in three and a half days, reaching sexual maturity. There is no increase in cell number however, only in cell mass (Brenner 1988).

The first task in achieving a comprehensive understanding of this animal was to identify every cell in the worm and trace cell lineages. These investigations were performed by John Sulston and colleagues who successfully described all the cell divisions that occur during development from the fertilized zygote to the mature adult hermaphrodite (Sulston and Horvitz 1977); (Sulston et al. 1983). John White, Sydney Brenner and colleagues went on to describe the complete anatomy and connectivity of all 302 neurones in the adult hermaphrodite and determined the positions of approximately 5000 synapses, 2000 neuromuscular junctions and 700 gap junctions (White et al. 1986). These monumental achievements were only possible because of *C. elegans* small size and essential invariance of both the lineage and neural connectivity. The invariance of the lineage provided the background with which to analyse mutants with developmental defects, and the invariance of the outgrowth, connectivity and placement of neurones allowed cell function and circuitry to be studied by cellular laser ablation.

Within ten years of Brenner's initial paper, several extensive screens were conducted to identify mutants with defects in the nervous system and its effectors. Amongst the first of these were screens for mutants for defective muscles (Waterston et al. 1980); (Zengel and Epstein 1980). Additional screens were conducted to identify mutants with defects in sensory modalities, for example, in thermotaxis (Hedgecock et al. 1990), chemotaxis (Lewis and Hodgkin 1977), osmotic avoidance (Culotti and Russell 1978), and touch (Chalfie and Sulston 1981). Other screens identified mutants in specific neurotransmitter pathways specifically, those of dopamine (Sulston et al. 1975), acetylcholine (Culotti et al. 1981); (Johnson et al. 1981); (Rand and Russell 1984), and serotonin (Trent et al. 1983). Later screens revealed mutants defective in the effector systems for the specific behaviours of egg-laying (Rand and Russell 1984), feeding (Avery 1993b), and defecation (Thomas 1990), and in olfaction (Bargmann et al. 1993). As a result of these and other screens, too numerous to mention,

approximately 7000 of the 19,000 genes contained within the *C. elegans* genome have now been assigned a function (Pennisi 1998).

The use of a relatively new technology known as RNA-mediated interference has now made it simpler to determine gene function within *C. elegans*. Double-stranded RNA (dsRNA) induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi) (Plasterk and Ketting 2000). This technique involves injecting worm oocytes with a piece of double-stranded RNA that matches the gene and this inhibits expression of homologous genes by a process involving messenger RNA degradation (Fire et al. 1998). Experimenters then look for changes in the worms that develop from the injected eggs. The specificity and potency of RNAi makes it ideal for investigating gene function beginning only with genomic sequence (Tabara et al. 1998). Furthermore, it appears that ingestion of dsRNA expressing bacteria results in RNAi of the targeted gene (Timmons and Fire 1998), making functional genomic analysis a simple procedure of feeding *C. elegans* bacteria that express dsRNA.

1.4. The *C. elegans* genome

In 1990 a collaborative effort between the Genome Sequencing Centre in St Louis, USA and the MRC Sanger Centre in Cambridge to sequence the entire *C. elegans* genome began. Eight years later, virtually all of the 97 million base pairs contained within six chromosomes were sequenced and published (The *C. elegans* Sequencing Consortium 1998). The project began with the development of a clone-based physical map (Coulson et al. 1991); (Coulson et al. 1988) to facilitate the molecular analysis of genes, which were been discovered at an ever increasing pace through the study of mutant worms. This, in turn, initiated a collaboration between the *C. elegans* Sequencing Consortium and the entire community of *C. elegans* researchers (Greenwald et al. 1987); (Ward et al. 1988). The free exchange of data and immediate access to the genome map were fundamental in the early completion of the project. The pioneering techniques developed throughout this collaboration not only increased *C. elegans* genome sequencing productivity but also laid the foundations for sequencing the 3 billion base pairs that make up the human genome.

The genome sequence predicts 19,099 protein-coding genes (The *C. elegans* Sequencing Consortium 1998), which is about three times that found in yeast (Mewes et al. 1997); (Goffeau et al. 1996). It's just under 5000 more than found in *Drosophila* (Adams et al. 2000), and approximately two thirds of the number determined for humans (Venter et al. 2001). Generally genes in *C. elegans* have smaller and fewer introns than their mammalian counterparts, and the gene density is high (Blumenthal and Thomas 1988). Many genes required for normal development and behaviour are currently under investigation. Furthermore, with the aid of an active transposon system (Emmons et al. 1983); (Eide and Anderson 1985); (Moerman et al. 1986) and a physical map of the genome (Coulson et al. 1988); (Coulson et al. 1991), they can be readily cloned. Methods for gene transformation and reintroduction have also been developed so that altered genes can now be expressed in the adult worm (Stinchcomb et al. 1985); (Fire 1986); (Fire and Waterston 1989). Furthermore, because the worm is transparent, the gene under study can be linked with the gene for green fluorescent protein (GFP) (Chalfie et al. 1994). Researchers can then discover when and where in the worm the hybrid gene is expressed by monitoring GFP's fluorescence.

Comparisons of the genes predicted from the *C. elegans* genome with that of other (non-nematode) organisms reveals that ~58% of the genes appear to be nematode-specific. A proportion of these nematode-specific genes have been functionally identified by genetic analysis, and many (34% of the total) form families with other nematode genes (Green et al. 1993). Thirty-six per cent of predicted *C. elegans* genes have a significant human match (The *C. elegans* Sequencing Consortium 1998); (Rubin et al. 2000) including many genes implicated in human disease (Rubin et al. 2000); (Ahringer 1997), and functional analysis of the *C. elegans* genome has shed light on many conserved biological processes and molecular pathways. The similarity of many *C. elegans* genes to those seen in mammals is often extensive, supporting the contention that information obtained in the nematode will be relevant to understanding the biology of humans. The genomic sequence has not only revolutionised *C. elegans* biology, but combined with genetic, developmental, and anatomical data, it has and should continue to provide a powerful resource for research in other systems.

1.5. *C. elegans* life cycle

A single adult hermaphrodite produces both sperm and oocytes and can produce around 280 hermaphrodite progeny by self-fertilisation and more than a 1000 males and hermaphrodite progeny when mated with males. *C. elegans* has a rapid life cycle: 14 hour embryogenesis and 36-hour post embryonic development through four larval stages, L1-L4 to the adult at 25°C (Sulston et al. 1983); (Sulston and Horvitz 1977) (Figure 1.2). Fertilised eggs are carried by the hermaphrodite worm and develop internally for several hours. This period can increase however depending on the age of the worm. Eggs are then laid in bursts over a 7-day period. Each egg develops into L1 larvae over a span of 14 hours at 25°C from the point of fertilisation until hatching. Newly hatched larvae grow rapidly through a series of four moults to become adult animals. Although the size and shape of the animal does not change markedly until the L4 stage, many postembryonic cell divisions and cell deaths are taking place internally (Sulston and Horvitz 1977). Males and hermaphrodites have mean life spans of 17.7 and 19.9 days at 20°C, respectively.

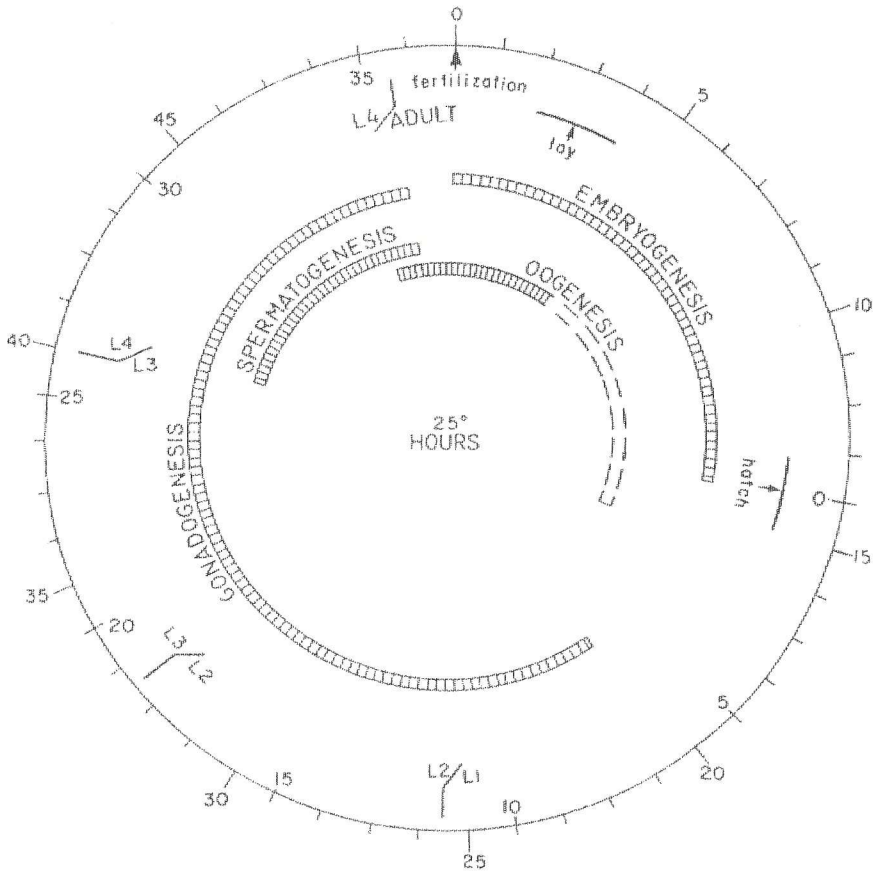


Figure 1.2. Diagrammatic representation of the *C. elegans* life cycle, showing durations of developmental stages. Numbers on the outside of the large circle indicate hour's post-fertilisation. Figure modified from Sulston & Horvitz, 1977.

1.6. Nervous system

The simplicity and structural consistency of the nematode's nervous system fascinated early neuroanatomists at the turn of the 19th century. It was the much larger parasitic nematode *Ascaris*, that was used in these early neuroanatomical reconstructions (Hesse 1892); (Goldschmidt 1908); (Goldschmidt 1909). They demonstrated that the number of neurones in this parasitic nematode is small, around 250. Later investigations provided a revised estimate of approximately 300 neurones (Stretton et al. 1978). The nervous system of *C. elegans* is nearly identical in terms of the number of neurones, their morphology and positioning. This similarity has allowed the *C. elegans* research community to extrapolate physiological and pharmacological data gained from *Ascaris* to further our understanding of the *C. elegans* nervous system. Although not an ideal approach, it has been necessary due to the technical difficulties associated with dissecting and making stable recordings from this tiny nematode. Recent developments in *C. elegans* electrophysiological techniques (Goodman et al. 1998), (Richmond and Jorgensen 1999) and calcium sensitive fluorescence (Kerr et al. 2000) have now made the characterisation of *C. elegans* nervous system and effectors a feasible project.

The adult hermaphrodite *C. elegans* nervous system contains exactly 302 neurones and each one has been completely described in terms of its location and morphology (Ward et al. 1975); (Ware et al. 1975); (White et al. 1986); (Hall and Russell 1991). Furthermore, the synaptic connectivity of approximately 5000 chemical synapses, 600 gap junctions, and 2000 neuromuscular junctions have also been described (White et al. 1986). The extensive genetic analysis of *C. elegans* has demonstrated that a number of basic neurobiological mechanisms, such as second-messenger signalling (Gross et al. 1990); (Lu et al. 1990); (Mendel et al. 1995); (Segalat et al. 1995), synaptic release (Nonet et al. 1993), and other cell-signalling events (Stern and DeVore 1994), are conserved between the worm and more complex biological systems. There are however distinct differences, for example, searches of the genome have shown that there are no voltage-activated sodium channels. Voltage-activated potassium and calcium channels are present however (Bargmann 1998). Rhodopsin molecules that are used for vertebrate and invertebrate vision are also absent in *C.*

elegans. Moreover, there are distinct differences in the sequences for gap junction and olfactory receptor genes although the encoded proteins have similar properties (Bargmann 1998). These differences may be a result of the phyletic separation of nematodes from other groups. Current best estimates of the time of divergence range from 1200 million to 600 million years ago (Philippe et al. 1994); (Raff 1996); (Raff et al. 1994); (Doolittle et al. 1996); (Wray et al. 1996); (Feng et al. 1997). Despite this early divergence, around 42% of the predicted protein products find significant matches in other organisms including humans with most of these matches containing functional information (Green et al. 1993).

The nervous system of *C. elegans* is separated into two parts: the pharyngeal and somatic nervous systems. The pharyngeal nervous system is composed of twenty neurones and connections between these are well documented by Albertson & Thomson, 1976. The somatic nervous system consists of the remaining 282 neurones, and is nearly completely isolated from the pharyngeal nervous system. The connections of the 282 somatic neurones have been studied by White *et. al.*, 1986. Most of the nematode's nervous system is situated in the head and is organised around the circumpharyngeal ring (Figure 1.3). The head is also richly endowed with sensory receptors, most of which have their endings near the mouth (Ward et al. 1975). There are two main classes of receptor in the head; one is generally considered to be mechanosensory and consists of two concentric rings of sensilla projecting back to cell bodies which are predominantly located in front of the nerve ring. The other class of receptors are arranged in two lateral sensilla, the amphids, and these are generally thought to be chemosensory (Maricq et al. 1995). The neurones in these sensilla project back to cell bodies that are in the lateral ganglia behind the nerve ring. The cell bodies for the sensory receptors, together with interneurones and some motor neurone cell bodies are situated between the corpus and terminal bulb of the pharynx. These cells send out processes that run circumferentially around the pharynx as a fibre bundle forming the nerve ring, which is the major neuropile in the animal.

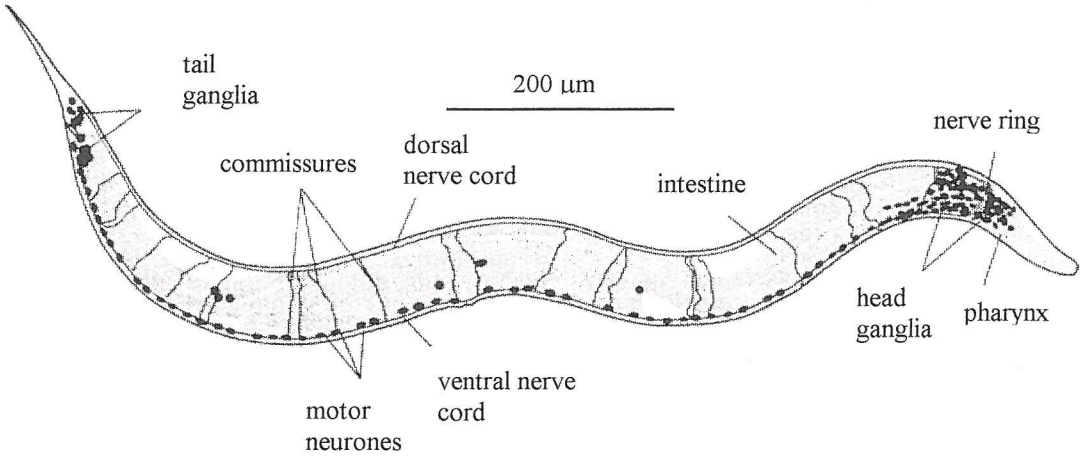


Figure 1.3. A diagram of the adult hermaphrodite nervous system. Note the large neuronal mass situated in the head and the motor neurone cell bodies that form the ventral cord. Figure modified from Jorgensen & Nonet, 1995.

Large proportions of the processes in the nerve ring enter and leave on the ventral side forming the ventral nerve cord (White et al. 1976). The ventral cord contains a linear sequence of 57 motor neurones. The motor neurones innervate the body wall muscles on both the ventral and dorsal sides. The ventral motor neurones have axons that run along the right hand side of the ventral cord and synapse onto muscle arms from the two sub-ventral quadrants of muscle. Motor neurones innervating the two sub-dorsal quadrants of muscle send out processes which leave the ventral cord and run around the outside of the animal as a commissure until they reach the dorsal hypodermal ridge where they turn and form the dorsal cord, innervating the dorsal muscles in the same manner as the ventral muscles. The dorsal cord is predominantly made up of these motor neurone axons whereas the ventral also contains groups of processes from interneurones. (White et al. 1976).

1.7. Neurotransmitters and receptors

In general, few of the chemical signals acting upon nerve cells are known in this animal, although several enzymes required for neurotransmitter synthesis, metabolism, and storage have now been identified in the genome sequence and their localisation is

helping in the characterisation of the nervous system. The two most comprehensively studied of the 'classical' transmitter substances in *C. elegans* are γ-aminobutyric acid (GABA) and acetylcholine (ACh). They both have a fundamental role in the functioning of the neuromuscular system.

1.7.1 γ-aminobutyric acid (GABA)

The presence of GABA, using GABA immunoreactivity, was initially demonstrated in 26 neurones within *C. elegans*, most of which are motor neurones (Mcintire et al. 1993b). Schaeffer and Bergstrom (1988) measured and detected the presence of (i) GABA, (ii) GABA binding sites, (iii) the GABA synthesising enzyme glutamate decarboxylase, and (iv) the GABA degrading enzyme, GABA transaminase. At present, five genes have been shown to be necessary either for GABAergic neuronal differentiation or pre- or postsynaptic GABAergic function. The gene *unc-30* is required for the differentiation of a specific subset of GABAergic neurone, the type D inhibitory motor neurone (Mcintire et al. 1993a). The gene *unc-25* is necessary for GABA expression and encodes glutamic acid decarboxylase (Jin et al. 1999). The genes *unc-46* and *unc-47* seem to be required for normal GABA release. Finally, the gene *unc-49* is necessary postsynaptically for the inhibitory effect of GABA on the body muscles (Richmond and Jorgensen 1999), and encodes multiple subunits of a heteromultimeric GABA receptor (Bamber et al. 1999).

The vast majority of GABAergic cells in *C. elegans* are inhibitory motor neurones. However, there are also a few excitatory GABAergic motor neurones (Mcintire et al. 1993b). Mutants with GABAergic transmission defects are viable, although they have several motor defects. The most obvious phenotype is a tendency to contract dorsal and ventral body wall muscle simultaneously in response to touch, which appears to result from lack of function of the GABA-containing DD and VD inhibitory motor neurones (Mcintire et al. 1993a); (Mcintire et al. 1993b).

1.7.2 Acetylcholine (ACh)

ACh appears to be the primary excitatory neurotransmitter controlling motor function in *C. elegans*. Cholinergic neurones have been identified using antibodies to choline acetyltransferase (ChAT), and the vesicular acetylcholine transporter encoded by the *unc-17* gene. Immunoreactivity to both proteins has been observed primarily in a punctate staining pattern in synaptic regions (Alfonso et al. 1993), as expected for synaptic or synaptic-vesicle-associated proteins. Almost all of the ChAT-positive cells appear to be motor neurones. Strongly staining cells include six of the eight classes of ventral cord motor neurones, three types of pharyngeal motor neurones, and six types of putative sub-lateral motor neurones (Alfonso et al. 1993); (Alfonso et al. 1994).

ACh is the only neurotransmitter so far identified in *C. elegans* that is essential for viability. Animals totally deficient for ACh synthesis (*cha-1* mutants) are inviable, whereas animals deficient in GABA, serotonin or dopamine are viable (Rand and Russell 1984). The *C. elegans* ChAT protein is 36% identical to pig ChAT and 34% identical to *Drosophila* ChAT (Alfonso et al. 1994). Viable *cha-1* mutants are small, slow growing and uncoordinated (Rand and Russell 1984); (Hosono et al. 1985). They also have slow pharyngeal pumping and a slow irregular defecation cycle (Avery 1993b); (Thomas 1990), and are also resistant to inhibitors of acetylcholinesterase (Rand and Russell 1984).

Nicotinic acetylcholine receptors (nAChRs) have been well described in nematodes and over 20 nAChRs sequences have been uncovered in the *C. elegans* genome sequencing project (Fleming et al. 1997); (Treinin and Chalfie 1995); (Ballivet et al. 1996); (Wiley et al. 1996). Pharmacological studies suggest that cholinergic transmission at *C. elegans* neuromuscular junctions is mediated postsynaptically by ligand-gated receptors of the nAChR family (Lewis et al. 1980); (Avery and Horvitz 1990). Two ACh receptors have been shown to mediate the postsynaptic neuromuscular response. One acetylcholine receptor was activated by the nematocide, levamisole. This response was eliminated in mutants lacking either the *unc-38* or *unc-29* genes, which encode alpha and non-alpha acetylcholine receptor subunits, respectively. The second acetylcholine receptor was activated by nicotine. The response desensitised rapidly and was selectively blocked by dihydro-beta-erythroidine, thus explaining the residual

motility of *unc-38* and *unc-29* mutants (Richmond and Jorgensen 1999). Although the neuromuscular nAChRs from *C. elegans* and vertebrates have pharmacological similarities, there are some important differences (Lewis et al. 1980); (Fleming et al. 1993). For instance, toxins such as α-bungarotoxin, which binds very tightly to vertebrate nAChRs, are not very effective against the *C. elegans* receptor, whereas the anthelmintic levamisole is a potent agonist of *C. elegans* neuromuscular nAChRs (Lewis et al. 1980); (Richmond and Jorgensen 1999).

Evidence also exists for muscarinic receptors in *C. elegans*. Cullotti and Klein (1983) described a membrane-bound, high affinity, saturable binding activity for N-methylscopolamine and quinuclidinyl benzilate, two potent muscarinic receptor blockers. In addition, Avery and Horvitz (1990) reported that muscarinic agonists and antagonists can modulate pharyngeal pumping. The first G protein linked ACh receptor was cloned by Lee *et. al.*, (1999). This gene encodes for a polypeptide of 682 amino acids containing seven transmembrane domains. When co-expressed in *Xenopus* oocytes with a G protein-gated inwardly rectifying K^+ channel (GIRK1), ACh was able to elicit a GIRK current (Lee et al. 1999). The ACh-induced current was substantially inhibited by the muscarinic antagonist atropine in a reversible manner. However, oxotremorine, scopolamine and pirenzepine had little or negligible effect (Lee et al. 1999). Together, these findings suggest that the cloned gene encodes a G protein-linked ACh receptor that is most similar to, but pharmacologically distinct from, muscarinic acetylcholine receptors.

1.7.3. L-glutamate

L-glutamate is a neurotransmitter in both vertebrates and invertebrates and several ionotropic receptors have been described gating either cation or anion channels. In *C. elegans*, glutamate can act as an excitatory or inhibitory neurotransmitter. For example the M3 motor neurones situated around the pharyngeal muscle appear to be glutamatergic and inhibitory and act by opening a chloride channel (Dent et al. 1997). These types of channels which are unique to the invertebrate phyla are described in greater detail in Chapter 4 . Degenerate PCR and classical genetic approaches have led to the cloning of an AMPA-like L-glutamate receptor gene, *glr-1* (Maricq et al. 1995);

(Hart et al. 1995) from *C. elegans*. Mutants of the *glr-1* are defective in mechanosensory behaviour (i.e. responses to touch) and are slightly sluggish in their movements. Interestingly, the ASH neurone, which mediates both touch and osmotic sensory information forms synapses with *glr-1* containing neurones, however only the touch sensory modality of this circuit is defective in *glr-1* mutants. The *glr-1* gene also appears to play a role in the RMD motorneurones that innervate head muscles. The subunit is 37-38% identical to rat AMPA (rat GluR1-4) subunits and 32-33% identical to rat kainate (GluR5-7) subunits (Hart et al. 1995).

1.7.4. Serotonin (5-HT)

Serotonin is also a common neurotransmitter in vertebrates and invertebrates. It has been identified in *C. elegans* neurones by formaldehyde-induced fluorescence (Horvitz et al. 1982) and by anti-serotonin staining (Desai et al. 1988); (Mcintire et al. 1992). There are at least ten cells in hermaphrodites and a greater number in males that demonstrate significant anti-serotonin immunoreactivity (Desai et al. 1988); (Loer and Kenyon 1993), but the NSM cells have the strongest and most consistent staining. These pharyngeal cells have varicosities, fine branches, and endings on the surface of the pharynx (Albertson and Thomson 1976), suggesting that serotonin might be released into the pseudocoelom and have a humoral function

Several genes have been identified that affect the response to exogenous serotonin (Segalat et al. 1995). The best characterised gene at present is *goa-1*, which encodes a Go subunit required for transduction of a signal from a metabotropic serotonin receptor (Mendel et al. 1995); (Segalat et al. 1995). Analysis of the *C. elegans* genome has revealed the presence of a single tryptophan hydroxylase gene (*tph-1*), the key enzyme for serotonin biosynthesis. Animals that have a *tph-1* deletion mutation do not synthesize serotonin but are nevertheless fully viable. The *tph-1* mutant shows abnormalities in behaviour and metabolism that are normally coupled with the sensation and ingestion of food. For example, rates of feeding and egg laying are decreased; large amounts of fat are stored; reproductive lifespan is increased; and some animals arrest at the metabolically inactive dauer stage (Sye et al., 2000).

1.7.5. Dopamine

The presence of dopamine has been demonstrated with the use of formaldehyde-induced fluorescence (Sulston et al. 1975). A small subset of neurones use dopamine as a neurotransmitter to control or modulate egg laying (Weinshenker et al. 1995). The *cat-1* gene encodes a vesicular monoamine transporter (VMAT) that is required for specific monoamine-dependent behaviours and yet *cat-1* knockouts are viable and grow relatively well (Duerr et al. 1999). The transporter is 47% identical to human VMAT1 and 49% identical to VMAT2.

1.7.6. Peptides

Many organisms, including mammals, use short peptides as neurotransmitters. The family of FMRFamide (Phe-Met-Arg-Phe-NH_2)-like neuropeptides, which all share an –RFamide sequence at their C-termini, have been shown to have diverse functions, including neuromodulation and stimulation or inhibition of muscle contraction. In *C. elegans*, FMRFamide-like peptides (FaRPs) are expressed in approximately 10% of the neurones, including motor, sensory, and interneurones that are involved in movement, feeding, defecation, and reproduction (Schinkmann and Li 1992). To date, forty-three *C. elegans* neuropeptide genes have been identified. Twenty-two genes, named *flp* (FMRFamide-like peptides) genes, encode FaRPs. Each *flp* gene encodes a different set of FaRPs, yielding a predicted total of 59 distinct FaRPs, although a few of these may also encode non-FaRPs (Li et al. 1999a). Twenty-one genes, named neuropeptide-like protein (*nlp*) genes, encode peptides distinct from the FaRP family. The predicted nlp-1 and nlp-2 neuropeptides have modest similarity to buccalin and myomodulin, respectively (Li et al. 1999b).

To date, only the *flp-1* gene has been characterised in *C. elegans*. Disruption of *flp-1* causes uncoordination and hyperactivity. Conversely, over expression of *flp-1* results in the reciprocal phenotypes (Nelson et al. 1998). Furthermore, Waggoner et. al., (2000) has demonstrated that *flp-1* is necessary for *C. elegans* to down-regulate their rate of egg laying in the absence of food.

1.9. Body wall muscle

There are 95 mononucleate muscles cells in the hermaphrodite adult worm and all are rhomboid in shape. The muscle cells can be divided into three groups based upon their source of synaptic input. The anterior group of four muscles in each quadrant are innervated by motor neurones in the nerve ring. The next group of four, which is dually innervated by motor neurones in the nerve ring and ventral nerve cord and the remaining muscles, which are innervated solely by the motor neurones of the ventral cord (White et al. 1986); (White et al. 1976). Nematode muscles are unusual in that they have a neurone-like process that extends from the muscle cell bellies to the neurone process bundles in which motor neurone axons reside (Rosenbluth 1965). Neuromuscular junctions are made by axons running along the surface of their process bundle, through the bounding basal lamina of the bundle and onto muscle arms. The muscle arms inter-digitate extensively and crowd around regions where neuromuscular junctions occur. There are often numerous gap junctions between the muscle arms in these regions (White et al. 1986). *C. elegans* body wall muscles are also unusual in that their sarcomeres have an oblique conformation with actomyosin filaments, aligned at an angle of about 10 degrees to the Z lines. This type of arrangement has been referred to as obliquely striated muscle (Rosenbluth 1965). The Z lines consist of longitudinally orientated lines of dense bodies that are stained darkly in electron micrographs (White et al. 1986).

1.10. Pharyngeal muscle

The pharynx is a large neuromuscular organ situated at the anterior of the worm and is a prominent feature of *C. elegans* and indeed all nematodes. It occupies approximately one fifth of the worm's structure and its role is to grind ingested bacteria and pump the mixture into the intestine against the high internal pressure that generates the "hydrostatic skeleton" (Crofton 1966). As a consequence of its size and importance for the animal it has become one of the most described organs of *C. elegans*. For example, its complete anatomy has been reconstructed from serial section electron micrographs (Albertson and Thomson 1976), and its role in feeding has been extensively studied (Doncaster 1962); (Seymour et al. 1983). Furthermore, 52 mutations in 35 genes have been described that play important roles in generating

pharyngeal morphology, pumping motion and contractile structures. These genes can be divided into 3 classes: those that affect gross pharyngeal structure (*pha* 1-2), those that affect contractile components (*phm* 1-7) and those that affect pharyngeal pumping (*eat* 1-26) (Avery 1993b).

1.11 Pharyngeal anatomy

The pharynx is two lobed, or of the rhabditoid type (Chitwood and Chitwood 1938). Photographed in the living animal (Figure 1.4), one can easily distinguish the buccal cavity, procorpus, metacorpus, isthmus, and terminal bulb. There are eight-muscle cells types, arranged end to end along the anterior-posterior axis of the pharynx. These muscle cells can be divided into three functional groups. The corpus, containing muscles pm1 to pm4, constitutes the anterior half of the pharynx. Its purpose is to take in and trap bacteria. The isthmus, muscle pm5, is the middle part of the pharynx. It regulates flow of food from the corpus to the terminal bulb. The posterior part of the pharynx, muscle cells pm6 to pm8, is the terminal bulb whose function is to grind up the bacteria.

The pharynx is composed of 34 muscle cells, 9 marginal cell, 9 epithelial cells, 5 gland cells and 20 neurones (Albertson and Thomson 1976). The nuclei of muscle, neuronal and epithelial cells of adult pharynxes are illustrated in Figure 1.5. Under Normarski optics the nuclei of different cell types have characteristic appearances that has proved invaluable in their identification and mapping. For instance, hypodermal and gut nuclei have a "fried egg" appearance in that they are round and smooth in texture with a large, prominent nucleolus. Neuronal nuclei are smaller and round, lack prominent nucleoli, and have a punctate nucleoplasm. Muscle nuclei are oblong, are intermediate in size between neuronal and hypodermal nuclei, and have a punctate nucleoplasm and a small nucleolus.

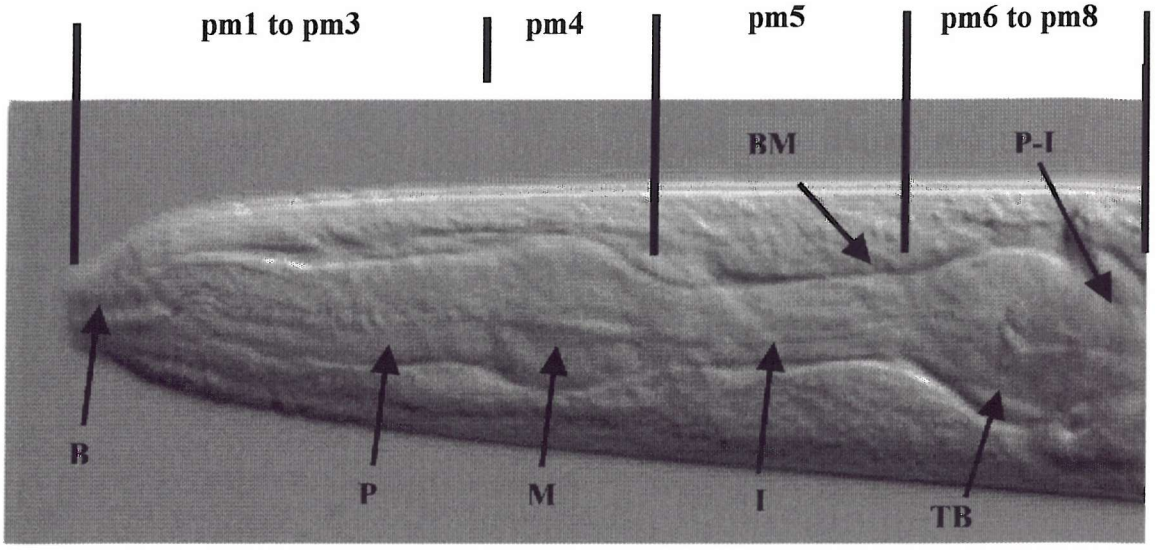


Figure 1.4. Light micrograph of the pharynx. Nematodes were anaesthetised with 10 mM sodium azide dissolved in modified Dent's saline and observed with Normarski optics. The pharynx is located at the anterior of the animal and opens to the outside by the buccal cavity (B). The procorpus (P) and metacorpus (M) together make up the structure called the corpus. The isthmus (I) connects the two bulbs of the pharynx. The terminal bulb (TB) connects to the intestine by the pharyngeal-intestinal valve (P-I), but is separated from the valve by the basement membrane (BM) surrounding the pharynx.

1.12. Pharyngeal role in feeding

Contractions of the *C. elegans* pharynx have been well described and consist of two motions, pumps and isthmus peristalsis. These actions bring food into the pharyngeal lumen, grind it up, and pass it to the intestine (Doncaster 1962); (Seymour et al. 1983). Near-simultaneous contractions of the corpus, anterior isthmus, and terminal bulb act to open the lumen of the pharynx, drawing in liquid containing suspended bacteria. This becomes trapped in the corpus because the posterior isthmus is closed. Isthmus peristalsis transports the bacteria from the corpus to the terminal bulb where it is broken up and passed into the intestine. Usually, only every fourth pump is followed by isthmus peristalsis (Avery and Horvitz 1987). The relaxation of the corpus, anterior isthmus, and terminal bulb returns the terminal bulb grinder to its resting position and allows the lumen of the corpus to close, expelling liquid whilst trapping bacteria. (Doncaster 1962).

1.13. Pharyngeal nervous system

A basement membrane isolates the pharynx from the rest of the animal, making the pharyngeal nervous system a self-contained unit that is composed of twenty neurones of 14 anatomical types (Albertson and Thomson 1976). Of the twenty pharyngeal neurones, 8 are unpaired and 6 are bilaterally symmetric. The pharyngeal and extrapharyngeal nervous systems are connected by a bilateral pair of gap junctions between the extrapharyngeal RIP neurones and the pharyngeal I1 neurones (Albertson and Thomson 1976). This connection can be severed with very little effect of pharyngeal function by killing the RIP neurones (Avery and Horvitz 1989). Although there may be a humoral communication between the pharyngeal and extrapharyngeal nervous systems, each of them can function without such interactions. For example, when the pharynx is exposed by dissection, presumably eliminating any humoral influences the extrapharyngeal nervous system might have, pharyngeal behaviour and electrophysiology are essentially normal (Avery et al. 1995).

