Nematode nicotinic acetylcholine receptors: a single-channel study in Ascaris suum and Caenorhabditis elegans

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Nematode nicotinic acetylcholine receptors: a single-channel study in *Ascaris suum* and *Caenorhabditis elegans*

by

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3.1. Abstract .................................................................................................................. 47
3.2. Introduction ........................................................................................................... 48
3.3. Materials and methods ......................................................................................... 51
  3.3.1. Maintenance of *Ascaris suum* ....................................................................... 51
  3.3.2. Vesicle Preparation ....................................................................................... 51
  3.3.3. Electrophysiology ......................................................................................... 52
  3.3.4. Data analysis .................................................................................................. 52
  3.3.5. Drugs ............................................................................................................ 53
3.4. Results .................................................................................................................. 54
  3.4.1. Levamisole activated conductance of three different channels ................. 54
  3.4.2. Levamisole preferentially activates G_{35} channels .................................. 58
  3.4.3. Bephenium activates only G_{35} and G_{45} channels .................................. 60
  3.4.4. Bephenium activates more G_{45} than G_{35} channels ............................... 61
  3.4.5. Different subtypes have different mean open-times.................................... 61
  3.4.6. Bath application of paraherquamide inhibits the opening of nAChRs ....... 64
  3.4.7. Paraherquamide has no effect on channel conductance or mean open-
times ......................................................................................................................... 67
  3.4.8. Inhibitory effects of paraherquamide and 2-desoxoparaherquamide are
selective and the % inhibition depends on the receptor subtype ....................... 71
3.5. Discussion ............................................................................................................. 73
  3.5.1. N-, L-, and B-subtypes identified at the single-channel level on body
muscle .......................................................................................................................... 73
3.5.2. Recognition of separate nAChR subtypes in parasitic nematodes is therapeutically significant................................................................. 76

3.5.3. Heterogeneity of nematode nAChRs ............................................................... 76

3.6. Acknowledgements .......................................................................................... 79

Chapter 4. The single-channel properties of levamisole-sensitive nicotinic acetylcholine receptors in *Caenorhabditis elegans* adults ............................................. 80

4.1. Abstract ............................................................................................................. 80

4.2. Introduction ...................................................................................................... 81

4.3. Materials and Methods ................................................................................... 83

4.3.1. Preparation ..................................................................................................... 83

4.3.2. Single-channel recording ............................................................................. 84

4.3.3. Data analysis .................................................................................................. 85

4.3.4. Drugs .............................................................................................................. 85

4.4. Results .............................................................................................................. 85

4.4.1. Nicotinic acetylcholine receptors were observed on the membrane of body wall muscle cells in *lev-10* mutants ......................................................... 85

4.4.2. Levamisole and acetylcholine activated nAChRs with a conductance of ~30 pS. ................................................................................................................ 87

4.4.3. The mean open-time of the nAChR was significantly shortened at high concentrations of levamisole ................................................................. 92

4.4.4. The mean closed-time of the nAChR presented an additional short component at high concentrations of levamisole ......................................................... 94

4.4.5. Single-channel kinetics of the levamisole-sensitive nAChR ......................... 97
4.4.6. The open probability of the channel ($P_{\text{open}}$) ........................................ 99

4.4.7. The nAChR located at the membrane of body wall muscle in lev-10 mutants were not targets of nicotine. ............................................................. 100

4.5. Discussion .................................................................................................. 101

4.6. Acknowledgments ....................................................................................... 104

Chapter 5. Changes in Caenorhabditis elegans levamisole receptor single-channel properties in lev-1 and lev-8 mutants ................................................................. 105

5.1. Abstract ..................................................................................................... 105

5.2. Introduction ................................................................................................ 106

5.3. Materials and methods ............................................................................. 108

5.3.1. Worm strains......................................................................................... 108

5.3.2. Movement assay................................................................................... 108

5.3.3. Cross breeding...................................................................................... 109

5.3.4. PCR ...................................................................................................... 109

5.3.5. Electrophysiology ................................................................................ 110

5.3.6. Data analysis ........................................................................................ 111

5.4. Results ...................................................................................................... 111

5.4.1. Levamisole resistance of lev-1 and lev-8 mutants .............................. 111

5.4.2. Single-channel recording of lev-1 and lev-8 mutants ...................... 112

5.4.3. Prediction of phosphorylation sites on LEV-1 and LEV-8 subunits .... 115

5.5. Discussion ................................................................................................ 119

5.6. Acknowledgments ....................................................................................... 121

Chapter 6. General discussion ............................................................................. 122
Chapter 1. Introduction

1.1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels in the post-synaptic plasma membrane that receive chemical signals released from presynaptic neurons. The opening of nAChRs is triggered by the neurotransmitter acetylcholine. nAChRs are broadly present in many tissues including nervous systems and muscle systems. In nematodes, nAChRs are targets of nicotinic anti-parasitic drugs, such as levamisole. Like acetylcholine, levamisole is a nAChR agonist. Two molecules of levamisole bind with the receptor to open the transmembrane tunnel, which is formed by five subunits. This drug is effective to treat parasitic nematode infections in humans and domestic animals. However, drug resistance emerges and spreads after long-term repeated use of levamisole. Drug resistance can occur naturally. The phenomenon of drug resistance may be due to many mechanisms linked to the ACh-signaling cascade (Jones and Sattelle, 2004).

My studies focused on levamisole action and diversity of nAChRs. It was known that multiple nAChR subtypes are present on somatic muscle cells in nematodes and the levamisole sensitivities of the subtypes are different. We used the patch-clamp technique to record ion channel signals of individual receptors and analyzed the single-channel properties. First we tested nAChR subtypes in the parasitic nematode *Ascaris suum*. Then we realized that genetically manipulated *C. elegans* could provide a good model for levamisole resistance. Therefore we developed single-
channel recording in *C. elegans* to investigate the levamisole sensitive receptor properties and receptor modulation. We believe that our results will help estimate how good *C. elegans* is as a model for studies of parasitic nematodes.

### 1.2. Thesis Organization

In chapter 2, the background information on the parasitic nematodes *Ascaris* spp. and the free-living nematode *C. elegans* is reviewed, as well as the molecular and genetic information on nematode nAChRs. My PhD study included three projects and each project was reported here as individual chapter (Chapter 3, 4 and 5). In chapter 3, I described the three nAChR subtypes observed in *Ascaris*. This study has been published (Qian *et al.*, 2006). I conducted all of the studies reported in this chapter, with the exception of Figure 3.6, which was made by Alan Robertson. In chapter 4, I described the levamisole sensitive nAChR observed in *C. elegans*. In chapter 5, I described the single-channel study of two nAChR subunits, LEV-1 and LEV-8. The studies described in these two chapters will be submitted for publication. Alan Robertson and Richard Martin will be co-authors for both of the studies. In chapter 6, the general discussion, I summarize the observations of my PhD studies and discuss the significance of the observations.
Chapter 2. Literature review

2.1. Nematode parasites and the model nematode

Parasites spend part of their life cycle in or on another living organism, such as humans. During the relationship, the host provides food for growth and survival of the parasite. The parasite induces parasitic disease in the host and can cause poor growth or even death. In a WHO report (WHO, 2000), it was observed that globally 17.3 million deaths of humans were due to infectious and parasitic diseases annually. Common parasitic nematoda include: round worms (*Ascaris lumbricoides*, *Strongyloides stercoralis*), hook worms (*Ancylostoma duodenale*, *Necator americanus*), pinworm (*Enterobius vermicularis*), filaria (*Dirofilaria immitis*, *Wuchereria bancrofti*, *Brugia malayi*) and whipworm (*Trichuris trichiura*). In my studies, the parasitic nematode *Ascaris suum* and the free-living nematode *C. elegans* currently are used as animal models. In this chapter, the life cycle and similarity of these two nematodes are discussed.

2.1.1. *Ascaris* and *ascariasis*

Nematode parasitic diseases commonly occur in human and domestic animals. One of the nematode parasitic diseases in man is ascariasis induced by *Ascaris lumbricoides*. *Ascaris* are large nematodes: adult females are 20-40 cm in length and 0.3-0.6 cm in diameter; adult males are 15-30 cm in length and 0.2-0.4 cm in diameter. Adult females are easily recognized by their large ovaries which occupy the posterior ~60% of the worm. There are two *Ascaris* species: *Ascaris*
lumbricoides (A. lumbricoides) in humans and Ascaris suum (A. suum) in pigs. The only anatomical difference between these two species is that A. suum has a row of small teeth on the lips (Sen et al., 1963). Although they are very similar, infection of man with A. suum or pigs with A. lumbricoides is rare (Urquhart et al., 1987). Both Ascaris species are distributed worldwide. Figure 2.1 shows the life cycle and infection pathway of A. lumbricoides. Adults are normally found in the lumen of the small intestine. A female is reported to release about 200,000 eggs per day, which are passed with the feces. Infective eggs contaminate food and water. Following ingestion of contaminated food or water, larvae hatch in the host intestine. Larvae migrate through the intestinal wall and then reach the lungs. After molting, L4 larvae migrate up the trachea and are swallowed. The L4 larvae then develop into adults in the small intestine. When infected by Ascaris, the host feels abdominal pain and nausea with disturbed function of the alimentary tract. When larvae arrive in the lungs, the host may have some respiratory symptoms.
Figure 2.1. (Modified from http://www.thelifetree.com/roundworms.com) The life cycle of *A. lumbricoides*. Adult *A. lumbricoides* are found in the lumen of the small intestine. The eggs produced by females are defecated with feces. Infective eggs contaminate food and water. Following ingestion of contaminated food or water, larvae hatch in the host intestine. Larvae migrate through the intestinal wall, pass through the liver and then reach the lungs. After molting, L4 larvae migrate up the trachea and are swallowed. L4 larvae then develop into adults in the small intestine.
2.1.2. *Caenorhabditis elegans*

*Caenorhabditis elegans* (*C. elegans*), a free-living nematode in soil environments, has both a hermaphrodite sex and a small male population. Since the distinguished studies done by Sydney Brenner in the 1970’s, this nematode is used extensively as an experimental model system for genetic and molecular studies. The reason for choosing this worm as the animal model is because:

- The life cycle of *C. elegans* is very rapid;
- It is easy to maintain in laboratory;
- It shows a large variety of behaviors and responses that can be tested;
- It has the advantage of being a multicellular organism as well as simple;
- The complete cell lineage has been determined;
- Many aspects of development have been studied;
- The entire genome has been sequenced;
- Many open sources are available;
- Advanced molecular technologies are available in *C. elegans*.

*C. elegans* has four larval stages (Figure 2.2), L1, L2, L3 an L4. Each stage lasts 10 - 14 hours at 20 °C. The complete life cycle is ~3.5 days at 20°C. Under certain environmental conditions the worm goes through a dauer larval stage and can survive for months. A single hermaphrodite can produce 300-350 progeny and a mated hermaphrodite can produce over 1000 progeny.
Figure 2.2. The life cycle of C. elegans. After hatching, the worm has 4 larvae stages and 1 adult stage. At 20°C the life time of the worm is 3 – 4 days. In certain environments, the worm may go through a dauer stage to survive for a long period.

C. elegans can easily be maintained on bacterial-seeded agar plates. The bacteria Escherichia coli (E. coli) are the food for the worm. The worm is transferred from one plate to another and grows to a large population in a few days. When large scale worm preparation is needed, liquid culture is an alternative. C. elegans also can be frozen and stored at -70 °C.

The animal is transparent and its development can be traced. The developmental fates of the somatic cells (959 in adult hermaphrodites; 1031 in the adult male) have been mapped (Sulston and Horvitz, 1977; Sulston et al., 1983). The development pattern of the simple nervous system (302 neurons in hermaphrodites) also has been reconstructed (White et al., 1983).
C. elegans shows many behaviors and responses, i.e. locomotion, pharyngeal pumping, defecation, touch response, chemosensation and thermosensation. Individual neurons associated with these behaviors have been studied in some detail (Riddle, 2001).

The C. elegans Sequencing Project was a collaboration between the Sanger Center in Cambridge, UK and the Genome Sequencing Center at the University of Washington, St. Louis, USA. The sequencing of the complete genome in 1998 makes C. elegans quite convenient for genetic analysis. As of March 2007 there were 24,019 genes in C. elegans released by WormBase W173. These genes distribute on 5 autosomes and 1 sex chromosome. The gene structure and sequence are available from WormBase (http://www.wormbase.org/) and nucleotide & protein databases (GenBank/EMBL/DDBJ/UniProt). The Caenorhabditis Genetics Center at the University of Minnesota (http://www.cbs.umn.edu/CGC/) maintains a huge database of C. elegans strains. As an addition to the genomic sequencing, the C. elegans EST Project helps to confirm the open reading frames. By the help of this project, the molecular information of more than 90% of the genes is available.

Since sequencing of the C. elegans genome was completed, many genetic and molecular methods have been found to reliably work in this animal. Forward genetics and genetic mapping help scientists to generate mutations to analyze gene function and phenotypes. As an important complement to forward genetics, reverse genetic methods allow us to knockout almost any gene in the genome. Among those techniques, RNA interference (RNAi) is one of the most successful. Up to March 2007, RNAi clones of 19,856 worm genes, which cover more than 80% of the
genome, are available. It is not clear that they all work. Some genes are not susceptible to RNAi, especially in neurons. DNA microarray is another advanced technique, which allows scientists to assess the expression level of every gene in *C. elegans* in a rapid and economical way.

### 2.1.3. *C. elegans* as a model for parasitic nematodes

*C. elegans* is commonly used as model for parasitic nematodes. However, *C. elegans* is a free-living animal not a parasite. This gives rise to the question whether *C. elegans* is a good model for parasite studies.

First we need to know how *C. elegans* is evolutionally similar to parasitic nematodes. To date the evolution positions of *C. elegans* and parasitic nematodes were not defined clearly. Usually parasites are classified as separate groups from free-living nematodes and evidences of morphological studies assumed that parasites developed from free-living species (Anderson, 1992). By comparing the similarity of homologous proteins from different nematode species, the relationships of *C. elegans* and the parasite groups are reconstructed. Molecular studies proposed other new schemes of nematode systematics (Lorenzen, 1994; Malakhov and Hope, 1994). For example, the molecular phylogenetic analysis of small subunit (18s) rRNA genes indicate that *C. elegans* belongs to the *Rhabditida* order (Figure 2.3), which is most closely related to parasitic *Strongylida* (Zarlenga et al., 1994; Zarlenga *et al.*, 1994; Fitch *et al.*, 1995; Baldwin *et al.*, 1997; Blaxter *et al.*, 1998; Burglin *et al.*, 1998).
Figure 2.3. (Duplicated from Burglin et al., 1998) The molecular phylogenetic tree of nematodes. The model nematode C. elegans belongs to the Rhabditida and the parasitic nematode Ascaris belongs to the Ascaridida order.

Another way to evaluate how good C. elegans is a model for parasites is the comparison of genomes between C. elegans and parasites. Inspired by the C.
*elegans* Genome Project, the World Health Organization sponsored the Filarial Genome Project since 1995. Initial focus was to generate the EST database of the human filarial parasite *Brugia malayi*. *B. malayi* shared the same ancestor with *C. elegans* 300-500 million years ago (Vanfleteren et al., 1994). It was predicted that *B. malayi* has at least 18,500 genes (Whitton et al., 2004). This number is close to that of *C. elegans*. More than 25,000 ESTs have been generated and grouped into 8348 clusters. About 40% of them show homologs in *C. elegans* (Geary and Thompson, 2001).

Furthermore, neurobiological aspects between *C. elegans* and parasites, such as neuropeptides, have also been studied. Neuropeptides act as signal transmitters in nematode nervous systems to modulate animal behaviors. More than 100 genes encoding over 250 neuropeptides have been discovered in *C. elegans*. The neuropeptides are conserved in nematode systems. Many of them are identical or highly similar between *C. elegans* and parasitic nematodes, such as *Ascaris*, *Haemonchus* (Geary et al., 1999; Li, 2005).

Overall, many evidences support that *C. elegans* is likely to be a good model for parasitic nematodes. This organism is commonly used in studies of pharmacology and development of antiparasitic drugs (Geary and Thompson, 2003; Brown et al., 2006). It is reasonable and necessary to consider this animal as the model for drug resistance studies. However, there are some limitations to using *C. elegans* as a model for studying of parasitic nematodes. Such as, although the peptides are similar in *C. elegans* and parasites, their expression and functions may vary between species (Maule et al., 2002). Another significant difference between *C.
elegans and parasites is the diversity of nicotinic receptors. Unpublished data indicate that C. elegans is not sensitive to the nematode nAChR antagonist paraherquamide, which potently inhibits the nicotinic receptors in Ascaris (Geary, pers. comm.).

2.2. Nervous system in nematodes

There are several ways to define the neuron class in nematodes. According to their physiological functions neurons are classified as sensory, interneurons or motoneurons. Based on the type of neurotransmitter produced by the neuron, neurons are classified as cholinergic, GABAergic, etc. A more refined method used to classify nematode neurons is based on their anatomical features. With this classification, neurons in C. elegans are defined into 118 classes and individual neurons are distinguished by their relative position to the anterior/posterior or left/right axis (White et al., 1986). Here, I briefly introduce the anatomical structure of nematodes using A. suum as example. Then the structure of commissures is explained using C. elegans as example.

2.2.1. Anatomical structure of nervous system in nematodes

The central nervous system of Ascaris is near the head and consists of a set of ganglia associated with a nerve ring (Figure 2.4). This ring receives the primary sensory input and gives rise to the cephalic muscle motor output. Two major nerve cords, dorsal and ventral, originate from the nerve ring (Figure 2.4 and Figure 2.5). There is also one small lateral nerve cord on each side, named the dorsal lateral and ventral lateral nerve cords (Del Castillo et al., 1989).
Figure 2.4. (Crompton and Joyner, 1979) The anterior part of the nervous system in *Ascaris*. The nerve ring receives the primary sensory input and gives rise to the cephalic muscle motor output. The two major nerve cords, dorsal and ventral, originate from the nerve ring.

The anatomical studies show that the cell bodies of motorneurons are located in the ventral nerve cord and connect with the dorsal nerve cord through one left-hand and three right-hand commissures (Figure 2.6). According to their anatomical features, there are seven classes of motorneuron: dorsal excitatory DE1, DE2 and DE3; ventral excitatory VE1 and VE2; dorsal inhibitory DI and ventral inhibitory VI. The connection commissures are arranged in a pattern along the nematode body forming five segments and each segment contains eleven motorneurons: one of DI, DE2 and DE3, two of DE1, VI, V1 and V2 (Stretton *et al.*, 1978).
Figure 2.5. (Thorn and Martin, 1987) Transverse section of *Ascaris*. The dorsal and ventral nerve cords are indicated as well as the two small lateral nerve cords. The two lateral lines separate somatic muscles into ventral and dorsal layers.

