CHAPTER ONE

INTRODUCTION

Ivermectin (IVM) resistance in parasitic nematodes of sheep has been observed in the field and found to be conferred by dominant single mutations in three instances. The mode of action of IVM in nematodes has also been studied, and found to involve activation of chloride ion channels. The genetics of IVM resistance in *Caenorhabditis elegans* has been investigated previously to some extent but dominant IVM resistant mutants have not been isolated previously. By isolating dominant IVM resistant mutants of *C. elegans* and characterising them candidate loci for IVM resistance in parasites can be identified. Also analyses of the phenotype of a range of dominant and recessive mutants and their interactions with other mutations might provide evidence for a mechanism of resistance to IVM in *C. elegans*. This report uses these two approaches to analyse IVM resistance in *C. elegans* as a prelude to studies using parasitic nematodes.

Macrocyclic lactones are a large group of compounds that can be isolated from a variety of fungi (Fisher and Mrozik, 1992). All compounds of the group contain a macrocyclic lactone ring structure (figure 1.1.). The group can then be divided into two depending on whether the molecule possesses a sugar group attached at carbon 13 (avermectins) or not (milbemycins). Avermectins have only been isolated from *Streptomyces avermitilis* whereas milbemycins have been obtained from a number of organisms. The structures of three avermectins and four milbemycins are given in figure 1. Much of the work described here is concerned with avermectins (AVM) and with ivermectin (IVM) in particular. This work is however equally applicable to milbemycins as the compounds are related. Ivermectin resistant strains of nematodes exhibit side-resistance to milbemycins (Shoop, Haines et *al.*, 1993) and a cloned IVM receptor from *Caenorhabditis elegans* is activated by a range of avermectins and milbemycins (Arena, Liu et *al.*, 1995) the action of the two drugs is therefore likely to be identical *in vivo* (Sangster, 1995).

Ivermectin is a mixture of two macrocyclic lactone compounds, 22,23-dihydro avermectin B_{1a} and 22,23-dihydro avermectin B_{1b} (Campbell, 1989). IVM and related compounds are used extensively to control nematode and arthropod pests in agriculture (Campbell, 1989; Fisher and Mrozik, 1992; Kennedy, 1992) and IVM also is used to treat filarial nematode parasites of humans (most notably *Onchocerca volvulus* - the causative agent of river blindness) (Fisher and Mrozik, 1992) and has been proposed for the control of ectoparasite arthropods which may also be vectors of nematode and other disease organisms (Ali and

Nayar, 1985; Miller, Garris et *al.*, 1989; Tesh and Guzman, 1990; Jones, Meisch et *al.*, 1992; Wilson, 1993) . Although macrocyclic lactone drugs are used to control a range of pests, reports of pest resistance have been almost exclusively limited to nematode parasites of livestock (*Haemonchus contortus*, *Trichostrongylus colubriformis* and *Ostertagia circumcincta.*), although field resistance of the two-spotted mite (*Tetranychus urticae*) has been reported (reviewed in (Clark, Scott et *al.*, 1994)). Field resistance of Trichostronylid parasites to ivermectin has now been reported from countries in Asia (Sivaraj, Dorny et *al.*, 1994; Sivaraj and Pandey, 1994), Africa (Wyk, Malan et *al.*, 1989), North America (Craig and Miller, 1990), South America (Echevarria and Trindade, 1989; Vieira, Berne et *al.*, 1992) and Oceania (Badger and McKenna, 1990; Craig and Miller, 1990; LeJambre, 1993; Swan, Gardner et *al.*, in press). In general IVM resistance has developed quickly, with field resistance being seen three to five years after the adoption of IVM as an anthelmintic. Resistance to ivermectin has developed more quickly in tropical countries where continual parasitic infections necessitate higher frequency of ivermectin use. These selection kinetics suggest that resistance alleles have reached high levels in 10-20 generations and imply that either (i) resistance is recessive and resistance alleles relatively frequent in "susceptible populations or (ii) resistance is dominant and resistance alleles are rare in "susceptible populations.

1.1. The mode of action of ivermectin.

Shortly after the discovery of the avermectins, studies began on the mode of action of these compounds in arthropods (Fritz, Wang et *al.*, 1979; Duce and Scott, 1985; Scott and Duce, 1985), and nematodes (Kass, Wang et *al.*, 1980).

The responsiveness of lobster muscle cells and neurons to applied intracellular or extracellular currents is reduced by IVM at 1-10 μg/mL (Fritz, Wang et *al.*, 1979). The authors proposed that the effect was mediated by the opening of chloride-ion channels. The effect of IVM on insect muscle cells has been attributed to interactions with two pharmacologically-distinct chloride ion channel receptors (Duce and Scott, 1985; Scott and Duce, 1985). One receptor is activated by as little as 0.5 ng/mL IVM, and belongs to the H-receptor class of chloride ion channels, which are activated by glutamate and ibotenate, with picrotoxin as an antagonist (Scott and Duce, 1985). Binding of IVM to the H-receptor appears to be irreversible. A GABA (γ-amino butyric acid) -induced chloride ion channel is also opened by IVM in insect muscle. This channel is even more sensitive to IVM, concentrations as low as 75 pg/mL producing chloride ion currents

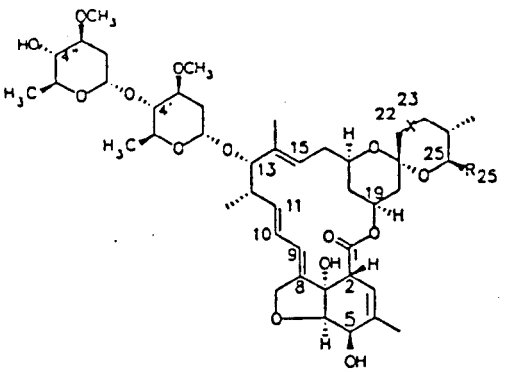
in muscle cells (Duce and Scott, 1985). The binding of IVM to the GABA channel is reversible and does not enhance GABA-induced responses. The action of IVM in arthropods therefore is most probably due to neuronal and/or muscular effects mediated by IVM binding to glutamate- and GABA-gated chloride ion channel receptors.

Three studies of AVM resistance in arthropods have recently been reviewed by Clark et *al.* (1994). Two studies concentrated on laboratory-selected resistant strains of the Colorado Potato Beetle (*Leptinotarsa decemlineata*) and the house fly (*Musca domestica*). The resistant beetle strain had increased levels of cytochrome P450, and as cytochrome P450 participates in detoxification of a range of chemicals, the strain most probably had an elevated capability to detoxify AVM leading to resistance. In contrast, the selected house flies were not observed to metabolise AVM, but had normal sensitivity to injected drug despite being up to 60,000 X more resistant than control strains to topically applied AVM. Decreased penetration of AVM in these flies is the most important resistance mechanism. The only resistant arthropod pest isolated from the field, the two-spotted mite, has not yet been intensively studied with respect to the mechanism of resistance. The synergists piperonyl butoxide (PBO) and *S*,*S*,*S*-tributyl phosphorotrithioate (DEF) had little effect on AVM resistance in the isolated lines of mites. Increased detoxification of AVM in these resistant mite strains is unlikely to occur as inhibition of oxidative or esteratic metabolism via PBO or DEF respectively would reduce resistance if drug resistance was due to metabolism of drug.

Studies involving IVM responses in arthropods therefore have revealed a major effect of the drug that is consistent with a neuronal or muscular effect mediated by chloride ion channels. The receptor molecules are very sensitive to the drug and may bind it reversibly or irreversibly. Mechanisms of resistance in arthropods may involve reduced cuticular penetration or increased metabolism of AVM. The study of IVM responses in nematodes is discussed in more detail below. Studies concerning the mode of action of the avermectins in nematodes have revealed many effects of this group of drugs (Kass, Wang et *al.*, 1980; Sani and Vaid, 1988; Martin and Pennington, 1989; Avery and Horvitz, 1990), with a variety of drug concentrations, biological preparations and techniques being used. Studies concerning the mechanism of resistance to IVM in nematodes

Figure 1.1.