Laser ablation studies has revealed that out of the twenty pharyngeal neurones, only the M4 is essential for life. Worms lacking all other nineteen remaining neurones are viable and fertile (Avery and Horvitz 1989). Although they are not essential for life,

these neurones are however important for normal feeding, normal growth rates and fertility levels (Avery and Horvitz 1989); (Avery 1993a). The following are three principal abnormalities, each of which have been attributed to a single motor neurone type: (1) muscle relaxation is delayed in the absence of M3; (2) pumping is slow in the absence of MC; and (3) there is no isthmus peristalsis in the absence of M4. Not only are these three motor neurones types necessary for normal feeding, they are also sufficient for nearly normal feeding; i.e., when all pharyngeal neurones except M4, MC, and M3 are killed, feeding is nearly normal (Avery 1993a); (Raizen et al. 1995). It is therefore believed that M4, MC, and M3 execute the principal functions of the pharyngeal nervous system. A fourth pharyngeal neurone type, NSM (neurosecretory motor neurone), may serve to communicate the presence of food to the rest of the worm. The other pharyngeal neurones may regulate these four, or they may have functions that are not visible under laboratory conditions.

1.13.1. M3 function

The M3s are inhibitory motor neurones that control the timing of pharyngeal relaxation (Avery 1993a). The two M3 neurones, located at the corpus-isthmus boundary, have ultra-structurally defined sensory endings in the posterior corpus and motor output to the corpus and anterior isthmus (Albertson and Thomson 1976). Two behaviours affected by M3, trapping of bacteria in the corpus and timing of pharyngeal relaxation, suggest that M3 is an inhibitory motor neurone with output to the corpus. (Avery 1993a); (Raizen and Avery 1994). This regulation of timing of relaxation seems to be important for effective transport of bacteria within the pharyngeal lumen (Avery 1993a).

Albertson and Thomson (1976) suggested that M3 has a sensory function because it has a process that ends under the cuticle of the lumen and is connected to neighbouring cells with desmosomes. This neuronal process may have a mechanosensory function in that is able to sense the contraction of the pharynx and then command its relaxation. In addition to M3, several other pharyngeal neurone types (Albertson and Thomson 1976), and at least one extrapharyngeal neurone type (Ward et al. 1975); (Ware et al. 1975), have been proposed on anatomical data, so it is likely that other *C. elegans* neurones have dual physiological functions.

J. A. Dent et. al., (1997) have tentatively identified the M3 neurotransmitter as glutamate. Although immunocytochemical localisation of glutamate within the M3s has not been successful, other lines of evidence support this hypothesis. Firstly, pulses of glutamate applied to the pharyngeal muscle mimic the effect of M3 and *avr-15* (avermectin-resistant) mutants, whose pharyngeal muscle does not respond to glutamate pulses, also lack M3 transmission.

1.13.2. MC function

The MC's neuronal cell bodies are located in the posterior part of the corpus (Figure 1.5), and are thought to be the main excitatory neurones necessary for rapid pumping. On an agar surface in the presence of abundant bacteria, normal wild-type worms pump nearly continually at an average rate of about 260 ± 7 pumps/min (Avery and Horvitz 1989); (Mcintire et al. 1993a). Laser ablation of the MC neurone reduced pumping to 45 ± 6 pumps/min, whilst ablation of the other 13 neuronal types had no observable impact on pumping rate (Avery and Horvitz 1989). Raizen et. al., (1995) have suggested that the MC neurotransmitter may be ACh. This is based on observations that worms who have mutations in *cha-1*, a gene required for ACh synthesis (Alfonso et al. 1994), have severely reduced pumping rates (Avery and Horvitz 1990). Partial loss-of-function mutations in *unc-17*, a gene required for vesicular packaging of ACh (Alfonso et al. 1993), also cause reduced pumping (Raizen et al. 1995). Furthermore, the nicotinic agonists ACh, carbachol, nicotine, and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), all caused pharyngeal contraction, whilst d-tubocurarine, a competitive nAChR antagonist (Taylor 1991), blocked pumping reversibly (Raizen et al. 1995). Despite these findings, no histochemical markers for ACh within the MC neurone have been observed, although staining in other pharyngeal neurones as well as cholinergic neurones in the ventral cord are clearly stained (Alfonso et al. 1993); (Alfonso et al. 1994).

1.13.3. M4 function

The M4 neuronal cell body is also located on the posterior part of the corpus but its process synapses onto the posterior m5 muscles of the isthmus (Albertson and Thomson 1976). The isthmus which connects the corpus to the terminal bulb (Figure 1.4), regulates the passage of bacteria between these two structures by propagating peristaltic waves. When M4 is killed, the posterior isthmus remains closed. The corpus and terminal bulb muscles still contract in synchrony, but since the isthmus is closed, the corpus quickly becomes full with bacteria (Avery and Horvitz 1987). Because food does not get into the intestine, these M4 deficient worms starve. They stop growing, and never get any bigger than a well-fed juvenile would be after 12 hr post-hatching. This pharyngeal defect is a specific consequence of killing M4. When any of the remaining nineteen neurones are killed, the corpus never becomes full, and the worms grow to adulthood (Avery and Horvitz 1987). Antibodies raised against choline acetyltransferase and the synaptic vesicle ACh transporter binds strongly to M4, suggesting that its transmitter is ACh (Alfonso et al. 1993); (Alfonso et al. 1994).

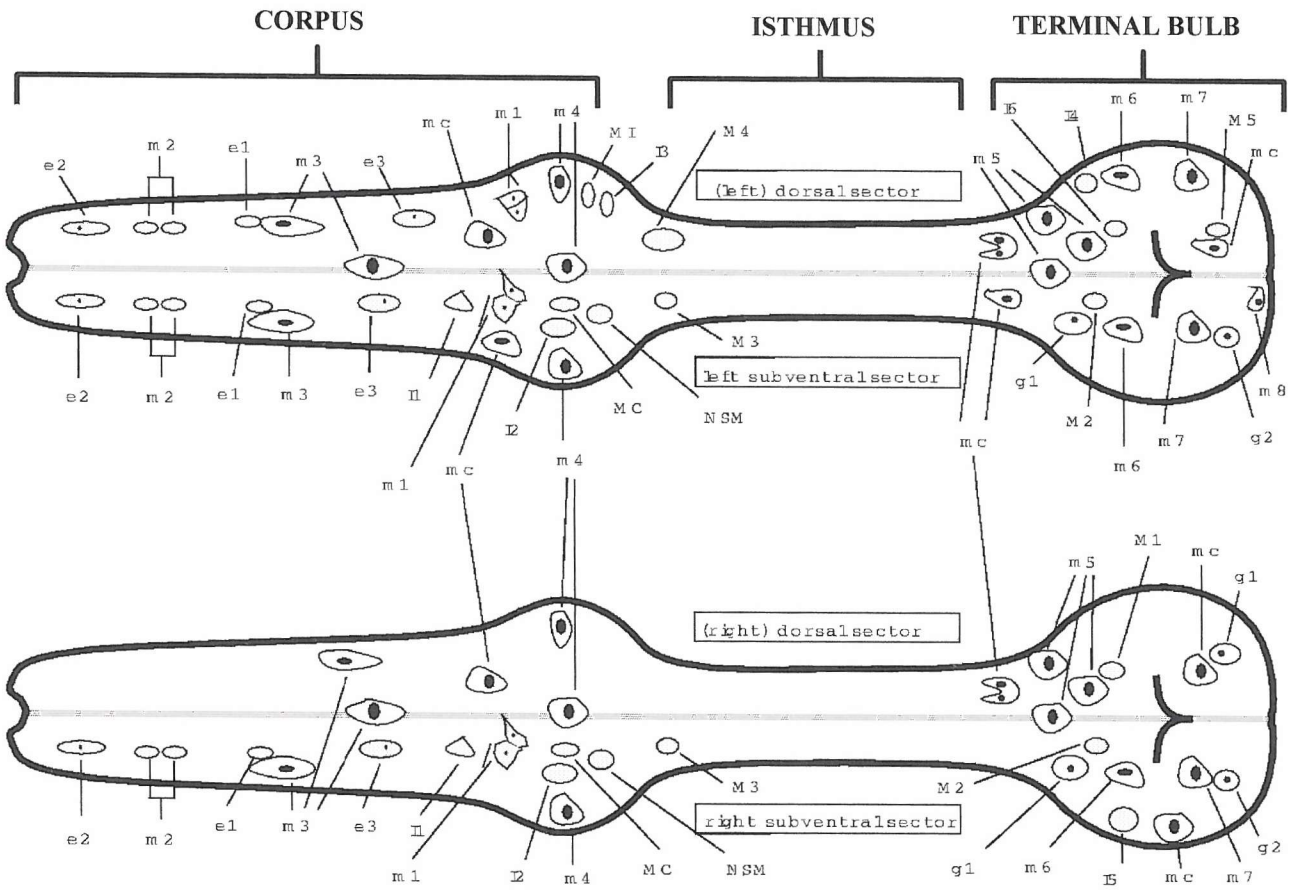


Figure 1.5. Locations of pharyngeal nuclei. Epithelial cell nuclei (e1-e3) are present in the procorpus only, whilst muscle (m1-m8) and neuronal (M1-M5, I1-I6, NSM and MC) nuclei are concentrated in the metacorpus and terminal bulb. Figure modified from Bargmann and Avery (1995).

1.14. Project Aims

Although a significant amount of information is available on the *C. elegans* pharynx, most of this relates to the anatomy and structure. There is little information on the physiology and pharmacology. For example, the ionic basis of the resting membrane potential and action potential have not been determined. This is partly due to the technical difficulties associated with making stable electrophysiological recordings from this nematode. Here, these difficulties have been addressed and a preparation for intracellular recording from the pharynx has been established. This has then provided the opportunity to

i) Explore the pharyngeal muscle's general pharmacology using a range of classical neurotransmitters/modulators.

ii) Investigate the ionic basis of the resting and action potentials responsible for pharyngeal muscle contraction

This information then provided the foundation for further studies in molecular genetics to identify ion channel, and neurotransmitter receptor function in *C. elegans*. In the remaining study, the preparation has been used to further the understanding of the mechanism of action of ivermectin. This anthelmintic is known from behavioural studies to have potent effects on the nematode pharynx.

Chapter 2: Methods

2. Materials and Methods

2.1. Experimental animals

The wild-type Bristol N2 strain was obtained from the Sanger Centre, Hinxton, UK. The *avr-14* (*ad1371*), *avr-15* (*ad1051*) and *avr-14:avr-15* strains were provided by Leon Avery, University of Texas, South Western Medical Centre, Dallas, Texas and Joe Dent, McGill University, Montreal, Canada. The *snb-1* (*md247*) strain was provided by the Caenorhabditis Elegans Genetic Centre, University of Minnesota, St. Paul, USA.

All strains were grown on NGM agar plates carrying a lawn of OP50, a uracil-requiring strain of *Escherichia coli* (Brenner, 1974). Generally 9 cm plates were used for experimental strains, whilst 5 cm plates were employed for the growing of all other strains. Poured agar plates were kept at room temperature for at least 2 days following pouring, so that excess moisture evaporates and those contaminated with fungi and bacteria can be detected and removed. Plates were then seeded with bacteria sufficient for 3 days requirements, i.e. 9 cm plates are streaked with 40 μl and 5 cm plates with 20 μl of an overnight OP50 culture. The OP50 lawn was allowed to grow overnight before the nematodes are added ensuring a plentiful supply of food for the growing nematodes.

Fresh nematodes were added to the new plates by removing approximately 1 cm^2 portion of agar from an old plate with the use of a sterilised scalpel. This was then lightly brushed along the agar surface to ensure adequate transfer of worms. Animals are immediately active after transfer and the plates are generally ready for experimental use following 2-3 days growth (Plate 2.1). All animals used in these investigations were grown at 19 °C.

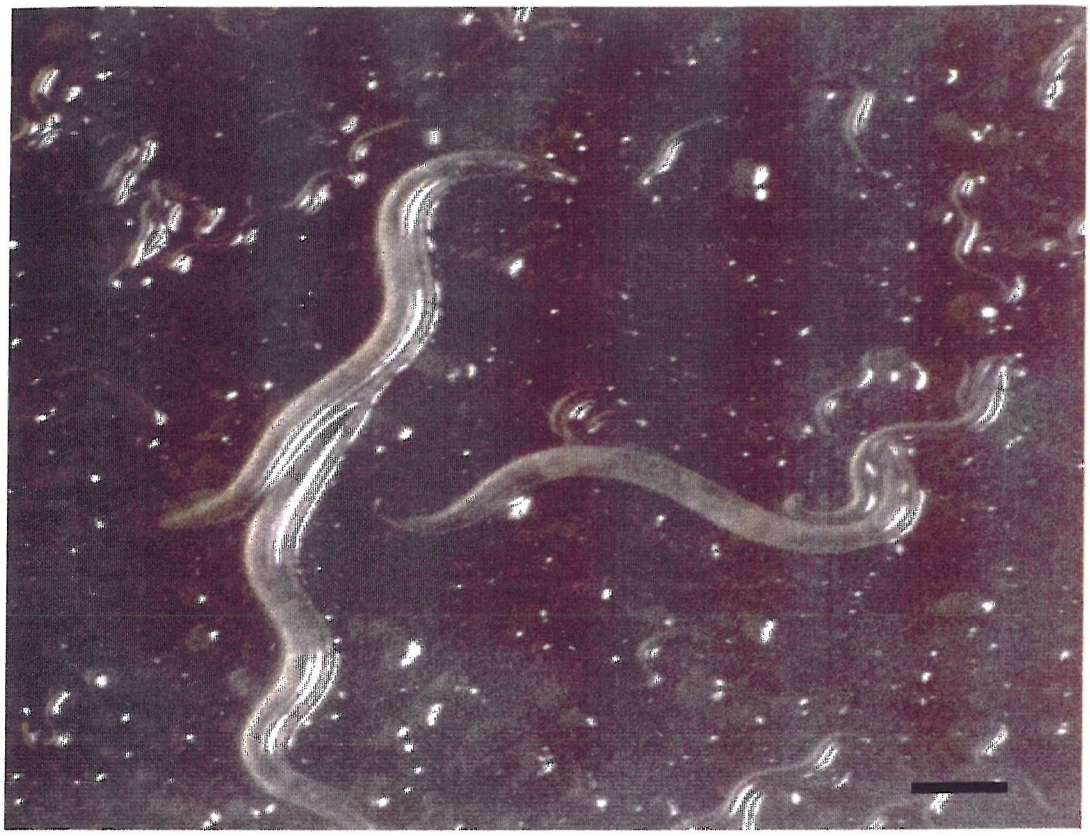


Plate 2.1. *Caenorhabditis elegans.* Light micrograph of adult and juvenile *C. elegans* feeding upon an agar plate. Scale bar (bottom right) is 100 μM.

2.2. Cleaning of contaminated plates

Normal bacterial sterile precautions at the open bench are adequate for transfer and seeding of nematodes, however contamination with foreign bacteria or yeasts does sometimes occur. A simple cleaning procedure was devised by Sulston and Hodgkin (1977) and involves picking a few adult gravid worms into a small drop of a caustic solution containing equal amounts of 2 M sodium hydroxide and 2% sodium hypochlorite solution on a fresh plate carrying an OP50 lawn. The solution destroys all unwanted contamination and dissolves the worm cuticle to expose the eggs. The plates are then left overnight to allow juvenile nematodes to hatch and crawl to the bacteria. Cleaned worms are then removed and transferred to fresh plates.

2.3. Physiological saline

Due to its small size, devising a physiologically relevant saline for *C. elegans* has proved more problematic than for other organisms. In general, creating a saline for *in vitro* experimental procedures is usually achieved by one of two methods. Either a small amount of extracellular fluid is withdrawn from the organism and analysed for its major ionic constituents. A saline is then composed with an ionic composition thatexactly matches the sample. This approach is unlikely to be successful in *C. elegans* because obtaining a sufficient quantity of uncontaminated fluid from this microscopic organism would prove to be an extremely difficult, if not impossible task. The second approach is trial and error, starting with salines derived from other species. One then varies the concentration of individual ions, using an easily observable physiological response to determine the effect.

Avery *et. al.*, (1995) started with a saline based on studies of the extracellular fluid from the much larger parasitic nematode *Ascaris Suum* (Hobson et al., 1952), (Brading and Caldwell, 1971), but this was shown to produce abnormal pharyngeal pumping and electropharyngeograms devoid of postsynaptic potentials (Avery et al., 1995). This has been attributed to abnormally high level of potassium in the saline making the reversal potential for this ion much more positive, thus depolarising neurones and muscles (Avery et al., 1995). Two saline solutions that gave essentially normal pumping in their investigations, Dent's and Tet's are described in Table 2.1

together with the *Ascaris* saline composition. The concentration of potassium found in these salines are consistent with the levels found in chemical analyses of the extracellular fluid in earthworms and other annelids that inhabits similar environments as *C. elegans* (Drewes and Pax, 1974); (Nicholls and Kuffler, 1964).

Modified Dent's saline composition used in standard recordings was: [mM]: NaCl 140, $MgCl_2$ 1, $CaCl_2$ 3, KCl 6, HEPES 5, D-Glucose 10; pH was adjusted to 7.4 with NaOH at room temperature. All experiments performed in the characterisation of pharyngeal GluCl channels were performed in saline that had 50% of NaCl replaced with Na isethionate. The isethionate ion is an impermeant ion and is therefore a useful substitute for chloride ions (Parri et al., 1991). In the study investigating the ionic basis of the resting membrane and action potential, NaCl was replaced by glucosamine hydrochloride whilst NaCl was used to replaced $CaCl_2$.

	ASCARIS	*DENT'S*	*TET'S*	*MODIFIED*	*EARTH-WORM*
NaCl	4	140	137	140	75.6
Na acetate	125	0	0	0	0
KCl	24.5	6	5	6	4
$CaCl_2$	5.9	3	1	3	3
$MgCl_2$	4.9	1	5	1	Not Calculated
HEPES	5	5	5	5	0
Glucose	0	0	10	10	0
pH	7.4	7.4	7.2	7.4	Not Calculated

Table 2.1. Summary of saline composition used for electrophysiological recordings in nematodes. Saline employed in all standard recordings within this work has been labelled "Modified" in the above table.

2.4. The *C. elegans* pharyngeal preparation

Under a binocular dissecting microscope, a few gravid adult worms were picked from 2-3 day old plates into a 3 cm petri dish containing approximately 8 ml of modified Dent's saline together with 0.1% bovine serum albumin (BSA). The addition of BSA prevents the worms from sticking to the walls of the petri dish and recording chamber. The nematodes were briefly immobilised by placing the petri dish within a freezer for approximately 3-5 minutes. A razor blade fragment held in a Weiss needle holder (WPI, Sarasota, FL) was then used to make a dissection at the level of the pharyngeal intestinal valve (Fig 2.1a). Due to the longitudinal nature of the bodywall muscle, the cuticle surrounding the pharynx rapidly retracts exposing the terminal bulb (Fig 2.1b).

Approximately 3-5 dissected preparations were transferred to a custom designed recording chamber (volume 500 µl) cut from cured Sylgard 184 (Dow Corning, UK) mounted upon a glass coverslip. The recording chamber was then mounted upon an Axiovert microscope (Carl Zeiss, Oberkochen, Germany) and perfused *via* gravity feed with modified Dent's saline at a rate of 5ml min^{-1}. High magnification visual assessments of dissections were performed to ensure that only undamaged muscle was used. A healthy dissection was then secured in position by sucking onto the pharyngeal terminal bulb with a 20-25 µm patch electrode under negative pressure delivered by a hand-held syringe. The muscle was then gently moved to the perfusion chamber bottom trapping the muscle between the cover slip and suction electrode (Figure 2.1b). A single terminal bulb muscle cell was then impaled with an aluminosilicate glass microelectrode (See below).

Drugs were applied by addition to the perfusate and rapid drug concentration changes were achieved by a method adapted from (Slater et al., 1984). The duration of drug application was typically 30 s to allow maximal responses to develop. All antagonists were applied 3 min before and concurrently with agonists. All experiments were conducted at room temperature.

2.5. Electrophysiological recordings

Sharp electrodes were pulled from 1 mm aluminosilicate glass (SM100C-10; Clarke Electromedical, UK) using a Sutter P-2000 horizontal laser puller (Sutter Instruments, Novato, CA). Electrodes typically had resistances of 35-70 MΩ and were filled with 4 M potassium acetate, 10 mM potassium chloride filtered through a disposable (0.1 μm pore size) Millix filter (Millipore, Watford, UK). The patch pipettes used for suction and EPG recordings were pulled from 1 mm borosilicate glass (GC100-10; Clarke Electromedical, UK) on a Narashige PP-830 vertical filament puller (Nikon UK Ltd, Surrey). The reference electrode was an agar bridge (4% agar in 3 M potassium chloride) connected to the system ground of the headstage via an Ag/AgCl pellet in 3 M KCl. The microelectrode was connected to the headstage (HS-2A 0.1× L) of an Axoclamp 2B (Axon Instruments, Foster City, CA) using Ag/AgCl wires and signals monitored on a PC running Axon laboratory Clampex 7.0. A hard copy of membrane potential and spike frequency were also obtained using a Gould RS 3200 two-channel chart recorder (Gould Nicolet Technologies, Essex, UK).

2.6. The electropharyngeogram (EPG) preparation

Dissected anterior *C. elegans* preparations were transferred to a non-perfused recording chamber mounted upon a Zeiss microscope. A patch pipette with an opening of approximately 30-35 μm was lowered into the recording chamber allowing the pipette to back-fill with saline by capillary action. The head of a worm was gently sucked into the pipette under negative pressure generated from a hand-held syringe. The voltage transients that occur during pharyngeal contraction were amplified and recorded. The reference electrode and recording apparatus are identical to that described above.

2.7 Drugs and antibodies

The *avr-14* antibody was a kind gift from Adrian Wolstenholme, University of Bath, UK. L-glutamic acid (sodium salt) was obtained from BDH (Poole, UK). Ibotenic acid, PDC (*trans*-4-carboxy-L-proline/L-*trans*-pyrrolodine-2,4-dicarboxylic acid), quisqualic acid and kainic acid were obtained from Tocris Cookson (Bristol, UK).

Common salts and other remaining compounds were obtained from Sigma, (Poole, UK). Hydrophobic drugs were made up as stock solutions in ethanol and subsequently diluted in modified Dent's saline to a final ethanol concentration of 0.1%. This vehicle had no detectable affect on the properties of the pharyngeal muscle. All drugs and ion-substituted salines were checked for pH prior to use.

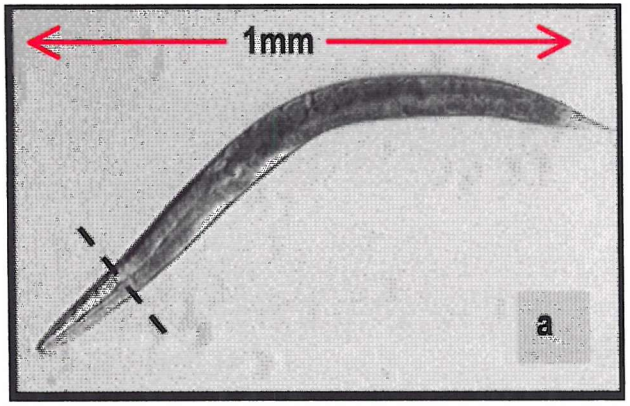
2.8 Analysis of data

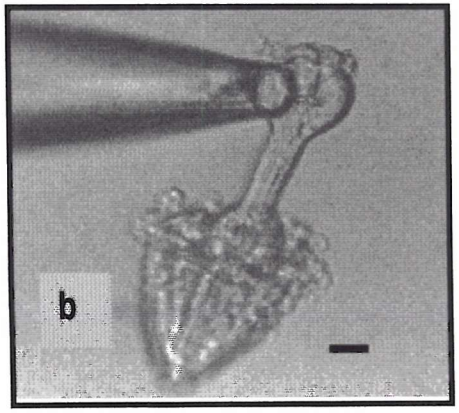
Concentration-response curves were fitted to the modified logistic equation using GraphPad Prism software, Version 3.0 (San Diego, CA) to generate EC_{50} values and maximal responses. Values are expressed as either mean with 95% confidence limits in brackets (for data pooled from n experiments) or the mean ± S.E.M. Statistical analyses were performed using either the paired on un-paired two-tailed Student's t test (as appropriate), with a significance level of $P < 0.05$.

2.9. *C. elegans* AVR-14 immunohistochemistry.

One hundred adult *C. elegans* were picked and dissected in modified Dent's saline using the same methods described earlier. The tissue was fixed in 4% paraformaldehyde (PFA) solution for 24 h at 4 °C. These were then washed three times with phosphate-buffered saline (PBS) containing 20% sucrose and left overnight at 4 °C. The worms were then washed again with PBS containing 0.1% Triton X-100 (PBST).

Immunohistochemical staining was carried out using AVR-14 affinity purified antibody, which was raised as described by Jagannathan *et. al.*, (1999). The primary antibody was diluted 1:20 in 0.1% (w/v) BSA, phosphate-buffered saline containing 0.1% Triton X-100 (PBST), and incubated for 24 h at 4 °C. Unbound antibodies were removed by extensive washing with PBS and the worms treated with a 1:40 dilution of FITC-conjugated affinity-isolated swine anti-rabbit IgG (DAKO, Denmark) overnight at 4 °C. The preparations were then extensively washed in PBST after which the worms were mounted and viewed under a BioRad MRC600 confocal microscope (BioRad, Hercules, CA). The immunostaining experiments were repeated on four separate occasions, each time using 100 worms. The control employed throughout these studies was the omission of primary antibody.





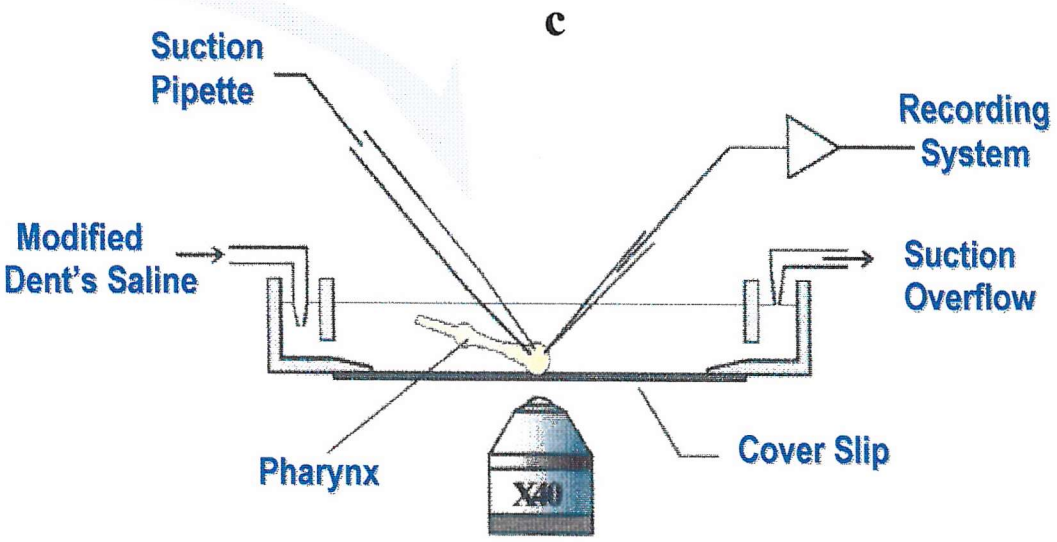


Figure 2.1. The electrophysiological set-up. Schematic diagram illustrating the dissection procedure together with the electrophysiological set up. Micrograph (a) shows an intact *C. elegans* with the dashed line representing the site of dissection. Micrograph (b) shows the pharyngeal preparation attached to a suction electrode. Note that the cuticle and somatic muscle have retracted exposing the terminal bulb (scale bar represents 10 μm). Thanks to Chris Franks for figure c.

Chapter 3: A study into the electrical and general pharmacological properties of the pharyngeal muscle of wild-type *C. elegans*

3.1. Introduction

Molecular cloning and sequence homology searches have revealed a surprisingly large number of ion channel genes and splicing variants. For instance, searches of the *C. elegans* genome have demonstrated the presence of more than eighty potassium channels (Wei et al., 1996), and at least ten calcium channels (Bargmann, 1998). Understanding the functional significance of this genetic complexity is one of the central problems facing neurobiologists today. Whilst one level of understanding has been derived from electrophysiological studies on cell lines expressing cloned ion channels, a complete understanding will require many additional type of studies, including genetic manipulations and their subsequent evaluation in intact animals.

C. elegans is an ideal organism for these type of studies. Moreover, the myogenic muscle cells of the pharynx are a model system with which investigate the contribution that individual genes make to cellular excitation. For example, a loss of function mutation in a putative calcium channel α1 subunit (*egl-19*), decreases the slope of the action potential rising phase and also shortens the action potential plateau phase (Lee et al., 1997). The gene *exp-2* encodes for a voltage-activated potassium channel (Kv type) and several loss of function mutations in *exp-2* leads to broadening of the action potential and a concomitant slowing of the pumping action of the pharynx (Davis et al., 1999). Furthermore, a mutation in a Na,K-ATPase α subunit (*eat-6*) causes feeble contractions and slow, delayed relaxations of pharyngeal muscle, presumably by reducing the ion gradients that power membrane potential changes (Davis M.W. et al., 1995).

Despite these extremely informative genetic and electrophysiological studies on the *C. elegans* pharynx, no investigations have been conducted to determine the ionic dependence of pharyngeal resting membrane and action potential. The lack of this essential information has obviously hindered interpretation of electrophysiological data from wild-type and mutant animals. For example, although a mutation in a putative calcium channel subunit alters action potential shape (Lee et al., 1997), it is unknown whether calcium ions are actually the main charge carrier. The study contained within the early part of this chapter provides a considerable proportion of this information, and thus lays the foundation for further detailed comparisons with mutant strains to

delineate the function of *C. elegans* ion channels in the control of excitable cell function.

The remaining section of this chapter is devoted to a pharmacological investigation of the pharyngeal muscle. Although it is clear that both classical transmitters and neuropeptides have pivotal roles in the normal physiology of nematodes (for review see Walker et. al., 1999), very little is known about *C. elegans* pharyngeal pharmacology. As the technical difficulties associated with making stable electrophysiological recordings have now been overcome within our laboratory, these voids in our knowledge about this important nematode can now be addressed. The aim of this study therefore, was to determine the effect of a few classical transmitters/modulators on pharyngeal action potentials. As the intention was simply to provide an overview of the muscle general pharmacology, determinations of EC_{50} values were not performed on any of the compounds examined.

3.2. Action potential recordings from wild-type *C. elegans* pharyngeal muscle cells

All dissected pharyngeal preparations were spontaneously active and generated action potentials with a frequency of around 0.2 Hz (Figure 3.1). Action potentials were observed to be coupled one-to-one with contractions of the terminal bulb. They were however, of a variable frequency, amplitude and duration, despite the fact that recordings were made from similar animals i.e. adult hermaphrodites from 3-5 day old plates. Analysis of 16 recordings from individual animals, measuring the average properties of at least 12 action potentials gave the following results. Mean resting membrane potential of terminal bulb muscle cells was -74.0 ± 0.8 mV. Mean amplitude of action potentials was 111 ± 4.4 mV with durations of 0.26 ± 0.23 s (n=16).

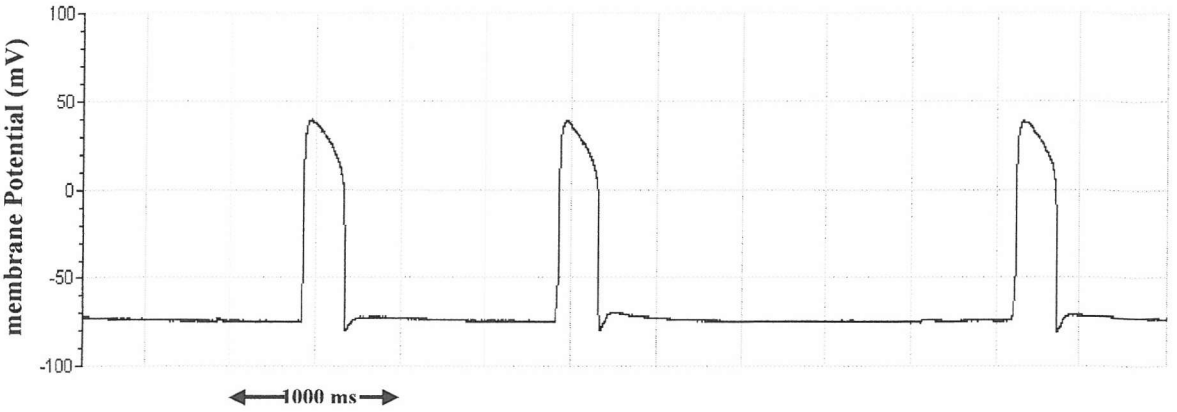


Figure 3.1. Action potentials recorded from wild-type *C. elegans* pharyngeal muscle. The trace shows a recording from a single muscle cell within the terminal bulb. The cell was impaled with a 45 MΩ microelectrode. In this particular recording, resting membrane potential was –74 mV with action potential amplitudes of 112 mV.

3.3. Properties of the resting membrane potential

All recordings were allowed a few minutes to stabilise following electrode impalement and typically the resting membrane potential was in the range of –80 to –65 mV. Ion replacement experiments were conducted to determine which ions contribute to the resting membrane potential. The reversibility of any effect on membrane potential was verified by reverting back to standard Dent's saline prior to experiment conclusion.

Decreasing extracellular Cl^- from 154 mM to 84 mM and then to 14 mM elicited a transient excitation and burst of action potentials, but no sustained effect on membrane potential was observed (Figure 3.2; n=7). Reducing extracellular Na^+, by replacement with glucosamine hydrochloride, from 140 mM to 70 mM (n=9) elicited a small depolarisation of 4.8 mV (Figure 3.2). Depolarisations of a similar magnitude were observed when Na^+ was completely removed (Figure 3.8). Reduction of extracellular Ca^{++}, by replacement with NaCl, from 3 mM to zero (n=4) elicited a small hyperpolarisation of 4.3 mV (data not illustrated).

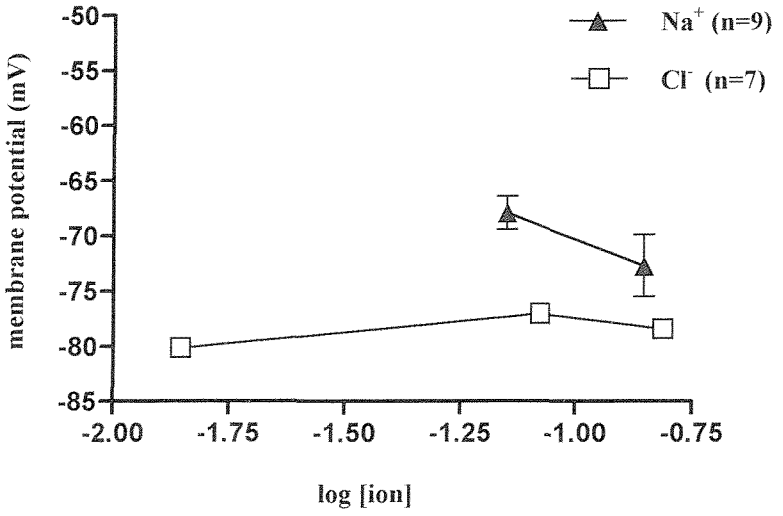


Figure 3.2. Effect of changing extracellular Na^+ and Cl^- concentrations on the resting membrane potential. Only small changes in membrane potential were observed, suggesting that ion(s) other than Na^+, Cl^- and Ca^{++} are mainly responsible for generating and/or maintaining resting membrane potential.