Figure 2.6. (Martin *et al.*, 1996) Diagram of the dorsal and ventral nerve cord in one segment of *Ascaris*. Seven anatomical types of motorneurons are
present: dorsal excitatory (DE1, DE2 and DE3), dorsal inhibitory (DI), ventral inhibitory (VI), ventral excitatory (V1 and V2).

- Inhibitory motorneuron
- Excitatory motorneuron
- Inhibitory output
- Excitatory output

2.2.2. Nervous commissures in nematodes

In the nervous system of nematodes, most neuron processes form ventral and dorsal nerve cords along the body axis. However, some processes make dorso-ventral transitions between the long nerve cords. These transitions are called commissures. In the head, there are two pairs of commissures, right & left amphid commissures and right & left deirid commissures. In the tail, there are three pairs of commissures, right & left lumbar, right & left dorsorectal commissures, and right & left dorsolateral commissures. An enlarged commissural region comprised with ~200 processes encircles the pharyngeal bulb and forms the nerve ring. In the pharynx, there are two commissures, pharyngeal nerve ring & terminal bulb commissures. Along the body, more than 40 commissures grow out from the ventral nerve cord to connect the dorsal nerve cord (White et al., 1986; Hedgecock et al., 1987).
2.3. Body wall muscle cell and neuromuscular junction in nematodes

Body wall muscle cells in nematodes are separated into ventral and dorsal layers (Stretton, 1976; Del Castillo et al., 1989). Each somatic muscle cell has three parts (Figure 2.7): a spindle region which is anchored to the hypodermis, a balloon-like bag which contains the nucleus, and arms which arise at the base of the bag region and extend to the nerve cord. The structure of the somatic muscle cell and neuromuscular junction in Ascaris is introduced here as an example.

2.3.1. Belly

The belly or bag region of the Ascaris muscle cell is in the pseudocoelomic space. The cortical region of the belly contains fibrillar bundles of cytoskeleton and a row of small mitochondria. The belly serves as an endoskeleton. The cytoplasm contains the nucleus and is filled with glycogen particles. These glycogen particles are depleted during starvation or exercise (Rosenbluth, 1965).

2.3.2. Arm

The arm is a thin process rising from the muscle belly. One muscle cell may have more than one and up to five arms (Rosenbluth, 1965). When the arms arrive at the nerve cord, they separate into several processes in bundles with gaps of 400-500 nm. The arms come together to form a structure called “syncytium” (Del Castillo et al., 1989). Synapses are formed between the syncytium and the nerve cord.
Figure 2.7. (Rosenbluth, 1965) Schematic diagram of the muscle cell and the neuromuscular junction. The muscle cell includes a spindle, a belly (or bag) and arms. The neuromuscular junction is formed between the end of the arms (which forms a syncytium) and the nerve cord. n: nerve cord; h: hypodermis; c: cuticle.

2.3.3. Spindle

The spindle region contains the contractile apparatus of the muscle cell forming a long tube lying against the hypodermis. It was suggested that the Ascaris
muscle cell is obliquely striated and has properties between smooth and striated muscle (Rosenbluth, 1965).

2.4. Physiology of the body-wall muscle cell in nematodes

2.4.1. Membrane potential of the muscle cell

The electrical activity of the somatic cells of Ascaris has been studied. The average resting membrane potential of the belly region is about -30 mV (Debell et al., 1963). The membrane potential varies with different bathing solutions. For example, the average value was -29.8 mV in diluted (30%) sea water and -34.5 mV in the perienteric fluid (Del castillo et al., 1964). One of the features of the resting membrane potential of Ascaris muscle cells is the relative insensitivity to change in the composition of extracellular concentrations of Na\(^+\) and K\(^+\) (Brading and Caldwell, 1971). However, changes of external Cl\(^-\) concentration affects the membrane potential (Del Castillo et al., 1964). It was proposed that there is a Cl\(^-\) battery in the membrane to maintain the potential. This hypothesis was doubted by Brading and Caldwell. A modified Goldman-Hodgkin-Katz equation

\[
E = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i + x}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o + y}
\]

was proposed to explain the insensitivity of membrane potential to K\(^+\) and Na\(^+\) (Brading and Caldwell, 1971). Two large addition components represented by \(x\) and \(y\) suggests the existence of an electrogenic ion transport system, which may be a Na\(^+\) transporter.
2.4.2. Spontaneous electrical activity of the muscle cell

The steady membrane potential is rhythmically interrupted by spontaneous depolarizations with a frequency of 1.5-7 per second (Debell et al., 1963). The spontaneous activity (Figure 2.8) has three phases: slow waves lasting up to 0.2 sec, spikes of ~22 ms duration and transient post-spike hyperpolarization (Del Castillo et al., 1989). It was observed that stimuli at the belly region of the muscle cell with external electrodes failed to induce propagated electrical activities, while electrical stimulation to the nerve cord gave rise to potential activities similar to the spontaneous activities. These observations suggest that the spontaneous postsynaptic activity is due to the release of neurotransmitters from the presynaptic nerve cord. These “pacemaker” potentials passively propagate from the arm regions of the muscle cells to the bag regions. Del Castillo concluded that the amplitude of the slow waves and the frequency of the spikes were modulated by the interplay of neurotransmitters ACh (excitatory) and GABA (inhibitory).

2.4.3. Patch-clamp technique in nematodes

The patch-clamp technique is a refinement of the voltage clamp technique. This technique was developed by Erwin Neher and Bert Sakmann in the late 1970s and early 1980s. They received the Nobel Prize in 1991 for this work. The development of a high resistance contact (up to 10 giga ohm) between a glass electrode pipette and cell membrane dramatically reduces the background noise and gives the possibility to study individual ion channels. Back in the 1980s, the patch-clamp technique was described to record the single-channel current of acetylcholine.
receptors (Hamill et al., 1981). Since 1985, this technique was used in nematodes to study the ion channel located on the body wall muscle (Martin, 1985). Later, it was found that single-channel recording is a very powerful technique to investigate the subtypes of ion channels and the channel properties of different subtypes were distinguishable (Pennington and Martin, 1990; Martin et al., 1991). Because of the small size, *C. elegans* was difficult to be dissected for electrophysiological investigations. Until 1999, Janet Richmond developed a *C. elegans* preparation to record the whole-cell current from the body wall muscle cell (Richmond and Jorgensen, 1999).

Figure 2.8. (Debell et al., 1963) An example of spontaneous electrical activities recorded from the bag region of Ascaris muscle cells. Each spontaneous activity has three phases: a slow wave, a spike and a transient post-spike hyperpolarization.
2.5. Neurotransmitters and their receptors in nematodes

In *C. elegans*, acetylcholine, glutamate, GABA, dopamine and serotonin have been found to exist as neurotransmitters. Other amine transmitters of vertebrates such as histamine, epinephrine and norepinephrine are not detected. In this chapter, the distribution and function of the neurotransmitters as well as their receptors are introduced.

2.5.1. Acetylcholine

Acetylcholine (Figure 2.9) is one major neurotransmitter in nematodes. More than a third of the neurons in *C. elegans* release acetylcholine (http://www.wormbook.org). Cholinergic transmission occurs both between neurons and at the neuromuscular junction. Acetylcholine is an excitatory transmitter. However, acetylcholine-gated chloride channels also were found. That suggests that acetylcholine could be an inhibitory transmitter in some *C. elegans* cells. Cholinergic transmission is involved in many nematode behaviors, including locomotion, egg laying, pharyngeal pumping, defecation cycling, and male mating (http://www.wormbook.org).

As in vertebrates, acetylcholine targets ionotropic ACh receptors and G-protein coupled ACh receptors in nematodes. The ionotropic nicotinic ACh receptors will be introduced in detail later. G-protein coupled ACh receptors are similar to vertebrate muscarinic receptors. This muscarinic AChR (mAChR) was found in 1983 (Culotti and Klein, 1983). Three genes, *gar-1*, *gar-2* and *gar-3*, encode mAChRs. The primary transcriptions from the genes are alternatively spliced and give different
receptor isoforms (Park et al., 2000; Suh et al., 2001; Park et al., 2003). The GAR-3 isoforms are the most similar to vertebrate muscarinic AChRs. It was found that gar-1 and gar-2 are expressed in sensory and motor neurons (Lee et al., 2000), and gar-3 is expressed in pharyngeal muscle (Steger and Avery, 2004).

![Chemical structure of acetylcholine](image)

Figure 2.9. The chemical structure of acetylcholine. Acetylcholine is synthesized from choline and acetyl-CoA by choline acetyltransferase (ChAT), and degraded by acetylcholinesterases (AChE).

In addition to excitatory ionotropic AChRs, four subunits (ACC-1, ACC-2, ACC-3 and ACC-4) were found to form ACh-gated chloride channels in *C. elegans*. No orthologs were found in vertebrate or *Drosophila* genomes (Putrenko et al., 2005). The expression patterns and functions of these ion channels remain to be studied.

### 2.5.2. Glutamate

Glutamate (Figure 2.10) exists in *C. elegans* as an excitatory and an inhibitory neurotransmitter. Glutamatergic neurotransmission mediates rapid excitatory synaptic signaling. At least 10 excitatory glutamate receptor subunits exist in *C. elegans*. GLR-1 – GLR8 are similar to non-NMDA receptors in vertebrates. NMR-1
and NMR-2 are similar to NMDA receptors (Brockie et al., 2001; Brockie and Maricq, 2003). There is also a family of glutamate-gated chloride channels (GluCl) in nematodes. Six genes encode multiple subunits of the GluCl receptors in *C. elegans*. GluCl receptors have been found in other nematodes such as *Ascaris* and *Haemonchus* (Cully et al., 1996; Forrester et al., 1999; Jagannathan et al., 1999), and are the target of the anti-parasitic drug avermectin (Cully et al., 1994).

![Chemical structure of glutamate](image)

Figure 2.10. The chemical structure of glutamate. Glutamate is also referred to as glutamic acid.

The distribution of GluR subunits has been studied with the help of GFP-tag expression. NMDA-like subunits are expressed in command interneurons, while non-NMDA-like subunits are expressed in both interneurons and the pharyngeal nervous system (Hart et al., 1995; Maricq et al., 1995; Brockie et al., 2001). Based on the receptor distribution it has been suggested that GluRs are involved in the avoidance of noxious stimuli and the control of pharyngeal pumping. GluCl receptors are found to be expressed in pharyngeal muscle and neurons and involved in pharyngeal...
pumping, sensory perception and locomotion (Dent et al., 1997; Laughton et al., 1997; Vassilatis et al., 1997; Dent et al., 2000).

2.5.3. GABA

GABA (γ-aminobutyric acid) is an amino acid neurotransmitter (Florey and McLennan, 1955; Bazemore et al., 1956). In C. elegans, GABA exists as a neurotransmitter in 26 of the 302 neurons. GABA neurons are conserved in nematodes (Johnson and Stretton, 1987; Guastella et al., 1991). GABA inhibits muscle contraction and foraging movements, but stimulates the enteric muscle during defecation (McIntire et al., 1993).

\[
\text{H}_2\text{N} - \text{C} - \text{H} \\
\text{CH}_2 \\
\text{CH}_2 \\
\text{COOH}
\]

Figure 2.11. The chemical structure of GABA. GABA is decarboxylated from glutamate by glutamic acid decarboxylase.

Two types of GABA receptors exist in nematodes, GABA\textsubscript{A} and GABA\textsubscript{B}. GABA\textsubscript{A} receptors are associated with Cl\textsuperscript{−} channels (Delcastillo et al., 1963). The activation of GABA\textsubscript{A} receptors inhibits cell activity. GABA\textsubscript{B} is a G\textsub{i/o}-coupled receptor
(Campbell et al., 1993). The activation of GABA_B inhibits voltage-gated Ca^{2+} channels or activates K^+ channels.

### 2.5.4. Dopamine

In hermaphrodites, eight neurons make dopamine (Figure 2.12) as a neurotransmitter. In males, six additional neurons in the tail make dopamine. Dopamine signaling is critical for learning and modulates locomotion behavior based upon the environment (Sawin et al., 2000). Dopamine signaling allows worms to search for food and alter their behavior by previous experience (Rose and Rankin, 2001). The dopaminergic system also contributes to another form of learning, odorant adaptation (Colbert and Bargmann, 1995; Bettinger and McIntire, 2004).

![Dopamine chemical structure](image)

Figure 2.12. The chemical structure of dopamine. Tyrosine is taken up into dopaminergic neurons by amino acid transporters, then hydroxylated into L-DOPA by tyrosine hyroxylase. Subsequently, L-DOPA becomes decarboxylased to dopamine by dopa-carboxylase.

Four dopamine receptors (DOP-1 – DOP-4) have been found in *C. elegans*. DOP-1 is similar to D1-like dopamine receptors in mammals (Suo et al., 2002).
DOP-4 also is highly homologous to D1-like receptors (Sugiura et al., 2005). DOP-2 and DOP-3 are D2-like receptors (Suo et al., 2003; Chase et al., 2004).

2.5.5. Serotonin

Serotonin (Figure 2.13) is synthesized in eight types of neurons in *C. elegans*. Treatment with exogenous serotonin (Horvitz et al., 1982; Weinshenker et al., 1995; Waggoner et al., 1998) or genetic knockout of serotonin synthetic enzymes (Loer and Kenyon, 1993; Waggoner et al., 1998) will cause defects of locomotion but stimulate egg laying. Serotoninergic motor neurons in the pharynx might release serotonin to communicate with other parts of the body. This signaling allows worms to remain in the area of food. Serotonin is also made in VC4/5 and HSN neurons that directly innervate the vulval muscle (Desai et al., 1988; Duerr et al., 1999).

Three G protein coupled serotonin receptors (SER-1, SER-4 and SER-7) and one serotonin-gated chloride channel (MOD-1) have been identified in *C. elegans* (Olde and McCombie, 1997; Hamdan et al., 1999; Ranganathan et al., 2000; Hobson et al., 2003). SER-1 is expressed on the vulval muscles and pharyngeal muscles (Dempsey et al., 2005). SER-4 and SER-7 are expressed in pharyngeal neurons and muscles (Hobson et al., 2003; Tsalik et al., 2003). The expression of MOD-1 has not been determined.
2.6. Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are ionotropic receptors activated by the neurotransmitter acetylcholine and nicotinic agonists, such as nicotine. nAChRs belong to the large superfamily of ligand-gated ion channels (LGIC) that includes 5-HT3, GABA, and glycine receptors (Cockcroft et al., 1990). The hierarchy of the ligand-gated ion channel family is shown in Table 2.1.
Table 2.1. Ligand-gated ion channels (modified from Le Novère & Changeux, 1999).

<table>
<thead>
<tr>
<th>Superfamily of nicotinicoid receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>family of nicotinic receptors</td>
</tr>
<tr>
<td>subfamily of epithelial receptors</td>
</tr>
<tr>
<td>subfamily of neuronal α-bungarotoxin sensitive receptors</td>
</tr>
<tr>
<td>subfamily of muscle receptors</td>
</tr>
<tr>
<td>subfamily of heteromeric neuronal receptors</td>
</tr>
<tr>
<td>subfamily of heteromeric protostomian receptors</td>
</tr>
<tr>
<td>family of serotonin receptors</td>
</tr>
<tr>
<td>family of GABA receptors</td>
</tr>
<tr>
<td>subfamily of GABA_A receptors</td>
</tr>
<tr>
<td>subfamily of GABA_C receptors</td>
</tr>
<tr>
<td>family of variable agonist receptors</td>
</tr>
<tr>
<td>subfamily of GABA receptors</td>
</tr>
<tr>
<td>subfamily of glutamate receptors</td>
</tr>
<tr>
<td>subfamily of glycine receptors</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Superfamily of excitatory glutamate receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>family of excitatory glutamate receptors</td>
</tr>
<tr>
<td>subfamily of NMDA receptor subunits</td>
</tr>
<tr>
<td>subfamily of AMPA receptor subunits</td>
</tr>
<tr>
<td>subfamily of kainate receptor subunits</td>
</tr>
<tr>
<td>subfamily of kainate binding proteins</td>
</tr>
<tr>
<td>subfamily of delta subunits</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Superfamily of ATP receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>family of ATP receptors</td>
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</table>

Each receptor is comprised of five subunits. The subunits surround a central pore. nAChRs are distributed in the nervous systems and muscle cells. The subunits of the receptor fall into two main categories: α and non-α subunits. A vertebrate muscle type of nAChRs has two α subunits, one β, one δ, one γ or one ε (Karlin, 1989). In vertebrate nervous systems, 12 subunits have been identified. Among them, α2-α7 and β2 - β4 were found in humans; α8, α9 and α10 were found in chick and rat CNS (Graham et al., 2002). In nematodes, nAChR subtypes seem
complicated and unclear. To date, in the gene database of the *C. elegans* Project 27 genes have been identified to encode nAChR subunits ([http://www.sanger.ac.uk/Projects/C_elegans/](http://www.sanger.ac.uk/Projects/C_elegans/) and [http://www.wormbase.com](http://www.wormbase.com)). Here we start with the *Torpedo* nAChR to introduce the ion channel structure. Then we focus on the diversity of nAChRs, especially in nematodes.

### 2.6.1. The structure of the subunit in *Torpedo*

The nAChR located on the electric organ of *Torpedo californica* was the first nAChR purified (Noda *et al.*, 1982; Noda *et al.*, 1983). This muscle type nAChR has been well studied from the protein sequences of the subunits to the global structure of the receptor.

The receptor is made up of four subunits α1, β1, γ and δ arranged with a 2:1:1:1 stoichiometry (Karlin, 1989). Each subunit (Figure 2.14) consists of: (1) a large hydrophilic N-terminal domain, (2) four hydrophobic transmembrane domains (M1-M4), (3) a small highly variable hydrophilic domain between M3 and M4 and (4) a hydrophobic C-terminal domain of approximately 20 amino acids. The large hydrophilic N-terminal domain is involved in ligand binding. The M2 transmembrane segment folds in an α-helix and contributes to the ion-channel pore (Galzi and Changeux, 1995). The nature of the charged amino acids on M2 determines whether the channel carries either cations or anions. Experimental evidence indicates a common transmembrane topology shared by all subunits (Hucho *et al.*, 1996).
Figure 2.14. The secondary structure of the nicotinic acetylcholine receptor subunit. Each subunit of the nicotinic acetylcholine receptor consists of a large hydrophilic N-terminal domain, four hydrophobic transmembrane domains (M1-M4), a small highly variable hydrophilic domain between M3 and M4, and a hydrophobic C-terminal domain.