A

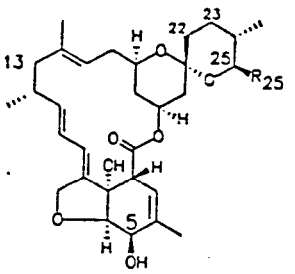


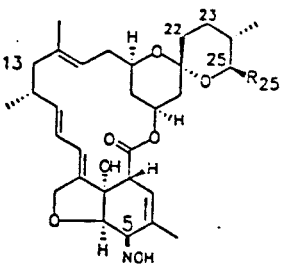
AVERMECTIN B1a : X = -CH=CH- R_{25} = $CH(CH_3)CH_2CH_3$

AVERMECTIN B1b : X = -CH=CH- R_{25} = $CH(CH_3)_2$

IVERMECTIN : X = $-CH_2CH_2-$ R_{25} = $CH(CH_3)CH_2CH_3$ > 80 % and $CH(CH_3)_2$ < 20 %

B





MILBEMYCIN $ALFA_1$ (A_3) R_{25} = CH_3

MILBEMYCIN $ALFA_3$ (A_4) R_{25} = CH_2CH_3

MILBEMYCIN D R_{25} = $CH(CH_3)_2$

MILBEMYCIN OXIME R_{25} = CH_3 and CH_2CH_3

Legend for Figure 1.1.

The structure of three avermectins (A) and four milbemycin (B) molecules are shown. Figure from (Fisher and Mrozik, 1992).

are preliminary at this stage. The work described in the following chapters will attempt to reveal something of the nature of IVM resistance in *C. elegans*.

1.1.1. Behavioural observations.

IVM has been shown to affect movement feeding and reproduction in nematodes. Kass et *al.* (1980) presented work showing inhibition by AVM of movement in *C. elegans*. The 50% effective dose value for paralysis was 100 ng/mL and 100% paralysis is seen at 1 µg/mL AVM (ten minute incubations). Paralysis was also observed in *Ascaris* adults injected with 1.5 µg of AVM. The nature of this paralysis was neither flaccid nor rigid, differing from that of 0.1 µg GABA, which caused relaxation and significant lengthening of the animal. Flaccid paralysis was not produced with AVM injections up to 5 µg, providing evidence that the effects of IVM are distinct from those of GABA in nematodes. Similarly, IVM mediated paralysis of *Haemonchus contortus* larvae is 50% effective at 0.35 µg/mL for a susceptible strain (Gill, Redwin et *al.*, 1991). 50% inhibition of resistant strains was seen at 0.9-3.0 µg/mL IVM.

In contrast to observations on whole body paralysis, IVM-mediated inhibition of feeding in nematodes is achieved at far lower concentrations of drug. Observable effects of IVM on *C elegans* pharyngeal pumping, measured by counting iron particles ingested from the media, were reported at concentrations as low as 5 ng/mL IVM (Avery and Horvitz, 1990). In *Haemonchus contortus*, pharyngeal activity, measured by liquid scintillation counting of whole worms after intake of tritiated inulin (Geary, Sims et *al.*, 1993), was observed at concentrations as low as 0.1 ng/mL. An interesting aspect of the *H. contortus* work was the apparent diphasic response of pharynx-pumping to IVM. A rapid drop in inulin uptake was observed between 0.01 ng/mL IVM and 0.12 ng/mL IVM (around 40% decrease), whereas inulin uptake remained constant between 0.12 and 1.15 ng/mL, dropping again between 1.15 and 11.5 ng/mL IVM (a further decrease of approx. 25%). The nematode pharynx therefore would appear to be much more sensitive to IVM than body musculature, and it appears that the response of the pharynx to IVM may be complex.

Effects of IVM on reproduction in *Dirofilaris immitis* have been reported (Lok, Harpaz et *al.*, 1988). The authors observed an accumulation of late stage embryos (stretched microfilariae) in the uteri of *D. immitis* adults taken from dogs treated with 250 µg/kg IVM. The effect of IVM in this case was slow in onset, with worms from dogs 42 days post-treatment being apparently

normal and a worm from a dog eighty days post-treatment showing increased numbers of late stage embryos *in utero*. The association between IVM and reduced reproductive capability in *D. immitis* must be seen as tentative, as it is based on the observation of only one worm. In *C. elegans*, animals with defective egg-laying capability have been shown to accumulate late stage embryos *in utero* (Trent, Tsung et *al.*, 1983), and therefore the putative effect of IVM on *D. immitis* reproduction could have been due to an effect on egg-laying ability. Reproductive performance has also been observed to be depressed by IVM in *Onchocerca volvulus* (Klager, Whitworth et *al.*, 1993) and patients treated with the drug remain free of infection for months. Female worms dissected from IVM treated patients one year after treatment were less likely to be gravid and more likely to be completely devoid of embryos *in utero* as compared to worms dissected from untreated patients. On closer examination, the number of oocytes was not significantly different between IVM treated and unexposed worms but there were significantly fewer embryos. Male worms similarly did not appear to have fewer sperm or reduced sperm production. The implication therefore is that the efficiency of fertilisation rather than gamete production is adversely affected by IVM. The authors propose that the accumulation of large numbers of arrested embryos *in utero* shortly after IVM treatment impedes sperm transfer. This does not explain reduced numbers of fertilised embryos after one year when most arrested microfilariae have been resorbed by the adult. In *C. elegans*, it has been shown that egg-laying behaviour can be modulated by neuro-active drugs such as serotonin, imipramine, octopamine, phentolamine (Horvitz, Chalfie et *al.*, 1982; Trent, Tsung et *al.*, 1983) and by the presence of bacteria (food source) (Chalfie and White, 1988). Serotonin and imipramine may act directly on the vulval muscle or the neurons which innervate it, but responses to environmental stimuli presumably require input from elsewhere in the nervous system. As the positions and connections of all the neurons in *C. elegans* are known (White, Southgate et *al.*, 1986) and there are neuronal links between the vulval muscles and a number of sensilla; such nervous input may mediate egg-laying responses to environmental stimuli. Effects of IVM could therefore be mediated at sites distant from the vulva and have an effect on egg-laying behaviour. Effects of IVM on fertilisation in *O. volvulus* have also been studied using similar preparations to those used by Klager et *al.* (1993) (Chavasse, Post et *al.*, 1993). This study revealed that numbers of sperm within the seminal receptacles of female *O. volvulus* were significantly decreased by multiple treatments with IVM. The authors proposed that the effect was

most likely due to failure of adult males to mate successfully. Failure of male mating in *C. elegans* has been linked with chemosensory failure (Dusenbery, 1976; Albert, Brown et *al.*, 1981; Perkins, Hedgecock et *al.*, 1986; Liu and Sternberg, 1995; Starich, Herman et *al.*, 1995). IVM might reduce the ability of males to successfully mate via direct action on muscle or neurons involved in mating or via modulation of sensory control of mating. Other studies have shown that IVM has no effect on sperm production in *O. volvulus* (Klager, 1988: referred to by (Chavasse, Post et *al.*, 1993)).

Inhibition of moulting in *Wuchereria bancrofti* third stage larvae *in vitro* by concentrations of IVM above 10 ng/mL has also been reported (Baird, R.Wiwiek et *al.*, 1991). In this experiment 10 ng/mL IVM slightly inhibited moulting, 50 ng/mL produced a response in 50% of worms and 500 ng/mL IVM prevented moulting in 100% of worms.