In earlier experiments performed by another member of the laboratory, the effect of varying extracellular K^+ on resting membrane potential was examined. Increasing extracellular K^+ concentration from 3 to 12 mM caused a depolarisation of membrane potential from –79.0 ±3.0 mV to –58.3 ± 0.5 mV; n=6. A Nernst plot of this data predicted a 39 mV depolarization for a ten-fold change in extracellular K^+, which is less than the 58 mV change predicted if the membrane was entirely dependent on K^+. Increasing extracellular K^+ also resulted in a decrease in the amplitude of pharyngeal action potential after-hyperpolarisations, and decrease in spike frequency (Chris Franks, personal communication).

3.4. Properties of the action potential: effect of varying extracellular Ca^{++}

Ion replacement experiments were performed to determine the role that individual ions play in generating pharyngeal action potentials. Once again, the reversibility of any effect on action potentials was verified by reverting back to standard Dent's saline prior to experiment conclusion. Replacement of extracellular Ca^{++} with NaCl had pronounced effects on action potentials. There was a transient increase in action potential frequency, followed by a decrease in spike amplitude and prolongation

of action potential duration (n=8; Figure 3.3). Complete cessation of action potentials was not observed however, although the membrane remained depolarised for considerable periods of time, greater than 6 seconds on some occasions. Similar effects on action potential amplitude and duration were also seen when Ca^{++} was decreased from 3 mM to 1.5 mM, although less marked than with total Ca^{++} removal (n=8; Figure 3.3).

The opposite effect on action potentials was seen when extracellular Ca^{++} was increased (Figure 3.5). Action potential overshoot (measured as the amplitude of spikes taken from 0 mV), increased as extracellular Ca^{++} was raised from 1.5 mM to 10 mM. For example, it increased from 30.0 ± 3.7 mV in 1.5 mM Ca^{++} to 42.9 ± 3.2 mV in 3mM Ca^{++} (n=8; mean + S.E.M.; p=0.0005, paired Students's t-test; Figure 3.4b). There was an inverse relationship between extracellular Ca^{++} concentration and spike duration (Figure 3.4a). In zero Ca^{++}, the effect on action potential duration was extremely marked (Figure 3.3), and resulted in prolonged plateau potentials. The mean action potential duration significantly decreased (measured from the first inflection from resting membrane potentials to the return to resting membrane potential) when extracellular Ca^{++} was increased from 1.5 mM to 3 mM (n=8; p=0.0247, paired Student's t-test; Figure 3.4a) and up to 10 mM (n=4).

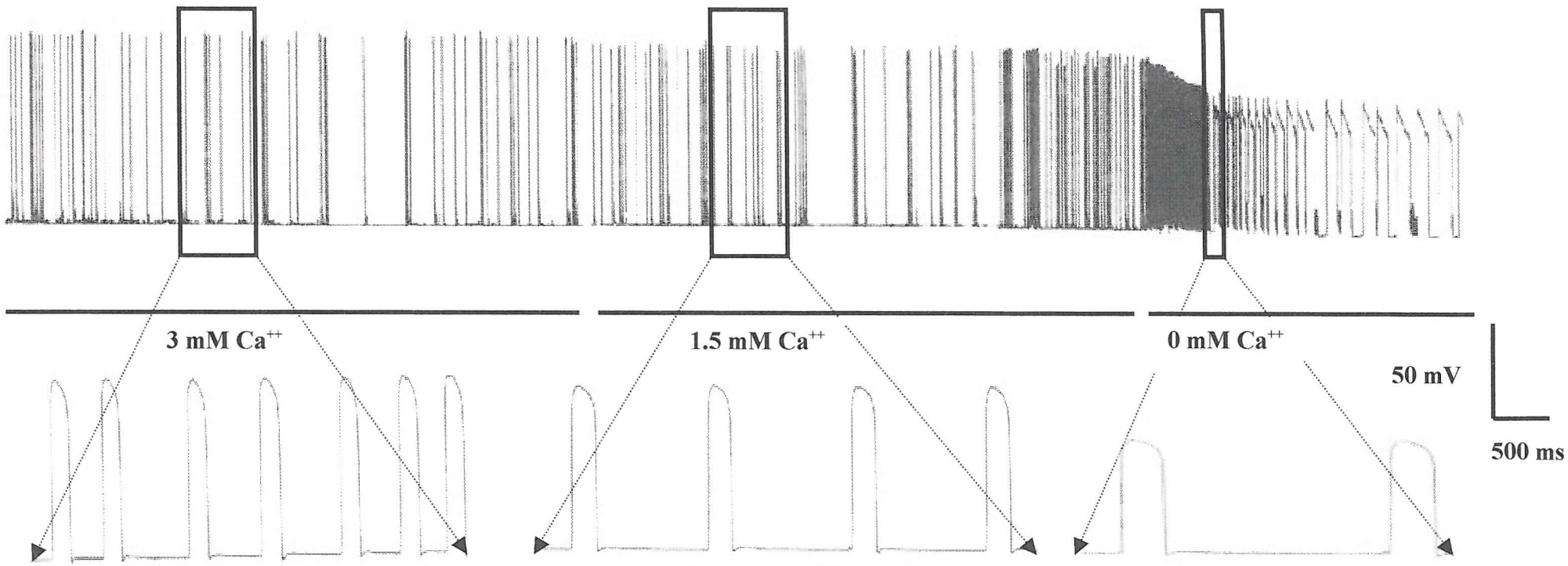


Figure 3.3. Effect of reducing extracellular Ca^{++} on pharyngeal action potential height and duration. Recording from a single cell within the terminal bulb. Resting membrane potential was –76 with action potential amplitudes of 104 mV. Ca^{++} removal elicited a concentration-dependent reduction in spike height together with an increase in action potential duration. Scale bar applies to expanded section only.

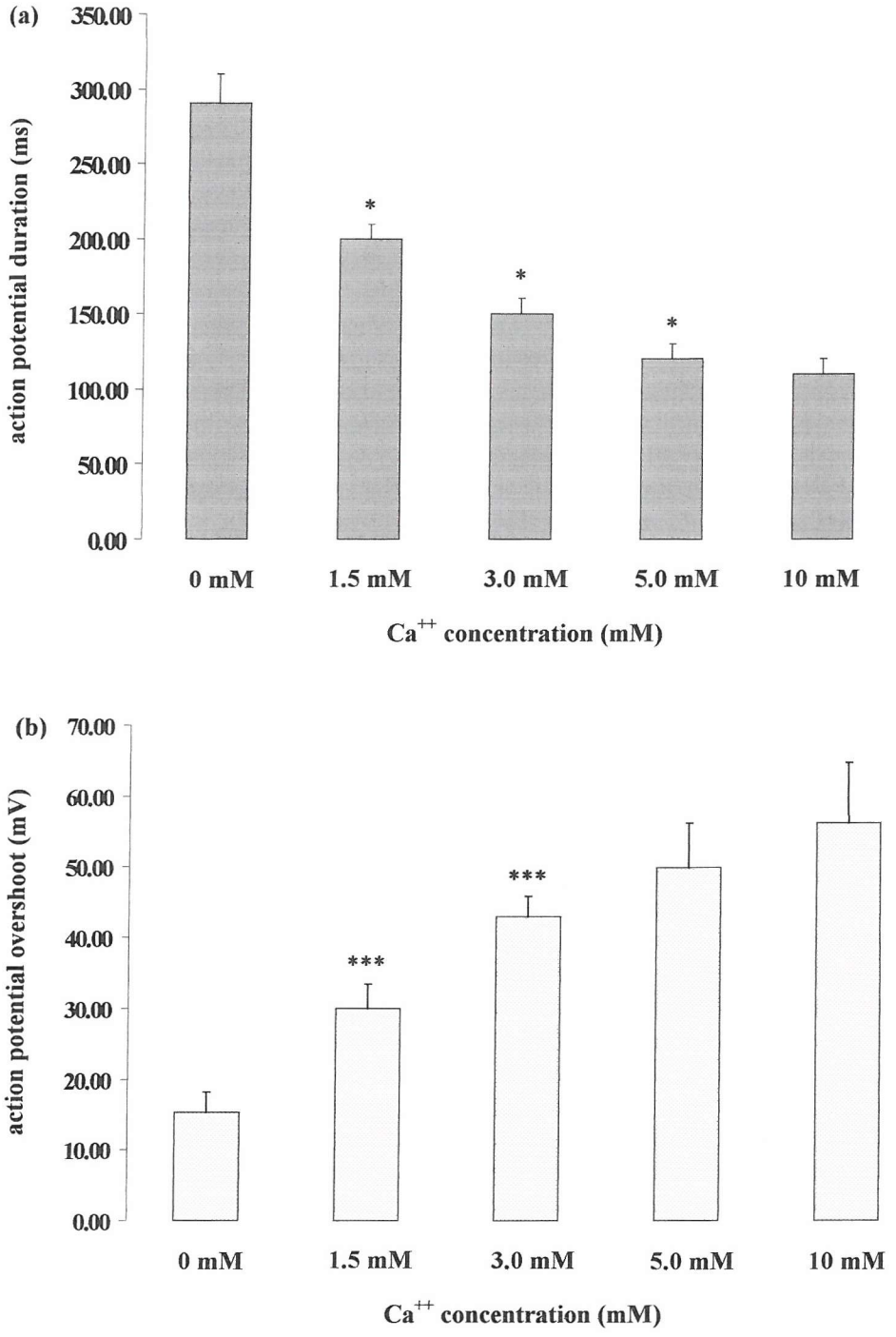


Figure 3.4. Effect of changing extracellular Ca^{++} concentrations on (a) action potential duration and (b) action potential overshoot (n=8). The duration was measured as the time between the first inflection from the resting membrane potential, to the resting membrane potential immediately after the spike. The effect on amplitude was measured as the change in action potential overshoot from 0 mV. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; compared to previous Ca^{++} concentration.

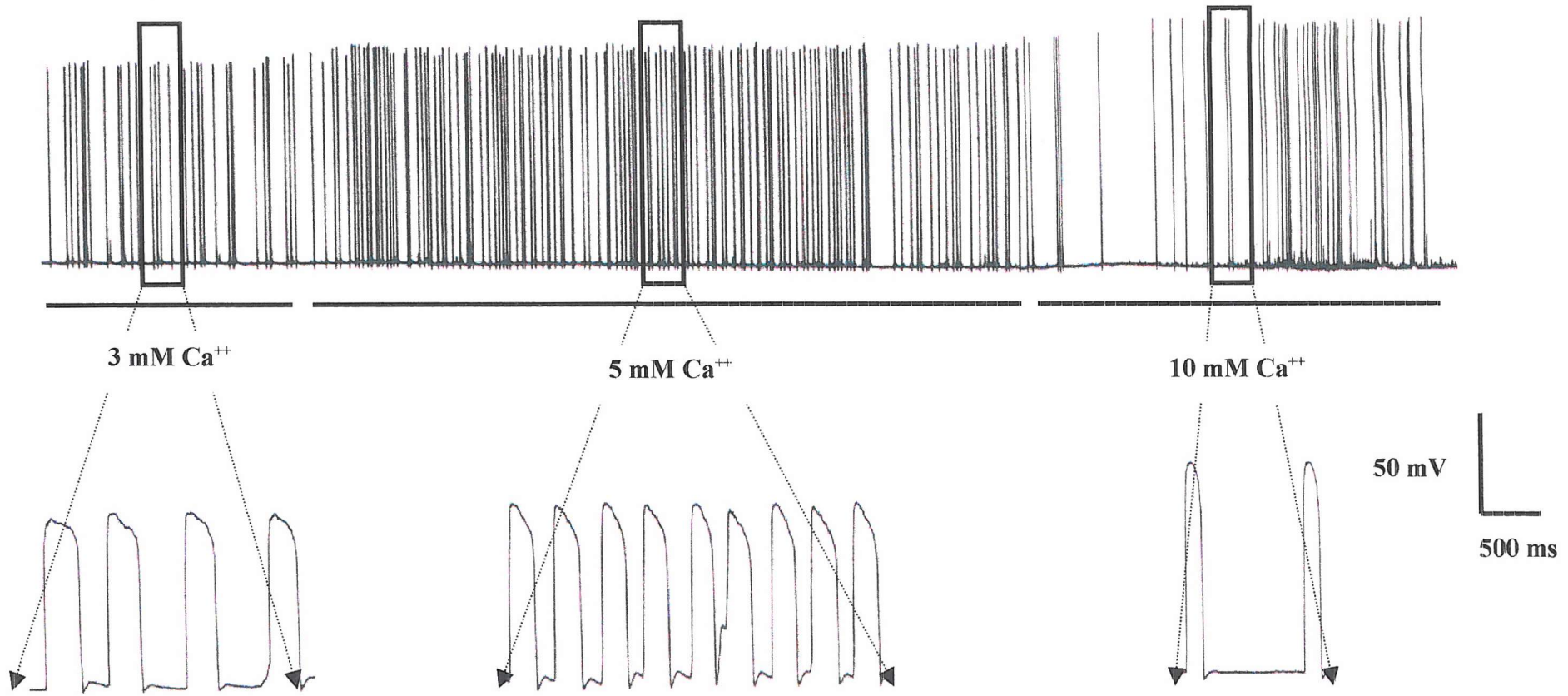


Figure 3.5. Effect of raising extracellular Ca^{++} on pharyngeal action potential height and duration. Resting membrane potential was –74 mV with action potential amplitudes of 102 mV. Increasing extracellular Ca^{++} elicited a concentration-dependent increase in spike height combined with a decrease in action potential duration. Scale bar applies to expanded section only.

3.5. Effect of L-type Ca^{++} channel blockers on pharyngeal action potentials

The contribution that L-type calcium channels may play in generating pharyngeal action potentials was examined with the use of the selective L-type calcium channel blockers, verapamil and nifedipine. Both of these compounds had similar effects, ie, a concentration-dependent increase in action potential frequency combined with a reduction in action potential duration and overshoot (Figure 3.6 & 3.7). Even at the higher concentrations examined, the preparation was not exposed to more than 0.1 % ethanol vehicle. This has previously been shown to have no effect on pharyngeal action potentials.

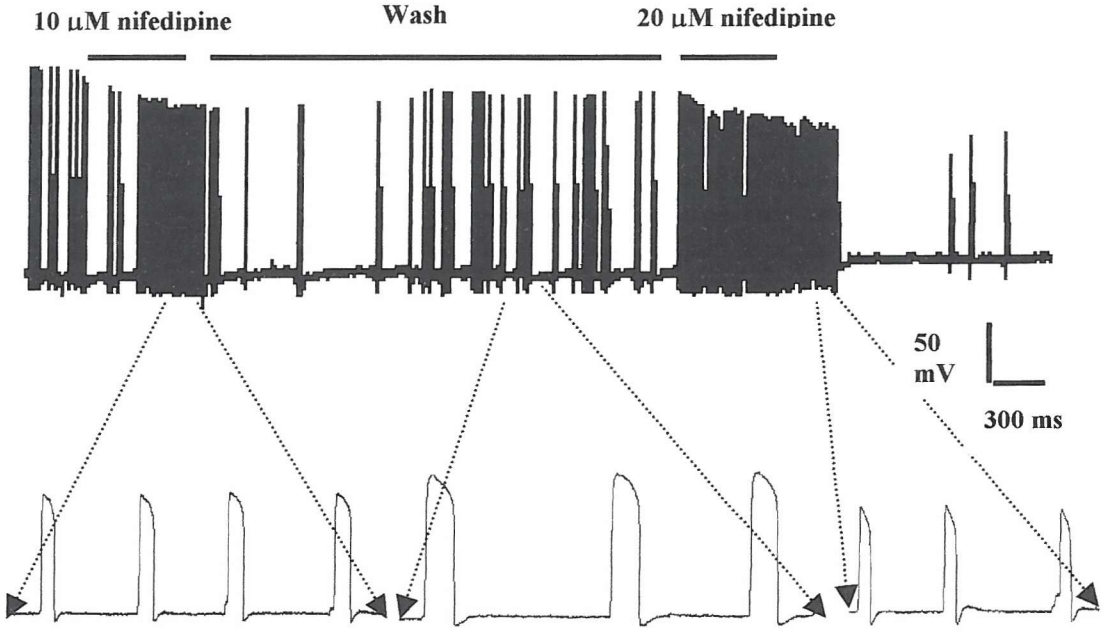


Figure 3.6. Trace demonstrating the effect of nifedipine on pharyngeal action potentials. Nifedipine caused a concentration-dependent reduction in action potential amplitude and duration. Resting membrane potential was –73 mV and action potential height was 110 mV in this preparation. Scale bar applies to expanded section only.

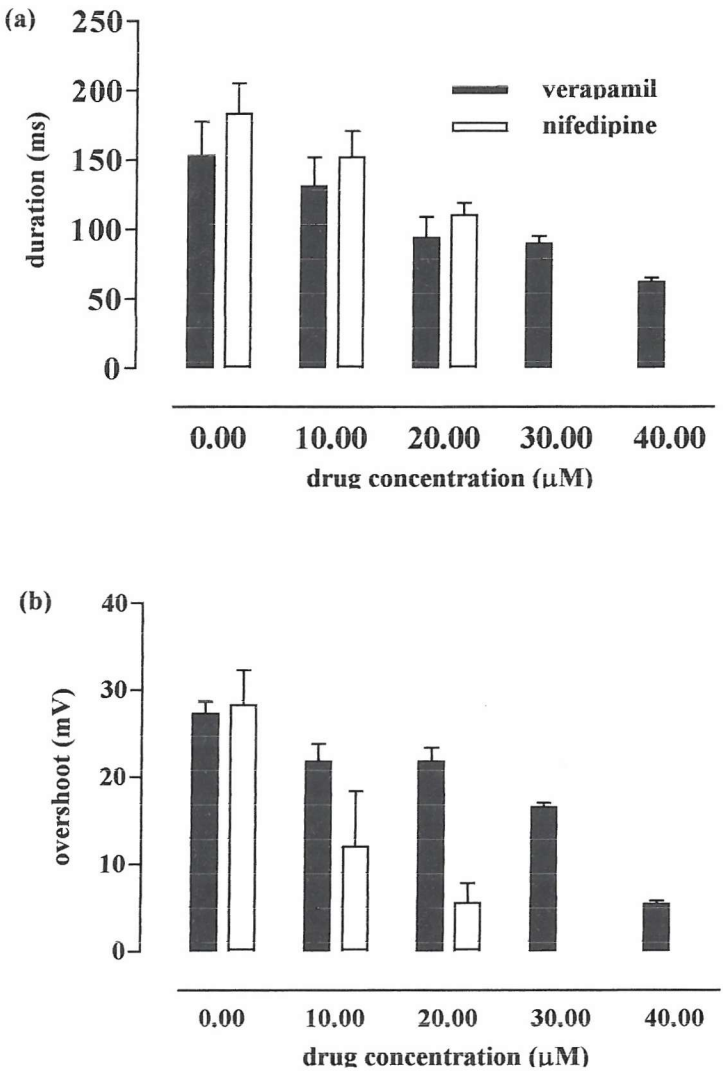


Figure 3.7. The effect of the calcium channel blockers, verapamil and nifedipine, on (a) action potential duration and (b) action potential overshoot. Drugs were applied at the concentration indicated, to the preparation for at least 5 mins before measurements were taken (n=8).

3.6. Effect of reducing extracellular Na^+ on pharyngeal action potentials

As action potentials persisted in zero Ca^{++}, the possibility that Na^+ ions may play a role was examined. Reducing extracellular Na^+, by replacement with glucosamine hydrochloride, from 140 mM to 70 mM (n=9) elicited a small depolarisation of 4.8 mV (Figure 3.2). Depolarisations were of a similar magnitude when Na^+ was completely replaced (Figure 3.8). Action potential rising slope and overshoot were reduced in 70 mM

and zero Na^+ and a complete, but fully reversible, cessation of spikes was observed (Figure 3.8; n=12).

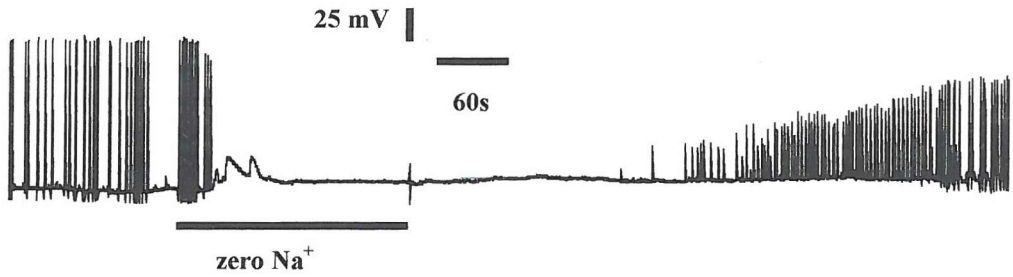


Figure 3.8. Trace demonstrating the cessation of action potentials in zero Na^+. Glucosamine hydrochloride was employed to replace Na^+ ions in this experiment. Resting membrane potential was –71 mV and action potential height was 106 mV. Spikes were gradually reinstated following introduction of standard saline.

3.7 Pharmacological properties of *C. elegans* pharynx

The response to a number of classical transmitters and peptides on pharyngeal action potentials were examined in the remaining section of this chapter.

3.7.1 Effect of cholinergic agonists on pharyngeal action potentials

Perfusion of ACh directly on the pharyngeal muscle produced a concentration-dependent depolarisation that was associated with an increase in action potential frequency at lower concentrations, threshold 500 nM (Figure 3.9). A gradual reduction in action potential overshoot was observed during periods of rapid action potential firing. The depolarising response slowly desensitised at all concentrations tested, although it was more evident at higher ACh concentrations. On introduction of the wash, membrane potential and action potential frequency rapidly returned to resting levels.

Nicotine produced an essentially identical response to ACh, although it appeared more potent. For example, in one experiment, 3 μM nicotine produced a 28 mV depolarisation compared to a 5 mV depolarisation to that seen with ACh (data not shown).

Muscarine at concentrations 10 to 500 μM produced a slight increase in action potential frequency associated with a gradual decline in spike overshoot. An initial small depolarisation of approximately 2-3 mV was observed but this returned to resting levels during drug application. Spike height and frequency soon retuned to resting levels following introduction of the wash (Figure 3.10).

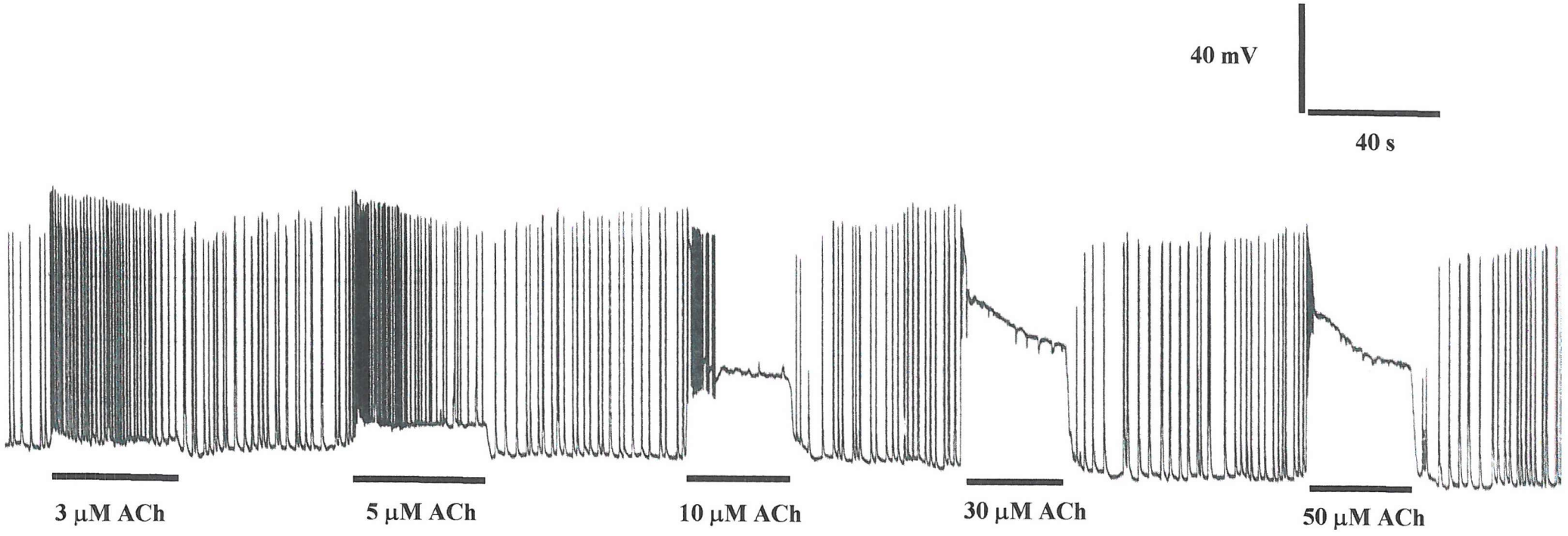


Figure 3.9. The effect of increasing concentrations of acetylcholine on pharyngeal action potentials. Voltage recording from a single cell within the *C. elegans* pharyngeal terminal bulb. The resting membrane potential in this experiment was –82 mV with action potentials amplitudes of 107 mV. Drug application indicated by horizontal bars. Acetylcholine produced a rapid concentration dependent depolarisation associated with an increase in action potential frequency at lower drug concentrations.

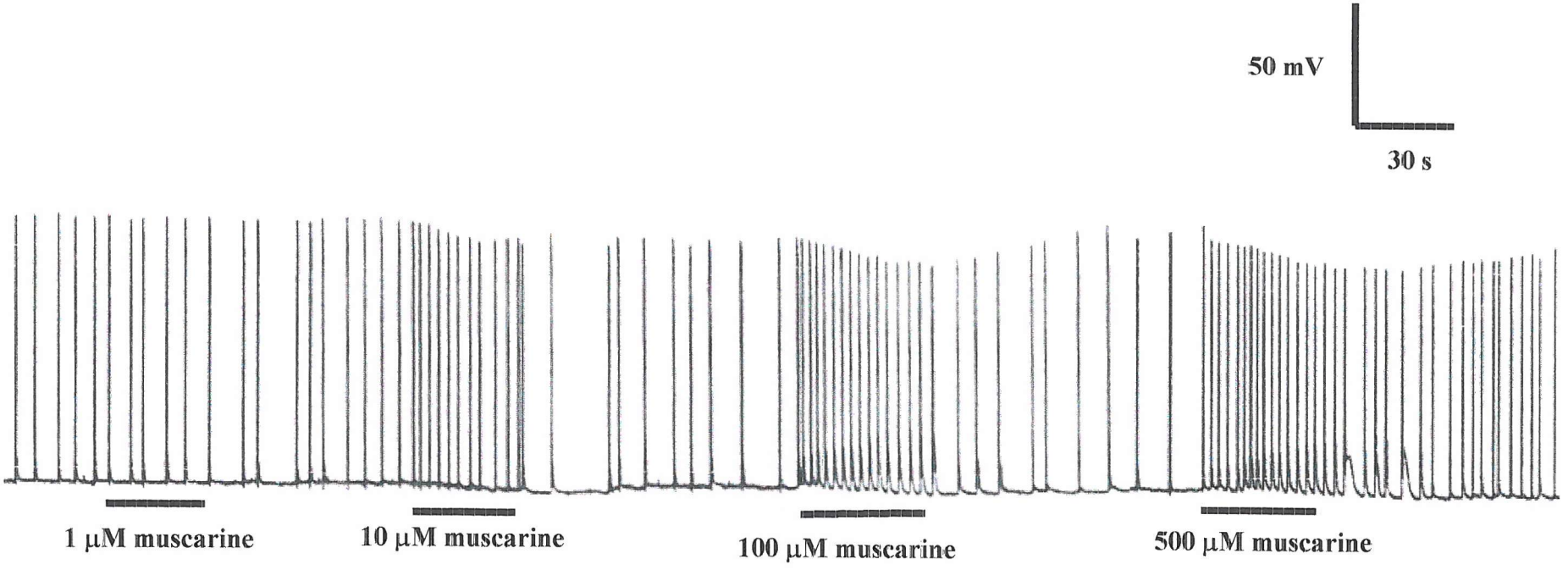


Figure 3.10. The effect of increasing concentrations of muscarine on pharyngeal action potentials. Voltage recording from a single cell within the *C. elegans* pharyngeal terminal bulb. The resting membrane potential in this experiment was –71 mV with action potentials amplitudes of 115 mV. Drug application indicated by horizontal bars. Muscarine produced a small concentration dependent depolarisation associated with a slight increase in action potential frequency. A gradual reduction in spike height was also observed.

3.7.2 Effect of biogenic amines on pharyngeal action potentials

Dopamine was examined at concentrations of 10 nM to 1 mM and produced a rapid increase in pharyngeal action potentials frequency with a threshold of between 1 and 10 μM (Figure 3.11). For example, in two experiments, action potential frequency was increased in the presence of 1 μM dopamine, whilst in another preparation; no effect was observed until 10 μM was applied (Figure 3.11). The excitatory effect of dopamine was identical to that seen with serotonin (data not shown), although the threshold for serotonin induced stimulation of pharyngeal pumping was lower (50 nM).

3.7.3 Effect of neuroactive peptides on pharyngeal action potentials

Application of the RFamide peptides, KNEFIRFamide (AF1) and KHEYLRFamide (AF2), both caused stimulation of pharyngeal pumping (Figure 3.12 & 3.13; n=3). This increase in action potential frequency was also associated with a slow decline in spike overshoot and a 3-5 mV depolarisation. Resting spike frequency and action potential overshoot was soon re-established following introduction of the wash.

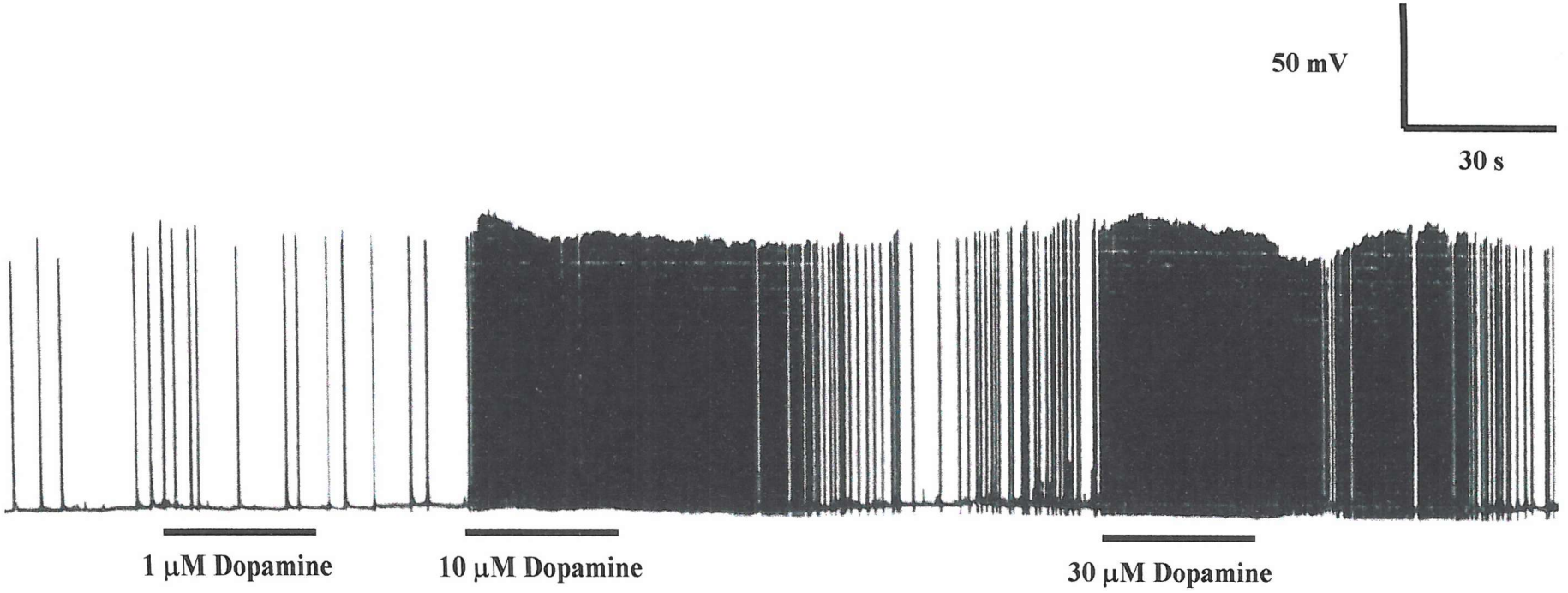


Figure 3.11. The effect of increasing concentrations of dopamine on pharyngeal action potentials. Voltage recording from a single cell within the *C. elegans* pharyngeal terminal bulb. The resting membrane potential in this experiment was –74 mV with action potentials amplitudes of 116 mV. Drug application indicated by horizontal bars. Dopamine produced a rapid concentration dependent increase in action potential frequency that was slow to recover following introduction of the wash.