Circular dichroism studies indicate that the N-terminal domain contains an abundance of β-strands. The computed structural model suggests that the extracellular N-terminal domain of nAChRs consists of a rigid core of β-strands and may spontaneously fold to become stabilized into a native-like conformation, which reduces the flexibility of the extracellular region (West et al., 1997). The structure of this region is changed when ligands, such as acetylcholine, bind with the receptor. The change of the N-terminal domain may occur between the subunits but not within the interface region. Within the transmembrane domain, electron microscopy reveals
that the binding of acetylcholine molecules induce the M2 segments to bend near the middle of the membrane and twist around the central axis at the lower part. In conclusion, structural changes occur in both the N-terminal and transmembrane regions during the allosteric transitions. The global conformational change within the transmembrane region is associated with the channel opening (Corringer et al., 2000).

2.6.2. The amino acid sequences of nAChR subunits in Torpedo

The amino acid sequences of this muscle nAChR are the best known. A database of the amino acid sequence of LGICs is accessible via the worldwide web site: http://www.pasteur.fr/units/neubiomol/LGIC.html. The α subunits contain vicinal cysteine residues near position 190 that form part of the acetylcholine binding site (Kao and Karlin, 1986). The amino acid sequences of the Torpedo acetylcholine receptor subunits are shown in Figure 2.15.
Figure 2.15. The amino acid sequences of *Torpedo* nAChR subunits.

Regions of sequence identity between the subunits are indicated by black coloring. Regions of sequence high similarity between the subunits are indicated by dark coloring. Regions of sequence low similarity between the subunits are indicated by light coloring. The vicinal cysteine
residues on the α subunit are indicated by red color. The positions of the four transmembrane domains (M1 – M4) are indicated.

2.6.3. The pentameric structure of the nAChR in Torpedo

The Torpedo nAChR is a pentamer. The three dimensional structure information of the receptor from electron microscopy at 4.6 Å resolution shows that the five transmembrane proteins create a central pore (Figure 2.16) with an inner diameter of ~25 Å (Miyazawa et al., 1999). The pore becomes narrower at the transmembrane level, which is suggested to correspond to the gate of the channel. Two cavities in the α-subunits located 30 Å above the lipid bilayer surface are believed to be the ACh binding sites. The cavities connect by tunnels about 10-15 Å to the central water-filled vestibule.

Figure 2.16. Diagram of the nicotinic acetylcholine receptor, depicting the ion channel and the ACh binding sites.
The crystal structure of an acetylcholine binding protein reveals the ligand-binding domain of nAChRs (Brejc et al., 2001). The ligand binding site, which is located at the subunit interfaces of α and the adjacent non-α subunits, is contributed by six loops: three from α subunit and three from the non-α subunits (Corringer et al., 2000). All amino acid residues in the binding site are conserved between most of the nAChRs except two loops. These two loops have low sequence conservations in the nAChR family and this change may lead to variations in ligand binding affinity. For example, it has been shown that the binding affinities of the α/γ and α/δ are pharmacologically different (Sine, 1993; Sine et al., 1995).

2.7. Nicotinic AChRs in Ascaris suum

>CAA09602
  1 araknyggvs vlyvpyemiw vpdivynna dsnyntist katlhysgev tweppaiufs
  61 mcqidrrwfp fdeqkchlkf gswtysedll vlelldgeph yeletnefge vdnitivddg
  121 idlbsdyypsv ewdismrsvai rrtknypsscc pqssdayidim yylelrrkpl fytvnlfpcc
  181 vgisfitiv fylpsdsgek vtlcisiiva ltvffillte iiipatsislp ligkyliftm
  241 vmvltvsvvt vislnlhfrt pthtrmpewv kwflkflpk vlflmrplad tddtlyrvsq
  301 rrgdncekva inyhehvrsr dieralstsp vderiqklyy spavvkafen vcfiaellkk
  361 kdrddkvded wkyvamvlvr lflillfsfac figtvtilq aptlydrrea ndlqyrpani
  421 stpvctq

Figure 2.17. The amino acid sequence of the UNC-38 subunit in Ascaris suum.

In the protein sequence database, the amino acid sequence (Figure 2.17) of an A. suum nAChR subunit is also available. This protein is 78% identical to the UNC-38 nAChR subunit in C. elegans. In EST library, a gene sequence that predicts to encode a UNC-38 subunit is also found in Ascaris lumbricoides. Although
information about the nAChR subunit is limited at the genetic and molecular level, our previous pharmacological and physiological studies in *Ascaris suum* indicated the existence of multiple subtypes.

### 2.7.1. Pharmacological observations

Using an *Ascaris* muscle contraction assay, three pharmacological types of nAChRs have been observed in *Ascaris* somatic muscle (Robertson *et al.*, 2002). All these types respond to acetylcholine and nicotinic agonists, such as nicotine, levamisole and bephenium. However, the sensitivities of the types to the agonists are different. According to the agonist sensitivity, we named them L-type (levamisole-sensitive), N-type (nicotine-sensitive) and B-type (bephenium-sensitive). The antagonists paraherquamide and 2-desoxoparaherquamide inhibit the muscle contraction induced by nicotinic agonists. The inhibitory effects are subtype selective and distinguishable with the competitive model. This evidence suggested the presence of multiple subtypes of nAChRs in *Ascaris* and different agonists and antagonists selectively target the different subtypes.

### 2.7.2. Physiological observations

The nicotine-sensitive (N-type) and levamisole-sensitive (L-type) nAChRs have also been observed at the single-single level (Levandoski *et al.*, 2005). With the patch-clamp technique, individual ion channel properties were observed. When nAChRs located at the somatic muscle bind with nicotine or levamisole molecules, the associated ion channel was opened and the single-channel current was recorded. In these single-channel recordings, the channel conductance exhibited at
least two levels, one was ~25 pS and the other one was ~40 pS. It suggests that two nAChRs were observed. Further analysis indicates that nicotine is more selective for the small conductance nAChR and levamisole is more selective for the large conductance nAChR. The evidence implies that N-type and L-type nAChR were observed at the single-channel level.

2.8. Nicotinic AChRs in C. elegans

Nematodes have a large family of nAChR subunits. In the genome of C. elegans, 27 genes were identified to encode nAChR subunits. There are also more than 20 orphan genes that are predicted to encode nAChR subunits (Jones and Sattelle, 2004; Brown et al., 2006). The knockout mutants of most nAChR subunits are available to study the function and phenotypes.

2.8.1. Receptor subunits in the genome

The 27 subunits are classed into 5 groups, UNC-38-like, UNC-29-like, ACR-16-like, DEG-3-like and ACR-8-like (Jones and Sattelle, 2004). The UNC-38-like group consists of three α subunits, UNC-63, ACR-6 and UNC-38, which are most similar to insect α subunits. The ACR-16-like group is highly homologous to the vertebrate α7 subunit and can form homomeric ion channels. The UNC-29-like group consists of four non-α subunits, ACR-2, 3, LEV-1 and UNC-29, which are similar to insect non-α and vertebrate skeletal muscle non-α subunits. The DEG-3-like and ACR-8-like groups appear to be unique in nematodes.
2.8.2. Levamisole receptor

Levamisole is a nematode specific nAChR agonist. Treatment of nematodes with levamisole can induce spastic paralysis of the muscle cells and stimulation of egg laying (Lewis et al., 1980). Studies of levamisole-resistant mutants make it possible to identify the genes that affect the function of the levamisole receptor (Fleming et al., 1997). The knockout of any of unc-38, unc-63 and unc-29 leads to the loss of levamisole sensitivity. The knockout of either lev-1 or lev-8 leads to a partial loss of levamisole sensitivity. These observations indicate that the UNC-38, UNC-63 and UNC-29 subunits are essential subunits for the functional levamisole receptor. The LEV-1 and LEV-8 subunits also contribute to the levamisole receptor but may not be critical (Fleming et al., 1997).

The properties of the levamisole receptor have also been studied using patch-clamp. The whole-cell current induced by levamisole or acetylcholine was observed at the body wall muscle cell. In unc-38, unc-63 or unc-29 knockout mutants, the whole-cell current was abolished (Richmond and Jorgensen, 1999; Culetto et al., 2004). In lev-1 mutants, the whole-cell current was reduced by ~90% (Culetto et al., 2004). In lev-8 mutants, the whole-cell current was reduced by ~70% (Towers et al., 2005). The function of levamisole receptors located at the neuromuscular junction have been reported to be involved in the body movement of nematodes, but the levamisole receptor null mutations only partially impair nematode movement. It is believed that other nAChR subtypes that are levamisole-insensitive are present at the neuromuscular junction, and also contribute to the excitatory neurotransmission between motor neurons and muscle cells.
2.8.3. Nicotine-sensitive nAChRs in *C. elegans*

The nicotine-sensitive nAChR was observed in 1999 (Richmond and Jorgensen, 1999), but the molecular and genetic basis of this receptor remained unknown until 2005. It was found that there is a levamisole-insensitive nAChR subtype at the neuromuscular junction. Nicotine and acetylcholine, but not levamisole, are agonists of this receptor and induce whole-cell currents. When acetylcholine depolarizes the body wall muscle cell, the nicotine-sensitive receptor contributes most of the inward whole-cell current. With a microarray searching strategy, among more than 40 putative nicotinic receptor subunit genes in the *C. elegans* genome, it was identified that ACR-16 is the only essential subunit of the levamisole-resistant nicotinic receptor (Touroutine *et al*., 2005).

2.9. Nicotinic AChRs in vertebrates

In human, rat and chick, multiple genes that encode nAChR subunits have been identified. Based on their evolutionary, pharmacological and physiological profiles, the subunits encoded by these genes are grouped into three classes: muscle type subunits (α1, β1, δ, ε and γ), standard neuronal subunits (α2-α6 and β2-β4) that form nAChRs in αβ combinations, and subunits (α7-α10) capable of forming homomeric nAChRs (Colquhoun & Patrick, 1997; Le Novere & Changeux, 1995; McGehee & Role, 1995).

Muscle nAChRs found at the neuromuscular junction are made up of two α1, one β1, one ε and one γ subunit. In vertebrate nervous systems, the neuronal nAChRs have many diverse combinations according to an α2β3 stoichiometry with
the possibility of more than one $\alpha$ subunit type in one nicotinic receptor (Conroy et al., 1992). In addition, $\alpha 7$, $\alpha 8$ and $\alpha 9$ subunits are known to form functional homooligomers (Couturier et al., 1990). However, heterologous expression of these subunits in *Xenopus* oocytes and mammalian cells has established certain rules to limit the subtypes of nAChRs that may functionally exist: (1) $\alpha 2$, $\alpha 3$ or $\alpha 4$ subunits can form functional nAChRs combining with $\beta 2 - \beta 4$; (2) The combination of only $\alpha 5$ and $\beta 3$ subunits cannot form functional nAChRs but they have to express with at least two other types of subunits (Lukas et al., 1999); (3) $\alpha 6$ subunits have been shown to form functional heteromeric nAChRs when co-expressed with $\beta 4$ or at least two other types of subunits including $\beta 3$ (Kuryatov et al., 2000); (4) $\alpha 7$, $\alpha 8$ and $\alpha 9$ are distinguished by their ability to form homomeric nAChRs in expression systems (Couturier et al., 1990; Keyser et al., 1993; Elgoyhen et al., 1994; Gotti et al., 1994). Evidence indicates that the $\alpha 7$ subunit can also form more complex combinations (Yu and Role, 1998; Girod et al., 1999).

In native systems, only a few major combinations of nAChRs have been identified. In the mammalian brain, most nAChRs contain either $\alpha 4\beta 2$ or $\alpha 7$ subunits (Charpantier et al., 1998; Nashmi and Lester, 2006). The $\alpha 4\beta 2$-containing nAChR is the major subtype existing in the central nervous system (CNS). Expression studies in *Xenopus* oocytes proposed the stoichiometry $(\alpha 4)_{2}(\beta 2)_{3}$ (Cooper et al., 1991). However, other combinations may present *in vivo* (Lukas et al., 1999). Another major subtype of nAChRs presenting in CNS and peripheral nervous system is comprised of $\alpha 7$ subunits (Chen and Patrick, 1997). The $\alpha 7$ subunit is generally
thought to form homomeric nAChRs. But in the chick brain and retina, it has been reported that $\alpha 7$ subunits also combined with $\alpha 8$ subunits to form heteromeric subtypes (Keyser et al., 1993; Gotti et al., 1994; Yu and Role, 1998). The $\alpha 8$ homomeric and heteromeric subtypes have been observed in avian brain and tissues (Couturier et al., 1990; Keyser et al., 1993; Elgoyhen et al., 1994).

2.10. The regulation of nAChR functions by receptor associated proteins

The function of ligand-gated ion channels is regulated by intracellular proteins, such as the stargazin protein required for normal action of glutamate receptors (Walker et al., 2006). SOL-1, another receptor associated protein, was recently found to regulate ionotropic glutamate receptor desensitization (Walker et al., 2006). Many proteins are also associated with nicotinic receptors (Gottschalk et al., 2005). The role of these proteins in receptor regulation is being studied. Here, two nAChR associated proteins are introduced. LEV-10 was found to be required for the receptor clustering. Another nAChR modulator recently has been found to regulate nAChR signal transduction.

2.10.1. Nicotinic receptor clustering and LEV-10 protein in C. elegans

LEV-10 was found to associate with the levamisole receptor in C. elegans (Gottschalk et al., 2005). The extracellular region of this protein has one LDL domain and five predicted CUB domains that may be involved in extracellular protein-protein interactions (Gally et al., 2004). A phenotype of weak levamisole- resistance was
observed in the *lev-10* knockout mutant (Fleming *et al*., 1997). In N2 wild type worms, levamisole receptors aggregate at the postsynaptic neuromuscular junction. When the *lev-10* gene is knocked out, the receptor cluster could not be detected with GFP labeling. However, the levamisole-induced whole-cell current remained similar to that in N2 animals. The evidence suggests that the receptor cluster diffuses but the receptor remains functional on the cell body. Due to this re-localization of nicotinic receptors, the synaptic cholinergic transmission is inefficient. In *C. elegans*, two nAChRs, levamisole-sensitive and nicotine-sensitive, are present at the neuromuscular junction. The LEV-10 protein is required for the clustering of the levamisole receptor but not the nicotine-sensitive receptor. Ongoing studies are investigating the clustering mechanism of LEV-10 (Gally *et al*., 2004).

### 2.10.2. Receptor modulation proteins in *C. elegans*

A novel mutant that showed partial levamisole resistance was recently isolated in Dr. Bessereau’s lab (pers. comm.). The gene associated with drug resistance was cloned. This gene encodes a transmembrane protein that seems to be synaptic in wild type animals. In mutants, levamisole receptor clusters were detected at the neuromuscular junction. It means that this protein is not required for receptor localization like LEV-10. Levamisole induced whole-cell currents were reduced by 50% compared to wild type, N2 animals. It is possible that this protein is directly modulating the activity of nAChRs, like the stargazin and SOL-1 proteins for glutamate receptors.
2.11. Anthelmintics: levamisole and paraherquamide

Nicotinic agonists, including levamisole, pyrantel, and morantel, are one of the major types of anthelmintic used to treat both adult and larval nematode infections of domestic animals. These compounds have been reported to cause depolarization and contraction of nematode muscle and produce spastic paralysis of parasites (Aceves et al., 1970; Aubry et al., 1970). Levamisole, pyrantel, and morantel act as agonists of nACh receptors on the muscle bag membranes of Ascaris suum. The relative potencies of levamisole, pyrantel and morantel have been determined from concentration-conductance relationships on whole cells and found to be: morantel = pyrantel > levamisole > ACh (Harrow and Gratien, 1985). These compounds also act as cholinergic receptor agonists in other invertebrates and vertebrates. The relative potencies of the drugs are different for receptors in the different species (Eyre, 1970). Paraherquamide is a novel anthelmintic, which was observed to induce flaccid paralysis of nematodes. The published data suggests that it behaves as a competitive antagonist of nicotinic acetylcholine receptors (Robertson et al., 2002).

2.11.1. Levamisole

Levamisole is (-)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole (Figure 2.18). Following oral administration in sheep and goats, levamisole is rapidly absorbed from the gastrointestinal tract and gives excellent results against both adults and larvae of most nematodes (Renoux, 1980).
Figure 2.18. Chemical structure of levamisole. Molecular Formula is $C_{11}H_{12}N_2S$. Levamisole is a potent anthelmintic used to treat nematode infestation. This compound has been shown to act as an agonist of nACh receptors.

After uptake of levamisole, there is an initial contraction followed by a paralysis of whole Ascaris (Aceves et al., 1970). Levamisole was found to induce an irreversible contraction that could not be inhibited by atropine, tubocurarine, hexamethonium or piperazine (Coles et al., 1974; Coles et al., 1975). This observation suggested that levamisole might act at ACh receptors without inducing the release of ACh. Using intracellular current- and voltage-clamp techniques, Harrow and Gratton (1985) showed that levamisole acted at ACh receptors located on the muscle bag membrane of *Ascaris suum* causing an increase in input conductance and depolarization of the muscle cells.

2.11.2. Paraherquamide

Paraherquamide is an oxindole alkaloid metabolite of *Penicillium paraherquii*. The first isolation of paraherquamide was back in 1981 (Yamazaki et al., 1981). Subsequently, Shoop (1990) detected the anthelmintic activity of paraherquamide.

Paraherquamide is effective against strains of parasites that are resistant to the
known broad-spectrum anthelmintic drugs (Shoop et al., 1990; Shoop et al., 1991; Shoop et al., 1992). The paraherquamide family consists of a group of analogues (paraherquamide A-G); among them paraherquamide A (Figure 2.19) is most active. Recently, some research groups including Pharmacia Upjohn investigated other new analogues, one of which, 2-desoxoparaherquamide was chosen for further study. Zinser et al. (2002) observed that paraherquamide and 2-desoxoparaherquamide applied in vitro induced flaccid paralysis of nematode somatic muscle without affecting ATP levels. Another research group also reported that these two compounds behaved as competitive antagonists of nAChRs in the nematode Ascaris suum (Robertson et al., 2002).

Figure 2.19. (Duplicated from Robertson et al., 2002) The chemical structure of the paraherquamide A. The O* is missing in 2-desoxyparaherquamide.
2.12. Drug resistance in parasitic nematodes

Anthelmintic drugs are used to treat parasitic nematode diseases in humans and domestic animals. Currently there are three major classes of anthelmintics on the market. Avermectins, such as ivermectin, target glutamate-gated and GABA-gated chloride channels. Ivermectin is effective against most intestinal worms, most mites and some lice (Hotson, 1982). Nicotinic agonists, such as levamisole and bephenium, target nicotinic acetylcholine receptors. Benzimidazoles, such as albendazole, bind to β-tubulins and inhibit the polymerization of tubulin to form microtubules (Lubega and Prichard, 1991).