Effects of IVM on reproductive performance and post-embryonic development could be secondary effects arising from inhibition of feeding by IVM. Alternatively, IVM treatment could primarily affect a central function with implications for feeding, reproduction and moulting. All these behaviours in *C. elegans* have been connected with chemoreception (Horvitz, Chalfie et *al.*, 1982; Trent, Tsung et *al.*, 1983; Golden and Riddle, 1984; White, Southgate et *al.*, 1986; Chalfie and White, 1988; Avery and Horvitz, 1990). A primary effect of IVM on chemoreception therefore could perhaps induce changes in a variety of other behaviours.

1.1.2. Biochemistry.

By showing that AVM (0.1 mg/mL) produced no change in production of lactate by *Dictyocaulus viviparous* or CO_2 by *Trichostrongylus colubriformis*, Kass *et al.* (1980) provided evidence that IVM has no effect on respiration over an eight hour incubation period.

Membrane preparations from *C. elegans* have been shown to bind IVM with high affinity (dissociation constant $=2 \times 10^{-8}$ M) (Turner and Schaeffer, 1989) with typical saturation kinetics expected for a specific binding site. IVM binding is proposed to be a two step process in which a rapidly reversible IVM/receptor complex is initially formed and this is transformed into a much more stable complex after longer incubation times (Turner and Schaeffer, 1989). In the same report, a number of avermectin analogues were analysed and the most potent anthelmintics had the highest affinity for the IVM binding site. GABA and some other neurotransmitters were found to have no effect on IVM binding in this system, and in a separate report (Schaeffer and

Bergstrom, 1988) it was shown that IVM did not interfere with GABA binding in a similar membrane preparation.

Muscle cell-derived vesicles from *Ascaris suum* were used by Martin and Kusel (1992) to show that a fluorescently-labelled IVM probe (4"5,7 dimethyl bodipy proprionylivermectin) was unable to cross lipid membranes. The implication of this work is that IVM-binding proteins must be situated within the cell membrane, with at least some portion of the protein protruding into the outer layer of the lipid bilayer (Martin, Kusel et *al.*, 1992). The receptor need dot be exposed at the cell surface, but must be located within the outer layer of the membrane. By observing the diffusion of ^{3}H-ivermectin across sections of *Ascaris* cuticle, it has been shown that IVM will diffuse through the extracellular cuticle layer (Ho, Geary et *al.*, 1990). In this study, pieces of cuticle were prepared by scraping muscle and hypodermal layers from the inside of cylindrical sections of adult worms. Extraction of the cuticle with chloroform/methanol greatly increased the amount of IVM passing through cuticle preparations. The studies of Martin et *al.* (1992) and Ho et *al.* (1990) indicate that IVM can diffuse through the cuticle but may not cross cell membranes. This suggests that IVM does not diffuse freely into the body of an intact worm.

A novel interaction of IVM with retinol binding proteins from filarial nematode species was observed when a range of anthelmintic compounds were tested for competitive inhibition of retinol and retinoic acid binding in membrane preparations (Sani and Vaid, 1988). Retinol (RBP) and retinoic acid binding proteins (RABP) from a variety of nematode parasites and vertebrate hosts were isolated. IVM-mediated inhibition of the binding of tritiated retinol or tritiated retinoic acid was measured for these proteins. 0.23 ng/mL IVM completely inhibited retinol binding to nematode RBPs whereas binding in a variety of rat tissues, including nodule walls surrounding *Onchocerca volvulus* and *Onchocerca gibsoni*, was uninhibited by IVM. Also, binding of retinol to *Schistosoma mansoni* RBP was unaffected by IVM and schistosomes are not sensitive to IVM. There is, therefore a relationship between affinity of IVM for retinol binding proteins and the species affected by the drug. The relevance of this information when considering the mode of action of the drug is yet to be determined.

1.1.3. Electrophysiology

Fritz, *et al.* (1979) (Fritz, Wang et *al.*, 1979) studied the effect of Avermectin B_{1a} (AVM) on dissected *Ascaris* muscle. They found no effect of the drug on the resting potential of the

tissue and no modified response of the tissue to acetylcholine-induced contraction or GABA induced relaxation.

Kass et *al.* (1980) investigated the effects of AVM on the electrophysiology of *Ascaris* neurons. In *Ascaris*, it was found that AVM blocked excitatory (depolarising) responses in muscle tissue. This effect was reversible using picrotoxin, but the AVM effect remained after washing picrotoxin from buffer, indicating that AVM binding was irreversible. Picrotoxin therefore must bind to the AVM receptor at a separate site to AVM and act antagonistically. The effect of AVM on muscle tissue preparations disappeared when neurons presynaptic to the muscle motor neuron were removed, indicating that the major effect of AVM was on a presynaptic interneuron. AVM also reduced hyperpolarising responses in muscle tissue mediated by the V1 motor neuron and this AVM response was not affected by picrotoxin. So IVM was observed to have major affects on neurons innervating muscle, affects observed on muscle cells were eliminated by removing the neurons and inhibition of both excitatory and inhibitory input suggests an action of the drug in neurons rather than muscle cells. The results were consistent with AVM acting to inhibit neuron activity by hyperpolarising cells, possibly via a chloride ion channel. The authors proposed that AVM acted to inhibit neuron activity by acting as a GABA agonist or by stimulating GABA release. This explanation does not explain their own observation that GABA stimulates the inhibitory motor neuron V1 whilst AVM acts to decrease its activity. It is therefore likely that AVM does act to inhibit neuron excitability, but its action is not likely to effect all GABA receptors (as at least some are excitatory in this system) and may effect receptors which are distinct from GABA receptor chloride ion channels.

Patch-clamp studies using *Ascaris suum* muscle cell vesicles (Martin and Pennington, 1989; Martin and Pennington, 1989; Turner and Schaeffer, 1989) have revealed two ionic channels that are very sensitive to 22,23-dihydroavermectin B_{1a} (IVM). The most sensitive channel found was a cation channel which was selective for large monovalent cations (permeability sequence: Cs>Rb>K>Na>Li>Ca) and which was situated only on the intracellular surface of the cell membrane. This channel could be activated by concentrations of IVM as low as 2.3×10^{-14} g/mL. A chloride ion channel in *A. suum* muscle was sensitive to 2.3×10^{-8} g/mL IVM, the channel was not induced by GABA and the response was not voltage-dependent (Martin and Pennington, 1989). Both these channels responded to IVM after a short delay which was shortened with increasing drug concentration; channel opening times for both were greater than 1 second. Antagonism of GABA-gated chloride ion channels was also studied. This effect was

observed at IVM concentrations in excess of 2.3×10^{-8} g/mL. The action of IVM as an antagonist of GABA-gated chloride channels or an agonist of other chloride ion channels occurred when the outside of the cell membrane was exposed to the drug, whereas cation channel activation by IVM was seen when the inner surface of the cell membrane was exposed. Evidence that IVM remains in the outer lipid layer (Martin, Kusel et *al.*, 1992) makes an action of IVM on the intracellular cation channels in live worms unlikely.

1.1.4. Molecular Biology of the IVM receptor from *Caenorhabditis elegans.*

Arena *et al.* (1991) expressed *C. elegans* mRNA in *Xenopus laevis* oocytes and observed an IVM sensitive chloride ion channel, which was not present in native oocytes. This channel was glutamate-gated and also responded to the glutamate agonist ibotenate. IVM-sensitive current generated by the channel could be blocked by picrotoxin and flufenamic acid. Neither GABA nor glycine activated a similar chloride ion channel. Pharmacologically, the channel appeared homologous with the H-class of glutamate gated chloride channels previously described in arthropods (Lea and Usherwood, 1973; Lea and Usherwood, 1973; Lingle and Marder, 1981; Horseman, Seymour et *al.*, 1988; Dudel, Franke et *al.*, 1989; Wafford and Sattelle, 1989).