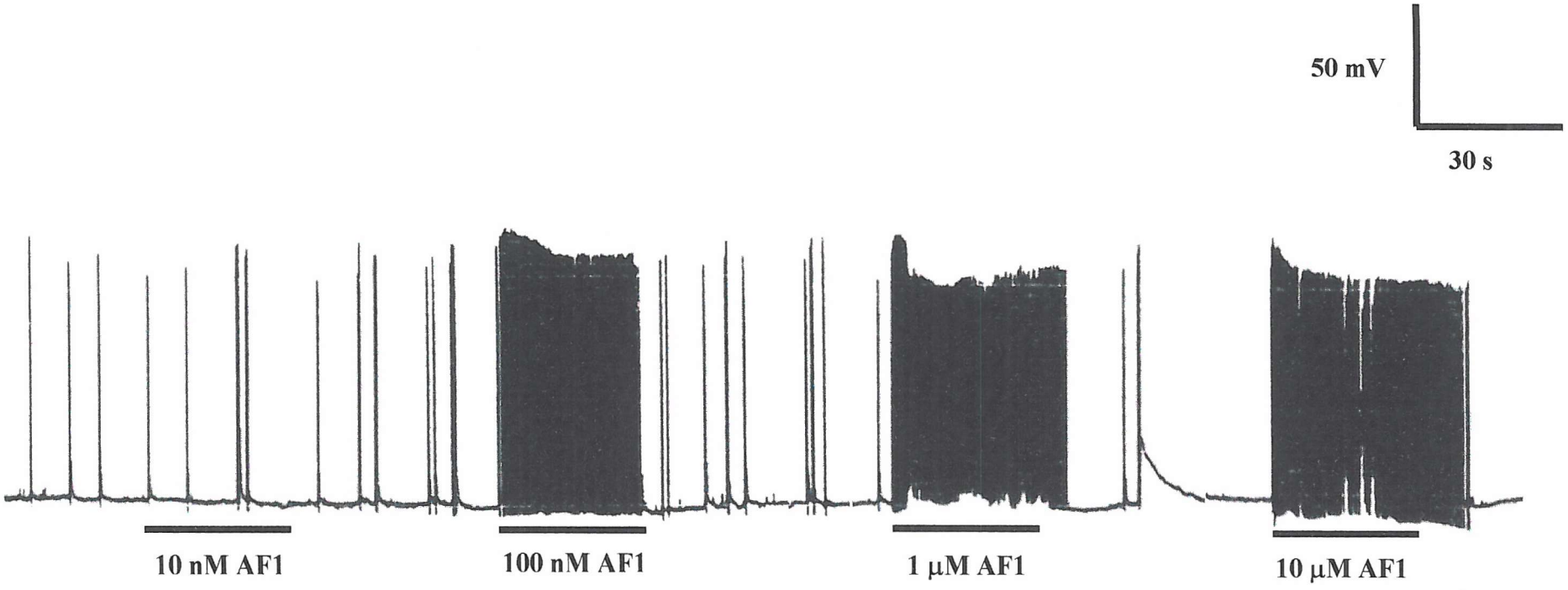


Figure 3.12. The effect of increasing concentrations of AF1 on pharyngeal action potentials. Voltage recording from a single cell within the *C. elegans* pharyngeal terminal bulb. The resting membrane potential in this experiment was –70 mV with action potentials amplitudes of 108 mV. Drug application indicated by horizontal bars. AF1 produced a rapid concentration dependent increase in action potential frequency associated with a small reduction in action potential height.

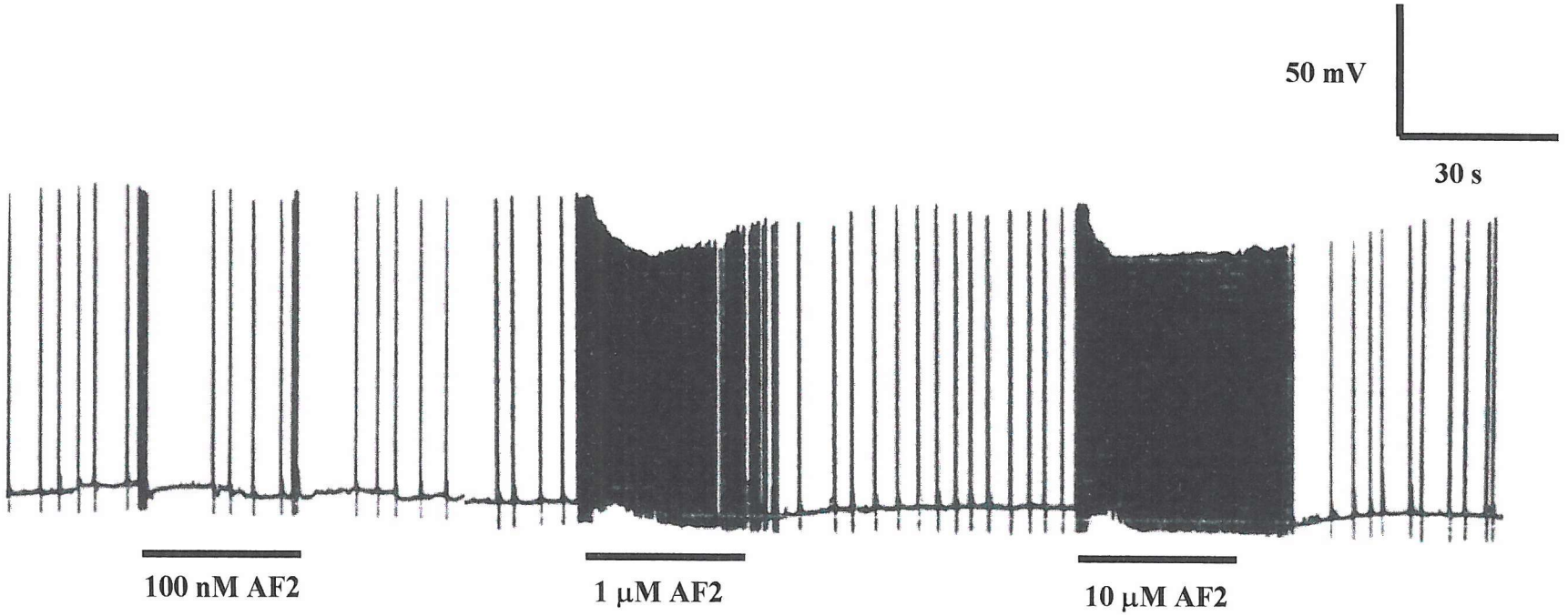


Figure 3.13. The effect of increasing concentrations of AF2 on pharyngeal action potentials in wild-type *C. elegans*. The resting membrane potential in this experiment was –75 mV with action potentials amplitudes of 115 mV. Drug application indicated by horizontal bars. AF2 produced a rapid concentration dependent increase in action potential frequency associated with a gradual reduction in action potential height.

3.7. Discussion

The first intracellular recordings from *C. elegans* pharyngeal muscle were made by Davis et. al., (1995). A resting membrane potential of –45 mV was described although the saline employed in their investigations was identical to that used here. This value differs by just less than 30 mV to that seen in these recordings, although action potential overshoot and duration are essentially identical. The reasons behind this large discrepancy remains unclear, but is likely to be due to the technical difficulties associated with making stable recordings from a contracting tissue. Lower resting membrane potentials were occasionally observed in these studies, but they have always coincided with bad electrode placement leading to damaged pharyngeal muscle. These preparations were easy to identify as terminal bulb contractions became feeble following impalement and the area around the electrode tip began to fibrillate. In all of the investigations described within this thesis, any preparations that displayed these characteristics were discarded.

Insights into the role that particular ions play in generating/maintaining resting membrane potential were gained from the experiments in which major cations and anions were removed or replaced. Decreasing extracellular Cl^- caused a transient depolarisation and increase in action potential frequency although resting membrane potential rapidly returned to the resting value. This suggests that although there is a resting Cl^- conductance, the equilibrium potential for Cl^- is tightly controlled. Studies on the chloride-dependent depolarisation of the pharynx by glutamate described in Chapter 4, have demonstrated that the equilibrium potential for Cl^- is maintained at a value 40 mV more positive than resting membrane potential (Pemberton et al., 2001). An inwardly-directed Cl^- pump is likely to maintain this positive value.

The membrane potential was extremely dependent on the extracellular concentration of K^+, although the depolarisation observed following elevating extracellular K^+ concentration was less than predicted from the Nernst equation. Earlier studies which demonstrated that ouabain produces concentration-dependent membrane depolarisations provides evidence for the contribution of an electrogenic Na^+-K^+ pump in generating resting membrane potential (Chris Franks, personal communication). This finding is in agreement with earlier investigations by Davis et.

al., (1995), who demonstrated that mutations in the *eat-6* gene, which encodes for a Na^+-K^+ ATPase, also produces changes in pharyngeal resting membrane potential.

Membrane potential was only slightly affected by changes in extracellular Ca^{++} and Na^+ concentration but the mechanisms behind these effects were not further investigated. It is probable however, that the small effects observed on membrane potential are caused by the effect of ion replacement on Na^+-Ca^{++} exchangers and/or Na^+-K^+ pumps.

In conclusion, the resting properties of *C. elegans* pharynx are different than those observed for the pharyngeal muscle of the much larger parasitic nematode, *Ascaris lumbricoides*. For this nematode, the pharyngeal resting membrane potential is around –40 mV and dependent on the concentration of extracellular anions (Del Castillo and Morales, 1967); (Del Castillo et al., 1964). As the habitats of these nematodes are very different (soil and gut), it is of no great surprise that their membrane potentials are generated by differing ionic species. Surprisingly, action potential shape and duration were very similar between these two nematodes, in that they both have a long plateau phase and also generate a K^+ dependent, hyperpolarizing potential which drives the membrane potential to around –90 to –100 mV (Del Castillo et al., 1964); (Byerly and Masuda, 1979); (Avery and Thomas, 1997).

The roles that individual ions play in generating/maintaining action potentials were also examined by ion replacement experiments. The effect of varying extracellular Ca^{++} levels had marked effects on action potential frequency, amplitude and duration. Reducing extracellular Ca^{++} caused a transient increase in action potential frequency combined with a reduction in spike amplitude and prolongation in spike duration. The increase in spike frequency can be explained by the stabilising effect of Ca^{++} on excitable cell membranes (Weidmann, 1955); (Shanes, 1958). Surprisingly spikes were still observed in Ca^{++} free saline. It is possible that enough Ca^{++} was present in the laboratory grade salts to generate these action potentials as no EGTA was added to the perfusate. The effect of reduced Ca^{++} on spike amplitude is consistent with the findings of Lee et. al., (1997), who suggested that an L-type Ca^{++} channel plays a role in action potential generation/maintenance. These conclusions were based on their observation that gain of function mutations in the $\alpha 1$ subunit of a

voltage activated L-type Ca^{++} channel caused prolongation of the spike plateau, whereas loss of functions mutations cause a decrease in the initial spike slope. Moreover, our findings that the L-type Ca^{++} channel blockers verapamil and nifedipine both reduced action potential duration and amplitude are consistent with the role of voltage-gated L-type Ca^{++} channels in generating/maintaining pharyngeal action potentials.

The effect of reducing extracellular Ca^{++} on spike duration was intriguing. A prolongation of action potentials was observed in low Ca^{++} whilst action potential duration was shortened in high extracellular Ca^{++}. This is the opposite effect to that which would be expected if the action potential plateau phase were Ca^{++} dependent. Identical effects of reduced Ca^{++} on spike duration have been observed in the molluscan heart (Wilkens, 1971) and on slow waves recorded from the somatic muscle cells of *Ascaris lumbricoides* (Weisblat et al., 1976). The most likely explanation is that a Ca^{++} dependent K^+ current controls the repolarisation phase in the *C. elegans* pharynx. Indeed, two genes that encode for these channels are present within the *C. elegans* genome (Wei et al., 1996) and these genes are expressed in the pharyngeal muscle (Yuan et al., 2000).

Another possible explanation of the effect of low Ca^{++} on action potential duration is that membrane repolarisation is controlled by Ca^{++} dependent neurotransmitter release. The glutamatergic motor neurone M3, is thought to be activated during pharyngeal muscle contraction (Avery, 1993). Subsequent glutamate release from M3 is then believed to hyperpolarize the muscle by its action upon glutamate-gated chloride channels (Dent et al., 1997). This hyperpolarisation would in turn activate the K^+ channel *exp-2* (Davis et al., 1999), thus facilitating muscle repolarisation. To test this hypothesis, these experiments would need to be conducted on animals that have had their pharyngeal nervous system laser ablated. As this technique was not established within the laboratory, it was impossible to investigate this further.

The most surprising observation of this study was the action potential dependence on extracellular Na^+, especially as no evidence of a voltage-gated Na^+ channel has been found within the *C. elegans* genome (Bargmann, 1998). The reduction or complete removal of Na^+ caused a rapid decrease in action potential rising

slope and overshoot followed by cessation of spikes. Action potentials were gradually reinstated following the return of Na^+ to the perfusate.

These findings were investigated further by another member of the laboratory using N-methyl-D-glucamine as the Na^+ ion substitute. Decreasing extracellular Na^+ to 35 mM (n=6), 50 mM (n=8), and 70 mM (n=6), all caused a similar reduction in amplitude and rising slope of action potentials to that seen with glucosamine. A complete, but fully reversible cessation of action potentials was also observed (Irina Vinogradova, personal communication).

All of these experiments were carefully monitored for pH effects and two different cations were employed to replace Na^+. It could be argued that a depolarising block is responsible for the eventual cessation of action potentials as a small depolarisation of approximately 5-10 mV was recorded in reduced and Na^+ free media. This explanation is unlikely however, as action potentials have been shown to occur when the muscle is significantly more depolarised than that seen with Na^+ removal (Figure 3.9). The simplest explanation for the above observation is that activation of a voltage-gated Na^+ channel is responsible for the early rising phase of pharyngeal action potentials and this possibility is currently been investigated. However, this preliminary study has provided tantalising evidence that a Na^+ current, through an as yet undescribed channel, plays a pivotal role in generating pharyngeal action potentials.

The pharmacological study has demonstrated that a number of classical transmitter/modulators have potent effects on the pharyngeal muscle. The nicotinic cholinergic agonists examined in this work, both caused an increase in action potential frequency, and at higher concentrations, concentration-dependent depolarisations were also observed. These effects are identical to that seen on the somatic muscle of *Ascaris summ* (Del Castillo et al., 1963) (Martin et al., 1991); (Segerberg and Stretton, 1993) and also on the vagina vera musculature of *Ascaris* (Fellowes et al., 2000). Patch clamp studies on *Ascaris*, have revealed that ACh increases the non-selective cation conductance of the membrane with a reversal potential near zero mV (Martin, 1982), (Harrow and Gration, 1985). This is consistent with ACh opening channels permeable to both Na^+ and K^+. Although the ionic basis of the *C. elegans* pharyngeal response to

ACh and nicotine was not examined within this study, it is not unreasonable to assume that these ions are also responsible for the membrane excitation and depolarisation seen here.

Previous genetic studies have shown that ACh plays a key role in pharyngeal excitation. For example, a putative null mutation in *cha-1*, a gene required for the synthesis of ACh (Alfonso et al., 1994), severely reduces pharyngeal pumping rates (Avery and Horvitz, 1990). Partial loss of function mutations in either *cha-1* or *unc-17*, a gene required for the vesicular packaging of ACh (Alfonso et al., 1993), also causes reduced pumping rates (Raizen et al., 1995). Furthermore, Avery & Horvitz (1990) have demonstrated that *cha-1* mutants can generate high pumping rates when placed on agar plates containing high concentrations of nicotine. Studies performed by Raizen et. al., (1995), have suggested that ACh released from the pharyngeal motor neurone MC, may act to stimulate pharyngeal pumping by its action upon muscle nAChRs. Mutations in two genes, *eat-2* and *eat-18*, eliminated MC neurotransmission. Subsequent cloning of *eat-2* revealed that it encodes for a β-subunit of a nicotinic acetylcholine receptor and its expression is restricted to pharyngeal muscle (McKay: personal communication). The effect of nicotinic agonists demonstrated in this study is therefore likely to be due to a direct effect on the pharyngeal muscle. The intracellular recordings made here are the first to demonstrate that ACh induces an increase in pharyngeal membrane excitability, which ultimately leads to increased action potential generation. This effect is likely to be mediated by opening of pharyngeal nAChRs leading to an increase in Na^+ and K^+ conductance across the membrane.

The effect of muscarine on pharyngeal action potentials was less pronounced than that seen with nicotinic agonists. Muscarine, at concentrations greater than 10 μM produced small depolarisations that returned to resting levels during drug application. Slight increases in action potential frequency associated with gradual reductions in spike height were also observed. The presence of muscarinic receptors was first demonstrated in *C. elegans* by the observation that the cholinergic antagonists [3H]N-methylscopolamine and quinuclidinyl benzilate ([3H]QBN) bound to crude tissue homogenates with high affinity (Culotti and Klein, 1983). The first G-protein linked ACh receptor was cloned a few years later and was shown to be similar in sequence but

pharmacologically distinct from the human muscarinic ACh receptor (Lee et al., 1999). In total, the *C. elegans* genome contains three open reading frames with significant homology to known muscarinic receptors. GFP-gene fusion constructs, has revealed that one of these genes, C15B12.5, is expressed in anterior pharyngeal muscle (Kate Steger, personal communication). Although the role that muscarinic receptors play in pharyngeal muscle excitation has yet to be fully examined, the finding here that muscarine has an effect on pharyngeal action potentials combined with the observation that at least one muscarinic receptor gene is expressed in the pharynx, suggests a possible physiological role for muscarinic receptors in feeding.

Dopamine at concentrations greater than 1 μM caused a concentration-dependent increase in pharyngeal action potential frequency with no consistent effect on action potential overshoot. This particular monoamine is thought to be the transmitter of at least eight neurones in the hermaphrodite form of *C. elegans* (Sulston et al., 1975). These include the mechanosensory cephalic and deirid neurones, which are located within the nerve ring. The other neurones that use dopamine as a transmitter include a small neural circuit that modulates the worms locomotory behaviour in response to a mechanical attribute of bacteria (Sawin et al., 2000), and a subset of neurones that control egg laying (Weinshenker et al., 1995). The parasitic nematodes *Ascaris lumbricoides* and *Ascaridia galli* also use dopamine as a transmitter (Mishra et al., 1984).

The stimulatory effect of dopamine on *C. elegans* pharynx is identical to that seen with serotonin although less potent. Serotonin has already been shown to stimulate pharyngeal pumping in *C. elegans* (Avery and Horvitz, 1990) and both of these compounds were also excitatory on *Ascaris summ* pharynx (Walker et al., 1999). Serotonin has been detected histochemically in whole mounts and is localised in two pharyngeal neurones that appear to be neurosecretory (Horvitz et al., 1982). One strong candidate for mediating the effects of serotonin on pharyngeal muscle is *ser-1*. This novel serotonin receptor is expressed predominantly in pharyngeal muscle and has recently been determined to be responsive to serotonin in a heterologous expression system (Hamdan et al., 1999). To date, no receptors for dopamine have been cloned from *C. elegans*. However, the observation here that dopamine stimulates pharyngeal

pumping suggests that dopaminergic receptors present either on the pharyngeal muscle or within the pharyngeal nervous system play a physiological role in modulating feeding behaviour.

Numerous FMRFamide-related peptides (FaRPs) have been isolated and sequenced from extracts of free-living and parasitic nematodes. The first two peptides isolated from *Ascaris summ* were AF1 and AF2 (Cowden et al., 1989); (Cowden and Stretton, 1990). Later studies have demonstrated the presence of these peptides in *C. elegans* (Marks et al., 1995); (Nelson et al., 1998). Both of these peptides were excitatory on *C. elegans* pharyngeal muscle with a threshold of around 100 nM. Surprisingly AF1 inhibits pharyngeal pumping in *Ascaris summ* whilst AF2 had no observable effect (Brownlee and Walker, 1999).

At the start of this pharmacological study, little was known about the actions of various classical transmitters and peptides upon the *C. elegans* pharyngeal preparation. All of our current understanding of nematode pharyngeal pharmacology has been derived from studies on the much larger parasitic nematode *Ascaris suum* and *Ascaris lumbricoides.* Whilst transfer of information between these nematodes has proved to be an essential and invaluable tool, it is still nevertheless an imperfect approach. The flaws in this extrapolation have been highlighted by the distinct differences in the *C. elegans* pharyngeal response to the peptides AF1 and AF2 when compared to *Ascaris.* This study has demonstrated that a detail pharmacological analysis of *C. elegans* pharyngeal muscle can now be performed. Furthermore, if used in conjunction with the genetic tractability that *C. elegans* has to offer; it will prove to be a powerful approach with which to determine the contribution that particular genes make to the observed pharmacology.

Chapter 4: *In vivo* characterisation of glutamate-gated chloride channels in the pharyngeal muscle of wild-type and mutant *C. elegans*

4.1. Introduction

The amino acid glutamate is thought to be one of the primordial neurotransmitter substances that were used in synaptic transmission before the evolution of specialised transmitters such as ACh and the catecholamines (Usherwood, 1978). Consistent with this view, glutamate is the major excitatory neurotransmitter of the mammalian central nervous system (Monaghan et al., 1989), and has been implicated in important physiological processes, including developmental plasticity, long term potentiation, excitotoxic damage in ischemia, and other neurodengenerative disorders (Nicoll et al., 1988); (Collingridge and Singer, 1990); (Kennedy, 1989); (Choi, 1988).

The diverse effects of glutamate are produced through a set of heterogeneous glutamate receptor subfamilies that are classified according to their pharmacological and electrophysiological properties. Glutamate receptors are primarily classified into metabotropic and ionotropic subtypes (Monaghan et al., 1989); (Watkins et al., 1990). Ionotropic glutamate receptors are multimeric assemblies of four or five subunits, and are divided into three groups, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate receptors, based on their pharmacology and structural properties. Metabotropic glutamate receptors are coupled to a variety of second messenger systems *via* G proteins. To date, eight different metabotropic glutamate receptors have been cloned and subsequently expressed in cell lines (Schoepp et al., 1999); (Pin and Duvoisin, 1995).

Glutamate also serves as a neurotransmitter in invertebrates, and several ionotropic receptors gating either cation or anion channels have been described (Sattelle et al., 1992); (Schuster et al., 1991); (Maricq et al., 1995); (Hart et al., 1995). Glutamate activated anion channels are permeable to chloride and are unique to the invertebrate phyla. These channels were first reported from locust (*Schistocerca gregaria*) leg muscle preparations in the 1970's (Cull-Candy and Usherwood, 1973); (Lea and Usherwood, 1973a); (Lea and Usherwood, 1973b). In this preparation, glutamate was shown to elicit a biphasic membrane potential response, indicating the presence of two classes of glutamate-sensitive conductances. The depolarising response is mediated by D-receptors, now known to be related to mammalian AMPA, kainate, and NMDA

glutamate-gated cation channels (Cull-Candy and Usherwood, 1973); (Lea and Usherwood, 1973a); (Lea and Usherwood, 1973b); (Schuster et al., 1991); (Ultsch et al., 1992). The glutamate-gated chloride mediated response could be selectively activated with the glutamate analogue ibotenic acid (Clark et al., 1979); (Cull-Candy and Usherwood, 1973); (Lea and Usherwood, 1973a); (Lea and Usherwood, 1973b).

The agonist profile of locust muscle glutamate-gated chloride channels (GluCl) is distinct from glutamate-gated cation channels and other ligand-gated chloride channels. They are activated with glutamate, ibotenate, and weakly with aspartate (Cull-Candy, 1976); (Cull-Candy and Usherwood, 1973); (Duce and Scott, 1985); (Dudel et al., 1989); (Lea and Usherwood, 1973a); (Lea and Usherwood, 1973b). They are insensitive to GABA, muscimol, NMDA, quisqualic acid and kainic acid, and are weakly sensitive to picrotoxin, requiring concentrations greater than 100 μM for complete inhibition (Cull-Candy, 1976); (Cull-Candy and Usherwood, 1973); (Duce and Scott, 1985); (Dudel et al., 1989); (Lea and Usherwood, 1973a); (Lea and Usherwood, 1973b).

Subsequent to the discovery of GluCl channels on locust leg muscle, insect GluCl channels with similar pharmacological profiles were reported in several insect neuronal preparations (Dubas, 1990); (Giles and Usherwood, 1985); (Horseman et al., 1988); (Wafford and Sattelle, 1989), in *Drosophila* larval muscle (Delgado et al., 1989), and have been expressed from *Schistocerca gregaria* leg muscle mRNA in *Xenopus* oocytes (Fraser et al., 1990). The presence of GluCl channels in nematodes was demonstrated in a series of studies that were investigating the mode of action of the anthelmintic compound, ivermectin (Arena et al., 1991); (Arena et al., 1992); (Arena et al., 1995); (Cully et al., 1994). Ivermectin is a member of the much larger avermectin family. This family of naturally occurring compounds are macrocyclic lactones that were originally isolated from *Streptomyces avermitilis* (Burg et al., 1979).

The introduction of ivermectin as a veterinary parasiticide in France in 1981 revolutionised animal anti-parasitic chemotherapy. Within five years, ivermectin was responsible for 16% of world-wide anthelmintic sales (Bloomfield, 1988), and by 1990 it was estimated that more than 800 million cattle had been treated with ivermectin

(Sutherland, 1990). Clinical trials in humans started in 1982 and ivermectin was shown to have excellent activity against microfilaria of *Onchocera volvulus* and to be generally well tolerated (Aziz et al., 1982). The low incidence of adverse reactions, prolonged post-treatment reductions in skin microfilarial counts, and potential drawbacks of alternative therapeutics (Goa et al., 1991), made ivermectin the drug of choice for human onchocerciasis and led to mass medication programmes in endemic areas (Aziz et al., 1982); (Remme et al., 1990).

Ivermectin appeared to interact in some way with all ligand-gated chloride channels including mammalian $GABA_A$ and glycine receptors (see Arena, 1994 for review). Early studies identified GABA-gated chloride channels as a site of action for ivermectin, but its main anti-parasitic activity is now thought to be due to its binding to GluCl channels in invertebrate nerve and muscle cells. This leads to an increase in chloride ion permeability of the cell membrane (Fraser et al., 1990); (Duce and Scott, 1985); (Scott and Duce, 1986); (Paiement et al., 1999), and paralysis and death result from irreversible hyperpolarisation or depolarisation of nerve and muscle cells.

Much of the work directed at elucidating the molecular action of ivermectin upon nematodes has benefited from the use of *C. elegans*. For example, Cully (1994) originally reported the cloning of two subunits of GluCl channels from *C. elegans*: an α-subunit, which when expressed in *Xenopus* oocytes formed chloride channels irreversibly gated by ivermectin, and a β-subunit which formed channels gated by glutamate. The sequences of the polypeptides showed all the characteristic motifs expected for inhibitory ligand-gated ion channels and possessed significant homology with vertebrate and invertebrate $GABA_A$ and glycine receptor subunits (Fig 4.1). When expressed together, the two subunits formed an ivermectin-potentiated, glutamate-gated chloride channel. Subsequent work showed that the α-subunit possessed a binding site for glutamate, but this was not coupled to the opening of an ion channel (Etter et al., 1996). Since then, the *C. elegans avr-15* gene has been demonstrated to encode, *via* alternative splicing, two forms of an α2 subunit; the longer of the two polypeptides can be expressed to form ivermectin and glutamate-gated chloride channels (Dent et al., 1997); (Vassilatis et al., 1997).

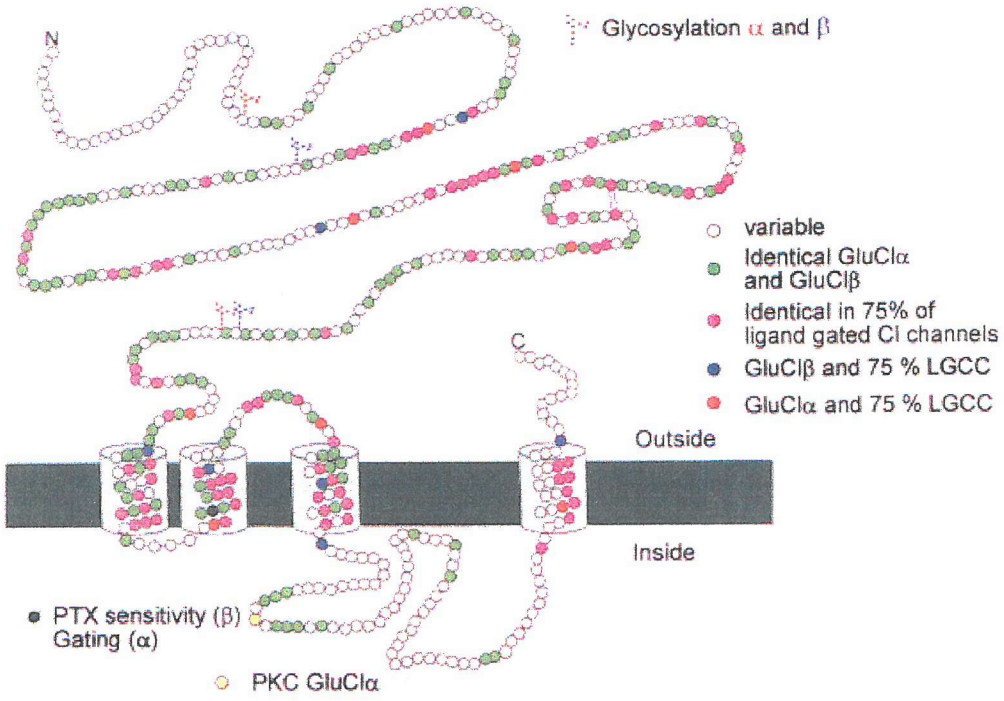


Figure 4.1. Schematic diagram of *C. elegans* GluClα and GluClβ proteins. The predicted protein sequences of GluClα and GluClβ were aligned with $GABA_A$ and glycine receptor subunits and related invertebrate proteins (Cully et al., 1994), and homologous amino acids are shown. Figure taken from Cully et. al., (1996).

The extensive genetic characterisation of *C. elegans* makes it a useful system to study the function of GluCl channels and the mechanisms of resistance to ivermectin (Simpken and Coles, 1981), (Ohba and Ishibashi, 1984), (Bennett and Pax, 1986). Although only distantly related to most parasitic species, *C. elegans* displays the morphological conservation of the phylum and shares structures found to be important to parasitic nematodes. It also has a characteristic nematode biochemistry and proved to be sensitive to all major nematicidal drugs. At present, eight GluCl subunits encoded by six genes have been identified from *C. elegans* (Cully et al., 1994), (Dent et al., 1997), (Vassilatis et al., 1997), (Cully et al., 1996), (Laughton et al., 1997). The family consist of at least two classes of subunit, α and β, which may co-assemble to form either

homomeric or heteromeric ligand-gated chloride channels. The *Xenopus* oocyte expression system has been used to characterise some of the GluCl subunits cloned from *C. elegans* (summarised in Table 4.1).

Although a large family of GluCl channels have been identified in *C. elegans*, the subunit configuration of the native channels is still a matter of conjecture. This is an important issue as it is evident that the subunit composition has a marked effect on the properties of the chloride channel. For example, the presence of an α subunit would appear to be necessary to confer ivermectin sensitivity on the channel (Cully et al., 1994). In this study, we have used a quantitative pharmacological approach and intracellular recording techniques of both wild-type and mutant *C. elegans* to characterise native GluCl channels.

	EC_{50} Glutamate	EC_{50} Ivermectin	Ibotenate Effect	IC_{50} Picrotoxin
α1	No effect at 1 mM [a,b]	140 nM [a,b] 970 nM [d,e]	N.D.	59 μM [c]
α2A	2 mM [f]	N.D.	Agonist [f]	N.D.
α2B	208 μM [g]	108 nM [b,g]	N.D.	>100 μM [g]
β	380 μM [a] 800 μM [d]	No effect at 1 μM [a,b]	N.D.	77 nM [c]
α1β	1.36 mM [a] 1.8 mM [b]	190 nM [a,b] 500 nM [d,e]	Agonist [a]	42 μM [c] 18.5 μM [b]
α2β	62 μM [g]	103 nM [b,g]	N.D.	>100 μM [g]
GLC3	1.9 mM [h]	>100 nM [h]	Partial agonist [h]	> 1 mM [h]
α3A	No response [i]	No response [i]		
α3B	No response to 10 mM [i]	Response to 10 μM [i]		
mRNA	300 μM [k]	90 nM [b,j]	4.5 × More potent than glutamate	180 μM [k]
Pharynx	166 mM [l]	2.7 nM [e,l]	10 × More potent than glutamate	>100 μM [l]

[a] Cully et. al., (1994)
[b] Using Ivermectin Phosphate
[c] Etter et. al., (1999)
[d] Bush (1998)
[e] Using Ivermectin
[f] Dent et. al., (1997)
[g] Vassilatis et. al., (1997)
[h] Horoszok et. al., (1999)
[i] Dent et. al., (2000)
[j] Arena et. al., (1991)
[k] Arena et. al., (1992)
[l] Current study
N.D., not determined

Table 4.1. A comparison of the pharmacological properties of GluCl channels. The individual subunits, or *C. elegans* mRNA, were characterised in the *Xenopus laevis* oocytes expression system. 'Pharynx' refers to the native pharyngeal GluCl channels.

4.2. Effect of glutamic acid on wild-type pharyngeal muscle cells.

Perfusion of L-glutamate directly over muscle cells of the pharyngeal terminal bulb elicited an immediate membrane depolarisation that was associated with a decrease in the frequency and amplitude, and eventual cessation of action potentials and terminal bulb contractions. Responses were rapid in onset and concentration-dependent. Increases in action potential frequency were often observed following introduction of the wash. Figure 4.2 shows a typical intracellular recording from a pharyngeal muscle cell exposed to increasing concentrations of glutamate (1 μM-20 mM).

The response to glutamate in wild-type animals showed rapid desensitisation. Quantification of this was achieved by determining the membrane potential before application of glutamate (A), the peak change in membrane potential (B) and the membrane potential immediately prior to the wash (Figure 4.2). Percentage desensitisation was then calculated by the following equation:

$$\% \text{ desensitisation} = \frac{(B-A) - (C-A)}{(B-A)} \times 100$$

Desensitisation in wild-type animals was calculated to be 80 ± 6% at 100 μM glutamate, 62 ± 7% at 1 mM glutamate and 56 ± 5% at 10 mM glutamate (n=10; Figure 4.4). Figure 4.3 shows the concentration-response curve for the depolarisation elicited by glutamate. The EC_{50} value for glutamate, determined from peak depolarisation was 166 μM (95% confidence limits: 132 to 207 μM; n=16; Figure 4.3). The maximal response was 31.5 mV (95% confidence limits: 29.5 to 33.5 mV).

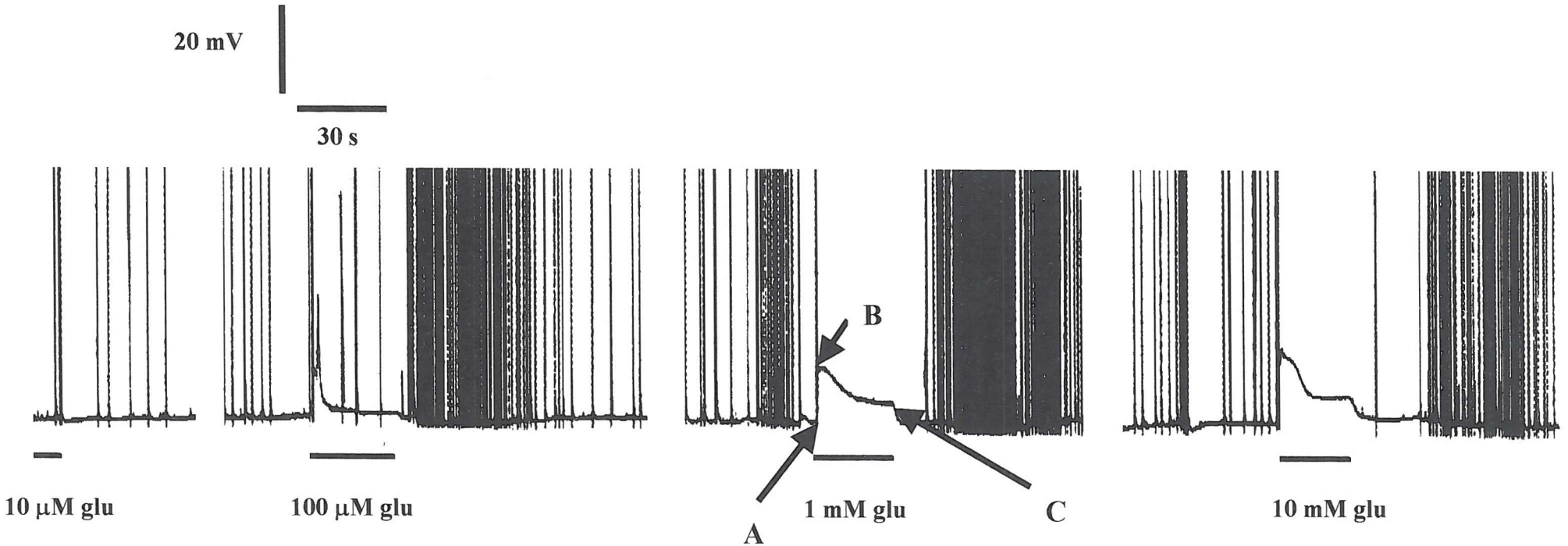


Figure 4.2. The effect of increasing concentrations of glutamate on membrane potential in wild-type *C. elegans*. Voltage recording from a single cell within the *C. elegans* pharyngeal terminal bulb. The resting membrane potential in this experiment was –77 mV with action potentials amplitudes of 106 mV. Drug application indicated by horizontal bars were for 30s in all cases. Glutamate produced a rapid concentration-dependent depolarisation that desensitised during drug application. Typically all cells demonstrated an increase in action potential frequency following the introduction of the wash. Points A-C refers to the method adopted for the quantification of desensitisation. Peaks of action potentials have been truncated in this trace.