The repeated intake of one anthelmintic compound will lead to resistance in the parasite population and resistance is inherited (Prichard et al., 1980). It is believed when one nematode population is resistant to one anthelmintic compound, they may also be resistant to other members of the same chemical class. It is also possible that one population develops multiple resistance to several chemical classes (Wolstenholme et al., 2004). Mechanisms of the development of resistance are varied. It may due to the loss of the drug target, the change in the molecular structure or the change in localization of the drug target. It also may be due to changes in the metabolism of the drug.

In my studies, I focused on the action of levamisole and levamisole resistance in nematodes. Levamisole acts as a cholinergic agonist. When levamisole molecules bind with the nicotinic acetylcholine receptor, the ion channel associated with the receptor is opened and induces cell depolarization. The depolarization of the body wall muscle cell leads to contraction and spastic paralysis of the animal (Aceves et
As with any other anthelmintic, the repeated intake of levamisole will lead to levamisole resistance in parasitic nematodes. The pharmacological and physiological studies of levamisole resistance in parasitic nematodes indicate that resistance is in part due to the diversity of nAChR subtypes (Robertson et al., 1999).
Chapter 3. Pharmacology of N-, L-, and B-subtypes of nematode nAChR resolved at the single-channel level in *Ascaris suum*

Modified from a paper to be published in *FASEB Journal* (Qian et al., 2006)

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2Graduate student & primary author

3Author for correspondence

4These authors contributed equally to this work

3.1. Abstract

Pharmacological experiments on *Ascaris suum* have demonstrated the presence of three (N-, L-, and B-) subtypes of cholinergic receptor mediating contraction of body wall muscle in parasitic nematodes (Robertson et al., 2002). In the present study, these ionotropic acetylcholine (ACh) receptors (nAChRs) were activated by levamisole and bephenium under patch-clamp conditions and competitively antagonized by paraherquamide and 2-desoxoparaherquamide. A number of recordings exhibited three separate current amplitude levels, indicating the presence of small, intermediate, and large conductance subtypes of receptor. The mean conductance of the small conductance subtype, $G_{25}$, was $22 \pm 1 \text{ pS}$; the intermediate conductance channel, $G_{35}$, was $33 \pm 1 \text{ pS}$; and the large conductance...
channel, $G_{45}$, was $45 \pm 1$ pS. The small channel was not antagonized significantly by paraherquamide and was identified as the N-subtype. The intermediate channel was preferentially activated by levamisole rather than bephenium and antagonized by paraherquamide: the intermediate channel was identified as the L-subtype. The large conductance channel was preferentially activated by bephenium, antagonized more by 2-desoxoparaherquamide than by paraherquamide and was identified as the B-subtype. These observations reveal that the three channel subtypes have different selectivity for cholinergic anthelmintics. The different selectivity of these compounds should be considered when dealing with drug resistant infections.

### 3.2. Introduction

Over one-third of the human population is infected with intestinal parasites. In a World Health Organization report (WHO, 2000), it was observed that 17.3 million deaths, of the global total of 52.2 million deaths, were due to infectious and parasitic diseases. A common nematode parasitic disease of humans is ascariasis, which has a prevalence of 30–60% in endemic areas, producing symptoms of malnutrition, retardation of growth in children, diarrhea, and abdominal pain and in a smaller proportion of cases, death (Holland et al., 1989). Gastrointestinal nematodes are the major cause of morbidity in schoolchildren when measured in disability adjusted life years (DALYs). Levamisole is a representative of an important group of anthelmintic drugs (including pyrantel) that is used for treatment of ascariasis as well as other human nematode parasite infections (Horton, 2003). Members of this group act rapidly by selectively gating pharmacologically distinctive ionotropic (nicotinic) ACh
receptor ion-channels (nAChR) on the body muscles of nematodes (Robertson and Martin, 1993; Martin et al., 1998).

Human studies show that anthelmintic treatment with cholinergic anthelmintics and other anthelmintics is <100% effective. Albonico et al. (Albonico et al., 2002) described the efficacy of cholinergic anthelmintics against Ascaris sp. (roundworm), Trichuris sp. (whipworm) and Ancylostoma sp. (hookworm). The cure rates for these parasite species is <100%. A proportion of parasites is innately resistant and unaffected by treatment. The regular use of anthelmintics produces a Darwinian selection pressure that increases the proportion of resistant individuals in the population (acquired resistance). This has been well established for nematode parasites of domestic animals. More recently, significant resistance of human parasites to cholinergic anthelmintics has been described making the drugs ineffective (Reynoldson et al., 1997; Sacko et al., 1999). The prospect for the development of new anthelmintics is very limited, so there is an urgent need to investigate the mechanisms of anthelmintic resistance and to find new ways to counter resistance.

We have focused on sites of action of the cholinergic anthelmintics to gain a better understanding of their modes of action and mechanisms of resistance. In contraction assays using muscle strips from the parasitic nematode Ascaris suum, we have observed that there are pharmacologically distinct nAChRs that can be activated by the different cholinergic anthelmintics (Robertson et al., 2002). We were able to separate three types of nAChR: the N-subtype that is preferentially activated by nicotine; the L-subtype that is preferentially activated by levamisole and
antagonized by paraherquamide; and the B-subtype that is preferentially activated by bephenium and antagonized by paraherquamide and 2-desoxoparaherquamide. Our studies also found that levamisole resistance is associated with a loss of sensitivity of the L-subtype receptors with no loss in the sensitivity of the N-subtype of receptors (Martin et al., 2003; Martin et al., 2004). These observations suggest that N-type and B-type selective cholinergic agonists may be useful for overcoming some types of levamisole resistance.

In addition to the muscle contraction assays, we have also compared the effects of nicotine and levamisole under patch clamp (Levandoski et al., 2005) to test our hypothesis that the selectivity of these two ligands is different. We found that both nicotine- and levamisole-activated channels had a wide and overlapping conductance range but that nicotine preferentially activates smaller, 26 pS, channels and that levamisole preferentially activates another, 39 pS, group of channels. In this study, we are able to separate out a third subtype to compare the effects of levamisole and bephenium at the single-channel level and test the effects of the novel competitive-antagonists paraherquamide and 2-desoxoparaherquamide (Yamazaki et al., 1981; Robertson et al., 1999; Zinser et al., 2002). We were thereby able to identify the single-channel properties of the N-, L-, and B-receptors. These observations are notable because they emphasize the different selectivity of the different cholinergic anthelmintics between the different subtypes of cholinergic receptor. This means, for example, that loss of the L-subtype with levamisole resistance (Robertson et al., 1999; Martin et al., 2004) might be overcome by using
other cholinergic agonists (methyridine: N-subtype) or antagonists (2-desoxoparaHerquamide; B-subtype) with selectivity for other subtypes of nAChR.

3.3. Materials and methods

3.3.1. Maintenance of *Ascaris suum*

Adult *Ascaris suum* were collected from the IBP Meat Packing Plant (Storm Lake, IA). The worms were maintained at 32°C in Locke’s solution (changed daily) for no longer than 5 days. Locke’s solution contains (mM): NaCl, 155; KCl, 5; CaCl₂, 2; NaHCO₃, 1.5; D-glucose, 5. Mature, active *Ascaris suum* ~10–20 cm in length were selected for experiments.

3.3.2. Vesicle Preparation

The muscle membrane vesicle preparation has been described previously (Robertson and Martin, 1993) and is briefly outlined here. The *Ascaris* were dissected and a muscle flap was prepared and pinned cuticle side down onto a plastic dish lined with Sylgard. The muscle flap preparation was washed with maintenance solution to remove fragments of the gut. The maintenance solution contains (mM): NaCl 35, NaAc 105, KCl 2, MgCl₂ 2, HEPES 10, glucose 3, L-ascorbic acid 2, EGTA 1. The maintenance solution was then replaced with collagenase solution containing (mM): NaCl 35, NaAc 105, KCl 2, MgCl₂ 2, HEPES 10, glucose 3, L-ascorbic acid 2, collagenase 1mg/ml. After collagenase treatment for 4–8 min at 37°C, the muscle preparation was washed and incubated at 37°C. Small membranous vesicles, 10–50 µm in diameter, grew out from the membrane of
the muscle cells. These membranous vesicles were transferred to a recording chamber.

### 3.3.3. Electrophysiology

The maintenance solution in the chamber was replaced with bath solution. To block potassium channels present in muscle cells, the bath solution contains high concentration Cs⁺ (mM): CsCl, 35; Cs acetate, 105; MgCl₂, 2; HEPES, 10; EGTA, 1; pH 7.2 with CsOH, at room temperature. The vesicle preparation was used within 3h.

The patch-clamp technique was used to record the single-channel currents activated by levamisole or bephenium from the vesicle preparation. The pipettes were filled with pipette solution that contained (mM): CsCl, 140; MgCl₂, 2; HEPES, 10; EGTA, 1; pH 7.2 with CsOH. Pipettes with resistances of 3–5 MΩ (Hamill et al., 1981) were used. The current signal was amplified by an Axopatch 200B amplifier (Axon Instruments, Union City, CA) filtered at 2 kHz (3-pole Bessel) and then sampled at 25 kHz digitized with a Digidata 1320A (Axon Instruments) and stored on a computer hard disk.

### 3.3.4. Data analysis

Data were idealized and analyzed using pCLAMP Ver 8.2 software (Axon Instruments). The generally low level of channel opening produced extremely rare multiple opening events; these were omitted from calculations of \( P_{\text{open}} \) and mean open time. The current amplitude histograms were fitted with the sum of one, two, and three Gaussian equation to determine the mean current amplitudes of the channel openings. The best Gaussian fits (confidence level > 0.95) were used to
help determine the number of amplitude populations present in one patch. This analysis and curve fitting were done using pCLAMP 8.2 and Prism V4 (GraphPad Software, San Diego, CA). To fit the distribution of the all the individual levamisole channel conductances, we used a simplex method to minimize the residual sum of squares with the aid of the NAG subroutine E040CCF to fit the sum of three Gaussian distributions (Robertson et al., 1999) and were able to obtain estimates of the areas, mean conductance, and SD of the three populations.

We measured $P_{\text{open}}$ as the patch-$P_{\text{open}}$ (the proportion of time channels in the patch were open during the recording). Results are mean ± SE and were tested for normality. Although the distribution of open times were exponential, we found that the distributions of the means of open times from different patches did not deviate significantly from normality so differences between three groups of means was tested by ANOVA and differences between two means were evaluated using a $t$-test.

3.3.5. Drugs

Paraherquamide and 2-desoxoparapherquamide were obtained from Pfizer Animal Health (Kalamazoo, MI). Other drugs and chemicals were purchased from Sigma Chemical Co (St. Louis, MO). Paraherquamide or 2-desoxoparapherquamide, dissolved in dimethyl sulfoxide (DMSO), was added to the bath solution to give final concentrations of 0.3–10 µM. The concentration of DMSO in bath was always < 0.3%. Levamisole or bephenium was present in the pipette solution to activate the channels.
3.4. Results

3.4.1. Levamisole activated conductance of three different channels

When levamisole (30 µM) was used as the agonist in the pipette solution, single-channel currents (Figure 3.1A) with a conductance of 18–53 pS and mean open times of 0.2 – 2 ms were observed at membrane potentials of ±75 and ±50 mV from inside-out patches. These channels were never recorded in the absence of levamisole. To confirm that these single-channel currents were indeed activated by levamisole, a series of alternating patch recordings was made from the same "active" vesicle: first with, and then without, levamisole present in the pipette solution. In six such paired recordings, channels were recorded in the presence of levamisole but not in the absence of levamisole. On four occasions, a third patch recording was made from the same "active" vesicle with pipette solution again containing levamisole. Under these conditions, characteristic single-channel currents were observed in all four of four patches. These observations confirmed that the currents were activated by levamisole.

In each patch, levamisole activated up to three populations of channel currents that were clearly separated by differences in their conductance. Figure 3.1A shows some representative channel openings at +75 mV in a single patch recording and the amplitude histogram that was fitted by the sum of three Gaussian distributions with mean currents of 2.0, 2.7, and 3.6 pA. In all experiments where three or more voltages were tested between +75 mV and −75 mV, it was found that the current-voltage relationships of the channels were linear. In some experiments
where only two potentials were available, it was also taken that the relationship was linear. We determined conductance from the slopes of the current-voltage plots using linear regression. In Figure 3.1B the channels had a conductance of 26, 36, and 48 pS.

We recorded levamisole-activated currents from 58 such channels from 38 patches: 22 had only 1 conductance level present, 12 had 2 conductance levels present, and 4 had 3 conductance levels present. The conductance ranged from 18 to 53 pS. The wide range of conductance values coupled with the low error associated with the measurement of individual channel conductance (SE ≤ 3 pS, $r^2 > 0.95$ with currents measured at 3 or more potentials) made it unlikely that there was only a single population of channels present. Since the experimental conditions were equal over an individual patch, the presence of the three types of conductance in single patches revealed the presence of separate and distinctive small-, intermediate-, and large-conductance channel populations. To determine the conductance ranges of the three populations, we determined the mean and 95% confidence intervals of the small, intermediate and large conductance populations from the 4 patches showing the three types of conductance (Table 3.1). The confidence limits for the small, 22 pS, channels were 17.8 and 26.3 pS; the confidence limits for the intermediate, 32.7 pS, channels were 26.5 and 38.9 pS; and the confidence limits for the large, 42.9 pS, channels were 36.6 and 49.3 pS.
Figure 3.1. Three levamisole-activated channel currents from a single patch.

A, top) Single-channel openings from a single patch recording at +75 mV showing 3 separate channel currents: O₁, O₂, and O₃ are open levels,
and C is closed level. A, bottom) Current amplitude histogram of patch recording best fitted by sum of 3 Gaussian distributions: peaks are 2.12 ± 1.47, 2.84 ± 0.05, and 3.83 ± 0.01 pA (mean ± SE). B) Current-voltage plots of conductance of 3 separate channels: linear regression was used to determine slope conductance: 26 ± 1, 36 ± 0, and 48 ± 1 pS (means ± SE). The 26 pS channel was not well resolved at –75 mV.

Next, we classified the remaining channel conductance in the other single or double channel patches into three populations according to their conductance using the 95% confidence intervals determined in the three channel patches. Channels classified as small all had a conductance of <26.5 pS; channels classified as intermediate had a conductance of 26.5–36.6 pS; and channels classified as large had a conductance of >38.9 pS. Only two types of conductance that had values between 36.6 and 38.9 could not be classified and were excluded in the following analysis.

Table 3.1. Conductance ranges and mean values of 3 populations for all patches.

<table>
<thead>
<tr>
<th></th>
<th>Small $G_{25}$</th>
<th>Intermediate $G_{35}$</th>
<th>Large $G_{45}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levamisole (30 µM)</td>
<td>18 - 27</td>
<td>28 - 36</td>
<td>39 - 52</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>23 ± 1</td>
<td>33 ± 1</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>N (number of channels)</td>
<td>15</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>Bephenium (1 µM)</td>
<td>-</td>
<td>31 - 40</td>
<td>42 - 49</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>-</td>
<td>36 ± 1</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>N (number of channels)</td>
<td>-</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>
For 30 µM levamisole experiments, 3 populations, \( G_{25} \), \( G_{35} \), and \( G_{45} \), were observed. For 1 µM bephenium, 2 populations, \( G_{35} \) and \( G_{45} \) were observed.

Table 3.1 shows that the mean ± SE of the conductance for all the small channels was 23 ± 1 pS (\( n = 15 \)), and for convenience we refer to this channel as \( G_{25} \). The mean ± SE of the conductance for all the intermediate channels was 33 ± 1 pS (\( n=22 \)), and we refer to this channel population as \( G_{35} \). The mean ± SE of the conductance for all the large channels was 45 ± 1 pS (\( n=19 \)), and we refer to this channel as \( G_{45} \).

**3.4.2. Levamisole preferentially activates \( G_{35} \) channels**

The confidence interval method showed that levamisole preferentially activated \( G_{35} \) channels over the other subtypes: 27% of the channels were \( G_{25} \) (15 of 56), 39% were \( G_{35} \) (22 of 56), and 34% were \( G_{45} \) (19 of 56). To estimate the proportion of each subtype present using another approach, we fitted the conductance histogram of all 58 channels activated by levamisole to the sum of three Gaussian distributions using the individual 58 conductance values and an iterative simplex method (58; Figure 3.2A); 28% of these channels were \( G_{25} \) channels with a mean ± SD of 24.5 ± 4.3 pS; 58% of the channels were \( G_{35} \) with a mean ± SD of 36.7 ± 5.3 pS; and 14% were \( G_{45} \) with a mean ± SD of 49.9 ± 1.6 pS. Both analytical approaches separated the channel subtypes and showed that levamisole activates more \( G_{35} \) channels than the other receptor channel subtypes.
The average conductance of the $G_{35}$ channels is close to channels identified as the L-subtype of receptor and the conductance of the $G_{25}$ channels is the same as N-subtype of nAChRs (Levandoski et al., 2005).

Figure 3.2. Frequency histogram of individual conductance of 58 channels activated by 30 µM levamisole. The histogram was fitted by the sum of 3 Gaussian distributions using a simplex method to minimize residual sum of squares; 28% of these channels were G25 channels with a mean ± SD of 24.5 ± 4.3 pS; 58% of channels were G35 with a mean ± SD of 36.7 ± 5.3 pS; and 14% were G45 with a mean ± SD of 49.9 ± 1.6 pS.
3.4.3. Bephenium activates only $G_{35}$ and $G_{45}$ channels

When levamisole in the pipette solution was replaced by 1 or 10 µM bephenium (a cholinomimetic anthelmintic), only two channel types, $G_{35}$ and $G_{45}$, were observed in the patches. Bephenium did not activate the $G_{25}$ channels. In 7 of the 19 patches with 1 µM bephenium in the pipette, $G_{35}$ and $G_{45}$ were recorded simultaneously; in the other patches (12 in 19 patches), only the $G_{35}$ or $G_{45}$ channel was present. The conductance range of all these channels was from 31 to 49 pS. Using the same approach that we used for the levamisole-activated channel currents, we again evaluated the mean conductance and 95% confidence intervals for the $G_{35}$ and $G_{45}$ channels. From the two channel recordings in the seven patches, we found that the $G_{35}$ channel had a 95% confidence interval of 31.6–40.2 pS and the $G_{45}$ channel had a 95% confidence interval of 41.6–47.1 pS. We used this information to sort the conductance of the single channel patches: conductance <40.2 pS was classified as $G_{35}$ channels; channels greater than 41.6 pS was classified as $G_{45}$ conductance channels. Overall we found that the $G_{35}$ channels activated by bephenium had a conductance of $36 \pm 1$ pS (mean $\pm$ SE; n=10). We found that the conductance of the $G_{45}$ activated by bephenium had a conductance of $43 \pm 1$ pS (mean $\pm$ SE; n=16). The means and the conductance ranges of these two bephenium-activated populations are similar to those of the $G_{35}$ and $G_{45}$ channels activated by levamisole.
3.4.4. Bephenium activates more $G_{45}$ than $G_{35}$ channels

The confidence interval method revealed that when 1 µM bephenium was the agonist 38% of the channel population was $G_{35}$ (10 of 26) and 62% was $G_{45}$ (16 of 26). When the agonist was 10 µM bephenium, the proportion of $G_{35}$ channels was 42% (8 of 19) and the proportion of $G_{45}$ channels was 58% (11 of 19). Thus bephenium activated more $G_{45}$ channels than $G_{35}$ channels, suggesting that these channels represent the B-subtype of receptors (Robertson et al., 2002).