The chloride ion channel observed by Arena *et al.* (1991) was fifty percent activated by 103 ng/mL IVM, with threshold concentrations for chloride channel activation close to 11.5 ng/mL IVM (Arena, Liu et *al.*, 1992). Reduced sensitivity of the chloride channel to pharmacologically-inactive IVM analogues was observed (Arena, Liu et *al.*, 1991) ie there was a correlation between in vivo efficacy and ability to open the Cl^- channel, implying that this channel is the biologically relevant drug target. IVM potentiated the action of glutamate on the chloride channel at concentrations below 10 nM: 5.8 ng/mL IVM increased the response to 100 µM glutamate 6.5 fold and 2.3 ng/mL IVM doubled the glutamate response (Arena, Liu et *al.*, 1992).

cDNA clones encoding two subunits of an IVM-sensitive glutamate gated Cl^- channel were isolated by successive rounds of sub-division and expression in *Xenopus* oocytes of pools of clones (Cully, Vassilatis et *al.*, 1994). Products from both clones are able to form a glutamate responsive, homomeric channel but only one of the two gene products binds ivermectin. Functional heteromeric channels sensitive to IVM are formed in oocytes expressing both subunit genes (Cully, Vassilatis et *al.*, 1994). The subunit composition of the native receptor is not known but the cloned receptor has also been shown to be more sensitive to

avermectins and milbemycins with high pharmacological activity and less sensitive to those with low anthelmintic activity, indicating that the *in vitro* effects observed in *Xenopus* oocytes injected with *C. elegans* mRNA reflect *in vivo* biological activity (Arena, Liu et *al.*, 1995).

1.1.5. Mode of action of IVM in nematodes.

The study of the response of nematodes to IVM has been undertaken by a number of laboratories using a variety of techniques, nematode species and drug concentrations. The result has been the accumulation of a large amount of data that is difficult to interrelate.

The most significant effect of IVM appears to be activation of chloride ion currents. The receptor has been cloned and shown to be part of a glutamatergic channel of the H-receptor type. This channel is activated by low concentrations of IVM, and the response to glutamate can be potentiated by IVM at even lower concentrations. Two channel subunits have been cloned, but the total number of subunits may be greater. A number of channel sub-types could feasibly exist, with different subunit numbers and types, and perhaps with differing responses to IVM. Also the cell types expressing different subunits may vary so that IVM receptors with different properties may be presented on the surfaces of different cells. The electrophysiology of the IVM response shows that both neuronal and muscle tissue of *Ascaris* respond to the drug.

GABA-gated chloride channels are generally not opened by IVM except at very high concentrations, whereas glutamate-gated chloride ion channels are very sensitive to IVM. The effects of the drug on pharyngeal function are also mediated at far lower concentrations than effects on body musculature. A glutamatergic neuron (M3) with inhibitory input onto pharyngeal muscle has recently been identified in *C. elegans* (Avery, Davis et *al.*, 1994). It seems likely that at least one site of action for IVM is the pharynx muscle, possibly the pharynx muscle membrane which receives inhibitory glutamatergic input from M3. One reason that a pharyngeal response to IVM might be seen at lower concentrations than effects on body muscle is that the cuticle of the pharynx directly overlays the muscle cells (Albertson and Thomson, 1976) whereas the body musculature is separated from the cuticle by a layer of hypodermal cells (White, 1988). As IVM can cross the cuticle but not the cell membrane, the drug might more easily gain access to the pharynx than the muscle cells of the body wall.

The application of much of the work on IVM is toward parasite control in humans, pets and livestock. For these reasons, studies on the action of the drug are most useful at drug concentrations comparable to those found *in vivo* in treated animals or people. IVM-mediated chloride ion conductances at GABA-gated channels are less likely to be involved in the primary lethal effect of the drug on nematodes *in vivo*, because GABA channels are not sensitive to "physiological" drug concentrations and the effects of IVM on glutamate-gated ion channels are seen at much lower drug concentrations.

Oral administration of IVM with commercially available formulations results in a peak blood concentration of 15-50 ng/mL IVM (over a range of vertebrate species) sometime within the first 1-5 hours (Fink and Porras, 1989). The drug concentration then tails off to less than 10 ng/mL within 12-48 hours after administration of the drug (Fink and Porras, 1989). Subcutaneous injections of IVM typically result in a slow onset but longer lasting peak drug concentrations. Intravenous injections result in much higher peak IVM concentration with a more rapid decline (Fink and Porras, 1989). Studies using high drug concentrations in the µg/mL range therefore are unlikely to be informative for practical applications. Many effects of IVM seen at high concentrations are probably unrelated to the primary anthelmintic activity of the drug.

The observation of IVM binding to retinol binding proteins in filarial nematodes may indicate a higher level of complexity for the mode of action of IVM in nematodes. One explanation for this interaction might be that glutamatergic chloride channels also bind retinol. Both extracellular receptors (such as ion channels) and retinol have been linked with neuronal development; it is possible that a glutamatergic receptor may play a developmental role with retinol as a ligand as well as a role in cell excitability with glutamate as a ligand. It would be possible to investigate such a hypothesis by treating transformed *Xenopus* oocytes expressing the IVM receptor with retinol and looking for inhibition of the IVM response. Effects of IVM on embryogenesis of *D. immitis* and moulting of *W. bancrofti* may indicate a developmental action of the drug in addition to neuromuscular effects.

There are still many unanswered questions with respect to the way in which the effects of IVM on a chloride channel are translated into anthelmintic activity. Also there are many IVM-mediated effects which have been observed *in vitro*, which have unclear relevance *in vivo*. One way of dissecting these problems is to investigate the genetics of IVM resistance. Using mutants with abnormal IVM responses, and analysing these with respect to

electrophysiology, molecular biology, biochemistry or behaviour, the link between IVM activity or behavioural effects and subcellular or cellular responses can be investigated.

1.2. Genetics of ivermectin resistance in *Caenorhabditis elegans* and intestinal parasites of sheep.

van Wyk *et al.* (1989) reported IVM resistance in five strains of *Haemonchus contortus* obtained from grazing properties on which anthelmintic failure had occurred. The resistance was verified using the faecal egg count reduction test (27.5%-67% reduction in drug efficacy) (Wyk, Malan et *al.*, 1989), a larval paralysis assay (3-8 times more resistant than sensitive control) (Gill, Redwin et *al.*, 1991) and an *in vitro* larval development assay (6-7 times more resistant than controls) (Lacey, Redwin et *al.*, 1990). One of the resistant strains isolated from South Africa by van Wyk *et al.* (1989), the White River Strain, was analysed by Martin and Turney (1992) in order to establish the mode of inheritance of the resistance gene(s). By analysing the resistance of F1 and F2 worms produced in a cross between White River and a susceptible strain it was determined that resistance in the White River strain was conferred by a single dominant mutation. A comparison of the resistance of F1 worms produced from resistant males crossed with sensitive females, and F1 worms produced from sensitive males crossed with resistant females, revealed no difference between the reciprocal crosses; indicating that the resistance gene was not sex-linked.

An Australian ivermectin resistant strain of *H. contortus* have also been analysed (LeJambre, 1993). The CAVR strain was isolated from the field through selection of progeny from a small number of females remaining after IVM treatment of sheep. CAVR adults were resistant *in vivo* to over four times the recommended dose of IVM and larvae were observed to be approximately twice as resistant to IVM in growth media when tested using a larval development assay as described in Lacey, et *al.* (1990) (LeJambre, Gill et *al.*, in press). Using the same methods as Martin and Turney (1992), LeJambre (1993) established that the IVM resistance of the CAVR strain was also conferred by a single dominant mutation that was most likely autosomal. The resistance of adults in treatment and slaughter assays was sex-influenced with males slightly more sensitive to IVM than females (LeJambre, Gill et *al.*, in press).