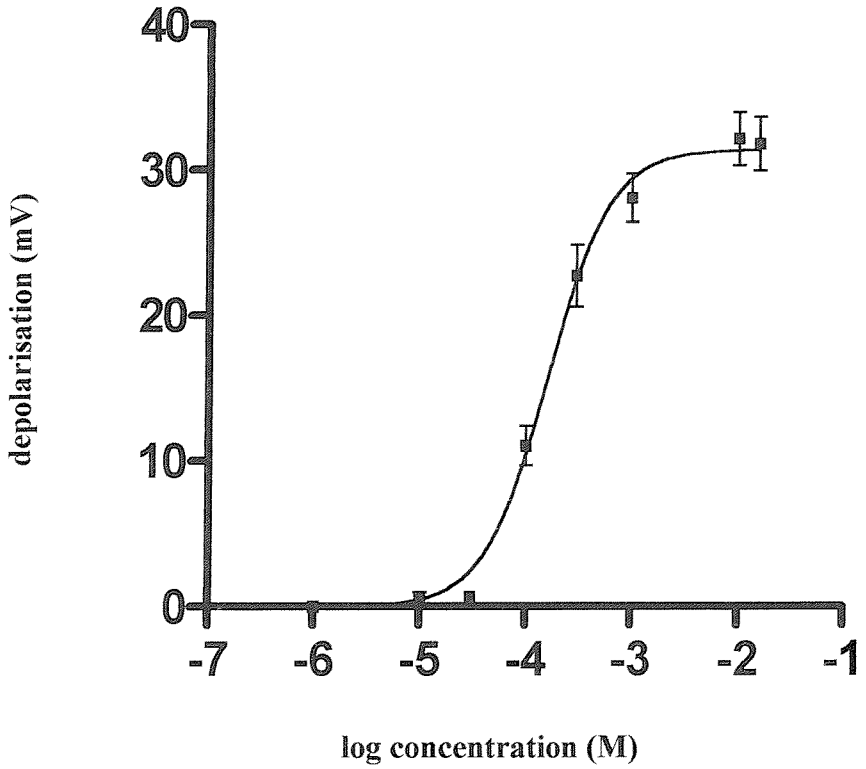


Figure 4.3. Concentration response curve for glutamate in wild-type *C. elegans*. The effect of increasing concentrations of glutamate (1 μM-20 mM) on membrane potential. Each point represents the mean of 16 individual determinations ± S.E.M.

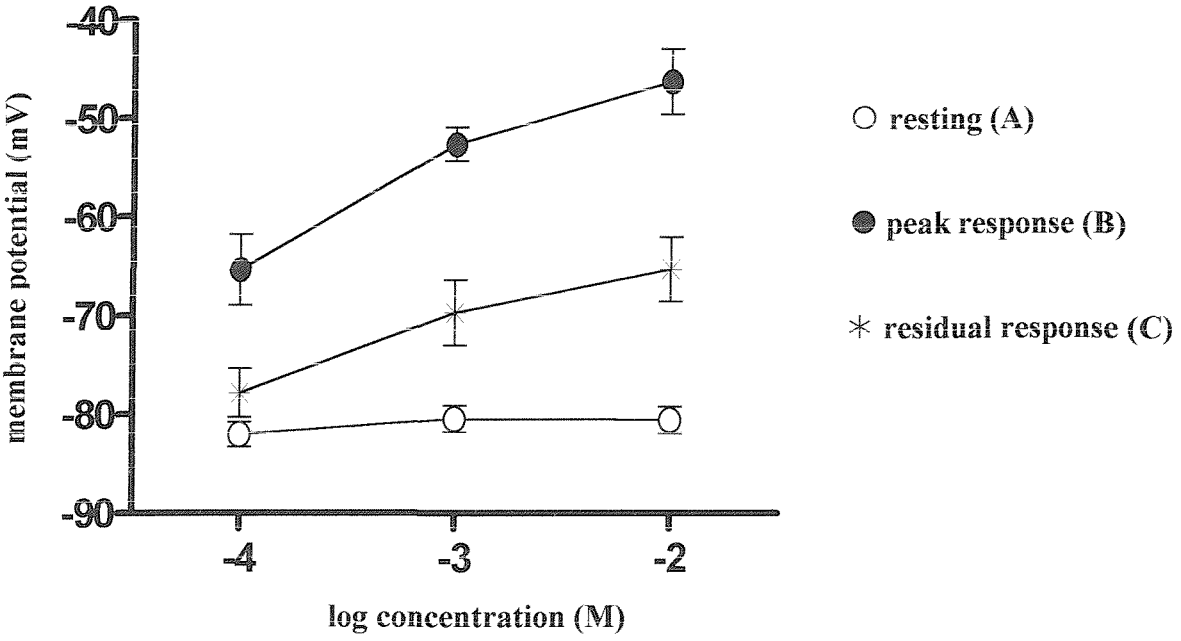


Figure 4.4. Graph demonstrating desensitisation of the glutamate response in wild-type *C. elegans*. Hollow circles indicate membrane potential prior to drug application. Filled circles indicate resting membrane potential at peak of response. Stars indicate membrane potential immediately prior to wash. Each point represents mean of 10 individual preparations ± S.E.M.

4.3. Ionic dependence of the glutamate response in wild-type *C. elegans*.

To test whether the depolarisation elicited by glutamate was produced by its action upon GluCl channels, a series of ion exchange experiments were conducted. Intracellular recordings were obtained from muscle cells bathed in saline whose extracellular chloride was replaced with the isethionate ion. Comparisons of the resultant glutamate responses were then made (Figure 4.6). The replacement of extracellular chloride by isethionate resulted in a transient increase in action potential frequency but had no significant effect on resting membrane potential (for extracellular chloride concentration 154 mM, membrane potential was –77.5 ± 1.5 mV, n=14; for extracellular chloride concentration 84 mM, membrane potential was –75.9 ± 1.1 mV, n=15; for extracellular chloride concentration 14 mM, membrane potential was –80.4 ± 1.5 mV, n=8; Figure 4.5). This indicates that whilst there exists

a resting chloride conductance within the muscle cells, the resting membrane potential of *C. elegans* pharyngeal muscle cells is not set by the equilibrium potential for chloride.

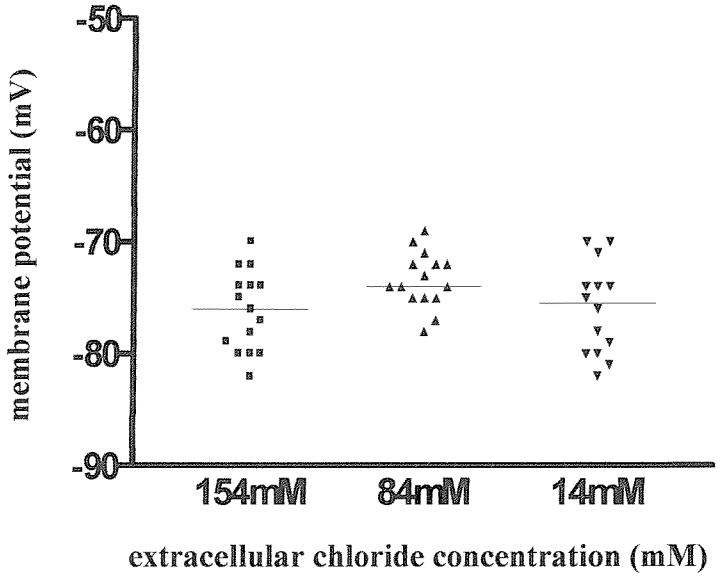
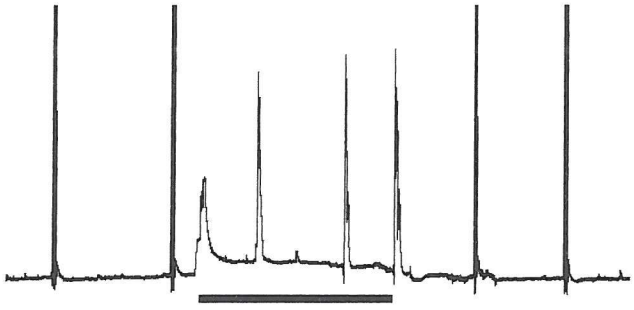


Figure 4.5. Graph demonstrating effect of reducing extracellular chloride on resting membrane potential in wild-type *C. elegans*. The resting membrane potential was determined in 154 mM (n=14), 84 mM (n=15) and 14 mM extracellular chloride (n=8). The data are paired for each concentration and each point represents an individual preparation. The horizontal bar indicates the median.

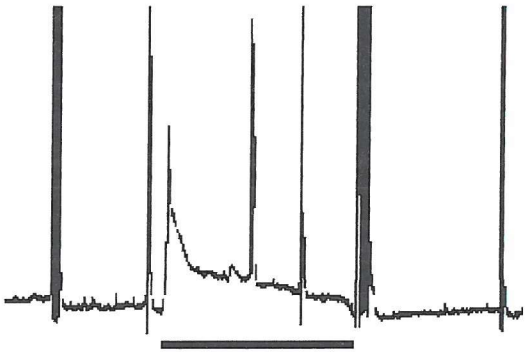
In saline containing 154 mM chloride, 100 μM glutamate caused a depolarisation of 14.7 ± 1.3 mV whilst in 14 mM chloride the same concentration of glutamate produced a depolarisation of 25 ± 1.5 mV (Figures 4.6 a to c). The glutamate depolarisation was inversely related to the logarithm of the extracellular chloride concentration (Figure 4.7), which is consistent the involvement of GluCl channels. All subsequent experiments were performed in 84 mM extracellular chloride.

(a) 154 mM extracellular chloride (Standard saline)



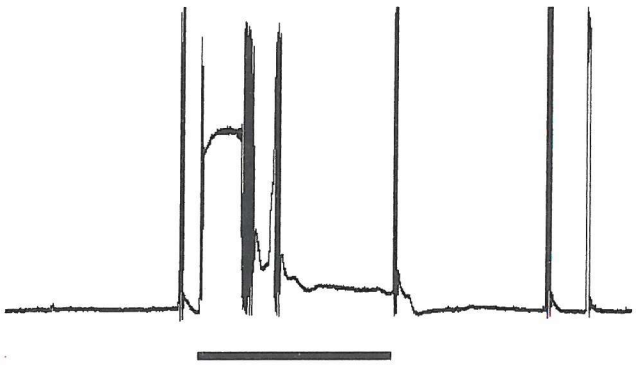
100 μM glu

(b) 84 mM extracellular chloride



100 μM glu

(c) 14 mM extracellular chloride



20 mV

30s

100 μM glu

Figure 4.6. Trace demonstrating effect of reducing extracellular chloride on the glutamate response in wild-type *C. elegans*. **(a)** The response to 100 μM glutamate in 154 mM extracellular chloride. **(b)** The response to 100 μM glutamate in 84 mM extracellular chloride. **(c)** The response to 100 μM glutamate in 14 mM extracellular chloride. These are consecutive recordings from the same cell. The resting membrane potential at the beginning of each trace was –72, -70 and –71 mV respectively.

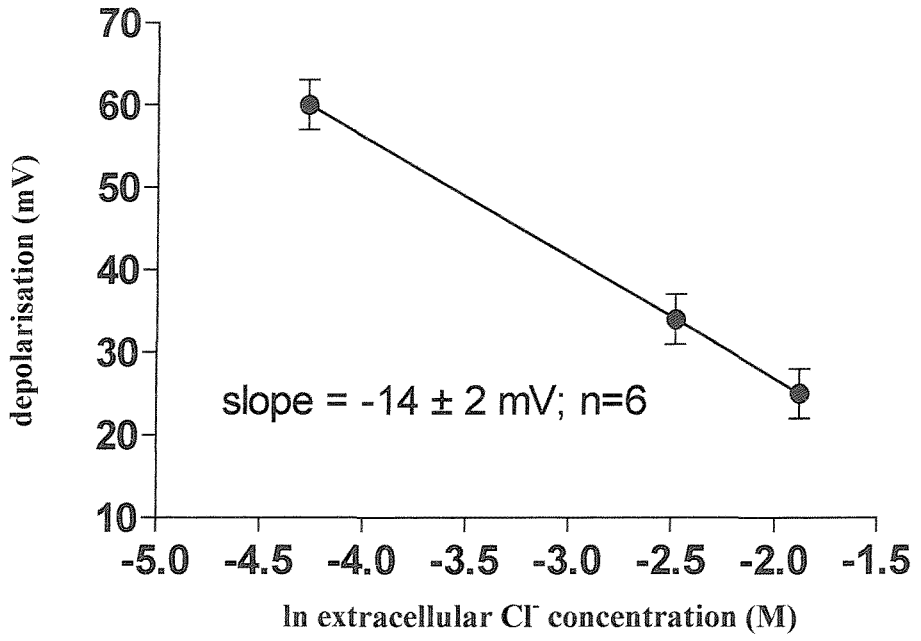


Figure 4.7. The chloride-dependence of the glutamate response. The glutamate depolarisation was inversely related to the logarithm of extracellular chloride concentration. Each data point is the mean of six preparations. NaCl was replaced with NaIsethionate to alter extracellular chloride concentration. The resting membrane potential did not change by more than 5 mV during the course of each experiment.

4.4. The pharmacology of the glutamate response in wild-type *C. elegans*.

The potency of glutamate receptor agonists on *C. elegans* pharyngeal muscle cells was evaluated. The depolarisation elicited by 100 μM glutamate was determined and a comparison was made with the responses generated by 100 μM of each particular agonist (Figure 4.8 a-c). The order of potency of agonists was ibotenate>glutamate>quisqualate=kainate. These agonists at 100 μM elicited depolarisations of 23.2 ± 5.5 mV (n=6; *p=0.0006* with respect to glutamate), 12.3 ± 1.5 mV (n=16), 0.7 ± 0.4 mV (n=6) and 0.5 ± 0.5 mV (n=4), respectively.

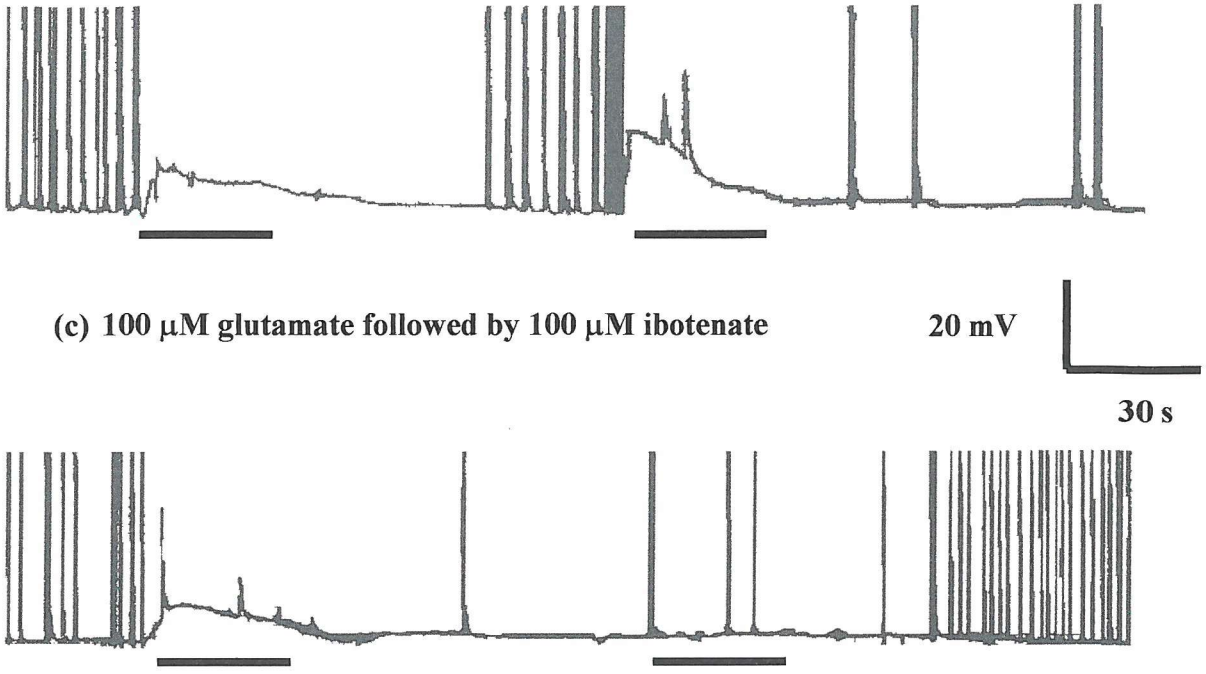


Figure 4.8. Trace demonstrating effect of selective glutamate receptor agonists on membrane potential in wild-type *C. elegans*. The resting membrane potentials were a) –78 mV b) –79 mV c) –76 mV. The agonists elicited depolarisations of 23.2 ± 5.5 mV (n=6; *p=0.0006* with respect to glutamate), 12.3 ± 1.5 mV (n=16), 0.7 ± 0.4 mV (n=6) and 0.5 ± 0.5 mV (n=4), respectively. All agonists were applied for 30 s.

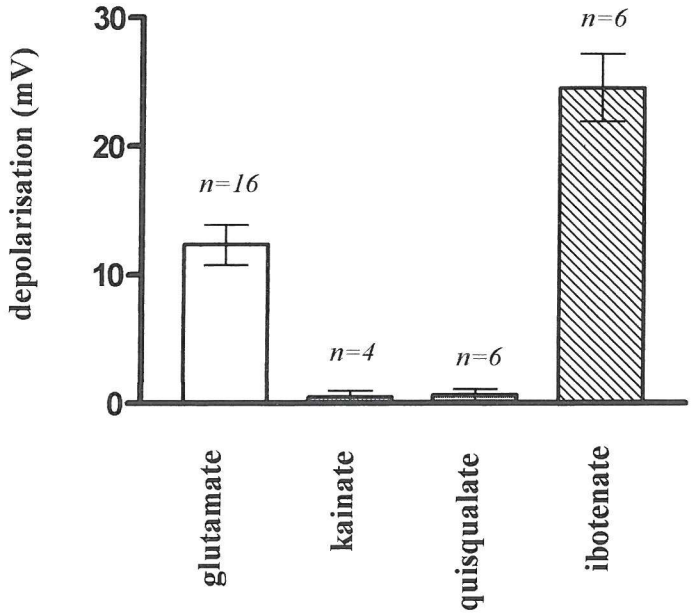


Figure 4.9. Graphical summary of glutamate receptor agonist activity in wild-type *C. elegans*. Kainate, quisqualate and ibotenate were all applied at 100 μM for 30 s. Each bar represents the mean of n individual preparations ± S.E.M.

In a further series of experiments the EC_{50} for ibotenate was determined. Perfusion of ibotenate directly over the muscle cells of the pharyngeal terminal bulb elicited an immediate depolarisation, which was concentration-dependent (Figure 4.10). The EC_{50} for ibotenate, determined from peak depolarisation was 17.8 μM (95% confidence limits: 11.1 to 27.2 μM; n=10; Figure 4.11). The maximal response was 36.5 mV (95% confidence limits: 32.9 to 40.1 mV). Ibotenate is therefore 10 times more potent than glutamate at eliciting a membrane depolarisation.

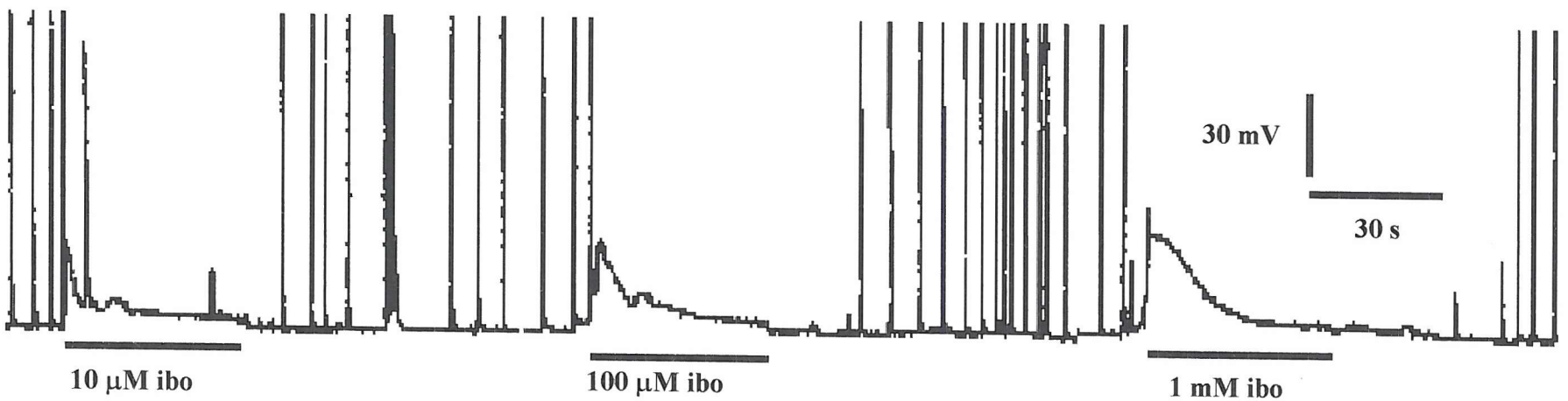


Figure 4.10. The effect of increasing concentrations of ibotenate on membrane potential in wild-type *C. elegans*. Voltage recording from a single cell within the *C. elegans* pharyngeal terminal bulb. The resting membrane potential in this experiment was –71 mV with action potentials amplitudes of 104 mV. Drug applications indicated by horizontal bars. Ibotenate produced a rapid concentration-dependent depolarisation that desensitised during drug application. Peaks of action potentials have been truncated in this trace.

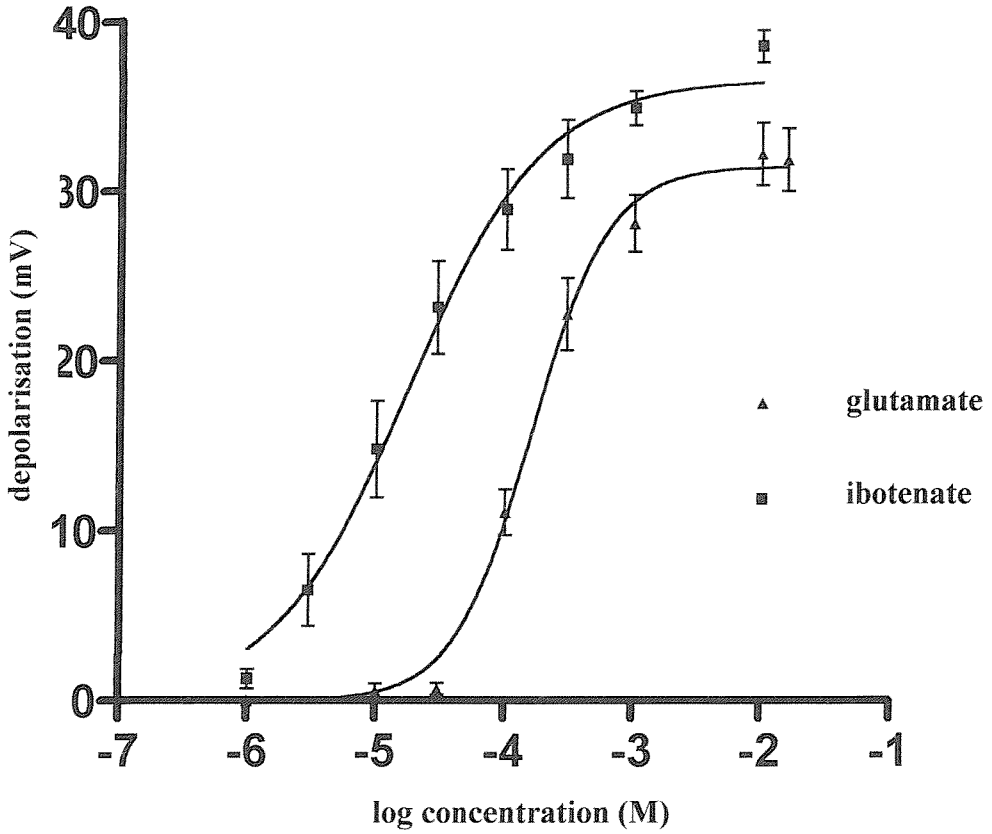


Figure 4.11. Concentration response comparison for ibotenate and glutamate in wild-type *C. elegans*. The effect of increasing concentrations of ibotenate and glutamate (1 μM-20 mM) on membrane potential. Each point represents the mean of 10 individual preparations ± S.E.M. for ibotenate and 16 individual preparations ± S.E.M. for glutamate.

4.5. Effect of chloride channel blockers/modulators on glutamate response in wild-type *C. elegans*.

The effect of picrotoxin, amobarbital, flufenamic acid, flurazepam and DNDS on the glutamate response was determined. The response to 100 μM glutamate without blocker was first obtained. Each compound was then added to the perfusate at a bath concentration of 100 μM for 60 s prior to and during the second application of glutamate (Figure 4.12). Picrotoxin at 100 μM only weakly reduced the response to glutamate, from 15.8 ± 1.7 mV to 9.6 ± 1.4 mV (n=5). None of the remaining compounds examined antagonised the glutamate response (Figure. 4.13).

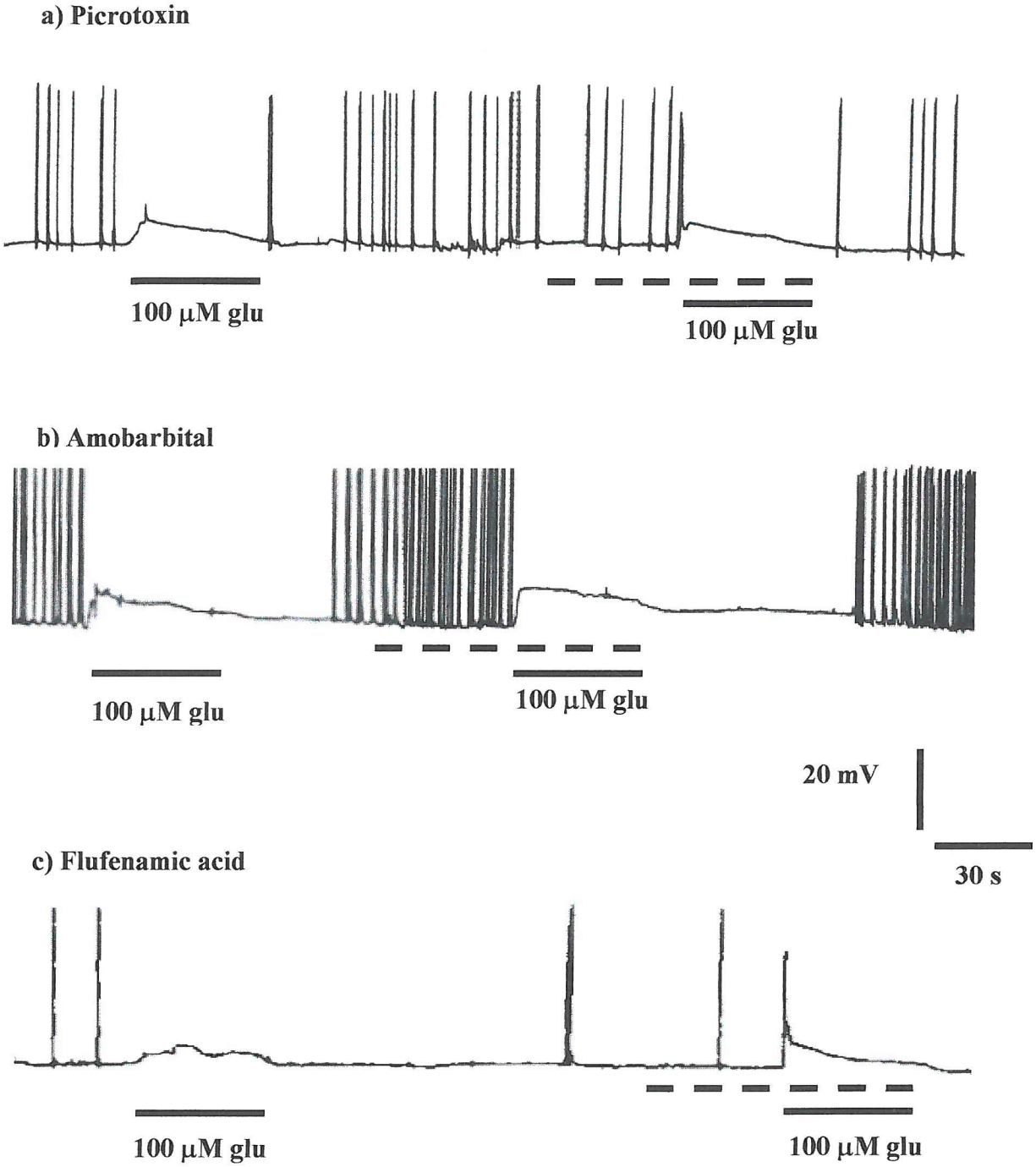


Figure 4.12. Trace demonstrating effect of a) picrotoxin, b) amo-barbital and c) flufenamic acid on the glutamate response in wild-type *C. elegans*. The trace shows the response to 100 μM glutamate independently, and in the presence of 100 μM antagonist/blocker. Dashed line indicates application of compound under examination whilst solid line shows application of glutamate.

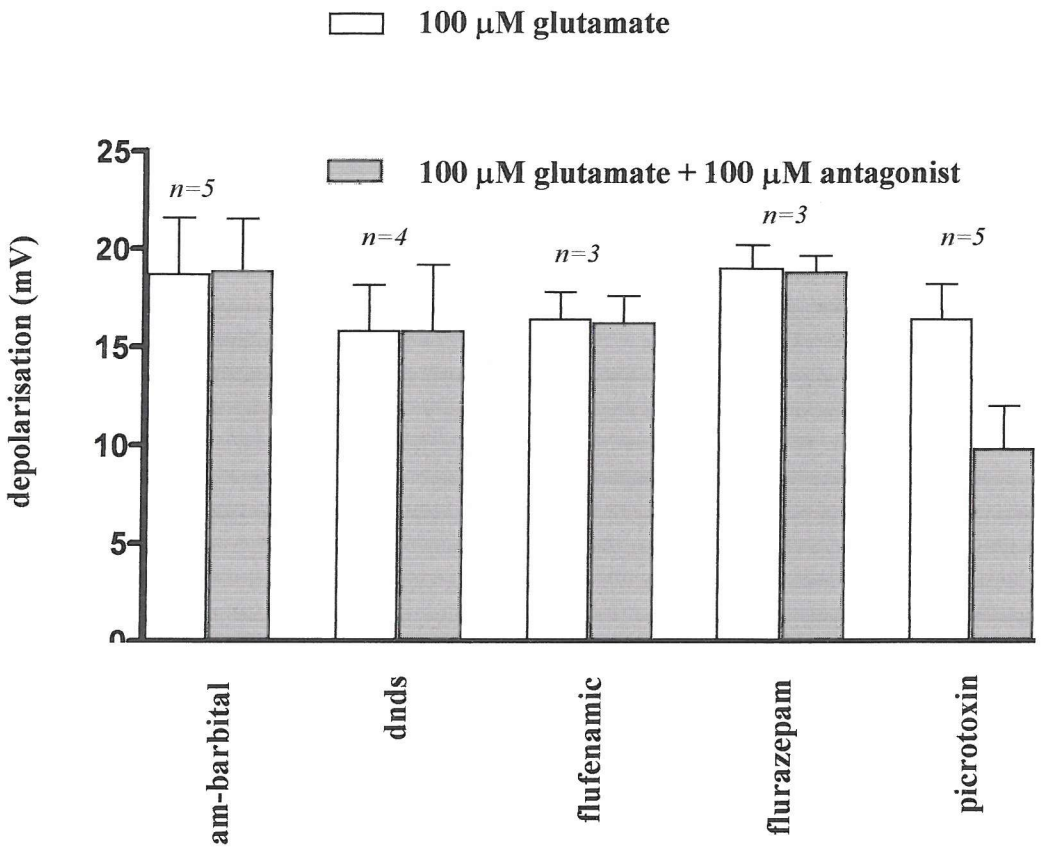


Figure 4.13. Graphical summary of the effect of amobarbital, dnds, flufenamic acid, flurazepam and picrotoxin on the glutamate response in wild-type *C. elegans*. The graph shows the response to 100 μM glutamate independently and in the presence of 100 μM antagonist. Picrotoxin at 100 μM was the only compound that antagonised the glutamate response. The response was reduced from 15.8 ± 1.7 mV to 9.6 ± 1.4 mV (n=5).

4.6. Effect of ivermectin on the pharyngeal muscle of wild-type *C. elegans*.

Ivermectin reduced the amplitude of the pharyngeal action potentials (Figure 4.16; at 10 pM this reduction was 10 mV; $p<0.05$ by Student's paired t test; n=7). This was accompanied by a slow and irreversible depolarisation. Co-application of glutamate (1 mM) during maximal depolarisation to ivermectin (1 μM) caused no further change

in membrane potential (Figure 4.15; -39.3 ± 1.2 mV with ivermectin alone, and –38.0 ± 0.6 mV upon addition of glutamate; n=3), which is consistent with these agonists sharing a common ionic mechanism.

In contrast to the response to glutamate, which had a reversible effect, the response to ivermectin was irreversible and the observed depolarisation continued to increase during the wash period, possibly due to the lipophilic nature of ivermectin and its propensity to accumulate in cell membranes. This complicated an accurate determination of an EC_{50} value. Therefore, the response that was measured was the maximum depolarisation from original resting membrane potential within 1.5 min of application of a given concentration of ivermectin.