3.4.5. Different subtypes have different mean open-times

Once we had separated the channels activated by 30 µM levamisole into $G_{25}$, $G_{35}$, and $G_{45}$, we were able to determine their mean open times by fitting a single exponential distribution to events >0.5 ms. We set the patch potential to +75 mV for these observations to minimize open channel block. In multiple conductance patches, each channel opening was separated into $G_{25}$, $G_{35}$, and $G_{45}$ and their mean open times determined separately. An example of the three open time histograms from one patch is shown in Figure 3.3A. The $G_{25}$ population had the shortest mean open times, and the $G_{45}$ population had the longest mean open times. The $G_{25}$ population had a mean open time of $0.6 \pm 0.1$ ms (mean ± SE; n=14); the mean open time of the $G_{35}$ population was $0.8 \pm 0.1$ ms (mean ± SE; n=18); and the mean open time of the $G_{45}$ population was $1.2 \pm 0.1$ ms (mean ± SE; n=17). The distributions of the mean open times of the three populations were not significantly different from a normal distribution. ANOVA was used to test the difference between the three ($G_{25}, G_{35}, G_{45}$) groups and significance was found ($F=8.9$, df 2, $P<0.005$). A t-test was
used to test the differences between the means of the G_{25} and G_{35} groups (P < 0.03),
the means of the G_{35}, and G_{45} (P <0.03), and the means of the G_{25} and G_{45} groups
(P < 0.001): they were all significant (Figure 3.3C).

A similar difference between the G_{35} and G_{45} channels was observed when
bephenium was the agonist, Figure 3.3B. The mean open times of the G_{35} channels
activated by 1 µM bephenium was 1.1 ± 0.2 ms (mean ± SE; n = 7), and this was
significantly (P < 0.05) shorter than the mean open time of the G_{45} population that
was 2.4 ± 0.2 ms (mean ± SE; n = 11) when tested with a t test, Figure 3.3C. The
differences between the mean open times of the different conductance populations
are consistent with these channel populations belonging to separable distinctive
subtypes groups and not being a single continuum.
Figure 3.3. The mean open-times of the three channel populations were significantly different. A) Representative open time histograms of distributions of G25, G35, and G45 channels activated by 30 µM levamisole at +75mV. Note that G25 have briefer openings than G35, which in turn are briefer than G45. B) Representative open time histograms of distributions of G35 and G45 channels activated by 1 µM bephenium at +75mV. Note that G35 have briefer openings than G45. C) Bar chart of mean open times of the G25, G35, and G45 of channels activated by levamisole and bephenium showing mean ± SE differences and the significance levels (*p < 0.05; **p < 0.01; ***p < 0.001).

3.4.6. Bath application of paraherquamide inhibits the opening of nAChRs

Our earlier muscle contraction experiments have demonstrated that paraherquamide is a competitive antagonist (Robertson et al., 2002). It is not possible to make outside-out patch recordings from the Ascaris muscle vesicles because they are under internal pressure and burst when whole-cell recordings are attempted. Fortunately, paraherquamide is lipid soluble and passes from the bath through inside-out patches to reach the extracellular receptor to act as an antagonist (Figure 3.4A). This is a technique that we have established and used for the application of lipid soluble ligands (Robertson and Martin, 1993; Levandoski et al., 2005). Here we measured and normalized the antagonism using paired patches. We recorded from two patches and measured the P_{open} values using the same protocol.
and time course to control for any rundown or effects of adding solution to the inner membrane. In the first control inside-out patch with channels activated by 30 µM levamisole in the pipette, we tested the effect of adding antagonist-free solution to the bath; there was sometimes a small decrease in $P_{\text{open}}$ in the control patch. In the second test patch, with channels also activated by 30 µM levamisole in the pipette, we observed the effect on channel opening of adding paraherquamide to the bath. As a normalized measure of inhibition, the percent change in $P_{\text{open}}$ produced by paraherquamide was divided by the percent change in $P_{\text{open}}$ produced by the antagonist-free solution.

The inhibitory effect of paraherquamide was dose dependent (Figure 3.4B) and was described by the logistic function with an IC50 of 3.1 µM. Thus, we established that paraherquamide produced a dose-dependent antagonism of levamisole-activated channels.
Figure 3.4. Paraherquamide inhibition of 30 µM levamisole-activated channels in inside-out patches. A) Low time and higher time resolution of an inside-out patch recordings at +75mV showing patch recording before the bath-application of 10 µM paraherquamide and after application. Note marked reduction in $P_{\text{open}}$ channel. B) Normalized $P_{\text{open}}$ ($NP_o$) paraherquamide ($X_B$) concentration-response plot.
To determine $N_{Po}$, the normalized patch $P_{\text{open}}$ in the presence of paraherquamide [$P_{\text{open}}(X_B)$] was divided by the patch $P_{\text{open}}$ before the application of paraherquamide [$P_{\text{open}}(X_B)$]; this ratio was then normalized by dividing by the ratio [$P_{\text{open}}(\text{before DMSO})/P_{\text{open}}(\text{DMSO})$] obtained from the DMSO control patch (see Materials and Methods). $n$, the number of patches, was 4 or greater for each concentration. Line was fitted using a logistic function: Where $NP_{\text{min}}$ is the bottom of the plateau; $NP_{\text{max}}$ is top of plateau; and IC50 is paraherquamide concentration producing 50% inhibition. IC50 = 3.1 µM.

3.4.7. Paraherquamide has no effect on channel conductance or mean open-times

Figure 3.5A and B, left, shows the representative effects of paraherquamide on channel conductance. It illustrates results from a patch at +75 mV with three channels ($G_{25}$, $G_{35}$, $G_{45}$) activated by 30 µM levamisole in the pipette before (Figure 3.5A) and after (Figure 3.5B) the addition of 1µM paraherquamide to the bath. The figure shows that the mean amplitude of each of the channels with a conductance of 27 pS, 37 pS, and 47 pS is the same before and after the addition of paraherquamide. Interestingly, Figure 3.5 also shows that although the amplitude of each of the three channels is unchanged by paraherquamide, the overall frequency of the events is reduced due to the inhibitory action of paraherquamide. In all patch
recordings, we found no effect of paraherquamide on the amplitudes of any of the channel subtypes.

Figure 3.5A and B, right, illustrates representative effects of 1 μM paraherquamide (Figure 3.5A, control; Figure 3.5B, paraherquamide) on the open time distribution of levamisole activated channels. It shows the distributions of open times of $G_{35}$ and $G_{45}$ channels activated by 30 μM levamisole in the pipette at +75mV before and after the addition of paraherquamide to the bath. There was no decrease of in the mean open times of either of the channel subtypes. There was no evidence in this patch or in any of the $G_{35}$ or $G_{45}$ channels recordings, that paraherquamide reduced the mean open time of the channels. The lack of effect on channel conductance or on open time is consistent with the competitive mode of action of paraherquamide (Colquhoun and Sheridan, 1982; Robertson et al., 2002).
Figure 3.5. Effect of paraherquamide on current amplitude and mean open times of $G_{25}$, $G_{35}$, and $G_{45}$ channels. A) Control 30 µM levamisole-activated single-channel amplitudes (left) and mean open times of $G_{35}$ and $G_{45}$ channels (center and right) from an inside-out patch at $+75$ mV; current amplitude distributions show 3 channels before addition of paraherquamide from a 1 min recording showing number openings and 3 populations with a mean conductance of 27 pS ($G_{25}$), 37 pS ($G_{35}$), and 47 pS ($G_{45}$). Channel amplitudes: $G_{25}$ of 1.8 pA, $G_{35}$ 2.7 pA, and $G_{45}$ 3.7 pA at $+75$ mV. B) Effect of 1 µM paraherquamide on the 30 µM levamisole-activated single-channel amplitudes (left) and mean open times of $G_{35}$ and $G_{45}$ channels (center and right); 4 min recording for levamisole current amplitude histogram. Note that paraherquamide reduced frequency of channel open so a longer-period recording was analyzed to collect enough channel open events to determine $P_{\text{open}}$. $P_{\text{open}}$ of 27 pS ($G_{25}$) population was 0.00027 in control and 0.00031 in 1 µM paraherquamide (no inhibition); $P_{\text{open}}$ of 37 pS ($G_{35}$) population reduced from 0.00807 in control to 0.00545 (68% reduction) in 1 µM paraherquamide; and $P_{\text{open}}$ of 47 pS ($G_{45}$) population was reduced from 0.04079 in control to 0.02010 (49% reduction) in the 1 µM paraherquamide. Channel amplitudes in the presence of 1 µM levamisole were $G_{25}$, 1.8; $G_{35}$, 2.7; and $G_{45}$, 3.7 pA: note that there was no change in channel amplitudes. $G_{35}$ had a mean open time of 0.8 ms and $G_{35}$ had a mean open time of 1.7 ms in the presence of 1 µM.
parahequamide: note that parahequamide could produce a reduction in $P_{open}$ without a reduction in mean open times. Thus, inhibitory effect of parahequamide on $P_{open}$ was not associated with a reduction in channel amplitude or mean open times. C) Selective inhibitory effects of parahequamide and 2-desoxoparahequamide are statistically different. Left) Bar chart of mean ± SE of normalized inhibition $NP1NP0$ by 1 µM parahequamide of $G_{25}$, $G_{35}$, and $G_{45}$ channels activated by 30 µM levamisole. Right) Normalized inhibition of 10 µM bephenium-activated channels by 3 µM 2-desoxyparahequamide of $G_{35}$ and $G_{45}$ channels. $NP_0$ is the patch $P_{open}$ before application of the antagonist; $NP_{XB}$ is patch $P_{open}$ in the presence of antagonist. 1 µM parahequamide had a significantly greater ($p < 0.05$, n=4, t test) effect on $P_{open}$ of both $G_{35}$ and $G_{45}$ (67.7% inhibition and 68.2% inhibition respectively) compared to no effect on $P_{open}$ of the $G_{25}$ population. 2-desoxoparahequamide inhibited opening of the $G_{35}$ (73.3%, n=4) and $G_{45}$ (36.4%, n=5) channel populations activated by 10 µM bephenium; the difference between the effects on the $G_{35}$ and $G_{45}$ population was significant, *$p < 0.05$, t test.

3.4.8. Inhibitory effects of parahequamide and 2-desoxoparahequamide are selective and the % inhibition depends on the receptor subtype

We have described the selective effects of parahequamide and 2-desoxoparahequamide on different nAChR subtypes in muscle contraction
experiments (Robertson et al., 2002). To investigate whether these two compounds show selective effects on the $G_{25}$, $G_{35}$, and $G_{45}$ populations at the single-channel level, we tested the inhibitory effects of 1 µM paraherquamide and 3 µM 2-desoxoparaherquamide on the $P_{\text{open}}$ of the $G_{25}$, $G_{35}$, and $G_{45}$ channels.

Figure 3.5C shows the effect of 1 µM paraherquamide on the three nAChR populations ($G_{25}$, $G_{35}$, and $G_{45}$) observed in seven patches at +75mV when activated by 30 µM levamisole. When 1 µM paraherquamide was present in the bath, ANOVA showed that the opening of the three channel populations was inhibited by different amounts ($F = 6.9$, df = 2, $P = 0.01$). Figure 3.5C shows that 1 µM paraherquamide had no effect on the $G_{25}$ population but had an inhibitory effect on the $G_{35}$ and $G_{45}$ channels (% control value: $G_{25}$ 106.1 ± 25.4%; $n = 5$; $G_{35}$ 32.3 ± 10.0%, $n = 5$; and $G_{45}$, 31.8 ± 7.0%, $n = 5$). A $t$ test showed that the inhibition of the $G_{35}$ and $G_{45}$ channel was significant when compared to the $G_{25}$ channels ($P < 0.05$). The lack of effect on $G_{25}$ channels is consistent with this channel population being the N-subtype because it is the least sensitive to paraherquamide (Robertson et al., 2002). The greater effect on the $G_{35}$ and $G_{45}$ channels is consistent with these channels belonging to the L- and B-subtype because paraherquamide is a more potent antagonist of these two subtypes than the N-subtype (Robertson et al., 2002).

We also used 10 µM bephenium to activate the $G_{35}$ and $G_{45}$ channels without activating the $G_{25}$ channels. Bephenium activated currents were recorded from 12 inside-out patches and the inhibitory effects on $P_{\text{open}}$ of bath-applied 3 µM 2-desoxoparaherquamide on the $G_{35}$ and $G_{45}$ channels observed, Figure 3.5C. 2-desoxoparaherquamide had a significantly greater inhibitory effect on the $G_{45}$
population than the G_{35} population (%reduction P_{open} of G_{45}: 26.7 ± 7.1, n = 5; %reduction in P_{open} of G_{35}: 63.6 ± 11.4, n = 4, P < 0.05). The greater effect of 2-desoxoparaherquamide on G_{45} channels is consistent with these channels being the B-subtype (Robertson et al., 2002) and the G_{35} channels being the L-subtype (Robertson et al., 2002).

3.5. Discussion

3.5.1. N-, L-, and B-subtypes identified at the single-channel level on body muscle

We have observed in muscle strip contraction assays of Ascaris suum (Robertson et al., 2002) that the cholinergic anthelmintics activate three subtypes of receptors on body muscle and that paraherquamide and 2-desoxyparaherquamide are competitive antagonists. There is the N-subtype that is preferentially activated by nicotine but not antagonized by paraherquamide and 2-deoxyparaherquamide; there is the L-subtype that is preferentially activated by levamisole and antagonized by paraherquamide; and there is the B-subtype that is preferentially activated by bephenium and antagonized by paraherquamide as well as 2-desoxoparaherquamide (Robertson et al., 2002).

In a previous study (Levandoski et al., 2005) at the single channel level, comparison of the effects of nicotine and levamisole revealed that nicotine and levamisole could activate a wide range of conductance but that nicotine preferentially activated small 26 pS channels and that levamisole preferentially activated larger channels with a conductance averaging 39 pS. In this current study,
we have been able dissect out the third, \( G_{45} \), subtype at the single channel level (Figure 3.6). Also in this study we found that levamisole activated channels over a wide conductance range and that in some patches we could identify 3 separate channels that we refer to as \( G_{25} \), \( G_{35} \), and \( G_{45} \) with mean conductance around 25, 35, and 45pS respectively. Levamisole activated more \( G_{35} \) channels than the other two subtypes. Paraherquamide had no effect on the \( G_{25} \) channels but antagonized the \( G_{35} \) and \( G_{45} \) channels in a manner that was consistent with the \( G_{25} \) channels being the N-subtype not the L- or B-subtype (Robertson et al., 2002).

When we used bephenium as the agonist, it did not activate the \( G_{25} \) channels but activated more \( G_{45} \) channels than \( G_{35} \) channels; furthermore, 2-desoxoparaherquamide selectively inhibited the \( G_{45} \) channels. We found that 2-desoxoparaherquamide is a more potent antagonist of the \( G_{45} \) channels than the \( G_{35} \) pS channels and that 1 \( \mu \)M bephenium preferentially activated the \( G_{45} \) channels. These observations allowed us to identify and confirm that the \( G_{45} \) channel is the B-subtype and that the L-subtype is the \( G_{35} \) channel. 2-desoxoparaherquamide is a more potent antagonist of the B-subtype than of the L-subtype (Robertson et al., 2002).
Figure 3.6. Summary diagram of channel properties and pharmacology of the N-, L-, and B-subtypes of AChR receptor channel found on the muscle cell of Ascaris showing their mean conductance, mean open time, and selectivity of agonists and antagonists.

In addition to separation of the subtypes by pharmacology and conductance, we found that there were significant differences in their mean open times with the N-subtype (G_{25}) having the briefest open time, 0.6 ms; the L-subtype (G_{35}) having an intermediate open time, 0.9 ms; and the B-subtype (G_{45}) having the longest open time, 1.3 ms, when levamisole was the agonist. These experiments have allowed us to identify the three subtypes of muscle nAChR at the single-channel level and to see their pharmacology at that level. The significance of the different subtypes of nAChR is discussed in the following paragraphs.
3.5.2. Recognition of separate nAChR subtypes in parasitic nematodes is therapeutically significant

We have observed that L-subtype channels decrease in frequency on the body muscle of the nematode parasite *Oesphagostomum dentatum* (Robertson *et al.*, 1999) and that levamisole resistant isolates of *Oesphagostomum dentatum* become less sensitive to levamisole (more selective for L-subtype channels) but that they remain sensitive to the N-subtype agonist methyridine (Martin *et al.*, 2003). If levamisole resistance is due to the loss of the L-subtype of receptors, a useful therapeutic approach may be to use cholinergic agonists or antagonists that have selective effects against N- and B-subtypes to treat levamisole resistance. This suggests that 2-desoxoparaherquamide or, a combination of methyridine or oxantel (N-subtype selective) and bephenium may be useful for the treatment of some types of levamisole resistance.

3.5.3. Heterogeneity of nematode nAChRs

We have observed three separate subtypes in the parasitic nematode *Ascaris suum*, but what is the molecular basis of the heterogeneity? Much information on the structure and function of nematode AChRs has advanced with the study of *C. elegans*, a soil nematode that has been subjected to detailed genetic analysis. We are encouraged to use *C. elegans* as a model for the levamisole receptor of parasitic nematodes by molecular phylogenetic analysis (Kennedy and Harnett, 2001). *C. elegans* belongs to clade V as do many nematode parasites like *O. dentatum*, and there are strong similarities between the single-channel properties of levamisole
receptors of A. suum (clade III) and O. dentatum (Jones and Sattelle, 2004). In both nematodes, channel conductance subtypes between 20 – 50 pS are observed with conductance subtypes that include the G$_{25}$, G$_{35}$, and G$_{45}$. In O. dentatum, however, we were able to separate a fourth G$_{40}$ conductance subtype (Robertson et al., 1999).