Thus, genetic analysis of resistance has shown that it is conferred by a single dominant allele in two strains. Modelling studies predict that selection of dominant alleles will be rapid, even from very low starting frequencies in "susceptible"

populations (Barnes, Dobson et *al.*, 1995). These predictions have been borne out by experience: the alleles are present at low starting frequencies (LeJambre Pers. Comm., 1995) and selection to levels sufficient to cause control failure has required only 10-20 generations.

IVM resistance in *C. elegans* had been analysed before the commencement of the work discussed in this thesis (Kim and Johnson, 1991; Novak and Vanek, 1992). The number of loci involved in IVM resistance at various drug concentrations and the mode of inheritance of many IVM resistance mutations had been ascertained. The frequency of spontaneous and ems (ethyl methanesulfonate) -induced mutations for IVM resistance had also been determined. The resistant strains studied in these reports however all carried recessive mutations. The experiments described herein are aimed at investigating dominant IVM resistant mutants, as these might be analogous to mutations carried by IVM resistant strains of *H. contortus*.

Wild-type N2 is sensitive to 2 ng/mL and above and many IVM resistant strains have been generated by growing *C. elegans* on agar media containing IVM at concentrations above 5 ng/mL (Day, Kim et *al.*, 1989; Kim and Johnson, 1991; Johnson and Clover, 1995). Three classes of IVM resistance were established on genetic and pharmacological grounds.

Mutations imparting low level resistance (5-25 ng/mL IVM in Nutrient Growth Media-agar (NGM)) were very frequent and genetic mapping and complementation studies indicated that there were approximately 30 loci involved (Johnson and Clover, 1995). Mutations conferring resistance to this range of IVM concentrations were found to occur in 1 out of every 204 mutagenised genomes (0.05 M ethyl methane sulfonate) and to occur spontaneously under laboratory conditions at a rate of 1.7×10^{-5} (Kim and Johnson, 1991). The majority of these mutations were recessive and had no apparent pleiotropic phenotypes. Mutations at two of the IVM resistance loci, however, conferred a severe uncoordinated phenotype and were later shown to be alleles of *unc-33* and *unc-44*. Another Unc mutation conferring resistance to 5 ng/mL IVM was also identified and mapped to linkage group I.

Medium level resistance mutations (10-50 ng/mL IVM) were less common and many were characterised by a pleiotropic Unc (uncoordinated) phenotype (Kim and Johnson, 1991; Johnson and Clover, 1995) Unc medium level resistance strains were found to carry mutations at the *unc-1*, *unc-9* and *unc-7* loci (Johnson, Pers. Comm., 1994). Some of these mutations can also confer IVM resistance to much higher concentrations (100-1000 ng/mL IVM) in combination with *avr-15*(nr395). Some *unc-1*, *unc-7* and *unc-9*

alleles do not confer resistance alone but still interact with *avr-15*, and others confer no resistance to IVM at all (Johnson, Pers. Comm., 1994): ie there is an allelic series of Avr/Unc interactions.

Mutants resistant to high levels of IVM (100 ng/mL) have been isolated, but mutations at two of three loci (*avr-14*, *avr-15* and *avr-20*) are required for resistance to high levels of IVM. These double events are very rare compared to mutagenesis rates for low level resistance, being less than 1 in 10^9 (spontaneous) and approximately one in 30 x 10^6 genomes (after EMS) (Kim and Johnson, 1991). Each mutation alone does not confer any drug resistance, suggesting redundant function of their gene products; possibly as subunits of a multimeric drug receptor molecule (Kim and Johnson, 1991).

As discussed above, a major effect of IVM may be inhibition of pharynx function. The electrical activity of the pharynx can be monitored using an apparatus which measures the current flowing out of the mouth of live worms; the resulting trace is named an electropharyngeogram (Raizen and Avery, 1994). *avr-15* mutations have been shown to eliminate inhibitory potentials mediated by the M3 glutamatergic neuron in the pharynx (Avery, Davis et *al.*, 1994), and *avr-15* is therefore likely to encode a component of the M3-pharynx IVM-sensitive pathway. *avr-14*, *avr-20*, *unc-1*, *unc-7* and *unc-9* mutations however were not observed to have any effect on electropharyngeograms.

All the IVM resistance mutations used in the studies reviewed above were isolated by growing worms on media containing IVM, thus achieving a continuous exposure of the animals to IVM throughout their growth. An alternative approach was taken by Novak and Vanek (1992) (Novak and Vanek, 1992), who exposed worms to high concentrations of IVM for short periods, selecting resistant strains from the progeny of surviving individuals. The dose required to kill wild type (N2) worms under these conditions was 200 ng/mL IVM, and two strains were selected with resistance to 3000 ng/mL (strain D5) and 2500 ng/mL IVM (strain D6). Genetic analysis of the strains generated will reveal the nature of the genes involved and establish whether any of these correspond to IVM-resistance mutations generated by other workers.

The method of selection used by Novak and Vanek (1992) closely approximates the kinetics of IVM exposure to which nematode parasites of livestock are exposed, however the concentrations used are higher than those seen *in vivo*. Typically, only adults come into contact with IVM and exposure consists of a rapidly achieved peak of drug concentration within the host followed by a slow decline in concentration over many weeks (Fink and Porras, 1989). The method used in the study described

above involves treating worms with IVM at high concentrations in liquid, removing small aliquots to be placed on agar plates; thus diluting the drug concentration after the initial exposure. The progeny of surviving worms were transferred to drug-free media on which they were allowed to grow and produce a third generation which was again selected for drug resistance: three cycles were used to establish resistant strains. Unfortunately this method is unattractive for further work. The method is more complicated, the larger number of steps involved making exact duplication of the published procedures difficult and the time used in isolating strains inconveniently long.

To investigate possible homologies between mutations imparting resistance in *C. elegans* and mutations imparting resistance in *H. contortus*, dominant IVM resistance mutations from *C. elegans* were isolated, genetically mapped and their pleiotropic phenotypes analysed extensively (Chapter 3). This analysis was expected to provide candidate loci for the generation of molecular probes which could be used in cloning IVM resistance genes from *H. contortus*. Additionally, it was hoped that identification of phenotypes associated with resistance would assist investigation of the mode of action of the drug. Drug concentrations chosen for use in this study were in the same range as those encountered by parasites *in vivo* (ie. 5 ng/mL - 20 ng/mL). This was in order to generate and study mutations imparting resistance levels that might be found in parasitic nematodes. Also, neither resistant parasites nor low-level *C elegans* resistant strains show decreased binding of the drug, indicating that these two groups of resistance mutations may be homologous (Johnson Per. Comm., 1990).

To answer more general questions about the mechanism of IVM resistance in nematodes, a study screening a large number of characterised mutations for IVM resistance or super sensitivity was undertaken (Chapter 2). By associating the known phenotypes and/or functions of identified resistance genes with the observed IVM phenotype, it was hoped that a mechanism of drug resistance could be put forward. This hypothesis then would provide a framework for further studies aimed at understanding IVM resistance (Chapter 5). One type of further study which could enhance knowledge of the mechanism of resistance is the characterisation of mutations which modify the drug resistance conferred by resistance loci. To this end, preliminary studies into mutations which suppress IVM resistance as conferred by the major class of low level IVM resistance loci were undertaken (Chapter 4). As low level resistance was found to be connected to sensory defects in *C. elegans* a description of the sensory structures of this nematode is given below.