Ivermectin was five orders of magnitude more potent than glutamate with an EC_{50} value of 2.7 nM (95% confidence limits, 1.1 to 6.3 nM; n=8) compared with an EC_{50} value of 166 μM for glutamate (95% confidence limits, 132 to 207; n=16; Figure 4.14). Furthermore, the maximum depolarisation for ivermectin was greater than that for glutamate (49 mV; 95 % confidence limits 42 to 55; n=8, compared with 32 mV; 95% confidence limits 30 to 34; n=16; Figure 4.14).

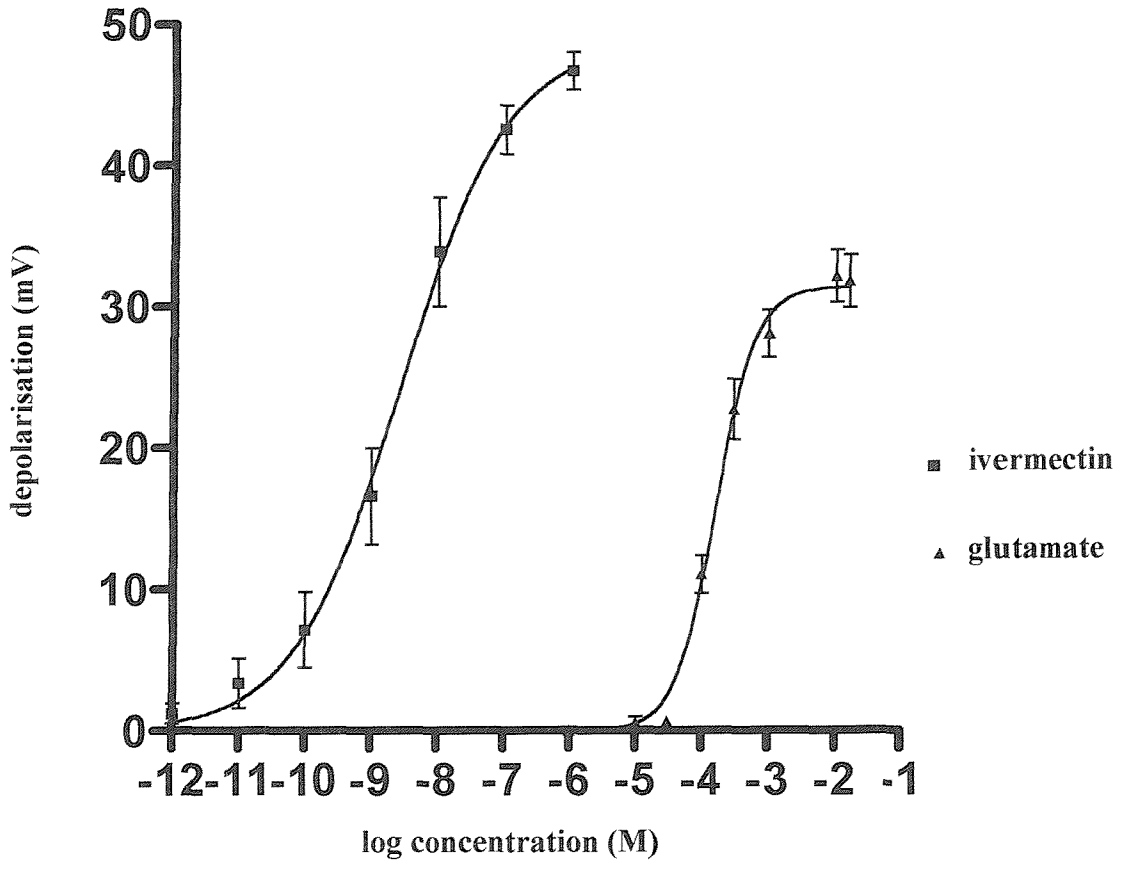


Fig 4.14. Concentration response comparison for ivermectin and glutamate in wild-type *C. elegans*. The effect of increasing concentrations of ivermectin (1 pM-1 μM) and glutamate (1 μM-20 mM) on membrane potential. Each point represents the mean of 8 individual determinations ± S.E.M. for ivermectin and 16 individual determinations ± S.E.M. for glutamate.

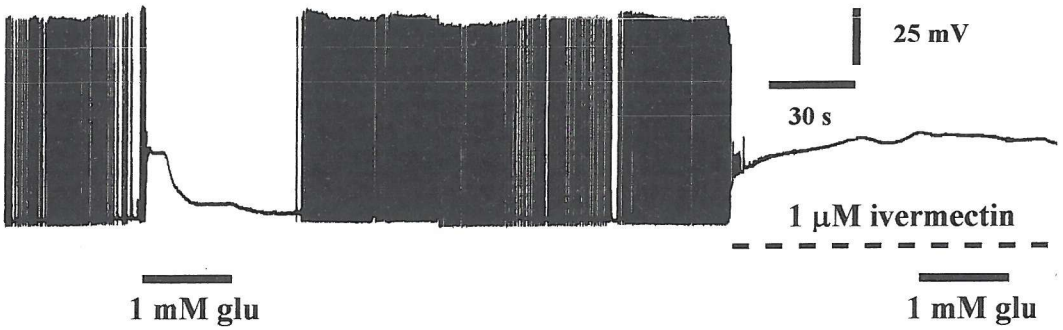


Figure 4.15. Trace demonstrating effect of co-application of 1 mM glutamate with 1 μM ivermectin in wild-type *C. elegans*. The dashed line indicates the continuous application of 1 μM ivermectin. Resting membrane potential was –79 mV with action potential amplitudes of 102 mV. No further depolarisation was observed when glutamate was co-applied with ivermectin thus supporting the contention that glutamate and ivermectin elicit their response *via* the same population of receptors.

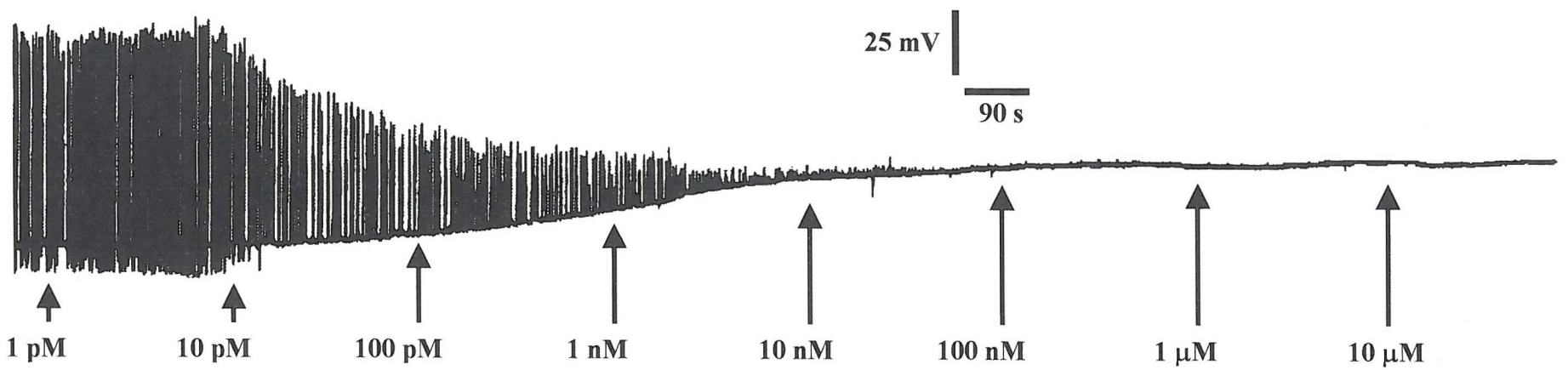


Figure 4.16. The effect of increasing concentrations of ivermectin on membrane potential in wild-type *C. elegans*. Voltage recording from a single cell within the *C. elegans* pharyngeal terminal bulb. The resting membrane potential in this experiment was –75 mV with action potentials amplitudes of 106 mV. Drug applications indicated by arrows were for 30 s in all cases. Ivermectin produced a slow, irreversible concentration-dependent depolarisation associated with a decrease in amplitude and eventual cessation of action potentials. The response for each individual concentration was taken as the change from resting membrane potential 1.5 min after its application.

4.7. Effect of glutamate uptake blocker PDC on membrane potential

The exceptional potency of ivermectin in depolarising the pharyngeal muscle suggested that endogenous glutamate may be involved in potentiating the response to ivermectin. To test whether tonic release of glutamate was occurring in the pharyngeal preparation, the effect of the glutamate uptake blocker PDC (*trans*-4-carboxy-L-proline/L-*trans*-pyrrolodine-2,4-dicarboxylic acid) was examined. At concentrations over 100 μM, PDC produced a concentration-dependent non-desensitising depolarisation that was rapidly abolished on introduction of the wash (Figure 4.17).

PDC (300 μM) when co-applied with 100 μM glutamate produced a response that was more than additive, suggesting that PDC may be synergistic with glutamate (100 μM glutamate 10.5 ± 1.6 mV; 300 μM PDC 5.7 ± 1.5 mV; 100 μM glutamate and 300 μM PDC 22.0 ± 2.6 mV; n=6; Figure 4.18).

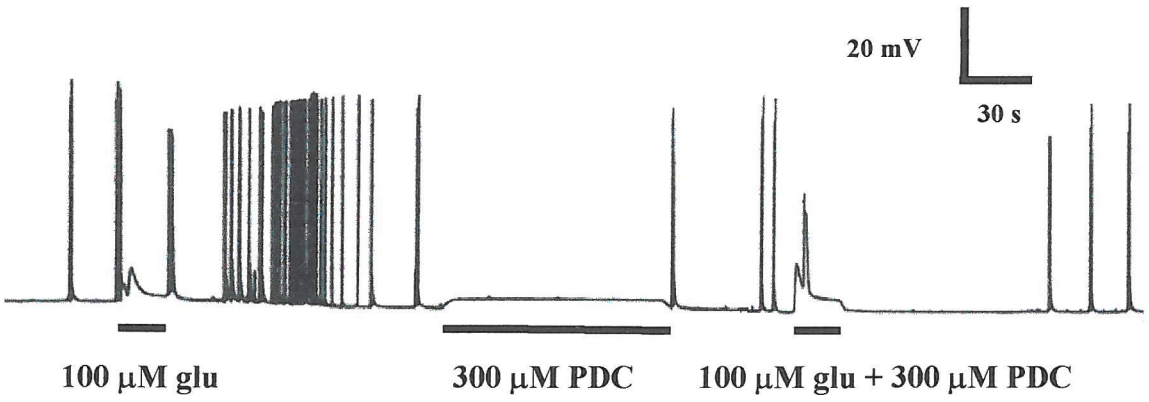


Figure 4.17. Trace demonstrating effect of glutamate and PDC in wild-type *C. elegans*. The resting membrane potential of this cell was –80 mV. The response to 100 μM glutamate and 300 μM PDC were determined independently. Glutamate and PDC were then co-applied and the maximal depolarisation recorded. Drug application is indicated by the horizontal bars.

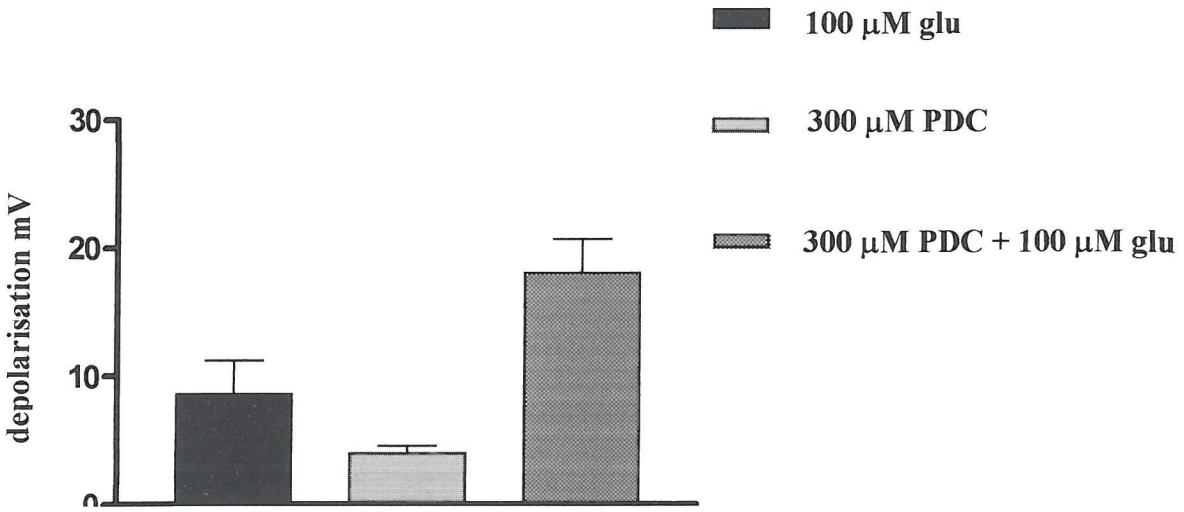


Figure 4.18. Graphical summary of the effect of PDC on the glutamate response in wild-type *C. elegans*. The response to 100 μM glutamate and 300 μM PDC were determined individually. PDC and glutamate were then co-applied and the response determined. Glutamate elicited a depolarisation of 10.5 ± 1.6 mV. PDC elicited a depolarisation of 5.7 ± 1.5 mV. When compounds were co-applied the response was 22.0 ± 2.6 mV; n=6.

4.8. Subunit contribution to observed pharmacology: role of GluCl-α2.

To investigate the role that individual channel subunits may play in generating the pharmacological profile of the pharyngeal preparation, a series of further experiments were performed on mutant strains of *C. elegans*. The first strain examined was the *avr-15* strain. This strain was originally isolated by screening for ivermectin resistance in the progeny of mutagenised wild-type animals. The *avr-15* gene encodes for an alternatively spliced chloride channel subunit, termed GluCl-α2a and GluCl-α2b (Dent et al., 1997). It has previously been shown to confer behavioural synthetic ivermectin sensitivity in conjunction with at least one other gene. It is also necessary for a functional IPSP generated by the M3 neurone (Avery, 1993). This synaptic activity is believed to play a role in the timing of pharyngeal muscle contractions. The expression

of GluClα2:GFP promoter fusion has revealed fluorescence staining in the pharyngeal muscles pm4 and pm5 as well as extrapharyngeal neurones (Dent et al., 1997).

Resting membrane potentials of *avr-15* were indistinguishable from wild-type (-78.9 ± 1.4 mV; n=12), as were the frequency, amplitude and shape of pharyngeal action potentials (Figure 4.19).

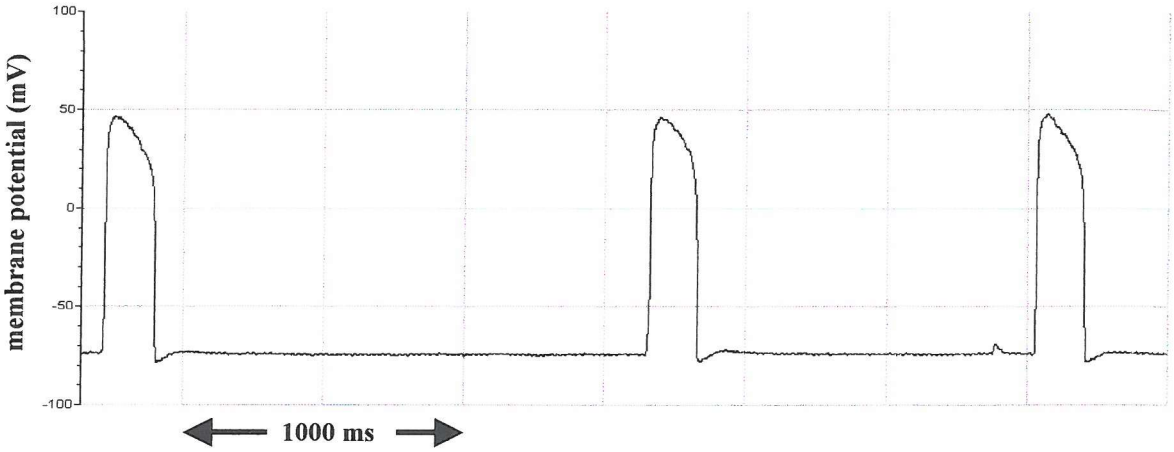


Figure 4.19. Resting properties of *avr-15 C. elegans* terminal bulb muscle cells. The trace shows a recording from a single muscle cell within the terminal bulb. The cell was impaled with a 40 MΩ microelectrode. In this recording, resting membrane potential was -73 mV with action potential amplitudes of 119 mV.

Glutamate induced membrane depolarisations were also observed in *avr-15*. The responses were rapid in onset and concentration-dependent (Figure 4.21). The EC_{50} value for glutamate, determined from peak depolarisation was 1.15 mM (95% confidence limits 0.52 to 2.55 mM); n=12; Figure 4.20. Thus, glutamate was 9 times less potent than wild-type; $p<0.001$ at 1 mM glutamate. The maximum depolarisation was not significantly different from wild-type; 31.0 mV (95% confidence limits 25.0 to 37.2 mV; n=12) compared to 32 mV (95% confidence limits 30 to 34) for wild-type ($p=0.08$).

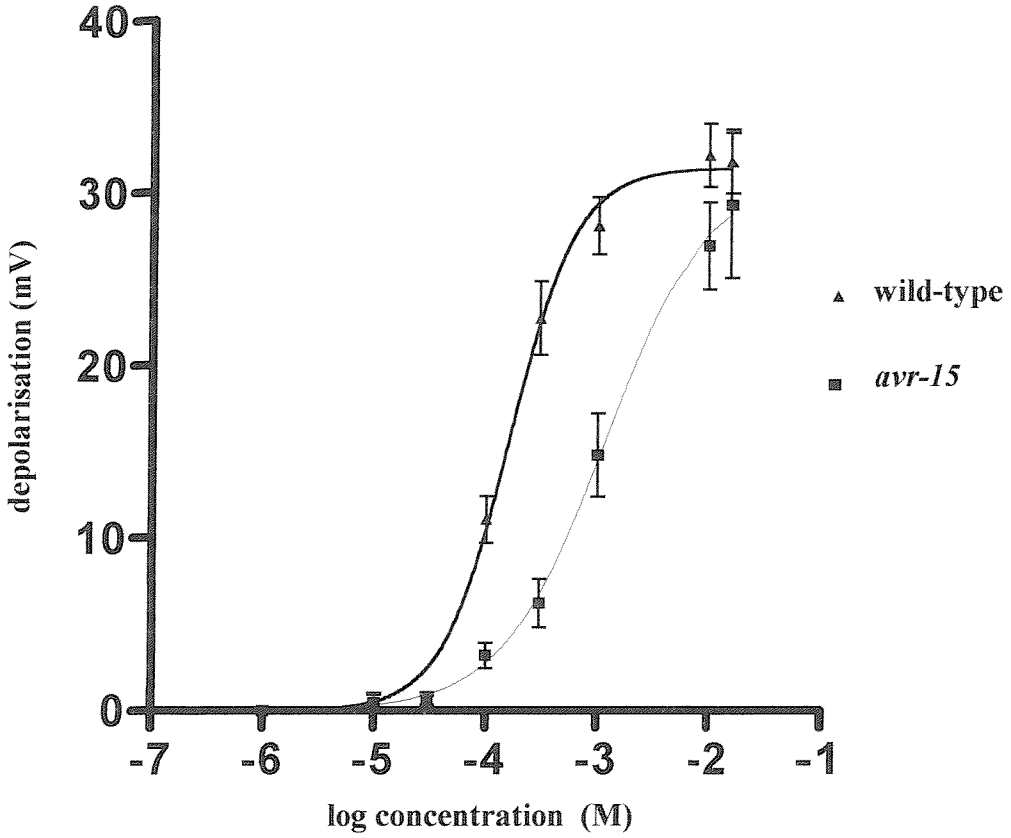


Figure 4.20. Concentration response to glutamate in wild-type and *avr-15 C. elegans*. The effect of increasing concentrations of glutamate (1 μM-20 mM) on membrane potential. Each point represents the mean of 12 individual preparations ± S.E.M. for *avr-15* and 16 individual preparations ± S.E.M. for wild-type.

Unlike wild-type, the glutamate response in *avr-15* did not show any desensitisation at either the lowest or maximal concentration of glutamate (Figure 4.22). For wild-type, the desensitisation was greatest at the lowest effective concentration of glutamate 80 ± 6% at 100 μM glutamate, 62 ± 7% at 1 mM glutamate and 56 ± 5% at 10 mM glutamate (n=10). This finding suggests that the GluClα2 subunit is necessary to initiate receptor desensitisation.

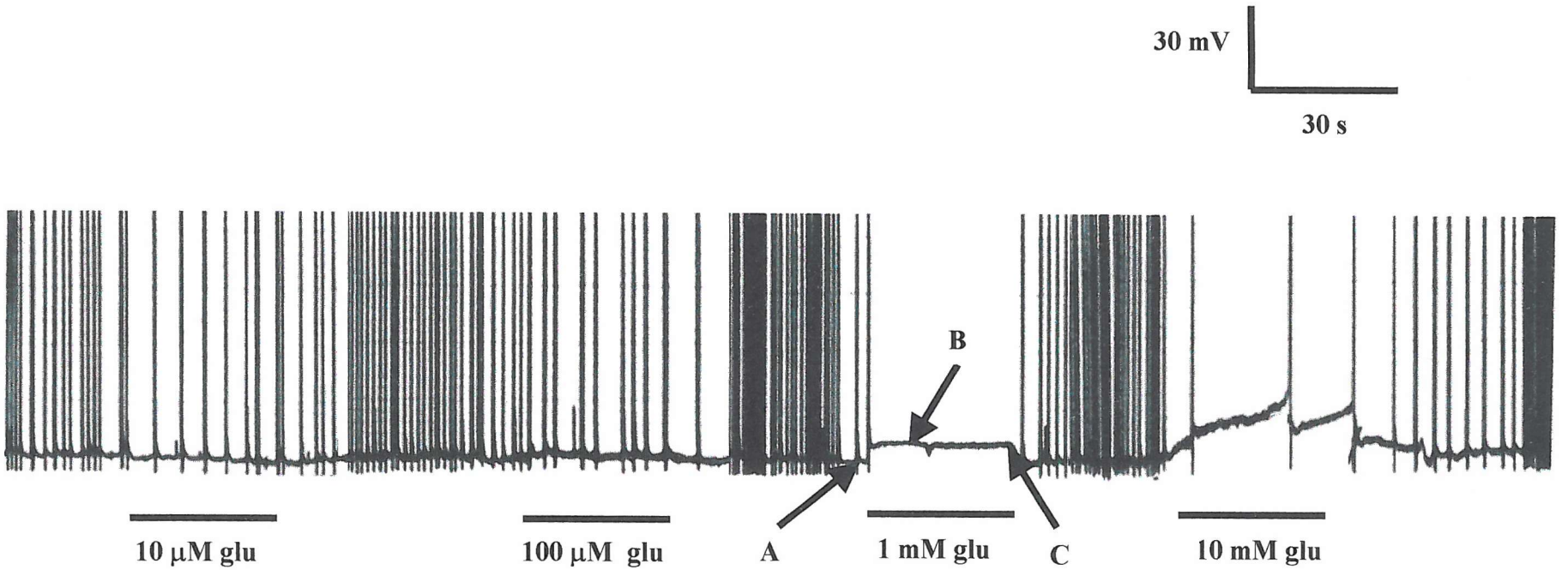


Figure 4.21. Trace demonstrating effect of glutamate on membrane potential in *avr-15 C. elegans*. Voltage recording from a single cell within the terminal bulb. The resting membrane potential was –76 mV with action potentials amplitudes of 111 mV. Drug applications indicated by horizontal bars were for 30 s in all cases. Glutamate produced a rapid concentration-dependent depolarisation that showed no desensitisation during drug application. Typically all cells showed an increase in action potential frequency following the response. Points A-C refers to he method adopted for the quantification of desensitisation. Peaks of action potentials have been truncated in this trace.

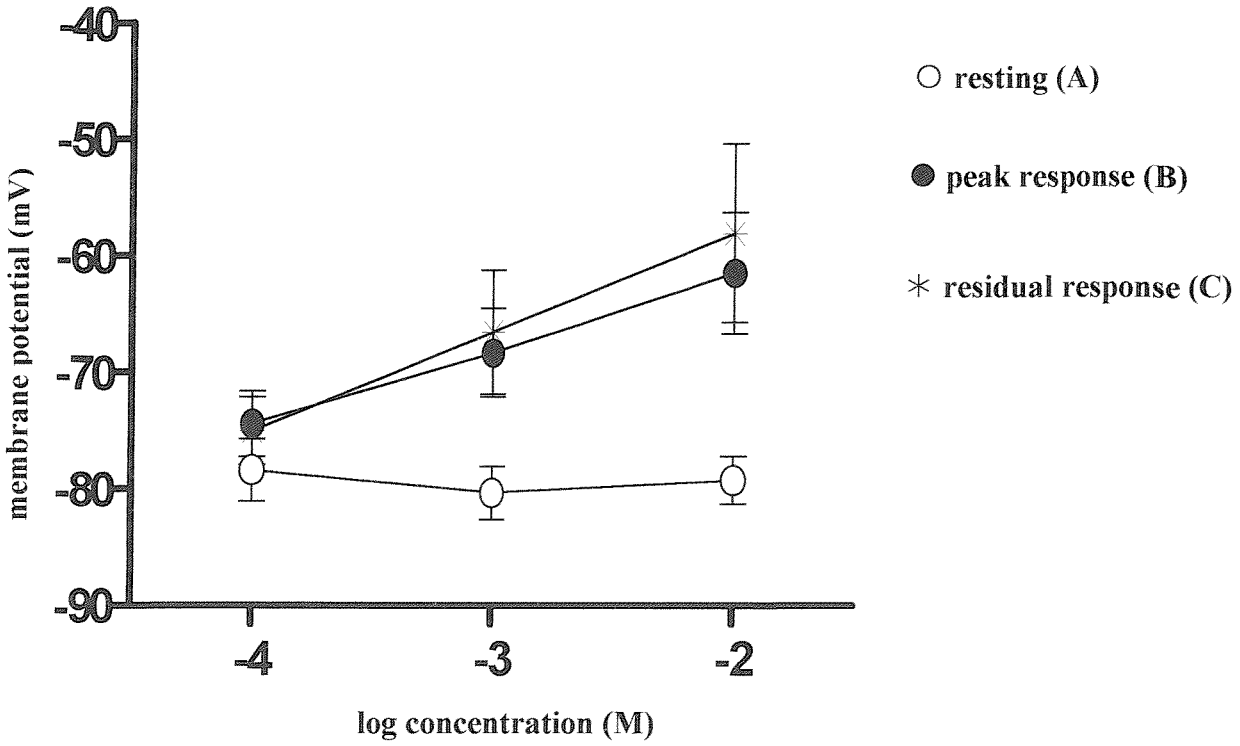


Figure 4.22. Graph demonstrating lack of desensitisation of the glutamate response in *avr-15 C. elegans*. Hollow circles indicate membrane potential prior to drug application. Filled circles indicate resting membrane potential at peak of response. Stars indicate membrane potential immediately prior to wash. There was no significant desensitisation observed of the glutamate response in *avr-15* (n=12).

4.9. Ionic dependence and pharmacology of the glutamate response in *avr-15 C. elegans*.

To determine that chloride ions were also the major charge carrier in the observed responses, ion exchange experiments were conducted as described earlier. Essentially, intracellular recordings were obtained from muscle cells bathed in saline for which extracellular chloride was replaced with the isethionate ion and comparisons of the resultant glutamate responses were made. As with wild-type, the response to glutamate was chloride-dependent (depolarisation to 1 mM glutamate was 12.1 ± 1.4

mV in 154 mM chloride compared to 20.9 ± 3.2 mV in 14 mM chloride, n=7; (Figure 4.23).

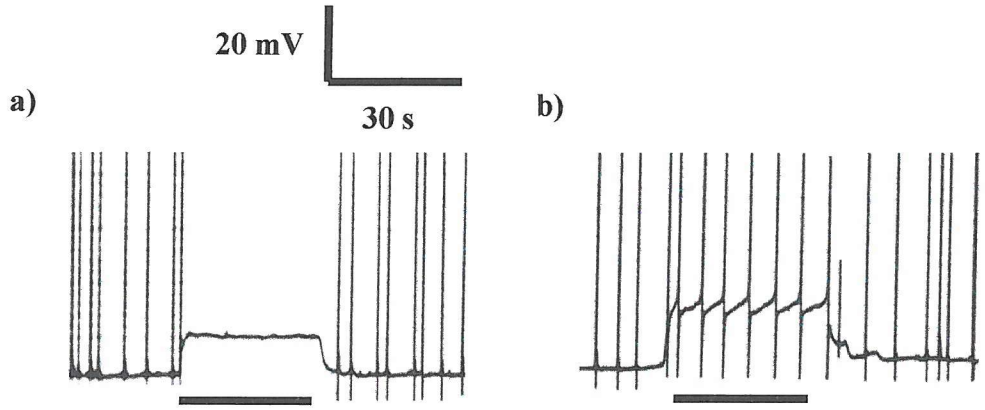


Figure 4.23. Trace demonstrating effect of reducing extracellular chloride on the glutamate response in *avr-15 C. elegans*. **(a)** The response to 1 mM glutamate in 154 mM extracellular chloride. **(b)** The response to 1mM glutamate in 14 mM extracellular chloride. These are consecutive recordings from the same cell. The resting membrane potentials at the beginning of each trace were -83 and -80 mV respectively. Horizontal line indicates application of 1mM glutamate. The peaks of action potentials are truncated in these traces.

The pharmacological properties of the response to glutamate in *avr-15* were further investigated to determine whether a GluCl channel was involved in mediating this response. As with wild-type, *avr-15* still responded to glutamate in the presence of 100 μM picrotoxin, (data not shown; n=2). Further evidence that a GluCl channel mediated the response was provided by the observation that the response to the potent GluCl agonist, ibotenate was reduced in *avr-15* in a quantitatively similar manner to the reduction in glutamate. The calculated EC_{50} value was 80.5 μM (95% confidence limits 56.9 to 113.9 μM; n=9); i.e. 4 times less potent than in wild-type (Figure 4.25). The maximal response for ibotenate in *avr-15* was also similar to that obtained for glutamate in *avr-15* (30.3 mV; 95% confidence limits 27.7 to 32.9 mV; n=9) compared to 31.0 mV (95% confidence limits 25.0 to 37.2 mV; n=12) for glutamate. As with glutamate,

no desensitisation was observed in the response to ibotenate at either the lowest or maximally effective concentration (Figure 4.24).

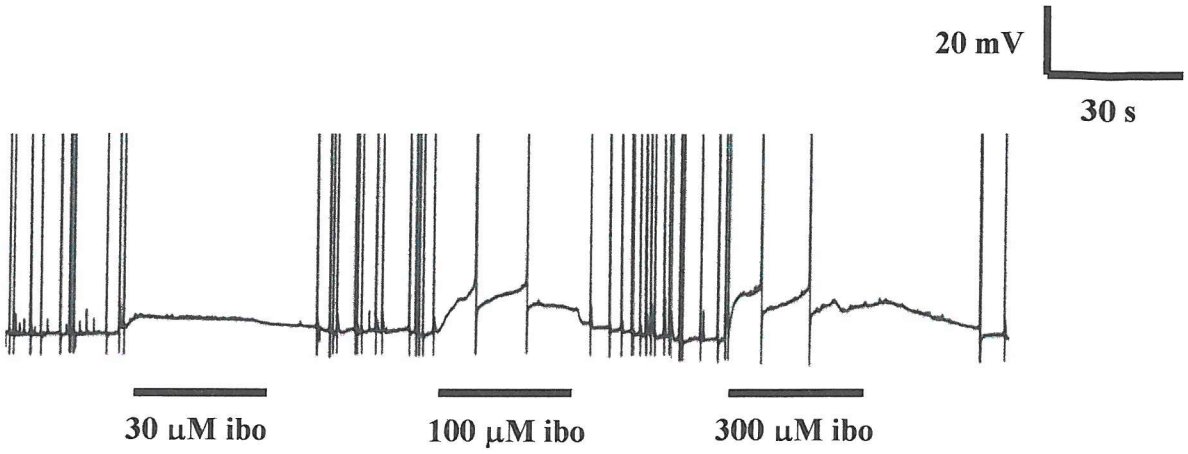


Figure 4.24. Trace demonstrating effect of ibotenate on membrane potential in *avr-15 C. elegans*. Voltage recording from a single cell within the terminal bulb. The resting membrane potential was –79 mV with action potentials amplitudes of 114 mV. Drug applications indicated by horizontal bars were for 30 s in all cases. Ibotenate produced a concentration-dependent depolarisation that showed no desensitisation during drug application. Peaks of action potentials have been truncated in this trace.

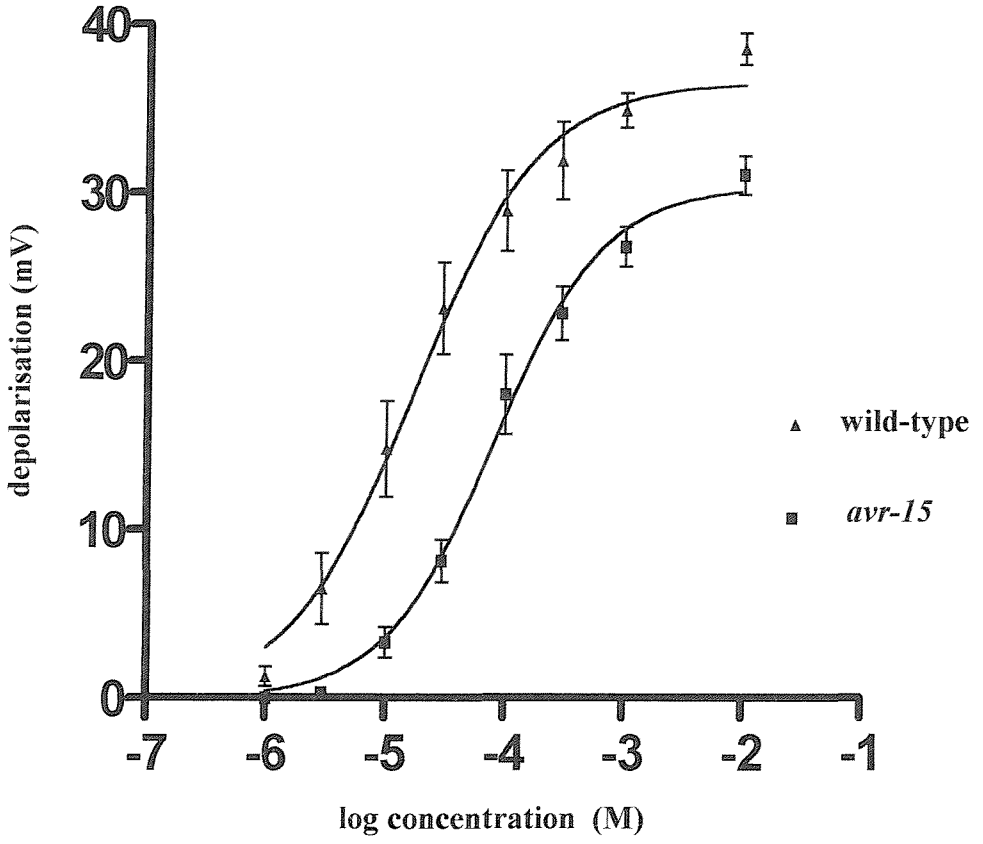


Figure 4.25. Comparison concentration response to ibotenate in *avr-15* and wild-type *C. elegans*. The effect of increasing concentrations of ibotenate (1 μM-20 mM) in *avr-15* and wild-type *C. elegans*. Each point represents the mean of 9 individual determinations ± S.E.M. for *avr-15* and 10 individual determinations ± S.E.M for wild-type.