With the completion of the C. elegans genome project, the full size of the nematode AChR gene family was finally revealed. There are now a total of 27 AChR subunit genes which is bigger than in mammals (13 genes, and is the largest number of AChR subunit genes in a single species (Jones and Sattelle, 2004). Homology has allowed these 27 subunits to be divided into five groups named: DEG-3-like; ACR-16-like; ACR-8-like; UNC-38-like; and UNC-29-like. The UNC-29-like subunits are non-α subunits and the remainders are mostly α subunits. If all combinations of α and non-α subunit were capable of forming functional AChRs, there could be over 25 (Robertson and Martin, 1993) or 14,348,907 receptor subtypes, but this is not likely. The molecular structures of levamisole UNC-38 subunit homologues in the parasitic nematodes, Trichostrongylus colubriformis (Wiley et al., 1997) and Haemonchus contortus (Hoekstra et al., 1997), are ~90% identical to C. elegans, suggesting that information derived from the model nematode may be a useful guide for parasitic nematodes. With a large number of AChR subunits in C. elegans, it is not surprising that we also see heterogeneity in our experiments on parasitic nematodes.

We can see the molecular potential for the heterogeneity, but what is the functional benefit of this extensive heterogeneity? If nematodes are like vertebrates, then the physiological reasons for the heterogeneity include a necessity to have
varying sensitivities to the agonist ACh, to vary desensitization, calcium permeability, and distributions in different cells (McGehee and Role, 1995). We know that expression of UNC-38, UNC-29, UNC-63, LEV-1, ACR-8, ACR 16, and ACR-13 (=LEV-8) GFP-tagged protein subunits occurs in body muscle cells of C. elegans (Fleming et al., 1997; Richmond and Jorgensen, 1999; Culetto et al., 2004; Touroutine et al., 2005; Towers et al., 2005), but details of the stoichiometric arrangements of the subunits and total number of receptor subtypes remain to be determined. In our study, we demonstrate the presence of three subtypes of nAChR in muscle of parasitic nematodes, each with a different sensitivity to cholinergic anthelmintics. The identification of multiple receptor subtypes in parasites has several practical implications. Firstly, it may help understanding of how resistance to the cholinergic anthelmintics can arise: changes in the relative of proportion of each subtype (by up- or down-regulation) will alter the sensitivity of the parasite to a specific anthelmintic. Secondly, therapeutics may benefit: using combinations of compounds that activate all receptor subtypes could enhance cure rates. Finally, it is predicted that, in some instances, parasites resistant to one type of cholinomimetic will retain sensitivity to others (e.g., levamisole resistant parasites can remain sensitive to bephenium or methyridine), thus offering an alternative strategy to deal with resistance.
3.6. Acknowledgements

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Chapter 4. The single-channel properties of levamisole-sensitive nicotinic acetylcholine receptors in *Caenorhabditis elegans* adults

4.1. Abstract

Nicotinic acetylcholine receptors (nAChRs) are important for synaptic neurotransmission. The roles of nAChR subtypes in the transmission are dependent on their channel properties and affinity to acetylcholine. In the nematode *C. elegans* the levamisole-sensitive nAChR is involved in many worm behaviors such as locomotion, egg laying and mating. Here we report the single-channel properties and kinetics of the levamisole receptor present at the neuromuscular junction (NMJ). In wild type animals nAChRs mostly aggregate at the NMJ. LEV-10 is a transmembrane protein that causes levamisole receptor clustering at the neuromuscular junction. In *lev-10* knockouts, functional levamisole receptors are dispersed over the muscle cell body and are accessible for study using the patch-clamp technique. In this study, we present the first study of the single-channel properties of synaptic nAChRs in adult *C. elegans*. Acetylcholine and levamisole activated the receptor with a single-channel conductance of ~30 pS and brief mean open-time. At high concentrations levamisole also behaved as an open channel blocker. We also tested for another neuromuscular nAChR subtype that is sensitive to nicotine. However, in *lev-10* mutants no nicotine-sensitive receptor was detected from the extrasynaptic region. It demonstrates that the LEV-10 protein is only
required for the clustering of levamisole-sensitive nAChRs but not nicotine-sensitive nAChRs. This provides an essential foundation for the understanding of synaptic transmission mediated by cholinergic receptors.

4.2. Introduction

Cholinergic neurotransmission mediated by nicotinic acetylcholine receptors (nAChRs) plays important roles in both vertebrates and invertebrates. In vertebrates, dysfunctions of certain subtypes of neuronal nAChRs lead to psychiatric disorders (Araki et al., 2002). The nAChRs at the neuromuscular junction in the free-living nematode Caenorhabditis elegans (C. elegans) share many similar characteristics with the neuronal nAChRs in vertebrates, including multiple subtypes. C. elegans is also a model for studies of nematode parasites. This organism has been used to discover anti-parasitic drugs and for studies of the mechanism of the drug action. The genome of C. elegans was completely sequenced, and the genetics of nAChR subunits was been studied extensively. However, due to the small size of the worm, the physiological properties of the receptor subtypes have not been investigated at the single-channel level.

At least two nAChRs are present at the postsynaptic neuromuscular junction of C. elegans body wall muscles: one is sensitive to levamisole and another is sensitive to nicotine (Richmond and Jorgensen, 1999). To date, it is known that the levamisole receptor is composed of three essential subunits (UNC-63, UNC-38 and UNC-29) and two nonessential subunits (LEV-1 and LEV-8). Behavioral studies indicate that the knockout of any essential subunits leads to strong levamisole
resistance, and the knockout of either nonessential subunits leads to partial resistance. Whole-cell patch-clamp studies showed the absence of the levamisole receptor in loss-function mutants of unc-63, unc-38, or unc-29, while the whole-cell current of lev-1 mutants was reduced to ~15% and the current of lev-8 mutants was reduced to ~1/3 of the wild type. Compared with the nicotine-sensitive nAChR, the inward current through the levamisole-sensitive nAChR only contributes a small proportion of the whole-cell current induced by acetylcholine, but the knockout mutants exhibit significantly uncoordinated motor behavior (Fleming et al., 1997). That suggests the important physiological role of this receptor.

Besides the nAChR subunits, some other proteins, such as LEV-10, may regulate levamisole resistance and synaptic transmission (Jones and Sattelle, 2004). LEV-10 is a transmembrane protein that associates with nAChRs in C. elegans. This protein has been shown to be required for the postsynaptic clustering of the levamisole receptor. When the lev-10 gene was knocked out, postsynaptic clustering could not be detected but the whole-cell levamisole-activated current was intact. It suggests that the functional levamisole-sensitive receptor remains in the cell membrane without post-synaptic aggregation (Gally et al., 2004). These observations give us the possibility that a large amount of levamisole-sensitive receptors could be detected on the extra-synaptic cell body. Based on this observation we decided, using the patch-clamp technique, to test the single-channel properties of the levamisole-sensitive nAChR on the cell bag of the body wall muscles.
Our previous studies showed that the single-channel recording technique could be used to investigate individual nAChR subtypes in nematodes (Robertson and Martin, 1993; Qian et al., 2006). The individual subtypes observed from body wall muscle cells of nematodes showed different drug kinetics, phosphorylation modulation and Ca\(^{2+}\) permeability (Levandoski et al., 2005; Martin et al., 2005; Qian et al., 2006). Here we present an initial single-channel investigation in adult *C. elegans*. The single-channel investigation along with genetic approaches will lead to major advances in our understanding of synaptic neurotransmission mediated by individual nAChR subtypes.

### 4.3. Materials and Methods

N2 and *lev-10*(x17) worms used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). All worms were grown on agar seeded with *Escherichia coli* OP50 at 20\(^{\circ}\)C using established methods (Brenner, 1974).

#### 4.3.1. Preparation

Adult worms were transferred into a recording chamber filled with extracellular solution containing (mM): NaCl 23, NaAc 110, KCl 5, CaCl\(_2\) 6, MgCl\(_2\) 5, HEPES 5, D-glucose 11 and sucrose 10. The pH of the solution was adjusted to 7.2 with NaOH. The osmolarity was adjusted to 330 mOsm with sucrose. The worms were immobilized onto a sylgard-coated coverslip with cyanoacrylate (GluSeal\({\textregistered}\) 510K# K030574, Glustitch Inc.). The cuticle was incised to expose anterior body wall muscle cells. The preparation was cleaned and enzyme-treated with extracellular
solution containing 0.5 mg/ml collagenase. This preparation method was modified from previous studies (Richmond and Jorgensen, 1999). The enzyme treatment was applied for ~15 seconds. Then the collagenase solution was replaced by recording bath solution containing (mM): CsCl 35, CsAc 105, MgCl₂ 4, HEPES 10, EGTA 1 and sucrose 25, pH 7.2 adjusted with CsOH, 330 mOsm.

4.3.2. Single-channel recording

The patch-clamp technique was used to record the single-channel currents activated by acetylcholine or levamisole from the C. elegans preparation. Fire-polished patch pipettes were pulled from capillary glass (G85150T, Warner Instruments Inc., CT). To block K⁺ channels (Yuan et al., 2000; Yuan et al., 2003) that may present on the patched membrane, we filled the recording pipettes with high Cs⁺ solution that containing (mM): CsCl 140, MgCl₂ 4; HEPES 10, EGTA 1 and sucrose 12, pH 7.2, 315 mOsm. Pipettes with resistances of 4 - 6 MΩ were used. The 1 cm near the tip of the electrode was covered with Sylgard to reduce background noise and improve frequency responses. All recordings were made using isolated inside-out patches. Single-channel currents were recorded at membrane potentials between -100 and +100 mV. The current signal was amplified by an Axopatch 200B amplifier (Axon Instruments Inc., CA, USA) filtered at 5 kHz (3-pole Bessel), and then sampled at 25 kHz digitized with a Digidata 1320A (Axon Instruments Inc., CA, USA) and stored on a computer hard disk.
4.3.3. Data analysis

Raw data was digitally-filtered at 2 kHz and analyzed using pCLAMP 9 software (Axon Instruments Inc., CA, USA). Histograms of the amplitude were fitted with Gaussian distributions. Histograms of the channel open-time and closed-time were fitted with exponential curves. All fittings were done in pCLAMP 9. All statistical analysis was done in Prism V4 (GraphPad software, Inc., San Diego, CA, USA). Results are presented as mean ± s.e.. Significant differences were determined by t-test ($p < 0.05$).

4.3.4. Drugs

NaCl, NaAc, KCl, CaCl$_2$ and MgCl$_2$ were obtained from Fisher. All the other drugs were obtained from Sigma-Aldrich.

4.4. Results

4.4.1. Nicotinic acetylcholine receptors were observed on the membrane of body wall muscle cells in lev-10 mutants

The neurotransmitter acetylcholine and nicotinic agonist levamisole activate multiple subtypes of nematode nAChRs (Richmond and Jorgensen, 1999; Martin et al., 2005; Qian et al., 2006). To investigate the single-channel properties of nAChRs at body wall muscle cells of C. elegans, first we used acetylcholine (30 µM) to detect receptor activity in N2 wild type animals. From 17 membrane patches, single-channel currents of 3 receptors were observed – in other words, 18% of the membrane patches showed some nAChRs. The observation indicates that in wild
type worms most nAChRs are not accessible. This is consistent with published studies that have shown nAChRs aggregate at the neuromuscular junction (Gally et al., 2004).

Because most nAChRs aggregate at the postsynaptic region of the neuromuscular junction where patch electrodes cannot access, we decided to use lev-10 mutants rather than N2 wild types for further investigation. Three concentrations (10, 30 and 100 µM) of acetylcholine and levamisole were used as agonists to activate nAChRs from lev-10 animals. Overall, 83% of patches exposed to acetylcholine and 91% of patches exposed to levamisole showed single-channel currents.

To confirm that these single-channel currents were indeed activated by acetylcholine or levamisole, two series of tests were made. First, several patches were isolated from the same cells (cell number = 5). One of the patches was tested with agonist-free pipette solution as a control and the other patches were tested with acetylcholine. With agonist-free control solution, no channel current was observed (n = 5). With acetylcholine test solution, 8 channels were observed from 11 patches. We also added levamisole into the bath solution of agonist-free control recordings. Four channels were observed from five patches. This is consistent with the expectation that lipophilic levamisole crossed membrane patches and bound with the extracellular agonist-binding sites to activate nAChRs. We conclude that the channel currents under our recording conditions were due to the activity of nAChRs.
4.4.2. Levamisole and acetylcholine activated nAChRs with a conductance of ~30 pS.

The single-channel properties of the receptor was tested with acetylcholine and levamisole at the concentrations of 10, 30 and 100 µM. Usually the channel currents were recorded when the membrane potential was held at -100, -75, -50 and +50 mV. Figure 4.1 shows a single-channel recording at -75 mV. In good worm preparations the membrane potential of some recordings was also held at +75 and +100 mV (Figure 4.2B). The histogram of the current amplitude was fitted with Gaussian curves to evaluate the mean amplitude value (Figure 4.2A).
Figure 4.1. Acetylcholine and levamisole induced single-channel cation currents on body wall muscle cells. (a) In an agonist-free control
recording, no Cs+ current was observed from the isolated inside-out patch at membrane potential -75 mV. (b) After levamisole was added into the bath solution, inward cation currents were observed from the same membrane patch. This demonstrated that the currents were induced by levamisole. (c) A typical single-channel recording trace recorded with 30 µM levamisole at -75 mV.

At negative membrane potentials, the relationship between membrane potential and current amplitudes was fitted with linear regressions to give the channel conductance. We calculated the channel conductance between -100 and -50 mV. When tested with acetylcholine, the conductances were $30.1 \pm 1.1$ (n = 6), $31.3 \pm 1.0$ (n = 5) and $31.5 \pm 0.99$ pA (n = 5), respectively, at concentrations of 10, 30 and 100 µM. When tested with levamisole, the conductances were $29.3 \pm 0.7$ (n = 8), $31.2 \pm 1.2$ (n = 5) and $28.9 \pm 1.2$ pA (n = 4), respectively, at concentrations of 10, 30 and 100 µM (Figure 4.2C, Table 4.1). The mean reversal potential calculated from the linear regression was $\sim 0$ mV (mean = $-1.1 \pm 0.6$ mV, n = 33). In our experimental conditions, symmetrical Cs$^+$ in pipette and bath solutions, the reversal potential predicted by the Nernst Equation is 0 mV. At positive membrane potentials, the relationship between membrane potential and current amplitude was not fitted with linear regression, but showed rectified $I$-$V$ characteristics (Figure 4.2B).
Figure 4.2. The single-channel conductance was calculated from the I-V plot. The channel current (I) was recorded at different membrane potentials (V) from -100 to +100 mV. (a) The histogram of amplitude at each potential.
was fitted with Gaussian distributions to determine the mean value. The example shown here was made at -75 mV. (b) The nAChR showed a rectified outward current at positive membrane potentials. The I-V relationship between -100 and -50 mV was fitted with linear regression, and the slope gave the single-channel conductance. The I-V plot of the sample recording in Figure 4.1c is shown here and the calculated conductance was 31.8 ± 0.3 pS. (c) Overall, the mean conductance of the ion channel was ~30 pS when acetylcholine or levamisole was used as agonist.

Table 4.1. The single-channel properties of the levamisole-sensitive receptors recorded from the body wall muscle of *C. elegans* adults.

<table>
<thead>
<tr>
<th></th>
<th>ACETYLCHOLINE (µM)</th>
<th>LEVAMISOLE (µM)</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td><strong>g (pS)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 ± 1 (n = 6)</td>
<td>31 ± 1 (n = 5)</td>
</tr>
<tr>
<td></td>
<td>0.31 ± 0.03 (n = 6)</td>
<td>0.44 ± 0.03 (n = 6)</td>
</tr>
<tr>
<td><strong>τc (ms)</strong></td>
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<tr>
<td></td>
<td>15.7 ± 2.7 (50%)</td>
<td>26.1 ± 9.6 (50%)</td>
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<tr>
<td></td>
<td>73.5 ± 9.6 (50%)</td>
<td>121.3 ± 49.0 (50%)</td>
</tr>
<tr>
<td><strong>NP_{open}</strong></td>
<td>0.002</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Note: g is single-channel conductance, τo is mean open-time, τc is mean closed-time, % is percentage of total area and NP_{open} is normalized P_{open}.
4.4.3. The mean open-time of the nAChR was significantly shortened at high concentrations of levamisole.

To determine the mean open-time ($\tau_o$) of the ion channel, the duration times of channel openings were sorted in histograms and the histograms were fitted with single exponential curves (Figure 4.3A). We set the patch potential to -75 mV for these observations to give good signal-noise ratio and membrane stability. When tested with acetylcholine, the mean open-times were 0.31 ± 0.03 (n = 6), 0.44 ± 0.03 (n = 6) and 0.31 ± 0.04 ms (n = 4), respectively, at concentrations of 10, 30 and 100 µM (Figure 4.3B, Table 4.1). When tested with levamisole, the mean open-times were 0.36 ± 0.03 (n = 8), 0.30 ± 0.02 (n = 3) and 0.20 ± 0.02 ms (n = 4), respectively, at concentrations of 10, 30 and 100 µM (Figure 4.3B, Table 4.1). Compared to the observations of the acetylcholine tests, the mean open-times of levamisole were significantly shorter at the concentrations of 30 and 100 µM. Furthermore, the mean open-time of 100 µM levamisole was also significantly shorter than that of 10 µM levamisole. These results indicated that the open time of the ion channel was decreased when the concentration of levamisole was increased.
Figure 4.3. The mean open-time of the nAChR at -75 mV. (a) The open-time distribution was fitted with a single exponential equation to estimate the mean open-time $\tau_\text{o}$. According to our experimental resolution, the dwell
times shorter than 0.2 ms were ignored. The sample recording showed here was tested with 30 µM levamisole (same recording shown in Figure 4.1c). $\tau_0 = 0.28$ ms. (b) Overall, the $\tau_0$ of the nAChR was between 0.2 and 0.44 ms. The $\tau_0$ of levamisole was significantly shorter than that of acetylcholine at 30 and 100 µM. It was also noted that the $\tau_0$ was decreased following the increase of levamisole concentrations.

4.4.4. The mean closed-time of the nAChR presented an additional short component at high concentrations of levamisole.

The mean closed-times ($\tau_c$) are an important single-channel property to estimate kinetics of drug action. To evaluate the closed state of the ion channel, the duration of channel closed-time at the membrane potential of -75 mV was sorted in logarithmic binned histograms (Figure 4.4). The histogram was fitted with exponential equations to give the mean value of $\tau_c$. The major component of the histogram was best fitted with two-exponential equation in 25 experiments and 3 experiments were best fitted with one exponential equation. This statistical analysis suggests that there are two closed states of the levamisole-sensitive nAChR. The mean values of the two closed-times are listed in Table 4.1 for each concentration of agonist. Interestingly, when the ion channel was activated by 100 µM levamisole, a large number (20% of total) of very short closed events were present and the histogram was best fitted with three-exponentials (Figure 4.4c). The additional brief $\tau_c$ with a mean value of $1.06 \pm 0.18$ ms indicated that the ion channels rapidly
switched between open and closed states at high concentration of levamisole. This channel behavior could be explained by levamisole acting as an open channel blocker (Robertson and Martin, 1993) and the brief closed-time represents the channel block-time.
Figure 4.4. The change of the mean closed-times at different concentrations suggested the channel block effect of levamisole. When tested with
acetylcholine or low concentrations of levamisole (a, b), the distribution of closed duration was best fitted with the two-exponential equation. At high concentrations (100 µM) of levamisole (c), an additional component was visualized from the distribution histogram. This brief $\tau_c = 1.2$ ms indicated the block-time. All histograms obtained from recordings held at -75 mV membrane potential.