1.3. Sensory structures and their functions in *Caenorhabditis elegans* .

As mutants with altered amphid structure are IVM resistant, (Chapter 2) a description of the sensory structures of *C elegans* and their functions is presented here. Various behavioural effects of ivermectin have been noted (see above) and one possible link between these effects could be a direct effect of the drug on a central nervous function. In particular, the ability of an animal to perceive environmental stimuli is likely to affect a variety of behaviours and an effect of IVM on sensory function could explain many observed effects of the drug. In addition, an increase in stimulatory input, or a decrease in inhibitory input, onto processes down-regulated by IVM, could possibly change the dose of IVM needed to inhibit those processes, thereby creating an opportunity for mutations which alter nervous or humoral functions to modulate the effects of IVM, or indeed other drugs.

C. elegans possesses an array of sensory structures. Of 302 neurons in the nervous system of the hermaphrodite, 100 have proven or putative sensory function (White, Southgate et *al.*, 1986; Chalfie and White, 1988). The male has 381 neurons and the majority of the additional neurons are sensory. A large part of the nervous system of *C. elegans* appears to have functions in perception of the nematode's environment, but the majority of putative sensory neurons in the nematode have been identified as such solely by morphological criteria. Alteration of neuron function via mutation or abolition of function via laser ablation, allows sensory role(s) of neurons to be established by analysing effects on behaviour, this type of work has been completed for a smaller subset of the putative sensory neurons in *C. elegans*.

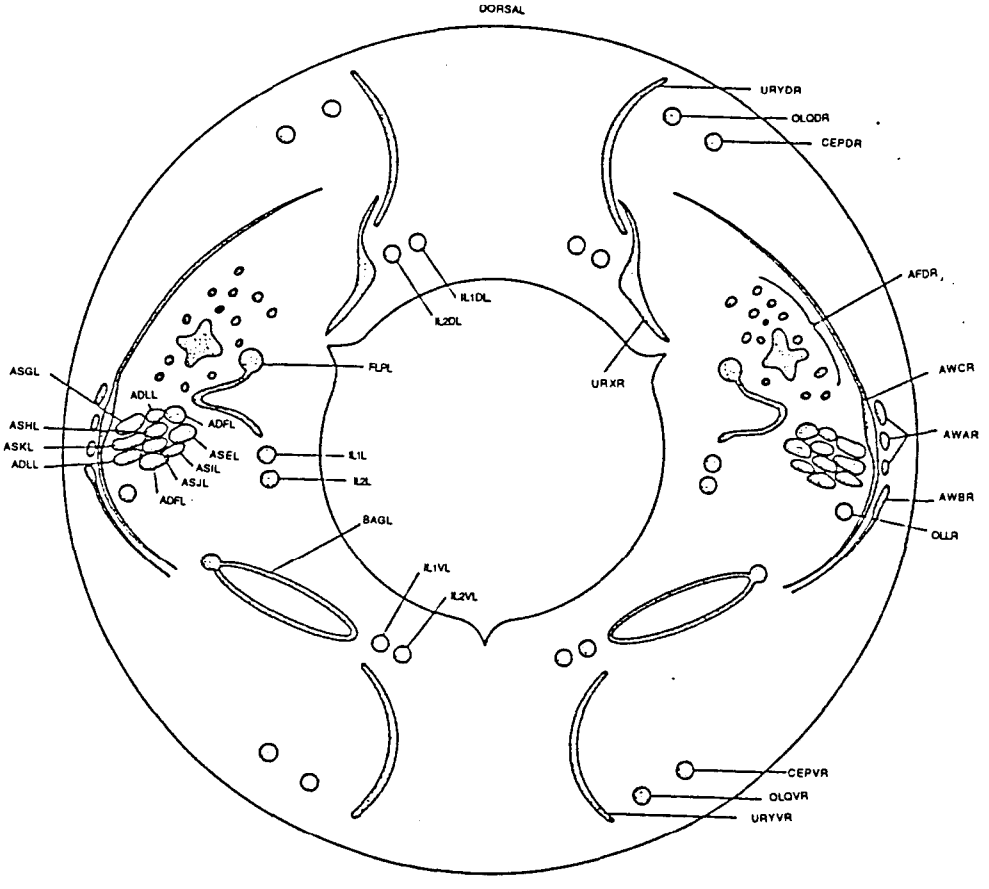
The best characterised sensory components of *C. elegans* are the sensilla of the head (Albert and Riddle, 1983; Bargman and Horvitz, 1991; Perkins, Hedgecock et *al.*, 1986; Ward, Thomson et *al.*, 1975), the phasmids and the specialised touch receptors which run along the body beneath the cuticle (Chalfie and Thomson, 1979; Chalfie, Sulston et *al.*, 1985; Chalfie, Dean et *al.*, 1986). The sensory structures associated with the male tail have also been analysed (Sulston, Albertson et *al.*, 1980; Liu and Sternberg, 1995) and a number of neurons in the pharynx are putative sensory-motor neurons and/or sensory interneurons (Albertson and Thomson, 1976). The other major class of sensory structures are the anterior and posterior deirids and there are also a range of other neurons with putative sensory function (White, Southgate et *al.*, 1986).

1.3.1. Anterior sensilla, phasmids and deirids.

The anterior sensory anatomy of *C. elegans* consists of a pair of amphids, four cephalic sensilla, six inner labial sensilla and six outer labial sensilla (Ward, Thomson et *al.*, 1975) (See figure 1.2.). All these sensilla consist of a channel formed by two supporting cells; the sheath cell and the socket cell. The socket cell ending lies directly beneath the cuticle and in sensilla that are open to the exterior through a cuticular pore, the interior surface of the channel formed by the socket cell is lined with cuticle. The sheath cell ending lies under the socket cell and is usually larger with multiple invaginations. The sheath cell also may secrete material into the channel which it forms. The channel formed by the sheath and socket cells contains one or more ciliated dendrite endings which may be exposed to the exterior through a pore in the cuticle or embedded in the cuticle if there is no opening. Other dendritic endings may invaginate the sheath cell.

The pattern of anterior sensory structures is highly conserved in nematodes, differences between species mainly affect the external appearance of the sensilla or the number of neuronal processes within the sensilla (Maggenti, 1981; Bird and Bird, 1991), while the number and position of the sensilla remains constant. The

Figure 1.2. - Anterior arrangement of sensory neurons in *C. elegans* (White *et. al.*, 1986).



Amphid channel cilia neurons:
ASE, ASG, ASH, ASI, ASJ, ASK, ADL, ADF.
Amphid channel cilia (ending within the sheath cell):
AWA, AWB, AWC.
Amphid neuron (non-channel) ending in sheath cell:
AFD.
Inner labial sensilla neurons:
IL1, IL2.
Outer labial sensilla neurons:
OLQ, OLL.
Cephalic sensilla neurons:
CEP.
Free ciliated neuron endings:
FLP, BAG.
Free neuron endings with specialised flattened endings:
URX, URY.
N.B. - **D** at the end of neuron name means dorsal, **L** left, **R** right and **V** ventral.

arrangement of the anterior sensory neurons of *C. elegans* is illustrated in figure 1.2. Diagrams of the amphid, inner labial, outer labial and cephalic sensilla are provided in figure 1.3.

Apart from the anterior sensilla, the most studied sensilla of nematodes are the phasmids and the deirids. *C. elegans* has a pair of phasmids near the end of the tail in both hermaphrodites and males. Also two pairs of deirids, one anterior and one posterior are present. These structures are also common to most nematodes (Maggenti, 1981; Bird and Bird, 1991) and have the same basic structure of sheath and socket cells forming a channel along which ciliated neuron endings run. The positions of the deirids and phasmids are given in figure 1.4.