Ivermectin was examined at concentrations between 1 nM and 10 μM. In contrast to glutamate, ivermectin failed to elicit any effect on spike amplitude, duration, or membrane potential in *avr-15* (n=4; Figure 4.26).

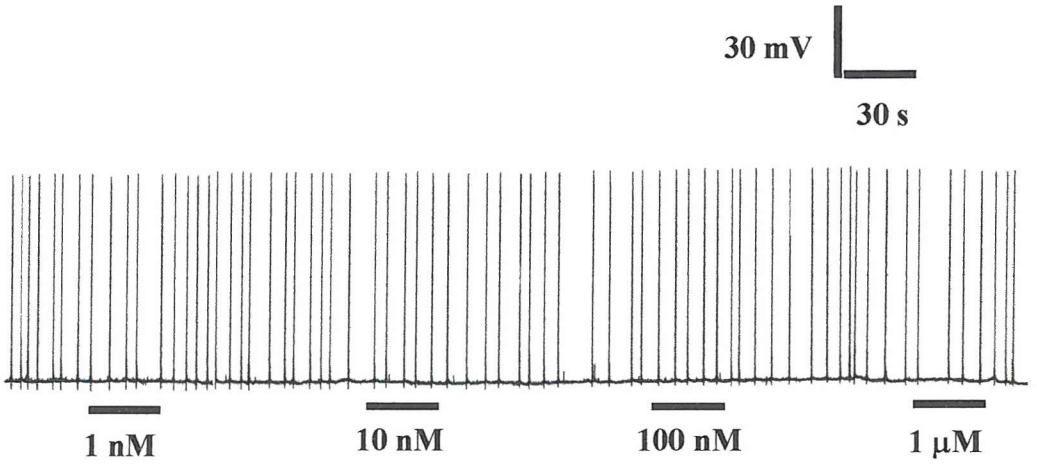


Figure 4.26. Trace demonstrating no action of ivermectin on membrane potential or spike amplitude in *avr-15 C. elegans*. Drug applications indicated by horizontal bars and were for 30 s in all cases. Ivermectin had no effect on resting membrane potential, spike amplitude or duration at concentrations up to 10 μM. Resting membrane potential in this preparation was –80 mV with spike amplitudes of 115 mV.

4.10. Role of GluCl-α3 in pharyngeal preparation pharmacology

The a*vr-14/gbr-2* gene encodes for alternatively spliced α subunit termed GluCl-α3a and GluClu-α3b. The *avr-14* (*ad1302*) strain is a miss-sense mutation of *avr*-14, predicted null for channel function (Dent et al., 2000). Resting membrane potentials of *avr-14* were indistinguishable from wild-type (-77.5 ± 1.0; n=12). The frequency and shape of action potentials were also identical to all other strains examined (Figure 4.27).

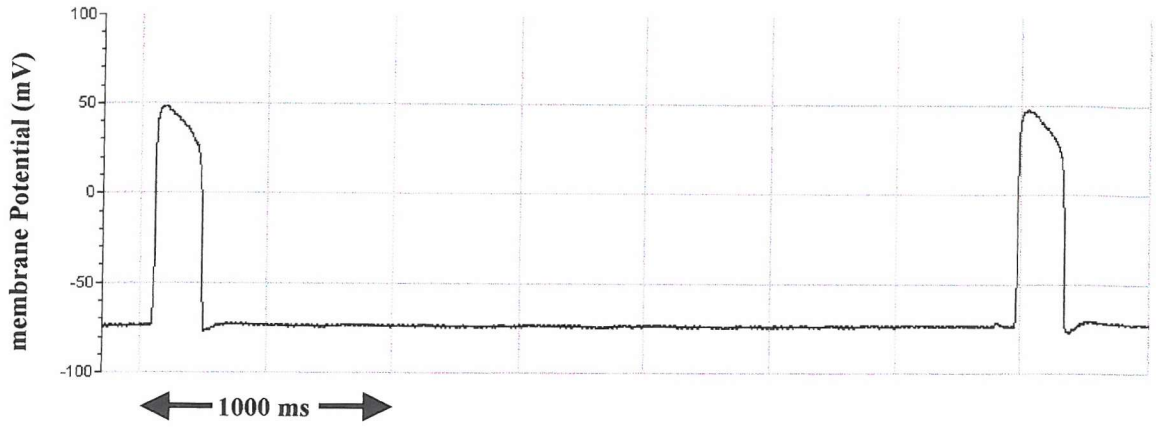


Figure 4.27. Resting properties of *avr-14 C. elegans* terminal bulb muscle cells. The trace shows a recording from a single muscle cell within the terminal bulb. The cell was impaled with a 45 MΩ microelectrode. In this recording, resting membrane potential was –74 mV with action potential amplitudes of 122 mV.

In the *avr-14* strain, glutamate produced a concentration-dependent depolarisation that was rapid in onset and showed desensitisation to the degree observed in wild-type (Figure 4.28). The determined EC_{50} value for glutamate in *avr-14*, from peak depolarisation, was 355.5 μM (95% confidence limits 233.5 to 541.1μM); n=12; Figure 4.29. Glutamate is therefore slightly less potent in *avr-14* than in wild-type by 1.4 times, ($p<0.001$) at 100 μM glutamate. The maximal response was 29.1 mV (26.0 to 32.3 mV; n=12), which was not significantly different from wild-type. As with wild-type, *avr-14* still responded to glutamate in the presence of 100 μM picrotoxin, (data not shown; n=3).

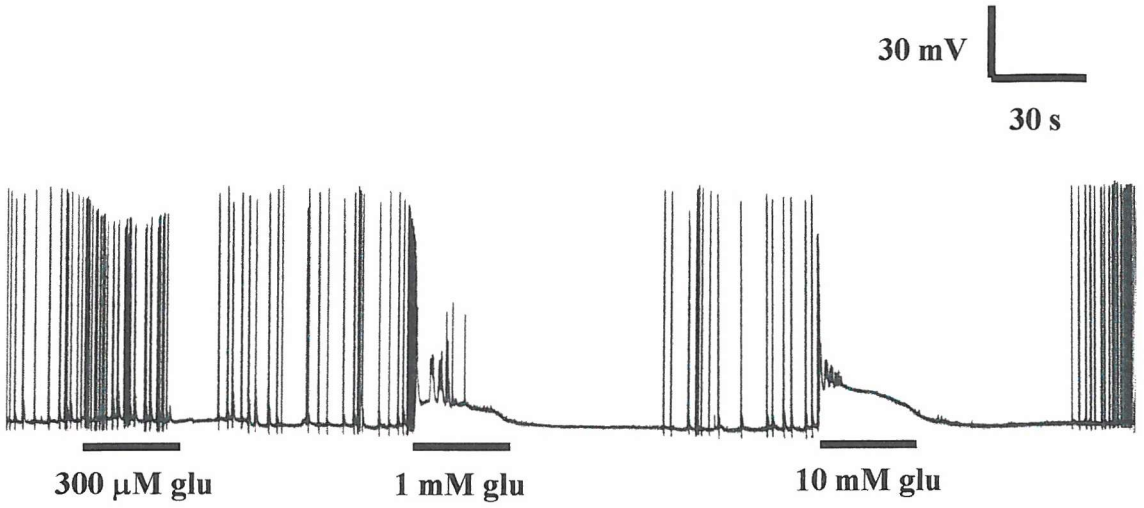


Figure 4.28. Trace demonstrating effect of glutamate on membrane potential in *avr-14 C. elegans*. Voltage recording from a single cell within the terminal bulb. The resting membrane potential was –82 mV with action potential amplitudes of 108 mV. Drug applications indicated by horizontal bars were for 30 s in all cases.

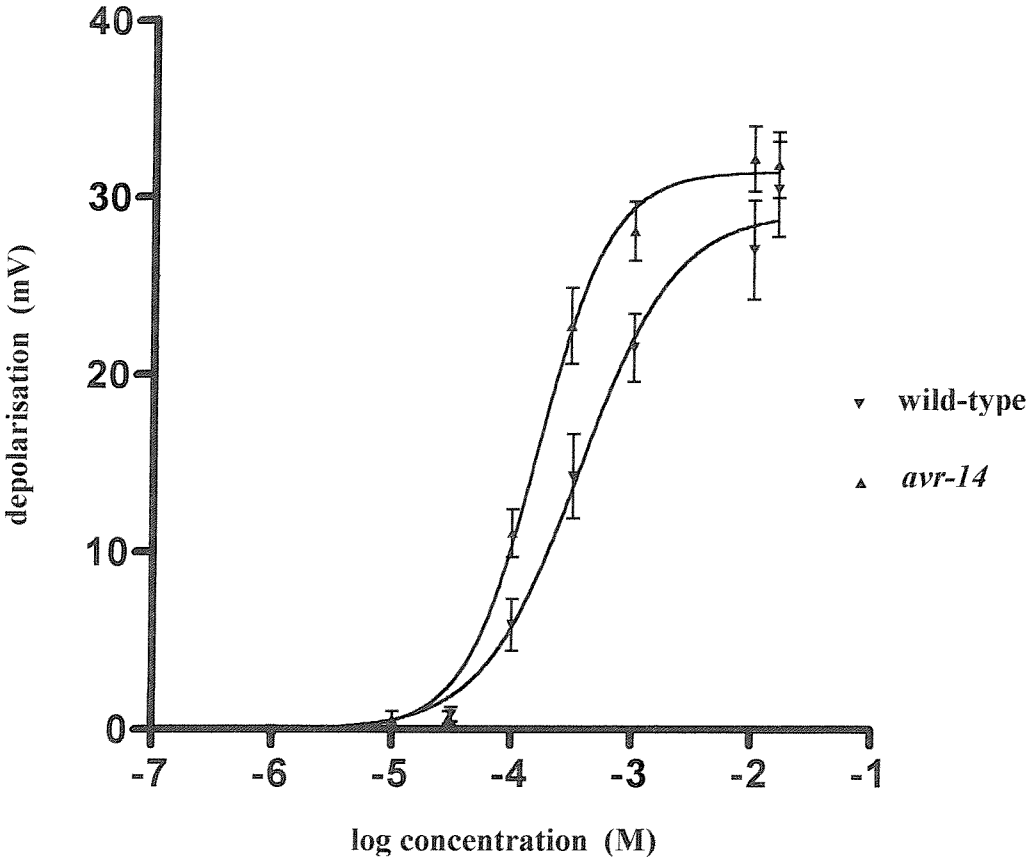


Figure 4.29. Comparison concentration response to glutamate in *avr-14* and wild-type *C. elegans*. The effect of increasing concentrations of glutamate (1 μM-20 mM) in *avr-14* and wild-type *C. elegans*. Each point represents the mean of 12 individual determinations ± S.E.M. for *avr-14* and 16 individual determinations ± S.E.M. for wild-type.

The small, yet significant reduction in potency of glutamate in the *avr-14* strain pharyngeal preparation was a surprising observation, as GluCl-α3 is not thought to be expressed in the pharyngeal muscle. Preliminary GFP-expression data has demonstrated expression of this subunit in neurones in the ring ganglia of the head, motor neurones in the ventral cord, and mechanosensory neurones (Dent et. al., 2000). Because GFP-expression may not always accurately represent expression of the native protein, a series of immunocytochemical studies were performed to determine expression of the GluCl-α3 subunit.

4.11. Immunohistochemical localisation of the AVR-14 protein.

Expression of the AVR-14 subunit was determined in dissected *C. elegans* preparations. Plate 4.1 and 4.2 shows anti-AVR-14 antibody staining of dissected pharynxes. AVR-14 immunoreactivity is present in pharyngeal neurones, and the pattern is consistent with labelling of neurones M4 and M1. No immunostaining was apparent on the muscle and in any of the control groups. The results described here are from three experiments each examining 100 animals. Immunoreactivity was consistent throughout.

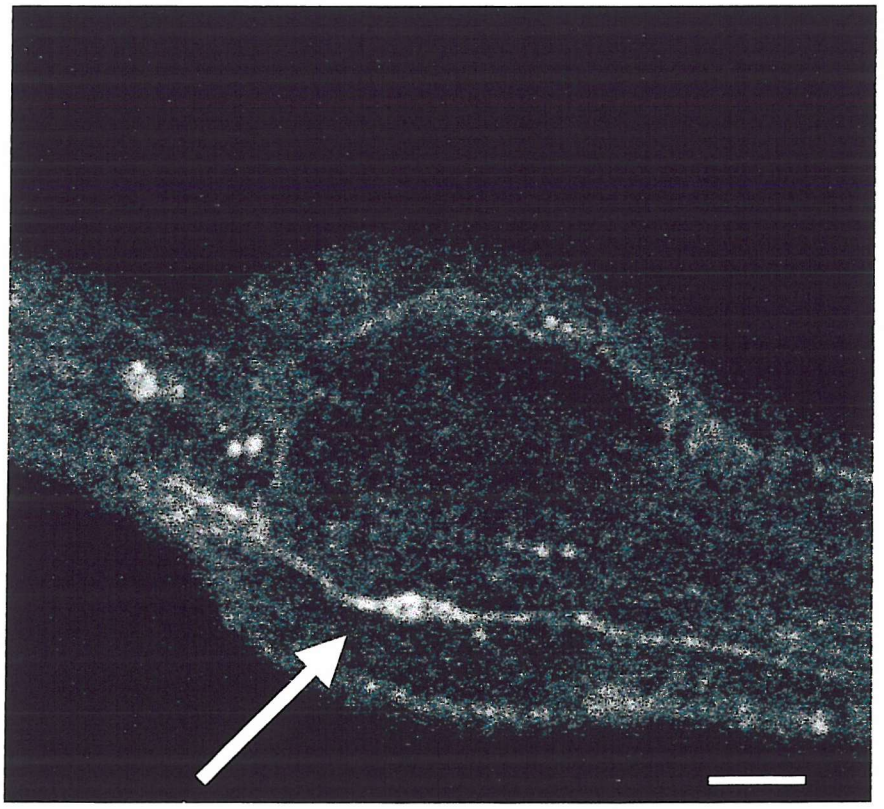


Plate 4.1. Confocal scanning laser micrograph image of anti-AVR-14 antibody staining of dissected *C. elegans* pharynx. Arrow indicates the staining of a single neurone whose cell body lies upon the corpus. Two processes can be seen that extend anteriorally and posteriorally. Affinity purified antibody was used at a 1:20 dilution. Scale bar=10 μM. The preparation is orientated so that the anterior of the animal is towards the bottom right hand corner of the plate. Signal was amplified to enable visualisation of the muscle.

4.12. Effect of ivermectin on *avr-14*

In marked contrast to the abolition of the ivermectin effect in *avr-15*, ivermectin was still a potent agonist on *avr-14*. Ivermectin depolarised the *avr-14* pharyngeal muscle in a concentration-dependent manner. This was accompanied by a decrease in the amplitude and eventual cessation of action potentials (Figure 4.31). The effect of ivermectin was essentially identical to that observed in wild-type, in that it was irreversible and the depolarisation appeared to increase during the wash period. Once again, this complicated an accurate determination of an EC_{50} value. The response that was measured was the maximum depolarisation from original resting membrane potential within 1.5 min of application of a given concentration of ivermectin.

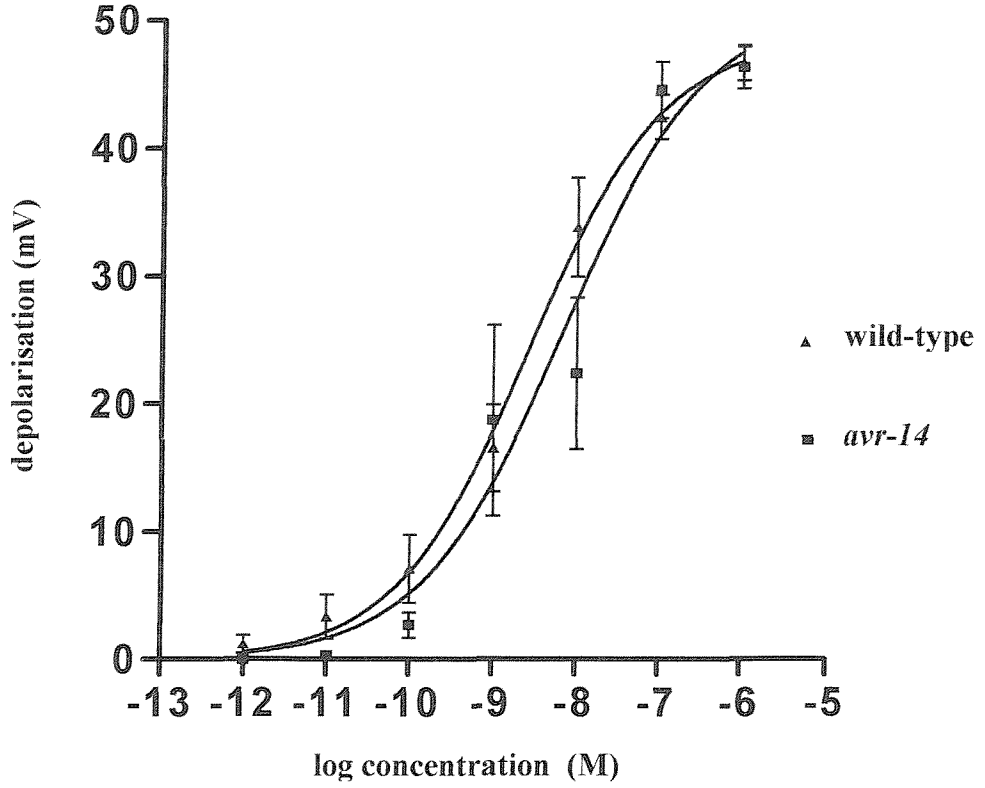


Figure 4.30. Comparison concentration response to ivermectin in *avr-14* and wild-type *C. elegans*. The effect of increasing concentrations of ivermectin (1 pM to 1 μM) in *avr-14* and wild-type *C. elegans*. Each point represents the mean of 10 individual determinations ± S.E.M. for *avr-14* and 8 individual determinations ± S.E.M. *for* wild-type.

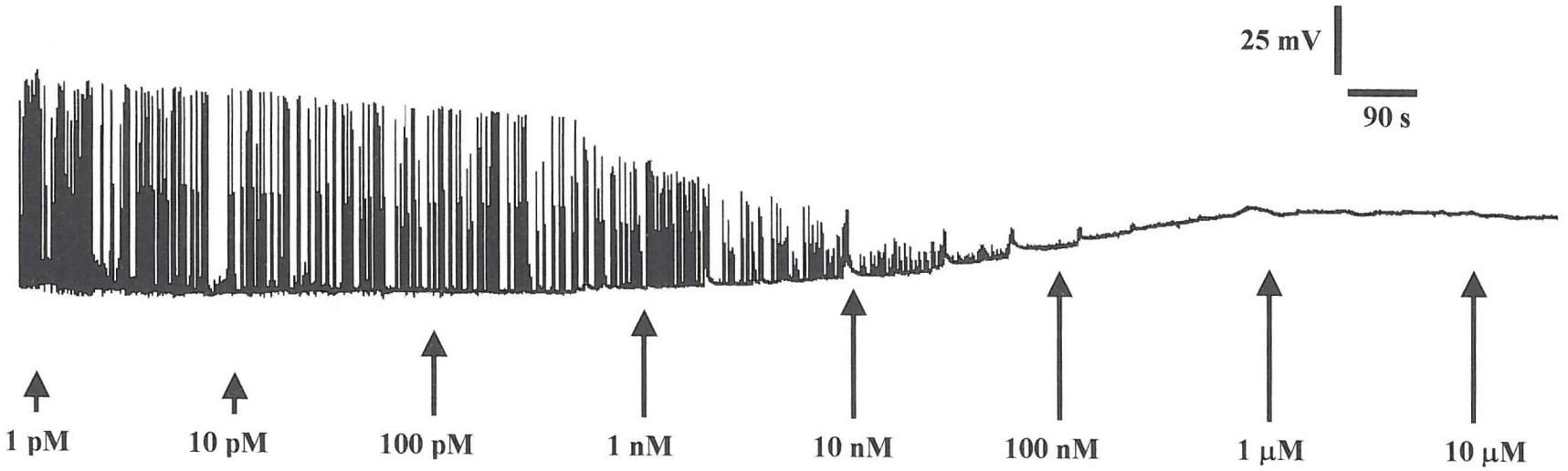


Figure 4.31. Trace demonstrating effect of ivermectin on membrane potential in *avr-14 C. elegans*. The resting membrane potential was –76 mV with action potentials amplitudes of 105 mV. Drug applications indicated by arrows were for 30 s in all cases. Ivermectin produced a slow, irreversible concentration-dependent depolarisation associated with a decrease in amplitude and eventual cessation of action potentials. The response for each individual concentration was taken as the change from resting membrane potential 1.5 min after its application.

The determined EC_{50} value for ivermectin on *avr-14* was 6.7 nM (95% confidence limits 1.2 to 37.0 nM), which was five orders of magnitude more potent than glutamate and close to the value determined in wild-type (Figure 4.30). Maximal depolarisation was 51 mV (95% confidence limits 37 to 65 mV), which was greater than that for glutamate 29.1 mV (95% confidence limits 26.0 to 32.3 mV; n=12).

4.13. Role of neuronal GluCl channels in observed pharyngeal pharmacology

As *avr-14* is expressed solely in neurones and yet alters the pharyngeal response to glutamate, it is quite likely that bath-applied glutamate is having a presynaptic action upon neuronal GluCl receptors to increase the pharyngeal muscle sensitivity to exogenous glutamate. To test for a role of neuronal GluCl receptors in the pharyngeal response to exogenous glutamate, a series of experiments were conducted on a synaptobrevin mutant that has previously been shown to demonstrate severe disruption in synaptic transmission (Nonet et al., 1998). The *C. elegans snb-1* (*md247*) gene encodes for a member of the synaptobrevin family, which are vesicle-associated proteins, implicated in neurotransmitter release. Mutants lacking synaptobrevin function die shortly after completing embryogenesis. However, viable hypomorphic mutants have been isolated that exhibit a variety of behavioural, pharmacological, and physiological defects, indicating that functional synaptobrevin is required for normal synaptic transmission.

To test whether the *snb-1* (*md247*) strain did in fact have altered synaptic transmission, electropharyngeogram recordings (EPG's) were performed to determine whether M3 inhibitory postsynaptic potentials were present (Figure 4.32). The first upward spike (E) marks the depolarisation of the muscle membrane that initiates muscle contraction. The large downward spike (R) marks the repolarisation of the corpus muscle that precedes corpus muscle relaxation. During the period of depolarisation, inhibitory postsynaptic potentials (IPSP's) from the M3 motor neurone are evident in the wild-type strain but are absent or are very weak in the *snb-1* strain, consistent with blocked or weak neurotransmission.

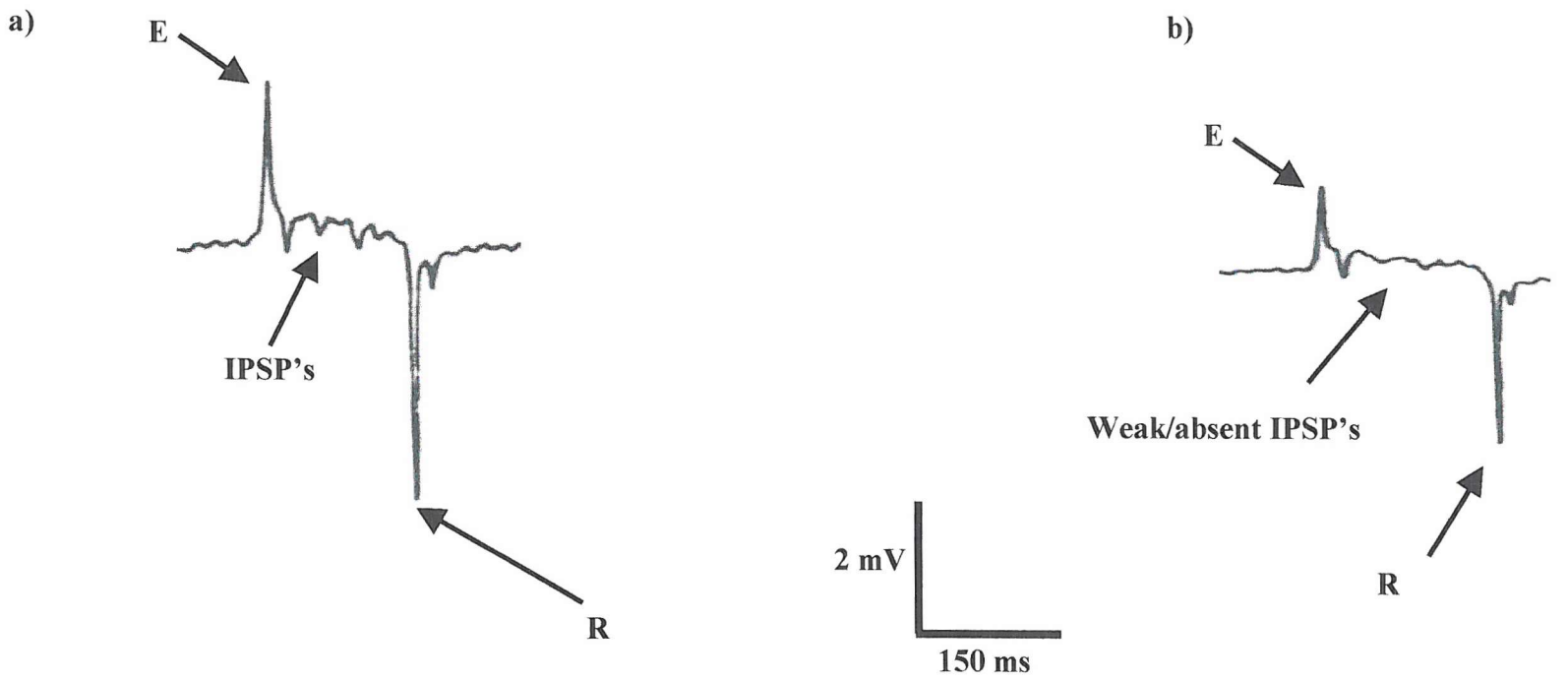


Figure 4.32. Extracellular recordings from a) wild-type and b) *snb-1 C. elegans* pharynegeal muscle. E and R correspond to excitation and relaxation phases of the pharyngeal muscle contraction whilst IPSP's are thought to arise from synaptic transmission generated by the M3's inhibitory motor neurone. IPSP's were very weak or absent in all *snb-1* animals examined (n=8), thus demonstrating that synaptic transmission is severely impaired in this strain.

4.14. Effect of glutamate on *snb-1* mutants

The mean resting membrane potential of *snb-1* pharyngeal muscle cells was -77.1 ± 1.7 mV (n=9). Action potentials were indistinguishable from wild-type (Figure 4.33) and were 118 ± 5.3 mV in amplitude with durations of 0.22 ± 0.19 s (n=9).

Figure 4.33. Resting properties of *snb-1 C. elegans* terminal bulb muscle cells. The trace shows a recording from a single muscle cell within the terminal bulb. The cell was impaled with a 75 MΩ microelectrode. In this recording, resting membrane potential was –74 mV with action potential amplitudes of 120 mV.

Perfusion of glutamate directly over the muscle cells of the pharyngeal terminal bulb elicited an immediate depolarisation, which was concentration-dependent (Figure 4.34). The calculated EC_{50} value for glutamate, determined from peak depolarisation was 371 μM (95% confidence limits 288 to 477 μM; n=16; Figure 4.35). The maximal response was 31.7 mV (95% confidence limits 29.5 to 33.8 mV). The response to glutamate in *snb-1* animals also rapidly desensitised and appeared similar to the degree of desensitisation observed in wild-type recordings.

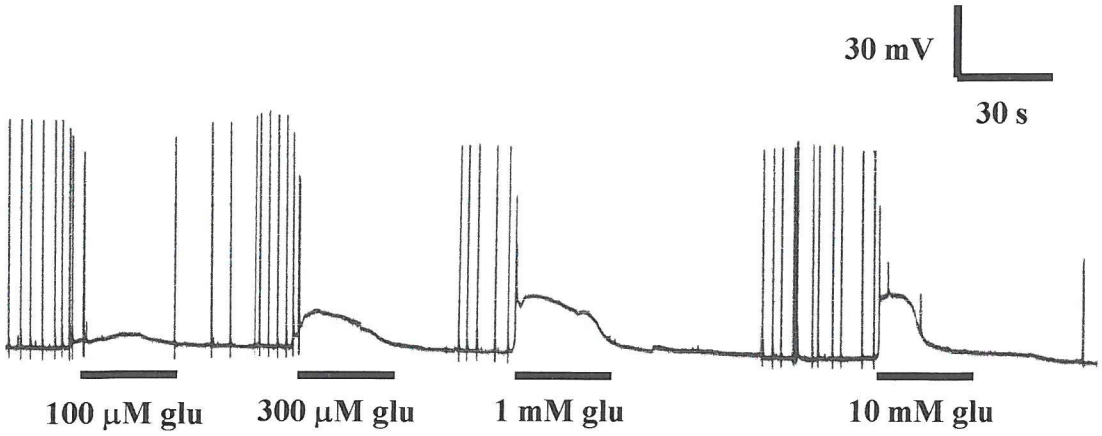


Figure 4.34. The effect of increasing concentrations of glutamate on membrane potential in *snb-1 C. elegans*. Voltage recording from a single cell within the *C. elegans* pharyngeal terminal bulb. The resting membrane potential in this experiment was –81 mV with action potentials amplitudes of 110 mV. Drug applications indicated by horizontal bars were for 30s in all cases. Glutamate produced a rapid concentration-dependent depolarisation that desensitised during drug application.

Comparisons of the glutamate concentration curves for *snb-1* and *avr-14* revealed a striking similarity (Figure 4.35). This finding lends further weight to the hypotheses that exogenous glutamate is having a direct action upon the pharyngeal muscle combined with an indirect action upon the pharyngeal nervous system. Mutations in *avr-14* or *snb-1* reduced the neuronal component of the glutamate response by abolishing the effect of glutamate on the neurone or by blocking neurotransmission respectively.

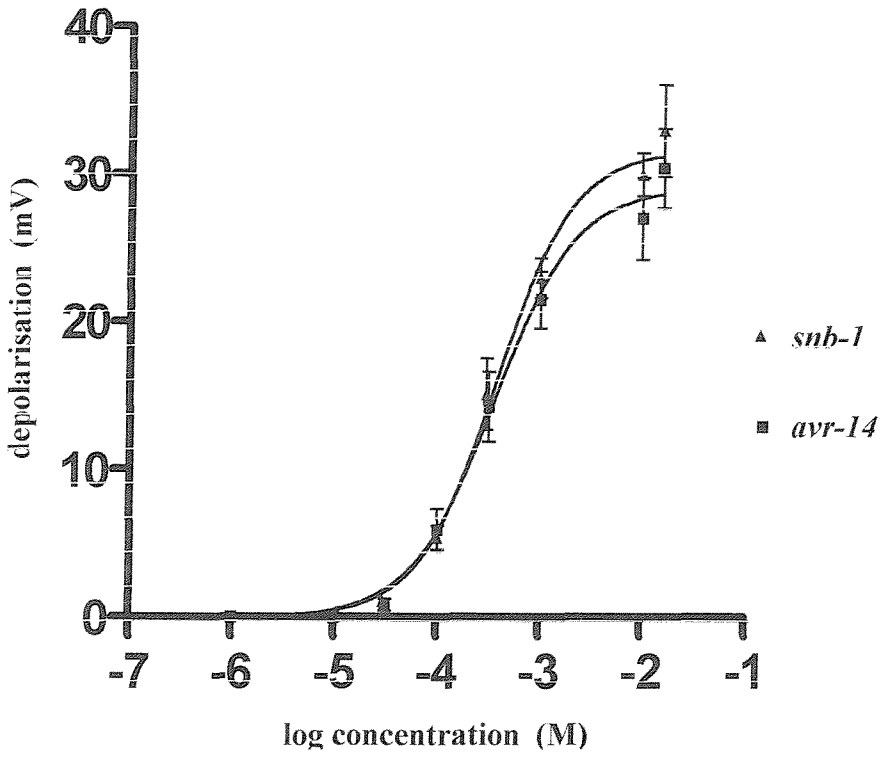


Figure 4.35. Comparison concentration response to glutamate in *avr-14* and *snb-1 C. elegans*. The effect of increasing concentrations of glutamate (1 μM-20 mM) in *avr-14* and *snb-1 C. elegans*. Each point represents the mean of 12 individual determinations ± S.E.M. for *avr-14* and 8 individual determinations ± S.E.M. for *snb-1*.

4.15. Effect of glutamate on *avr-14/15* double mutant *C. elegans*.

The resting membrane potentials of *avr-14/15* were indistinguishable from wild-type (-73.4 ± 2.0; n=7). The frequency and shape of action potentials were also identical to all other strains examined (data not shown). Perfusion of glutamate directly over the cells of *avr-14/15* pharyngeal muscle caused an immediate depolarisation. The response to glutamate was rapid in onset and concentration-dependent. The response was identical to the glutamate response in *avr-15* in that it showed no desensitisation (Figure 4.36).

The calculated EC_{50} value for glutamate in *avr-14/15*, determined from peak depolarisation was 732.2 μM (95% confidence limits 390.3 μM to 1.37 mM); n=8; Figure 4.37. The maximal response was 19.5 mV (95% confidence limits 16.17 to 22.75 mV). Identical to all the other strains examined, *avr-14/15* still responded to glutamate in the presence of 100 μM picrotoxin (data not shown; n=3).

Ivermectin at concentrations up to 10 μM had no effect on spike height, duration or membrane potential (Figure 4.38).