4.4.5. Single-channel kinetics of the levamisole-sensitive nAChR

When the levamisole-sensitive nAChR was activated by acetylcholine, the ion channel showed a single open-state and two closed-states. We estimated the channel kinetics with a two closed-state model

\[
\begin{array}{c}
\text{C}_1 \\
\text{C}_2 \\
X
\end{array}
\begin{array}{c}
\text{O} \\
\text{O}
\end{array}
\begin{array}{c}
\alpha \\
\beta
\end{array}
\begin{array}{c}
\gamma_+ \\
\gamma_-
\end{array}
\begin{array}{c}
k_{++} \\
k_{--}
\end{array}
\]

where $C_1$ is the 1st closed state, $C_2$ is the 2nd closed state, $O$ is the open state, $X$ is the acetylcholine concentration, $\alpha$ is the channel closing-rate and $\beta$ is channel opening-rate. In this model $\alpha$ was estimated by $\alpha = 1 / \tau_0 = 2900$ s$^{-1}$. Unlike many other nAChRs, this levamisole receptor lacks bursts of openings. The limited separation of the two major closed-times prevented accurate estimate of $\beta$, $k_-$ and $k_{++}$. 
When the receptor was activated by a high levamisole concentration, the ion channel showed an additional block-state. The channel kinetics were predicted by a channel-block scheme (Adams, 1976; Colquhoun and Sakmann, 1985):

\[
\begin{array}{c}
\text{C}_1 \quad \text{C}_2 \\
\text{k}_-^1 \quad \text{k}_+^1 \cdot X \\
\text{B} \quad \text{O} \\
\text{B} \quad \text{X} \\
\text{k}_-^B \quad \text{k}_+^B \\
\end{array}
\]

where B is the blocked state, X is the levamisole concentration, \( k_+^B \) is the blocking-rate constant, \( k_-^B \) is the unblocking-rate constant. As the previous discussion, the short mean closed-time represents the mean block-time. Therefore, \( k_-^B \) is estimated by the reciprocal of the short mean closed-time. At -75 mV, the value of \( k_-^B \) is calculated as 1 ms\(^{-1}\). The \( k_+^B \) was determined from the plot of the reciprocals of \( \tau_o \) against drug concentrations (Figure 4.5). The relationship between \( 1/ \tau_o \) and levamisole concentration at -75 mV was fitted with linear regression and the slope represents the \( k_+^B = 2.6 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \). Therefore, the channel-block dissociated constant, \( K_B \) is predicted by \( k_B/k_+^B = 38 \text{ µM} \). The channel closing-rate \( \alpha \) can be estimated by \( \tau_o = 1/(\alpha + k+BX) \). The \( \alpha \) is \( \sim 2500 \text{ s}^{-1} \) and is independent of drug concentration.
Figure 4.5. Reciprocal plot of $\tau_0$ against levamisole concentration. When levamisole targeted the nAChR, it behaved as an open channel blocker. The mean open-time $\tau_0$ was shorter following the increase of concentrations. The relationship of $1/\tau_0$ and levamisole concentration was linear. The slope represents the blocking rate $k_{+B}$. At a membrane potential of -75 mV, the blocking rate calculated from this plot was $2.6 \times 10^7$ M$^{-1}$s$^{-1}$.

4.4.6. The open probability of the channel ($P_{\text{open}}$)

Unlike the neuronal nAChRs in vertebrates, we showed here that the channel open-time of the levamisole-sensitive nAChR in *C. elegans* is brief and it was rare to observe the overlap of multiple channel openings when more than two channels presented in one isolated membrane patch. Therefore, the $P_{\text{open}}$ could not be normalized according to channel numbers present in the patch. The $P_{\text{open}}$ values
listed here are the patch $P_{\text{open}}$ rather than the channel $P_{\text{open}}$. For 10, 30 and 100 µM acetylcholine, the values were 0.016 ± 0.007, 0.005 ± 0.003 and 0.008 ± 0.003, respectively. For 10, 30 and 100 µM levamisole, the values were 0.005 ± 0.002, 0.009 ± 0.006 and 0.015 ± 0.007, respectively. To evaluate the overall $P_{\text{open}}$, another factor to be considered is the percentage of active patches at each dose of agonists. For 10 µM acetylcholine among total 45 patches, 11 of them did not show any channel opening, 28 could not be analyzed due to the low frequency of channel opening. This means that only 13% of the patches were considered as active. The active patches of 10, 30 and 100 µM acetylcholine were 13%, 64% and 71%, respectively. The active patches of 10, 30 and 100 µM levamisole were 32%, 28% and 33%, respectively. Therefore we evaluate the overall channel activity of the levamisole receptor at different agonist concentrations by $NP_{\text{open}} = P_{\text{open}} \times$ proportion of patches active. The values are listed in Table 4.1. The $NP_{\text{open}}$ indicated that when activated by acetylcholine or levamisole, the activity of the nAChR observed on the lev-10 body-wall muscle was similar.

4.4.7. The nAChR located at the membrane of body wall muscle in lev-10 mutants were not targets of nicotine.

Two nAChRs were found to present at neuromuscular junctions of body wall muscle cells in C. elegans (Richmond and Jorgensen, 1999). One is sensitive to levamisole and another one is sensitive to nicotine. To investigate whether the nAChR observed on the lev-10 body wall muscle was the levamisole-sensitive or the nicotine-sensitive receptor, we patched some cells with nicotine-filled pipettes. At the
concentration of 10 μM nicotine, no channel events were observed. For further confirmation, we also pulled some cell membrane patches with 10 μM levamisole to obtain active channels. Then a high concentration of nicotine (2 mM) was added into the recording chamber (5μl in approx. 1ml). Like levamisole, lipophilic nicotine is membrane permeable and crosses the inside-out membrane patch to target extracellular ligand-binding sites of the receptor (Levandoski et al., 2005). We assumed that if the nicotine-sensitive receptor existed in the membrane patch or nicotine targeted the levamisole-sensitive receptor, the addition of nicotine would increase the activity of the receptor or change the properties of the single-channel currents. However, in our experiments there was no significant change in channel activity or the single-channel properties from three recordings. We conclude that nicotine did not target the levamisole-sensitive receptor.

4.5. Discussion

In wild type C. elegans, nAChRs aggregate at the neuromuscular junction. The receptor cluster could be visualized using fluorescent labeling techniques (Gally et al., 2004; Gottschalk and Schafer, 2006). However, the existence of unclustered extra-synaptic receptors has not been demonstrated. In this single-channel study, 18% of the membrane patches from the non-synaptic region of muscle cells showed acetylcholine-activated channel currents. This observation suggests that a small amount of extra-synaptic nAChRs exist in wild type animals. The receptor-associated protein LEV-10 is critical for the receptor clustering (Gally et al., 2004). When the lev-10 gene was knocked-out, the receptor cluster was diffused from
postsynaptic sites but the whole-cell current remains unchanged. This suggests that unclustered nAChRs broadly distribute at non-synaptic regions. Based on this hypothesis we adapted the single-channel recording technique to investigate the single-channel properties of nAChRs in *lev-10* mutants.

Nicotinic receptors were observed on body wall muscle cells in *lev-10* mutants. The receptor-associated ion channel was activated by levamisole but not nicotine. This exclusive levamisole sensitivity indicated that the receptor observed in *lev-10* mutants was the levamisole-sensitive receptor. Previous studies indicated that the levamisole-sensitive receptor contains five nAChR subunits, UNC-63, UNC-38, UNC-29, LEV-1 and LEV-8 (Fleming et al., 1997; Richmond and Jorgensen, 1999; Culetto et al., 2004; Towers et al., 2005; Brown et al., 2006). However, it is unknown that the five subunits form one or multiple subtypes (Brown et al., 2006). In our previous studies from other nematodes, multiple nAChR subtypes were activated by levamisole (Robertson et al., 1999; Qian et al., 2006) and the single-channel properties of the multiple subtypes were physically distinguishable. The observations in this *C. elegans* study indicated that there was no evidence of more than one levamisole-sensitive receptor subtype. All ion channels showed a single conductance and single mean open-time. The results imply that we observed a single subtype of the levamisole receptor on body wall muscles. Previous studies indicate that the UNC-63, UNC-38 and UNC-29 are required for the functional levamisole receptor. The roles of the LEV-1 and LEV-8 subunits remain to be studied (Fleming et al., 1997; Culetto et al., 2004; Towers et al., 2005).
In wild type animals, most nAChRs aggregate at the synaptic region. In \textit{lev-10} mutants, the levamisole receptor, but not the nicotine-sensitive receptor, was highly observed at the extra-synaptic region. This observation is consistent with the previous suggestion that the LEV-10 protein only affects the clustering of the levamisole-sensitive receptor but not the nicotine-sensitive receptor (Richmond and Jorgensen, 1999).

At low concentrations, levamisole, like the neurotransmitter acetylcholine, acts as a potent nAChR agonist. At high concentrations, levamisole also behaves as an open channel blocker at hyperpolarized potentials. This channel block effect of levamisole was also observed in other nematodes (Robertson and Martin, 1993). Compared with the levamisole-sensitive nAChRs in other nematodes, such as \textit{Ascaris} and \textit{Oesophagostomum} (Robertson \textit{et al.}, 1999; Qian \textit{et al.}, 2006), the receptor in \textit{C. elegans} is exclusively sensitive to levamisole but not nicotine. It suggests the diversity of nAChRs in different species.

Compared with the nicotine-sensitive receptor at neuromuscular junctions, the levamisole-sensitive receptor contributes a small percentage of the postsynaptic excitatory currents, but it plays a greater functional role in locomotion (Touroutine \textit{et al.}, 2005). It will be interesting to know the physiological functions of this receptor in neurotransmission. Another thing that remains to be studied is the subunit composition. The physiological roles of two subunits, LEV-1 and LEV-8, are unclear. The knock-out of either of the subunits leads to partial loss of the levamisole-sensitive receptor but not all. It was questioned that there may be more than one subtype of levamisole-sensitive receptors. However, our observations are against
this hypothesis. We predict that a single-channel study in lev-1 and lev-8 mutants will help to shed light on this controversy.

4.6. Acknowledgments

We would like to thank Dr. Janet Richmond for the help of worm dissections. We are pleased to acknowledge National Institute of Health for financial support (R01 AI47194) to RJM.
Chapter 5. Changes in *Caenorhabditis elegans* levamisole receptor single-channel properties in *lev-1* and *lev-8* mutants

5.1. Abstract

Nicotinic acetylcholine receptors (nAChRs) play an important role in synaptic transmission. A large family of nAChR subunits has been discovered in *C. elegans*. The post-synaptic levamisole receptor, one nAChR present at the neuromuscular junction, is composed of essential subunits (UNC-63, UNC-38 & UNC-29) and non-essential subunits (LEV-1 and LEV-8). Behavioral studies indicated that knockout of any essential subunit leads to strong levamisole resistance. Knockout of either non-essential subunit leads to weak levamisole resistance. Previously we used *lev-10* mutants to study the single-channel properties of muscle levamisole receptors. LEV-10 is a transmembrane protein that causes levamisole receptor clustering at the neuromuscular junction. In *lev-10* knockouts, functional levamisole receptors are dispersed over the muscle cell body and are accessible for study using the patch-clamp technique. In this study, we used *lev-1;lev-10* and *lev-8;lev-10* double mutants to investigate the single-channel properties of receptors lacking these non-essential subunits. In both double mutants we detected levamisole activated single-channel currents. The single-channel conductance and mean open-time of the double mutants were similar to that of the *lev-10* single mutant, but single-channel events occurred at a lower frequency. We found that in the *lev-1;lev-10* mutant, less
than 10% of the membrane patches were active and that channel opening was rare with NP\textsubscript{open} = 0.0001. In the lev-8;lev-10 mutants, single-channel currents were present in ~40% of the experiments and NP\textsubscript{open} was significantly less than that observed from the lev-10 single mutant. These observations predict a reduction in current flow in response to levamisole and are in close agreement with previous studies using the whole-cell voltage-clamp technique.

5.2. Introduction

Nicotinic acetylcholine receptors (nAChRs) are important for excitatory synaptic transmission in C. elegans. At neuromuscular junctions (NMJ) of muscle cells, the neurotransmitter acetylcholine released from presynaptic neurons targets postsynaptic nAChRs to induce muscle cell depolarization and cause muscle contraction. At the NMJ of body wall muscle cells, two nAChRs have been studied (Richmond and Jorgensen, 1999). One is sensitive to the nicotinic anthelmintic levamisole and named as levamisole receptors. One is sensitive to nicotine and has been found to contain ACR-16 as the only essential subunit (Touroutine et al., 2005). The levamisole receptor has been studied for its important roles in worm locomotion. The anti-parasitic drug levamisole targets this receptor to induce muscle contraction and leads to paralysis of the worm. Some genes have been found to associate with levamisole resistance (Fleming et al., 1997). Among these genes, unc-38, unc-63, unc-29, lev-1 and lev-8 were identified to encode the levamisole-sensitive nAChRs. The knockout mutants of unc-38, unc-63 and unc-29 are strongly resistant to levamisole. The subunits encoded by these genes are thought to be essential for the
levamisole receptor. The knockout mutants of lev-1 and lev-8 still present some levamisole sensitivity (Lewis et al., 1987; Fleming et al., 1997; Culetto et al., 2004; Towers et al., 2005). Additional to the observations of behavior phenotypes, levamisole-induced whole-cell currents were recorded from body wall muscle cells. The current recorded in the lev-1 mutant was reduced by ~85% compared with wild type (Culetto et al., 2004). The current recorded in the lev-8 mutant was reduced by ~65% compared with wild type (Towers et al., 2005). The remaining part of the whole-cell current in lev-1 or lev-8 mutants may suggest more than one levamisole receptor subtype. However, our previous single-channel study disagreed with this suggestion. At the single channel level, only one levamisole receptor population was observed at muscle cells in C. elegans (chapter 4). We conclude that a single levamisole receptor subtype present on body wall muscle in C. elegans and this receptor comprises five subunits, one UNC-38, one UNC-63, one UNC-29, one LEV-1 and one LEV-8. To investigate the roles of the non-essential subunits, LEV-1 and LEV-8, here we use the single-channel recording to test the lev-1 and lev-8 mutants.

We noted that levamisole receptors were rarely observed on the muscle body of wild type worms. To make single-channel recording at the cell body, we used lev-10 knockout mutants (Chapter 4). The lev-10 gene encodes a transmembrane protein in C. elegans (Gally et al., 2004). This protein is critical for the synaptic aggregation of the levamisole receptor. In knockout animals, receptor clusters are diffused from NMJs and the receptor was easily detected at the surface of cell body at the single-channel level. In this study we made lev-1;lev-10 and lev-8;lev-10 double mutants to detect the LEV-1 or LEV-8 lacking levamisole receptor. All the lev-
1, lev-8 and lev-10 mutants we used here contained a Mos1 sequence insert. Mos1 is a transposon isolated from Drosophila mauritiana. This unique sequence can be inserted into the C. elegans genome and provides an easy way to rapidly detect the mutated gene (Bessereau, 2006; Boulin and Bessereau, 2007). Previous studies showed Mos1 as a tool to insert a mutagenesis into the lev-10 gene (Gally et al., 2004). In this study Mos1 was inserted into lev-1, lev-8 and lev-10 genes. More information about Mos1-mediated mutagenesis and the lev-10 gene are available from Dr. Jean-Louis Bessereau’s lab (http://www.biologie.ens.fr/bcsgnce/).

5.3. Materials and methods

5.3.1. Worm strains

N2, lev-10(kr26::Mos1) and unc-38(e264) were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). Single mutants lev-1(kr105::Mos1) and lev-8(kr136::Mos1) were provided by Dr. Bessereau’s lab at Institut National de la Santé et de la Recherche Médicale. All worms were grown on NGM plates seeded with Escherichia coli OP50.

5.3.2. Movement assay

Worms from the stock plates were examined using a stereo microscope. L4 larvae were identified and incubated for 24 hours at 20°C. Then the worms were transferred into M9 buffer with varying concentrations of levamisole. The M9 buffer contains (g/L): Na₂HPO₄ 6, KH₂PO₄ 3, NaCl 5, MgSO₄ 0.12. After 1 hour, worms
were checked for paralysis or non-paralysis. The worms showing no movement or curling were considered as paralyzed. Four groups of worms (n > 10 per group) were tested for each levamisole concentration.

5.3.3. Cross breeding

L4 stage hermaphrodites were transferred to OP50 seeded NGM plates and heat shocked for 6 hours. Then the worms were returned to 20°C for self-fertilizing. The males in the F1 progeny were picked out for cross breeding. The offspring of cross breeding was self-fertilized and genotyped by PCR to screen for double mutated homozygotes.