1.3.1.1. Amphids

Each amphid of *C. elegans* consists of a socket cell, sheath cell and twelve sensory neurons. There are eight neurons which run along the amphid channel and which are exposed to the exterior, two of these produce paired cilia (ADF, ADL) and the remainder have a single cilium (ASE, ASG, ASH, ASI, ASJ, ASK), so that there are ten cilia in the amphid channel (Ward, Thomson et *al.*, 1975). There are also three dendrites which have ciliated endings in the sheath channel, but which diverge from the channel and have their endings embedded in the sheath cell. Within the sheath cell one of these neurons forms five branches (AWA), another forms two branches (AWB) and a third (AWC) creates a large sheet-like "wing" structure which spans across the sheath cell from within. Finally there is a neuron ending which does not run in the amphid channel at all, but penetrates the sheath cell from outside and forms multiple microvilli or "finger" structures (AFD). The different amphid neurons and their structures are shown in figure 1.2. and some of their characteristics are summarised in table 1.1.

A characteristic of some amphid channel cilia is that they can take up FITC (fluorescein isothiocyanate) or the lipophilic dye DiO (3,3'-dioctadecyloxacarbocyanine perchlorate - (Haugland, 1992)) and accumulate them in the cell bodies further posterior in the animal (Hedgecock, Culotti et *al.*, 1985; Starich, Herman et *al.*, 1995), thus allowing the cells to be visualised under fluorescent light. The neurons ADL, ASH, ASI, ASJ, ASK and ADF fill with FITC (Hedgecock, Culotti et *al.*, 1985) while DiO is taken up by the neurons ADL, ASH, ASI, ASJ, ASK and AWB (Starich, Herman et *al.*, 1995) (see table 1.1.). Mutants which fail to take up these dyes or which exhibit fainter than normal staining are said to have a Dyf (for Dye Filling Defective) phenotype (Hedgecock, Culotti et *al.*,

1985; Perkins, Hedgecock et *al.*, 1986; Hall and Hedgecock, 1989; Starich, Herman et *al.*, 1995).

The function of the amphid in chemoreception has been widely examined. Mutations affecting amphid structure also inhibit the ability to orient toward attractants such as sodium ions (Che) (Lewis and Hodgkin, 1977; Albert, Brown et *al.*, 1981; Starich, Herman et *al.*, 1995) or volatile compounds such as Pyrazine (Bargmann, Hartwieg et *al.*, 1993; Sengupta, Colbert et *al.*, 1994), the ability to avoid concentrations of high osmotic strength (Osm) (Culotti and Russell, 1978; Perkins, Hedgecock et *al.*, 1986) and the ability of males to find hermaphrodites and so mate successfully (Perkins, Hedgecock et *al.*, 1986; Starich, Herman et *al.*, 1995). A large amount of work has been done regarding mutations which change responses to chemoattractants and repellents in *C. elegans* (Dusenbery, Sheridan et *al.*, 1975; Dusenbery, 1976; Lewis and Hodgkin, 1977; Culotti and Russell, 1978; Dusenbery, 1980; Dusenbery, 1980; Albert, Brown et *al.*, 1981; Perkins, Hedgecock et *al.*, 1986; Bargmann, Hartwieg et *al.*, 1993; Sengupta, Colbert et *al.*, 1994; Starich, Herman et *al.*, 1995). A summary of the majority of studies is presented in table 1.2. The table shows the loci for which chemotaxis (or amphid morphology) mutants have been characterised. Only the most commonly used attracting and repellent compounds are given in the table. The response of wild type and mutant animals has also been analysed for *E. coli*, pyridine, lysine, OH^-, CO_2, and serotonin which are attractants and garlic extract, extract of dead *C. elegans*, acid and alkali solutions which are repellents. Also some chemotaxis-deficient mutants which have not been tested against many different chemicals or for which ultrastructural studies have not been completed have been left off the table; for example *unc-33*, *unc-44*, *unc-101*, *unc-31*, *unc-86*, *lin-32*, *vab-3*.

Some of the work summarised in table 1.2. has not been complemented with electron microscope reconstruction. A number of mutants including *tax-2*, *tax-3*, *tax-4*, *tax-5* and *tax-6* are defective for attraction to NaCl, OH^-, CO_2, pyridine and cAMP (Dusenbery, 1976). None of these have been analysed for ultrastructural defects, but Perkins *et al.*, (1986) reported that none of these are defective in the uptake of FITC; presumably, these mutants either affect non-amphid chemosensory neurons, or the defects do not result in abolishment of FITC uptake in amphid neurons and are good candidates for mutations downstream in

Table 1.2. - Some chemosensory mutants of *C. elegans* : their morphological and behavioural characteristics.

	Morphology of amphid neuron endings					Chemotaxis toward aqueous compounds				Chemotaxis toward volatile compounds						Avoidance of:				
Gene	Channel Cilia	AWA AWB AWC	AFD	Dyf	Daf	Na^+	Cl^-	NaCl	cAMP	Benz.	2-But.	Isoamyl Alcohol	Thiazole	Pyrazine	Diacetyl	High Osmotic Strength	D-trp	MM	Other sensilla	Comments
che-1	bundling different[a]	AWB branches abnormal[a]	malformed[a]	no[d]	NA	-[a]	-[a]	NA	NA	+[b]	+[b]	+[b]	NA	NA	NA	NA	NA	NA	IL2 cilia sometimes shortened or missing[a]	Variably thermotaxis deficient. Also is ts sterile[a]
che-2	shorter[a]	atrophy of wing structures[a]	shorter, microvilli normal[a]	yes[d]	d[a]	-[a]	-[a]	NA	NA	-[b]	-[b]	-[b]	NA	NA	NA	-[d]	NA	0[d] -[a]	All anterior cilia shortened[a]	smaller than wild type[a]
che-3	shorter[a]	atrophy of wing structures[a]	shorter, microvilli normal[a]	yes[d]	d[a]	-[a,g]	-[a,g]	NA	-[a,g]	-[b]	-[b]	-[b]	NA	NA	NA	-[d k]	-[g]	0-3[d] -[a]	All anterior cilia shortened[a]	
che-5	NA	NA	NA	no[d]	NA	+[a]	-[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	other anterior sensory structures normal[a]	erratic movement[a]
che-6	normal[a]	normal[a]	normal[a]	no[d]	r[a]	-[a]	-[a]	NA	NA	+[b]	+[b]	+[b]	NA	NA	NA	NA	NA	NA	IL2 cilia often malformed or missing[a]	
che-7	normal[a]	normal[a]	normal[a]	no[d]	NA	-[a]	-[a]	NA	NA	-[b]	-[b]	-[b]	NA	NA	NA	NA	NA	NA	other anterior sensory structures normal[a]	smaller than wild type[a]
che-10	malformed[d]	shorter, wing structures normal[d]	shorter, microvilli normal[d]	yes[d]	sd[d]	NA	NA	±[d]	NA	+[b]	+[b]	+[b]	NA	NA	NA	-[d]	NA	0[d]	Striated rootlets of IL1, OLQ & BAG are missing	
che-11	abnormal cytoplasm[d]	atrophy of wing structures[d]	normal[d]	yes[d]	d[d]	NA	NA	±[d]	NA	-[b]	-[b]	-[b]	NA	NA	NA	-[d]	NA	0-2[d]	CEP cilia shorter, other cilia have minor defects[d]	Amphid cilia in this mutant contain abnormal dark material[d]
che-12	normal[d]	normal[d]	normal[d]	yes[d]	sd[d]	NA	NA	+[d]	NA	+[b]	+[b]	+[b]	NA	NA	NA	-[d]	NA	3[d]	Other anterior sensory structures normal[d]	Matrix surrounding amphid cilia not produced[d]
che-13	shorter[d]	atrophy of wing structures[d]	shorter, microvilli normal[d]	yes[d]	d[d]	NA	NA	±[d]	NA	-[b]	-[b]	-[b]	NA	NA	NA	-[d]	NA	0[d]	All anterior cilia shortened[d]	
che-14	normal[d]	normal[d]	normal[d]	no[d]	sd[d]	NA	NA	±[d]	NA	+[b]	+[b]	+[b]	NA	NA	NA	±[d]	NA	4[d]	cuticle embedded structures in mechanosensilla are abnormal[d]	Extra neurons: CEP, ADE and PDE, stain with FITC[d]
daf-6	normal[l]	normal[l]	microvilli displaced[l]	yes[d]	d[l]	-[l]	-[l]	NA	NA	+[b]	+[b]	+[b]	NA	NA	NA	-[d l]	NA	4[d]	lateral outer labial cilia shortened[l]	sheath cell hypertrophy seen in all sensilla[l]
daf-10	shorter and malformed[l]	AWA missing, AWB, AWC, rearranged[l]	normal[l]	yes[d]	d[l]	-[l,g]	-[l,g]	NA	-[g]	-[b]	-[b]	-[b]	NA	NA	NA	-[d l k]	-[g]	0-1[d]	CEP cilia enlarged and abnormal[l]	
daf-19	no cilia[d]	no cilia[d]	shorter, microvilli normal[d]	yes[d]	c[d]	NA	NA	-[d]	NA	NA	NA	NA	NA	NA	NA	-[d]	NA	0[d]	All sensory cilia are missing[d]	The only Dyf mutation known to be Daf-c[d]