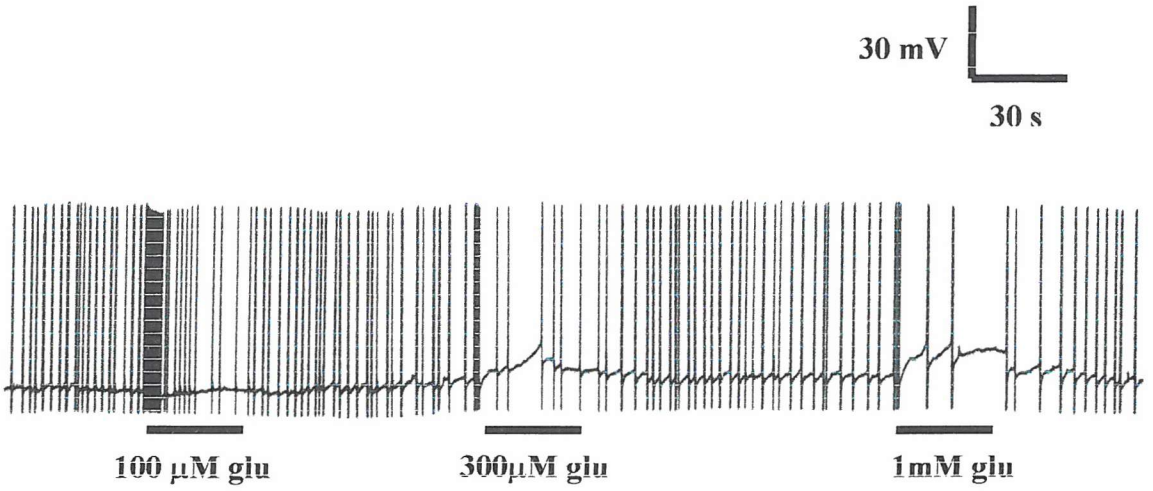


Figure 4.36. Trace demonstrating effect of glutamate on membrane potential in *avr-14/15 C. elegans*. Voltage recording from a single cell within the terminal bulb. The resting membrane potential was –81 mV with action potential amplitudes of 103 mV. Drug applications indicated by horizontal bars were for 30 s in all cases. Glutamate produced rapid concentration-dependent depolarisation that did not desensitise during drug application.

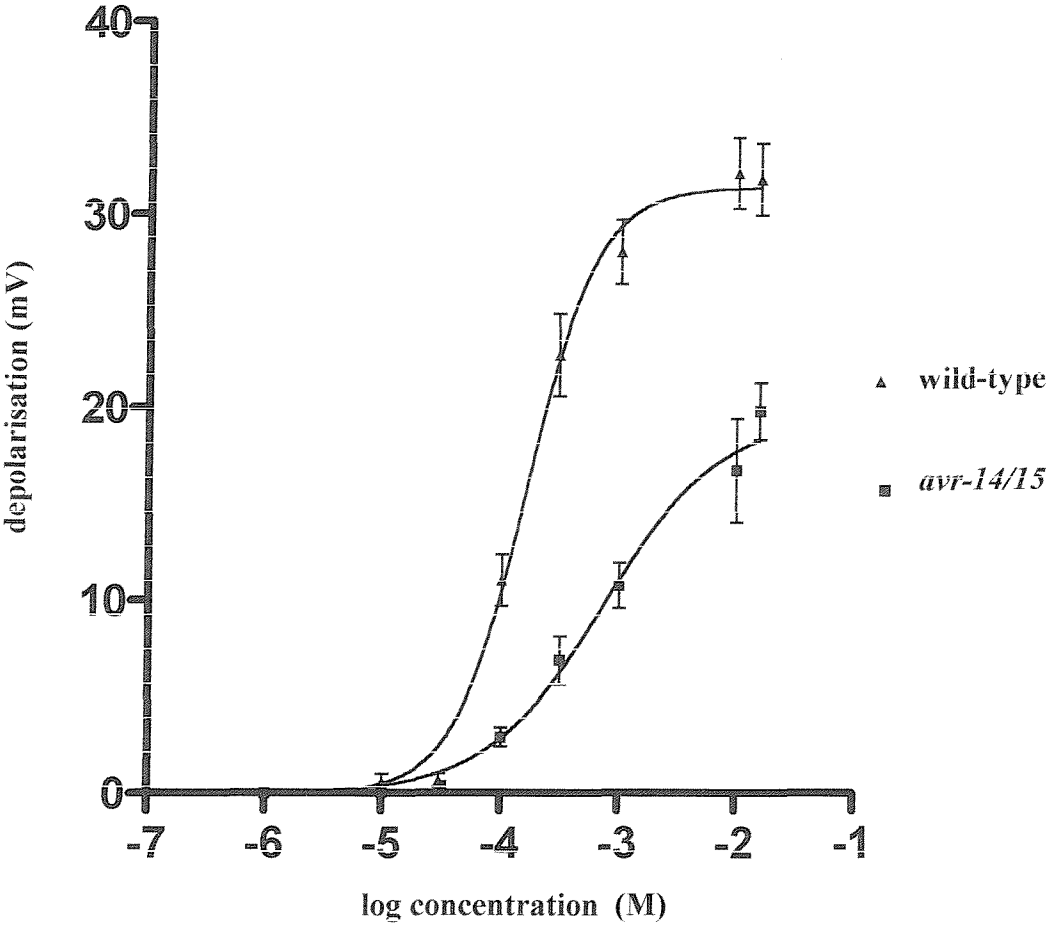


Figure 4.37. Comparison concentration response to glutamate in *avr-14/15* and wild-type *C. elegans*. The effect of increasing concentrations of glutamate (1 μM to 20 mM) in *avr-14/15* and wild-type *C. elegans*. Each point represents the mean of 8 individual determinations ± S.E.M. for *avr-14/15* and 16 individual determinations ± S.E.M. *for* wild-type.

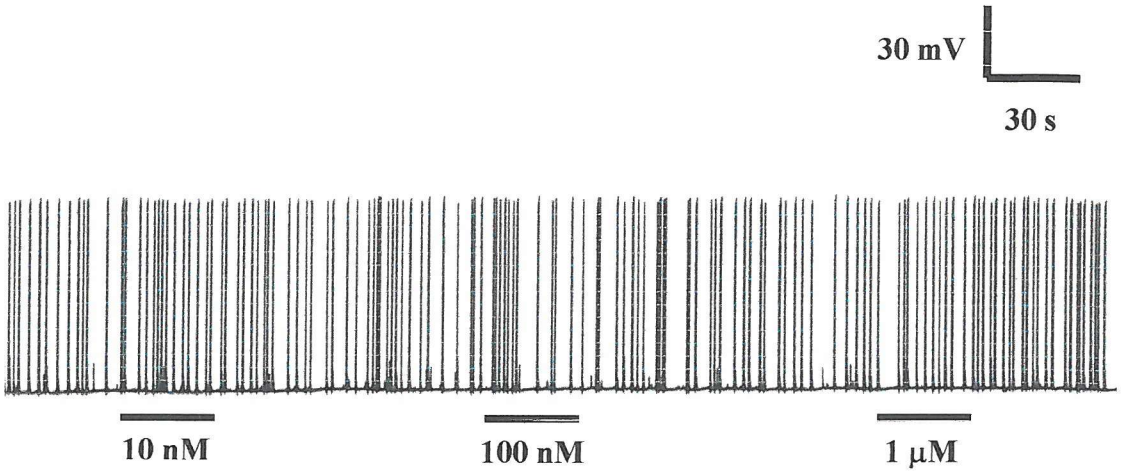


Figure 4.38. Trace demonstrating no effect of ivermectin on membrane potential in *avr-14/15 C. elegans*. Drug applications indicated by horizontal bars and were for 30 s in all cases. Ivermectin had no effect on resting membrane potential, spike amplitude or duration at examined concentrations. Resting membrane potential was –80 mV with spike amplitudes of 115 mV.

Figure 4.39 shows the glutamate concentration response curves for wild-type, *avr-15*, *avr-14* and *avr-14/15*. The response curves were significantly shifted to the right compared to wild-type for all three mutant strains examined (e.g. at 300 μM glutamate $p=0.03$ for *avr-14* and $p<0.0001$ for *avr-15* and *avr-14/15*). The maximum response was also reduced in *avr-14/15* ($p<0.0001$).

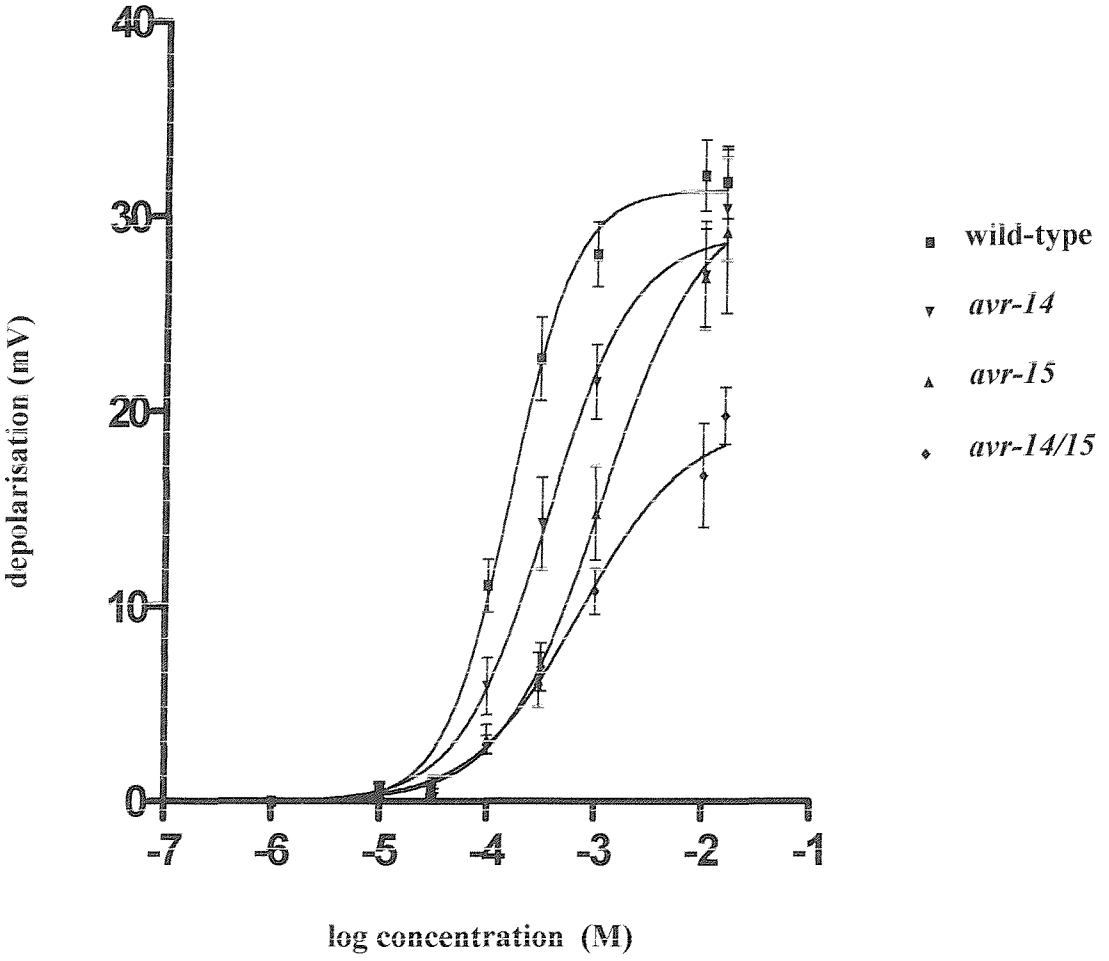


Figure 4.39. Comparison of concentration response curves to glutamate in wild-type, *avr-14*, *avr-15*, *avr-14/15*. The effect of increasing concentrations of glutamate (1 μM to 20 mM) in wild-type, *avr-14*, *avr-15*, *avr-14/15 C. elegans*. Each point represents the mean of 16 individual determinations ± S.E.M. for wild-type, 12 for *avr-14*, 12 for *avr-15* and 8 for *avr-14/15*.

4.16. Discussion

Laser ablation studies have demonstrated that the *C. elegans* pharyngeal muscle is myogenic in nature (Avery and Horvitz, 1989). The twenty neurones that comprise the pharyngeal nervous system probably acts therefore to regulate the frequency of pumps and precise timing of muscle contractions during a pump in response to environmental, physiological, and proprioceptive cues. As described earlier, three of these neurones have been shown to have important roles in regulating normal feeding behaviour (Avery and Horvitz, 1989). One such neurone, the glutamatergic M3, is thought to facilitate membrane repolarisation, and therefore aid rapid muscle relaxation by generating inhibitory postsynaptic potentials (IPSP's). These IPSP's are generated by glutamates action upon pharyngeal muscle GluCl channels (Avery, 1993). In this study, bath applied glutamate caused a decrease in the frequency and amplitude of action potentials followed by an eventual cessation of action potentials and terminal bulb contractions. At higher concentrations, membrane depolarisations were also observed. These effects were fully reversible and as they were clearly chloride-dependent, opening of muscle GluCl channels are likely to be responsible.

If it were assumed that the maximum glutamate response would be approaching the equilibrium potential for chloride (E_{Cl}), then estimates of this value would be approximately –40 mV. As glutamate always caused depolarisations, even at the highest extracellular chloride concentration examined (154 mM), E_{Cl} must always be more positive than resting membrane potential. This unusually positive value must be actively maintained by an inwardly-directed chloride pump. The properties of the pharyngeal chloride pump were not examined in this study, but it is likely to be a very important regulator of muscle function.

In the parasitic nematodes, *A. suum* and *A. galli*, pharyngeal responses to glutamate are less pronounced. For example, Martin (1996) observed that glutamate at concentrations greater than 100 μM caused, in the most part a small transient hyperpolarisation of approximately 1 mV in *A. suum* pharynx, although occasionally a depolarisation was sometimes observed. In *A. galli*, 100 μM glutamate produces a small depolarisation of only 0.2 mV (Holden-Dye: unpublished observations). The small

changes in membrane potential observed in these preparations to glutamate is because resting pharyngeal membrane potentials for these preparations are between –27 mV to –44 mV (Martin, 1996); (Del Castillo and Morales, 1967); (Holden-Dye 1999: personal communication), and is close to the calculated reversal potential for chloride ions in the bathing medium employed.

Contrary to that seen in *A. suum* and *A. galli*, resting membrane potential in *C. elegans* was unaffected by changes in extracellular chloride concentration. This suggests that the *C. elegans* pharyngeal muscle has a low resting conductance to chloride. However, altering of extracellular chloride concentration has a marked effect upon resting membrane potential in the pharyngeal muscle of *A. galli*. For example, a reduction from 84 mM to 14 mM extracellular chloride reduced resting membrane potential from –44 mV to –32 mV (Holden-Dye: unpublished observations). The somatic muscle of these two parasitic nematodes also has a high resting chloride conductance, and E_{Cl} is more negative that resting membrane potential. Consequently, activation of GluCl channels is these preparations consistently produces hyperpolarising responses (Del Castillo et al., 1964); (Parri et al., 1991).

Further evidence that activation of GluCl channels are responsible for the observed responses was provided by the finding that the agonist profile determined for the *C. elegans* GluCl channel in this work is identical to that seen for GluCl channels in other invertebrate preparations (Cull-Candy, 1976); (Cull-Candy and Usherwood, 1973); (Duce and Scott, 1985); (Dudel et al., 1989); (Lea and Usherwood, 1973a); (Lea and Usherwood, 1973b). This study is the first determination of *in vivo* agonist activity of GluCl channels in *C. elegans*. Qualitatively, the response to ibotenate was similar to that seen with glutamate, but it was 10 times more potent. Quisqualate and kainate were both extremely weak agonists. This is also similar to the profile of GluCl-α1:GluCl-β heteromer when expressed in *Xenopus* oocytes (Cully et al., 1994).

Picrotoxin and flufenamic acid are chloride channel blockers that have weak blocking action on invertebrate GluCl channels and on the expressed *C. elegans* GluCl-1α1:GluCl-β heteromer (Cully et al., 1994). In this preparation, these compounds were weaker still: flufenamic acid was ineffective and picrotoxin at high concentrations

(>100 μM), only partially reduced the glutamate response. None of the remaining chloride channel blockers/modulators examined in this study antagonised the glutamate response.

Ivermectin was also a potent agonist at these channels, although its effect was strikingly different to that observed with other GluCl agonists examined in this study. At low concentrations, significant reductions in action potential amplitude were seen. At higher concentrations, slow irreversible membrane depolarisations were observed. No recovery was seen, even after 60 minutes of continuous washing. The depolarising effect of ivermectin seen here is contrary to that assumed by Dent et. al., (2000), who suggested that the main anthelmintic activity of ivermectin in *C. elegans* was due to cellular hyperpolarisation.

The effects of ivermectin upon *C. elegans* pharyngeal muscle seen here are similar to its action in other invertebrate preparations. For example, Martin (1996) and Duce and Scott (1983, 1985a, 1985b), all described an irreversible chloride-dependent increase in conductance that continued to rise for at least 60 minutes. From patch clamp studies in *Ascaris* it has been shown that ivermectin (1-100 pM) activates a Cl^- conductance. (Martin and Pennington, 1989). The channels remain open for about 100 ms and can be seen to open sequentially in a stepwise manner. Furthermore, the work of Arena (1991, 1992) on *Xenopus* oocytes injected with poly A^+ RNA isolated from *C. elegans* demonstrated that an irreversible chloride current was generated following ivermectin application.

The most intriguing difference in responses between glutamate and ivermectin were the significant difference in maximum depolarisation. As this disparity could not be explained by a difference in the resting membrane potentials of the pharynxes in these two sets of experiments, it could be interpreted as indicating some difference in ionic mechanism between these two agonists. To test this further, glutamate was applied to the muscle during the maximal ivermectin depolarisation. As no further depolarisation was observed, it seems most probable that the ionic mechanism for the both the glutamate and ivermectin response is the same (i.e. chloride). Differences in E_{Cl}, or more likely, an underestimate of the glutamate maximum response due to rapid

receptor desensitisation could explain the difference in maximum depolarisations between these two agonists.

Ivermectin was considerably more potent on the native *C. elegans* pharyngeal GluCl channel than at any of the GluCl subunits expressed in *Xenopus* oocytes For example, the threshold for ivermectin's effect in this study was 10 pM, compared to between 10 and 100 nM for the expressed GluClα2a and α2b subunits (Cully et al., 1994); (Vassilatis et al., 1997). It could be that the effect of ivermectin is potentiated by endogenous glutamate in much the same way that low concentrations of ivermectin can potentiate the glutamate response in *Xenopus* oocytes expressing either *C. elegans* mRNA or the GluCl-α1:GluCl-β heteromer (Arena et al., 1992); (Cully et al., 1994). The finding here that the glutamate uptake blocker PDC depolarised the pharyngeal muscle provides evidence for tonic glutamate release, which would be required for this to happen. Although not examined in this study, it would be interesting to see whether PDC can potentiate the effect of ivermectin on this preparation. An alternative explanation for the significant difference in ivermectin potency between the native GluCl channel and expressed subunits could be that the native receptor is intrinsically more sensitive to ivermectin. Certainly, binding assays on *C. elegans* membrane preparations suggests that this may well be the case as the *K*d value for ivermectin has been estimated to be as low as 3 pM (Dent et al., 2000).

The experiments on the *avr-15* (*ad1051*) strain provide an insight into the role of the GluCl-α2 subunit in pharyngeal GluCl receptor function. Previous behavioural analysis on *avr-15* demonstrated that dissected pharyngeal preparations continued to pump in the presence of ivermectin. Furthermore, iontophoretically applied glutamate had no effect on *avr-15* (Dent et al., 1997). This study demonstrated that ivermectin has no effect on pharyngeal action potentials or resting membrane potential in the *avr-15* strain, although the resting membrane potential and action shape and duration of *avr-15* were identical to that seen in wild-type animals. Glutamate however, still produced membrane depolarisations, but at higher concentrations than required in wild-type. This observation raised the question whether a GluCl channel is still mediating the observed responses, and if so what subunits comprise the channel? As the response to glutamate

in *avr-15* was still chloride-dependent and there was also a similar decrease in potency for ibotenate, it is likely that GluCl channels are involved in these responses.

It may be possible that a GluCl-β homomeric channel is responsible; especially as the GluCl-β subunit is expressed in the pharyngeal muscle (Laughton et al., 1997). However, two observations argue against this; firstly, the glutamate response in *avr-15* has a lower affinity for glutamate than would be expected if a GluCl-β homomeric channel was involved (Cully et al., 1994). Secondly, the response was not significantly blocked by 100 μM picrotoxin, whereas the GluCl-β homomeric channel would be predicted to be blocked by nanomolar concentrations of picrotoxin, based on the pharmacological profile of the subunit when expressed as a homomer in *Xenopus* oocytes (Etter et al., 1999). Two possible interpretations of this data are that either the GluCl-β subunit does not play any role in mediating the pharyngeal response to glutamate, or it is co-assembled with another, as yet unidentified subunit, which alters it properties. The latter explanation seems most likely in view of the reported expression of the GluCl-β subunit in the pharynx. The pharyngeal GluCl channel may therefore be a hetero-oligomer comprising of GluCl-α2, GluCl-β, and at least one other isoform of GluCl subunit. This subunit would be predicted to be ivermectin-insensitive, unlike all of the GluCl subunits characterised in oocytes so far (with the exception of GluCl-β). However, one putative GluCl gene, C27H5.8, remains to be pharmacologically characterised; therefore, the possible contribution of this to the pharyngeal receptor should be considered.

The response to glutamate and ibotenate showed rapid desensitisation in wild-type animals. This was quantified for glutamate, and the greatest desensitisation occurred at the lowest glutamate concentration. This observation argues against the possibility that desensitisation is caused by a change in the ionic gradient (e.g., for chloride) as a consequence of the opening of GluCl channels. If this were the case, then it would be expected that the greatest desensitisation would occur at the maximally effective glutamate concentration. It is more likely to reflect an intrinsic mechanism for receptor regulation. The lack of desensitisation in the *avr-15* strain suggests a pivotal role for the GluCl-α2 subunit in the desensitisation process. However, this observation does not shed any light on which subunits are likely to be assembled in the mutant to

form the low affinity receptor. In *Xenopus* oocytes, all of the subunits characterised to date (see Table 4.1) have exhibited receptor desensitisation as homomers. The GluCl-α1:β heteromer does not appear to desensitise (Cully et al., 1994), but it is unlikely that this is the 'residual' receptor in the *avr-15* pharynx, because this would be expected to respond to ivermectin.

In conclusion, because the responses to glutamate, ibotenate, and ivermectin were all affected by a mutation in GluCl-α2, all of these agonists must interact with the same population of native channels, to depolarise the pharynx and inhibit the activity of the muscle. However, whereas this subunit is essential for the effect of ivermectin, it only modulates the response to glutamate and ibotenate, conferring high affinity, and desensitisation. The persistence of a reduced glutamate and ibotenate response in *avr-15* demonstrates that the native channel must be a heteromer. The data presented here indirectly suggests it is most likely to consist of three isoforms of GluCl subunits. This receptor has a low nanomolar affinity for ivermectin. Notably, this affinity is much greater than that determined from expression of GluCl subunits in the *Xenopus* oocytes expression system.

The experiments on the *avr-14* (*ad1302*) strain illustrate the role that this other α-like subunit plays in mediating the pharyngeal response to glutamate. The immunocytochemical localisation of the *avr-14* gene product confirmed earlier findings by Dent (2000) who showed that this subunit was specifically expressed within the pharyngeal nervous system. In marked contrast to the abolition of ivermectin's response in the *avr-15* strain, ivermectin was still a potent agonist on the *avr-14* strain, with no discernable difference in potency when compared to wild-type animals. However, the response to glutamate was significantly reduced, suggesting a role for this subunit in the glutamate response. The most likely explanation for the reduction in glutamate potency seen in *avr-14* is that this particular GluCl subunit is mediating a presynaptic action of glutamate to increase the overall pharyngeal sensitivity to exogenous glutamate. Further support for this hypothesis was provided by the finding that the concentration-response curves for the synaptic transmission impaired strain (*snb-1*) and *avr-14* were identical. It would appear therefore, that bath applied glutamate is having not only a direct action upon the pharyngeal muscle, but also an indirect action upon the pharyngeal nervous

system. It was possible to remove the indirect action of glutamate upon the pharyngeal nervous by examing the glutamate response in the synaptic transmission impaired *snb-1* strain.

In summary, these intracellular recordings provide the first detailed description of native *C. elegans* GluCl channels. This is an important step forward in understanding the physiological role of these channels, and in identifying the genetic determinants of receptor function. This study has revealed some surprising results. Firstly, glutamate and ivermectin depolarise the pharynx, rather than hyperpolarise as was expected on the basis of extracellular recordings (Dent et al., 1997). If this depolarisation is sufficient to open voltage-gated Ca^{++} channels, as seems likely, then this raises the intriguing question that ivermectin may also be excitotoxic, which may be an important contributory factor to its potent nematocidal action. Secondly, both *avr-14* and *avr-15* determine the pharyngeal response to glutamate; *avr-15* contributes to the high affinity, desensitising properties of the channel in the muscle, whilst *avr-14* increases the sensitivity of the pharynx to glutamate by contributing to GluCl channel function in the pharyngeal motor neurones, possibly neurones M4 and M1. However, only *avr-15* is essential for the pharyngeal response to ivermectin. These observations will undoubtedly inform future studies on the GluCl subunit family aimed at resolving the stoichiometry of a therapeutically relevant receptor.

Summary

5.1 Summary

As described earlier, *C. elegans* is renowned for its advanced genetic information and complete descriptions of its cellular anatomy, cell lineage, and neuronal wiring diagram. However, due to its small size and protective cuticle, information concerning its physiology and pharmacology are lacking. The first task of this study therefore was to address the technical difficulties associated with making stable electrophysiological recordings from this nematode. Once these were overcome, detailed investigations into the ionic basis of the pharyngeal resting membrane were then conducted. The data given here, demonstrates that the resting membrane potential of pharyngeal muscle cells are relatively unaffected by changes in the extracellular concentration of Cl^-, Na^+ or Ca^{++}, demonstrating that these ions play little, or no part in generating the resting membrane potential. Variations in extracellular concentrations of K^+, or exposure to ouabain however, had profound effects on resting membrane potential, although the depolarisations observed during elevations of extracellular K^+ were less than would be predicted if the membrane potential were completely dependent on K^+. It can be concluded therefore, that the resting membrane potential of *C. elegans* pharyngeal muscle is largely determined by a K^+ permeability, and a ouabain-sensitive, electrogenic pump.

Studies into the ionic basis of pharyngeal action potentials revealed that action potential height and duration are dramatically affected by changes in extracellular Ca^{++} concentrations. For example, reducing extracellular Ca^{++} caused an initial, transient increase in action potential frequency, combined with a significant reduction in spike amplitude and prolongation in spike duration. The opposite effects were seen when extracellular Ca^{++} levels were increased. These observations adds to earlier findings by Lee et. al., (1997) who suggested that the pharyngeal action potential is Ca^{++} dependent. Surprisingly however, action potentials persisted in Ca^{++} free saline. As these experiments were carried out in 'nominally' free Ca^{++} saline, as no chelator was included in the perfusate, it may be possible that sufficient Ca^{++} was still present to support a Ca^{++}-dependent action potential.

The effect of changing extracellular Ca^{++} concentration on action potential duration was intriguing, as action potential duration increased as extracellular Ca^{++} was

decreased. This is the opposite effect to that which would be expected if the plateau phase of the action potential were determined by a Ca^{++} current. The most probable explanation for these observations is that the repolarisation phase is partly determined by a Ca^{++}-dependent K^+ channel. In fact, searches of the *C. elegans* genome have revealed the presence of two genes that encode for putative Ca^{++}-dependent K^+ channels (Wei et. al., 1996), and one of these is expressed in the pharyngeal muscle (Yuan et al., 2000). Previous studies indicated that a major determinant of the pharyngeal action potential is an L-type Ca^{++} channel (Lee et. al., 1997). In agreement with this, it was found that the L-type Ca^{++} channel blockers, verapamil and nifedipine, both reduced action potential amplitude and duration.

Possibly the most surprising finding of these studies was the absolute dependence of pharyngeal action potential generation on extracellular Na^+ concentration, especially as extensive searches of the *C. elegans* genome have failed to find any voltage-gated Na^+ channel. All of these experiments were carefully monitored for pH effects and two different cations were employed to replace Na^+. It could be argued that a depolarising block is responsible for the eventual cessation of action potentials as a small depolarisation of approximately 5-10 mV was recorded in reduced and Na^+ free media. This explanation is unlikely however, as action potentials have been shown to occur when the muscle is significantly more depolarised than that seen with Na^+ removal. The simplest explanation for the above observation is that activation of a voltage-gated Na^+ channel is responsible for the early rising phase of pharyngeal action potentials and this possibility is currently been investigated using voltage-clamp techniques.

The pharmacological study demonstrated that a number of classical transmitters/modulators have potent effects on the pharyngeal muscle. The responses ranged from the subtle effects of dopamine, muscarine, AF1 and AF2 on membrane excitability, ultimately leading to increases in action potential frequency to the large membrane depolarisations seen with nicotinic agonists and glutamate. As receptor gene expression has previously been shown to occur in the muscle for many of the compounds under test, most if not all of the effects are likely to be due to the compounds direct action upon the muscle. However, an indirect action *via* the pharyngeal nervous system cannot be ruled out. To summarise, perfusion of ACh,

nicotine or glutamate directly on the pharyngeal muscle produced a concentration-dependent depolarisation that was associated with an increase in action potential frequency at lower concentrations. Application of the RFamide peptides, (AF1) and (AF2), both caused stimulation of pharyngeal pumping. Similar effects on action potential frequency occurred following perfusion of muscarine, dopamine or serotonin.

These preliminary studies demonstrated that a detailed pharmacological investigation could be performed on the pharyngeal muscle of *C. elegans*. The remaining series of experiments described went on to pharmacologically characterise pharyngeal GluCl channels in wild-type and mutant animals, with the intention of determining the subunit stoichiometry of the native receptor. Glutamate was observed to cause chloride-dependent membrane depolarisations. From this data, it was estimated that the equilibrium potential for chloride (E_{Cl}) was –40 mV. This value is significantly more positive than the determined E_{Cl} values for parasitic nematodes investigated by other groups. This positive value must be actively maintained by an inwardly-directed chloride pump. Further evidence that activation of GluCl channels were responsible for the observed responses was provided by the finding that the agonist profile determined for the *C. elegans* GluCl channel in this work is identical to that seen for GluCl channels in other invertebrate preparations. Moreover, the weak or absent antagonism of the glutamate response by the chloride channel blockers/modulators examined in this study is similar to that seen with other *in vivo* invertebrate GluCl channels and expressed *C. elegans* GluCl subunits.

Ivermectin was also a potent agonist at these channels, although its effect was strikingly different to that observed with other GluCl agonists.. The depolarising effect of ivermectin is contrary to that assumed by Dent et. al., (2000), who suggested that the main anthelmintic activity of ivermectin in *C. elegans* was due to cellular hyperpolarisation. Ivermectin was considerably more potent on the native *C. elegans* pharyngeal GluCl channel than at any of the GluCl subunits expressed in *Xenopus* oocytes. The hypothesis that endogenous tonic release of glutamate was potentiating the response to ivermectin was supported by the observation that the glutamate uptake blocker PDC depolarised the muscle in a concentration-dependent manner.

The experiments on the *avr-15* strain provided an insight into the role of the GluCl-α2 subunit in pharyngeal GluCl receptor function. In agreement with the behavioural studies performed by Dent et. al., (1997), it was found that ivermectin had no observable effect on resting membrane or action potentials on the pharyngeal muscle of *avr-15*, although the resting membrane potential and action shape and duration were identical to that seen in wild-type animals. However, glutamate, still produced membrane depolarisations, but at higher concentrations than required in wild-type.

The presence of the GluCl-β subunit in the pharyngeal muscle (Laughton et al., 1997), initially suggested that perhaps a GluCl-β homomeric channel was responsible the residual glutamate response. However, two observations argued against this; firstly, the glutamate response in *avr-15* has a lower affinity for glutamate than would be expected if a GluCl-β homomeric channel was involved (Cully et. al., 1994). Secondly, the response was not significantly blocked by 100 μM picrotoxin, whereas the GluCl-β homomeric channel would be predicted to be blocked by nanomolar concentrations of picrotoxin, based on the pharmacological profile of the subunit when expressed as a homomer in Xenopus oocytes (Etter et al., 1999). It is more probable that the GluCl-β subunit is co-assembled with another, as yet unidentified subunit, which alters it properties. The pharyngeal GluCl channel may therefore be a hetero-oligomer comprising of GluCl-α2, GluCl-β, and at least one other isoform of GluCl subunit. This subunit would be predicted to be ivermectin-insensitive, unlike all of the GluCl subunits characterised in oocytes so far (with the exception of GluCl-β). However, one putative GluCl gene, C27H5.8, remains to be pharmacologically characterised; therefore, the possible contribution of this to the pharyngeal receptor should be considered.

The response to glutamate and ibotenate showed rapid desensitisation in wild-type animals. The lack of desensitisation in the *avr-15* strain suggests a pivotal role for the GluCl-α2 subunit in the desensitisation process. In the *Xenopus* oocyte expression studies, all of the subunits examined to date (see Table 4.1) have exhibited receptor desensitisation as homomers. The GluCl-α1:β heteromer does not appear to desensitise Cully et. al., (1994), but it is unlikely that this is the 'residual' receptor in the *avr-15* strain as it would be expected to respond to ivermectin.

In conclusion, because the responses to glutamate, ibotenate, and ivermectin were all affected by a mutation in GluCl-α2, all of these agonists must interact with the same population of native channels. However, whereas this subunit is essential for the effect of ivermectin, it only modulates the response to glutamate and ibotenate, conferring high affinity, and desensitisation. The persistence of a reduced glutamate and ibotenate response in *avr-15* demonstrates that the native channel must be a heteromer. The data presented here indirectly suggests it is most likely to consist of three isoforms of GluCl subunits.

The experiments on the *avr-14* strain illustrate the role that this other α-like subunit plays in mediating the pharyngeal response to glutamate. The immunocytochemical localisation of the *avr-14* gene product confirmed earlier findings by Dent et. al., (2000) who showed that this subunit was specifically expressed within the pharyngeal nervous system. In marked contrast to the abolition of ivermectin's response in the *avr-15* strain, ivermectin was still a potent agonist on the *avr-14* strain, with no discernable difference in potency when compared to wild-type animals. However, the response to glutamate was significantly reduced, suggesting a role for this subunit in the glutamate response. The most likely explanation for the reduction in glutamate potency seen in *avr-14* is that this particular GluCl subunit is mediating a presynaptic action of glutamate to increase the overall pharyngeal sensitivity to exogenous glutamate. Further support for this hypothesis was provided by the finding that the concentration-response curves for the synaptic transmission impaired strain *snb-1* and *avr-14* were identical. It would appear therefore, that bath applied glutamate is having not only a direct action upon the pharyngeal muscle, but also an indirect action upon the pharyngeal nervous system.

In conclusion, this study has addressed some of the voids in our knowledge of the *C. elegans* pharyngeal muscle. The techniques demonstrated here, if used in conjunction with the genetic tractability that *C. elegans* has to offer will provide important information regarding the genetic determinants of its physiology and pharmacology. This study has also demonstrated that the accepted practice of extrapolating data gained from studies on *Ascaris* into *C. elegans* is fundamentally flawed. These nematodes differed not only in their ionic basis of resting membrane and

action potential, but also in their general pharmacology. The framework for further detailed investigations into this nematode has now been provided and with some further technical development, it should be possible to extend the electrophysiological investigations into the *C. elegans* nervous system. These techniques combined with studies using voltage-clamp techniques on the somatic and pharyngeal muscle are currently under development.

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