5.3.4. PCR

The lev-1 gene was amplified using primers oTB263 and oTB265. The lev-8 gene was amplified using primers oTB264 and oTB266. The lev-10 was amplified by primers LEV10up and LEV10dwn. The Mos1 was detected by primer oJL103. The primer sequences were listed in Table 5.1. The primers oTB263/265, oTB264/266 and oJL103 were provided by Dr. Jean-Louis Bessereau. To amplify lev-1 and lev-8, the PCR conditions were as following: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 66°C for 1 min, 72°C for 1 min. To amplify lev-10, the PCR conditions were as following: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min.
Table 5.1. Primers for PCR tests of lev-1, lev-8 and Mos1.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>oTB263</td>
<td>CGTCCAGCTTCCAAAGTCAAACCTGC</td>
</tr>
<tr>
<td>oTB265</td>
<td>GAGGATCGCGCTGATGGTCGACC</td>
</tr>
<tr>
<td>oTB264</td>
<td>GTCAGACCAGTTCATAATGCATCAG</td>
</tr>
<tr>
<td>oTB266</td>
<td>GTTGTAAAGTACAATGTCAGGGATCC</td>
</tr>
<tr>
<td>LEV10up</td>
<td>AAAATTAATGAAAAACTCAGCCATGA</td>
</tr>
<tr>
<td>LEV10dwn</td>
<td>CAAGCTATTACCATTGAGTAAGC</td>
</tr>
<tr>
<td>oJL103</td>
<td>TCTCGGAGTTTGTGTTTTGCGTTTGAG</td>
</tr>
</tbody>
</table>

5.3.5. Electrophysiology

Adult worms were transferred into the recording chamber. The extracellular solution contained (mM): NaCl 23, NaAc 110, KCl 5, CaCl$_2$ 6, MgCl$_2$ 5, HEPES 5, D-glucose 11 and sucrose 10. The pH of the solution was adjusted to 7.2 with NaOH. The osmolarity was adjusted to 330 mOsm with sucrose. The worm was immobilized with cyanoacrylate (GluSeal® 510K# K030574, Glustitch Inc.) onto a sylgard-coated coverslip. Sharp glass needles were used to cut open the cuticle. Exposed muscle cells were cleaned with 0.5 mg/ml collagenase solution. The dissected worms were used for patch-clamp experiments. The recording solution contained (mM): CsCl 35, CsAc 105, MgCl$_2$ 4, HEPES 10, EGTA 1 and sucrose 25, pH 7.2 adjusted with CsOH, 330 mOsm. The recording pipette was filled with solution containing (mM): CsCl 140, MgCl$_2$ 4; HEPES 10, EGTA 1 and sucrose 12, pH 7.2, 315 mOsm. Pipettes with resistances of 4 - 6 MΩ were used. All recordings were made using isolated inside-out patches. The current signal was amplified by an Axopatch 200B
amplifier (Axon Instruments Inc., CA, USA) filtered at 5 kHz. This single-channel recording method is described in our previous study (Chapter 4).

5.3.6. Data analysis

The raw data was digitally-filtered at 2 kHz and analyzed using pCLAMP 9 software (Axon Instruments Inc., CA, USA). To analyze the amplitude of the single-channel current, amplitude histograms were fitted with Gaussian equations. To analyze the mean open-time of the current, histograms of open duration were fitted with one-exponential equations. All the fits were obtained using pCLAMP 9 software. Statistical analysis was done with GraphPad Prism. Results are presented as mean ± s.e.. Significance was tested by t-test and defined by $p < 0.05$.

5.4. Results

5.4.1. Levamisole resistance of lev-1 and lev-8 mutants

To test how the LEV-1 and LEV-8 subunits affect the locomotion of worms, we used a paralysis assay to examine the levamisole induced paralysis of N2, lev-1(kr105::Mos1) and lev-8(kr136::Mos1) animals. Compared to wild type animals, both lev-1 and lev-8 exhibited levamisole resistance (Figure 5.1). The EC50 of N2 animals was 9 µM. The EC50 of lev-8 mutants was 40 µM. The dose dependent curve shifted to the right. A low percentage of lev-1 mutants showed paralysis at low concentrations. At high concentrations, the maximum percentage of paralyzed worms was 14%. As a negative control, unc-38(e264) worms showed rare paralysis between 1 and 100 mM of levamisole. This paralysis study is consistent with
previous observations that *lev-1* mutants have a strong levamisole resistance and
*lev-8* mutants have a weak levamisole resistance.

![Graph showing the percentage of paralyzed animals at different log concentrations of levamisole](image)

Figure 5.1. *lev-1* and *lev-8* animals are partially resistance to levamisole.

The concentration relationship of N2, *lev-1* and *lev-8* were fitted with
dose-dependent curves. The EC50 of N2 animals was 9 µM. The EC50
of *lev-8* mutants was 40 µM. The maximum of *lev-1* dose-dependent
curve was 14% with logEC50 of 50 µM. The relationships of *lev-1;lev-8*
and *unc-38(e264)* could not fitted with dose-dependent curves.

### 5.4.2. Single-channel recording of *lev-1* and *lev-8* mutants

To examine the single-channel properties of LEV-1 or LEV-8 lacking
levamisole receptors, we made *lev-1; lev-10* and *lev-8;lev-10* double mutants. In
these mutants, the mutated levamisole receptor distributed at the cell bag of body
wall muscles and was detectable with patch-clamp. After getting high resistance seals, the membrane patch was isolated and air-exposed to confirm inside-out configuration.

In the *lev-1(kr105::Mos1);lev-10(kr26::Mos1)* double mutants, rare levamisole induced channel opening was observed. Considering that the low channel opening may be due to the receptor desensitization, we tested acetylcholine and levamisole as agonists at different concentrations between 10 and 100 µM. According to our previous experience, agonist-induced channel single-currents were observed from ~90% of patches and ~30% were considered as active patches. In the *lev-1;lev-10* mutants, only ~20% of recordings showed convincing channel events with a low frequency of opening. We also used agonist-free pipette solution to patch cell membranes and then added levamisole into the bath solution to test whether desensitization caused the low frequency of channel opening. No improvement in channel opening was observed. From a total of 40 patches, only 2 recordings could be analyzed at the membrane potential of -75 mV.

The cord conductance of the receptor is listed in Table 5.2. Not enough opening events were available for analysis of mean open-time. The NP\textsubscript{open} (Normalized P\textsubscript{open}) was calculated according to the average P\textsubscript{open} of the two patches and the percentage of the active patches, which showed enough opening events to be analyzed. The NP\textsubscript{open} at 10 µM levamisole was 0.0001 and dramatically lower than the NP\textsubscript{open} observed in *lev-10* single mutants (NP\textsubscript{open} = 0.0016, Table 5.2).
Table 5.2. The single-channel properties of the levamisole receptor.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>ACH (10 µM)</th>
<th>LEV (10 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lev-10</td>
<td>30.1 ± 1.1 (n = 6)</td>
<td>29.3 ± 0.7 (n = 8)</td>
</tr>
<tr>
<td>g (pS)</td>
<td>lev-1;lev-10</td>
<td>-</td>
</tr>
<tr>
<td>lev-8;lev-10</td>
<td>27.4 ± 0.3 (n = 3)</td>
<td>30.1 ± 0.7 (n = 3)</td>
</tr>
<tr>
<td>τ (ms)</td>
<td>lev-10</td>
<td>0.31 ± 0.03 (n = 6)</td>
</tr>
<tr>
<td></td>
<td>lev-1;lev-10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>lev-8;lev-10</td>
<td>0.34 ± 0.02 (n = 3)</td>
</tr>
<tr>
<td>NP_open</td>
<td>lev-10</td>
<td>0.0021</td>
</tr>
<tr>
<td></td>
<td>lev-1;lev-10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>lev-8;lev-10</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

* lev-1;lev-10 double mutants were tested with 10 uM acetylcholine. However, no convincing channel opening observed from 8 patches. When 10 uM levamisole was used to test lev-1;lev-10 mutants, 6 of 21 patches showed channel opening and 2 were analyzed. The conductance of the channels were listed in the table. Due to small amount of open events, mean open time of the channels could not be analyzed.

In the lev-8(kr136::Mos1);lev-10(kr26::Mos1) double mutants, levamisole and acetylcholine induced channel openings were observed frequently (Figure 5.2). 50% of the patches (8 of 16) showed channel openings with 10 µM acetylcholine or levamisole as agonists. The cord conductance was 27 and 30 pS when activated by acetylcholine and levamisole, respectively (Table 5.2). The conductance observed here was very similar to our previous results in lev-10 single mutants. It suggests that the channel permeability was not changed when the LEV-8 subunit was lacking. The channel also showed a similar mean open-time with the wild type receptor. The NP_open was lower in the double mutants, especially when levamisole was used as agonist (Table 5.2). However, channel openings were frequently observed in the
LEV-8 lacking mutants. It suggests that the \textit{lev-8} mutated receptor is still partially functional for synaptic transmission.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.2}
\caption{The single-channel recording of \textit{lev-8} mutants. Channel opening events were detectable in \textit{lev-8;lev-10} double mutants with low frequency. No change of single-channel conductance and mean open-time were observed. [LEV] = 10\,\mu\text{M}. Membrane potential was held at -75 mV.}
\end{figure}

5.4.3. Prediction of phosphorylation sites on LEV-1 and LEV-8 subunits

To further investigate the differences between LEV-1, LEV-8 and other nAChR subunits required for the levamisole receptor, we compared the protein amino acid sequences (Figure 5.3). The second transmembrane domains (M2) line
the tunnel of the ion channel and are important for ion selectivity and permeability. The amino acid sequences of the M2 region are highly conserved except for the LEV-8 subunit. At the cytoplasmic end, the other subunits have a conserved acidic glutamate, but the LEV-8 subunit has a basic His288. The positive charged tunnel was suggested to form an anion channel instead of a cation channel (Jones and Sattelle, 2004). The change of this amino acid residue from Glu to His in neuronal α7 subunit resulted in a non-functional receptor (Towers et al., 2006). However, the LEV-8 containing levamisole receptor is a non-selective cation channel and the knockout of this subunit did not improve the cation permeability. This observation indicates that the LEV-8 subunit is not important for ion permeability.
Figure 5.3. Amino acid sequences of the five levamisole receptor subunits.

The protein alignment was constructed using Vector NTI. The six loops of ligand binding domains and four transmembrane domains are indicated. The histidine at the cytoplasmic ends of the subunits is conserved except LEV-8. Between M3 and M4 each subunit has a large intracellular loop, which has multiple putative phosphorylation sites. The region is predicted to be important for receptor regulation.

All the subunits have a large intracellular domain between M3 and M4. The amino acid sequences of this region are less conserved and important for the modulation of receptors. Phosphorylation sites of the subunits were predicted using NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) and NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/). The putative phosphorylation sites are listed in Table 5.3. At the intracellular loop of LEV-1 and LEV-8 subunits, multiple tyrosine and serine sites were expected to be phosphorylated by CaM kinases or other protein kinases, especially protein kinase C. In nematodes, CaM kinase has been shown to potentiate levamisole responses of somatic muscle cells (Trailovic et al., 2002). PKC is also highly involved in the modulation of nAChRs. It has been demonstrated that in C. elegans PKC encoded by the tpa-1 gene is essential for the down-regulation of levamisole receptors (Waggoner et al., 2000). The intracellular loop of the UNC-29 subunit, which is essential for functional levamisole receptors, also has three putative phosphorylation sites for PKC and one site for PKA or PKG. The UNC-63 and UNC-38 subunits showed relatively fewer phosphorylation sites.
The analysis implies that LEV-1, LEV-8 and UNC-29 may play important roles in channel modulation.

Table 5.3. The phosphorylation sites of the five subunits.

<table>
<thead>
<tr>
<th>UNC-63 Site</th>
<th>Kinase</th>
<th>UNC-38 Site</th>
<th>Kinase</th>
<th>UNC-29 Site</th>
<th>Kinase</th>
<th>LEV-1 Site</th>
<th>Kinase</th>
<th>LEV-8 Site</th>
<th>Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 395</td>
<td>PKC</td>
<td>S 397</td>
<td>PKC</td>
<td>T 324</td>
<td>PKC</td>
<td>S 332</td>
<td>PKC</td>
<td>S 352</td>
<td>PKC</td>
</tr>
<tr>
<td>S 418</td>
<td>CaMK</td>
<td>S 350</td>
<td>PKA</td>
<td>S 366</td>
<td>PKC</td>
<td>S 386</td>
<td>CaMK</td>
<td>S 350</td>
<td>PKA</td>
</tr>
<tr>
<td>S 423</td>
<td>PKA</td>
<td>T 372</td>
<td>PKC</td>
<td>T 389</td>
<td>CaMK</td>
<td>T 394</td>
<td>PKC</td>
<td>S 400</td>
<td>PKA</td>
</tr>
<tr>
<td>S 436</td>
<td>PKG</td>
<td>S 398</td>
<td>PKC</td>
<td>S 397</td>
<td>PKC</td>
<td>S 425</td>
<td>PKA</td>
<td>S 425</td>
<td>PKB</td>
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5.5. Discussion

The levamisole receptor located at the NMJ of body wall muscles has a critical role in locomotion of nematodes. The levamisole sensitive nAChR exhibits worm specific characteristics compared with vertebrate nAChRs and is important for studies of anti-parasitic drugs and drug resistance. One question of interest to scientists is: is there only one subtype of levamisole receptor or more than one subtype? Previous results from levamisole resistance assays and whole-cell patch clamp suggest the existence of more than one subtype, because the knockout of lev-1 or lev-8 only causes partial loss of levamisole responses. Given the facts that LEV-1 containing receptors contribute ~85% and LEV-8 containing receptors contribute ~65% of the whole-cell levamisole current (Culetto et al., 2004; Towers et al., 2005), we believe that the majority of levamisole receptors is one subtype of
nAChRs, which contains both LEV-1 and LEV-8 subunits. Our previous single-channel study of the levamisole receptor in the *lev-10* single mutant supported this hypothesis. We only observed one ion channel population. The LEV-1 and LEV-8 subunits are not necessary for forming pentameric ligand-gated ion channels, but they may play important roles to modulate the ion channel activity. As we showed in this study, although the levamisole receptor was observed in *lev-1* and *lev-8* mutants, the channel opening was much less than the normal levamisole receptor. We also determined that the percentage of active membrane patches was significantly lower in *lev-1* lacking receptors than normal receptors. This may suggest the number of the levamisole receptors on the cell surface was decreased.

The intracellular loop between M3 and M4 is important for receptor subunit modulations. Multiple putative phosphorylation sites exist on the intracellular loop of LEV-1 and LEV-8 subunits. These sites are predicted to be targets of protein kinases and CaM kinases. In nematodes the nAChR activity could be modulated by kinases directly (Trailovic *et al.*, 2002). Furthermore, these tyrosine, serine and threonine sites may also be phosphorylated by other kinases, i.e. tyrosine kinases and G-protein-linked receptor kinases (GRKs), to modulate the receptor endocytosis, desensitization and protein association (Gainetdinov *et al.*, 2004). Nicotinic AChRs in *C. elegans* are known to associate with many transmembrane or intracellular proteins (Gottschalk *et al.*, 2005). Some of the nAChR-associated proteins serve as modulators to regulate the channel activity. Some of the proteins, such as LEV-10 (Gally *et al.*, 2004), are related to receptor expression and localization. To conclude, nAChRs lacking either LEV-1 or LEV-8 are open less frequently than normal
levamisole sensitive nAChRs. We hypothesize that normal levamisole sensitive
receptors are positively modulated by phosphorylation. The lack of channel activity
in *lev-1* and *lev-8* mutants corresponds to a lack of phosphorylation sites present in
channels containing those subunits.

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Chapter 6. General discussion

Three pharmacological nAChR subtypes have been described in our previous studies (Robertson et al., 2002). Here we observed the three subtypes at the single-channel level. We tested the single-channel properties and pharmacological properties. Each subtype exhibited individual channel conductance, mean open-time and $P_{\text{open}}$. Levamisole sensitivities of the subtypes are also different. It has been found that levamisole resistant nematodes lost some levamisole-sensitive nAChR subtypes (Robertson et al., 1999). The L-type nAChR in Ascaris is the most sensitive subtype and may play an important role in producing levamisole resistance, because the opening of L-type receptors contributes most of the levamisole induced inward current. If this subtype is missing, the worm will exhibit levamisole resistance. Paraherquamide and 2-desoxyoparaherquamide, two novel nicotinic anthelmintic compounds, act as competitive antagonists of nematode nAChRs and the inhibitory effects are subtype selective. Paraherquamide treatment inhibits normal cholinergic transmission at neuromuscular junctions and induces flaccid paralysis of parasitic nematodes. Paraherquamide and 2-desoxyoparaherquamide are potential anthelmintic drugs.

The free living nematode C. elegans is commonly used as a model animal for drug resistance studies. More than 20 nAChR subunits have been found in C. elegans. When some of the subunits were knocked out, the nematode produced levamisole resistance. It is believed that a levamisole sensitive nAChR composed of these subunits is involved in levamisole resistance. We observed this levamisole
receptor at the single-channel level. The single-channel properties of the levamisole receptor in *C. elegans* showed some similarity with L-type nAChRs in *Ascaris suum*, but were not identical. In *Ascaris*, two other nAChR subtypes, N- and B-type, are also activated by levamisole. In *C. elegans*, we observed one levamisole sensitive nAChR type. An additional levamisole-insensitive nAChR exists at the neuromuscular junction of *C. elegans* (Richmond and Jorgensen, 1999). In our experiments we could not detect this subtype and test its single-channel properties. We estimate that both *Ascaris suum* and *C. elegans* have multiple nAChR subtypes at the neuromuscular junctions. The levamisole sensitivity of these subtypes is different. Loss of levamisole sensitive subtypes is one mechanism for levamisole resistance.

Two levamisole receptor subunits, LEV-1 and LEV-8, are not essential to form levamisole receptor pentamers. They may play important roles in receptor modulation. When LEV-1 or LEV-8 was absent, the ion channel was less active. nAChR subunits have a large intracellular loop between transmembrane domain 3 and 4. We analyzed putative phosphorylation sites on the intracellular loop. The LEV-1 and LEV-8 subunits have multiple phosphorylation sites for CaM Kinases and protein kinases, especially PKC. Our previous studies in parasitic nematodes indicated that CaM Kinases and protein kinases directly regulate nAChR channel activity (Trailovic *et al.*, 2002). It is reasonable to hypothesize that the LEV-1 and LEV-8 play important roles in receptor modulation, and receptor activity was inhibited in *lev-1* or *lev-8* knockout mutants. Another possible modulation is protein association. It was known that nAChRs are associated with many transmembrane
and intracellular proteins, which regulate nAChR functions. We believe that further understanding of receptor modulation will give us the possibility to enhance nematode responses to anthelmintic drugs and reverse drug resistance.

Phosphorylation of nAChRs has been showed to modulate receptor activity in the nematode Ascaris suum. We expect that protein kinases also modulate levamisole receptors in C. elegans. For further investigation of this hypothesis, we can either apply protein kinase catalytic subunits and ATP to test channel activity, or use some genetic manipulated mutants such as TAX-6 to investigate effects of phosphorylation. Another future study interesting to us is that the role of nicotine sensitive nAChRs. This nAChR subtype has been showed to contribute ~80% of the whole-cell current induced by acetylcholine, but the knockout of this nAChR has no observable phenotype. The nicotine sensitive receptor and levamisole sensitive receptor may have different Ca\(^{2+}\) permeability or induce different Ca\(^{2+}\) cascade. To investigate the Ca\(^{2+}\) permeability of nicotine sensitive nAChRs, I propose to use Ca\(^{2+}\) imaging or patch-clamp technique to test the Ca\(^{2+}\) permeability in levamisole receptor knockout mutants. To investigate the Ca\(^{2+}\) permeability of levamisole sensitive nAChRs, I propose to use same techniques in acr-16 knockout mutants. The results will indicate the contribution of the two nAChR subtypes to whole-cell Ca\(^{2+}\) entry and the roles of the two nAChRs in muscle contraction.
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