dyf-1	shorter[j]	normal[j]	normal[j]	yes[f]	sd[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	–[j]	NA	2[f]	other sensory structures normal[j]	
dyf-2	NA	NA	NA	yes[f]	d[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4[f]	NA	
dyf-3	NA	NA	NA	yes[f]	d[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2-4[f]	NA	
dyf-4	NA	NA	NA	yes[f]	sd[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3-4[f]	NA	
dyf-5	NA	NA	NA	yes[f]	sd[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2[f]	NA	
dyf-6	NA	NA	NA	yes[f]	d[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3-4[f]	NA	
dyf-7	NA	NA	NA	yes[f]	d[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1[f]	NA	
dyf-8	NA	NA	NA	yes[f]	d[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1[f]	NA	
dyf-9	NA	NA	NA	yes[f]	sd[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1-3[f]	NA	
dyf-10	NA	NA	NA	yes[f,m]	+[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4[f]	NA	
dyf-11	NA	NA	NA	yes[f]	sd[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3[f]	NA	
dyf-12	NA	NA	NA	yes[f,m]	+[fm] c[m]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3[f]	NA	
dyf-13	NA	NA	NA	yes[f]	+[f]	NA	-[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2[f]	NA	
mec-1	bundling different[a]	normal[a]	normal[a]	no[d]	NA	±[a]	+[a]	NA	NA	NA	NA	NA	NA	NA	NA	+[d]	NA	2[d] +[a]	abnormally large number of vesicles in sheath cells of all head sensilla[a]	
mec-2	normal[a]	normal[a]	normal[a]	no[d]	NA	-[a]	-[a]	NA	NA	-[b]	-[b]	-[b]	NA	NA	NA	NA	NA	+[a]	other anterior sensory structures normal[a]	
mec-8	misdirected[d]	normal[d]	normal[d]	yes[d]	+[d]	NA	NA	+[d]	NA	NA	NA	NA	NA	NA	NA	NA	+[d]	NA	2-3[d]	other anterior sensory structures normal[d]	mechanosensory defective.
mor-1	normal[a]	normal[a]	normal[a]	NA	NA	+[a]	-[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	other anterior sensory structures normal[a]	
mor-2	normal[a]	normal[a]	normal[a]	NA	NA	±[a]	-[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	other anterior sensory structures normal[a]	
odr-1	normal[b]	normal[b]	normal[b]	no[b]	NA	+[b]	+[b]	NA	+[b]	-[b]	-[b]	-[b]	+[b]	+[b]	+[b]	+[b]	NA	NA	other anterior sensory structures normal[b]	
odr-2	normal[b]	normal[b]	normal[b]	no[b]	NA	+[b]	+[b]	NA	+[b]	-[b]	+[b]	-[b]	+[b]	+[b]	+[b]	+[b]	NA	NA	other anterior sensory structures normal[b]	

odr-3	normal[b]	AWC is reduced[b]	normal[b]	no[b]	NA	+[b]	+[b]	NA	+[b]	-[b]	-[b]	+[b]	±[b]	±[b]	-[b]	-[b]	NA	NA	other anterior sensory structures normal[b]	
odr-4	normal[b]	normal[b]	normal[b]	no[b]	NA	+[b]	+[b]	NA	+[b]	-[b]	+[b]	+[b]	-[b]	+[b]	-[b]	+[b]	NA	NA	other anterior sensory structures normal[b]	
odr-5	normal[b]	normal[b]	normal[b]	no[b]	NA	+[b]	+[b]	NA	+[b]	-[b]	-[b]	-[b]	+[b]	+[b]	+[b]	+[b]	NA	NA	other anterior sensory structures normal[b]	
odr-7	normal[c]	normal[c]	normal[c]	NA	NA	+[c]	+[c]	NA	+[c]	+[b]	+[b]	+[b]	+[b]	-[b]	-[b]	+[c]	NA	NA		
osm-1	shorter[d]	atrophy of wing structures[d]	shorter, microvilli normal[d]	yes[d]	d[d]	NA	NA	-[d]	NA	-[b]	-[b]	-[b]	NA	NA	NA	—[dk]	-[g]	0-2[d]	All anterior cilia shortened[d]	
osm-3	shorter[d]	normal[d]	normal[d]	yes[d]	d[dm]	NA	NA	±[d]	NA	+[b]	+[b]	+[b]	NA	NA	NA	—[dk]	-[g]	3-4[d]	Other anterior sensory structures are normal[d]	
osm-5	shorter[d]	atrophy of wing structures[d]	shorter, microvilli normal[d]	yes[d]	d[d]	NA	NA	-[d]	NA	-[b]	-[b]	-[b]	NA	NA	NA	—[dk]	-[g]	1[d]	All anterior cilia shortened[d]	
osm-6	shorter[d]	atrophy of wing structures[d]	shorter, microvilli normal[d]	yes[d]	d[d]	NA	NA	-[d]	NA	-[b]	-[b]	-[b]	NA	NA	NA	—[dk]	-[g]	1[d]	All anterior cilia shortened[d]	defects are less severe than for *che-13*, *osm-1* & *osm-5* [d]
tax-1	NA	NA	NA	no[d]	NA	-[i]	-[i]	-[ik]	-[i]	NA	NA	NA	NA	NA	NA	NA	-[g]	NA	NA	
tax-2	NA	NA	NA	no[d]	NA	-[i]	-[i]	-[ik]	-[i]	-[b]	-[b]	-[b]	NA	NA	NA	NA	-[g]	NA	NA	
tax-3	NA	NA	NA	no[d]	NA	-[i]	-[i]	-[ik]	-[i]	NA	NA	NA	NA	NA	NA	NA	-[g]	NA	NA	
tax-4	NA	NA	NA	no[d]	NA	-[i]	-[i]	-[ik]	-[i]	-[b]	-[b]	-[b]	NA	NA	NA	NA	-[g]	NA	NA	
tax-5	NA	NA	NA	no[d]	NA	-[i]	-[i]	-[ik]	-[i]	NA	NA	NA	NA	NA	NA	NA	±[g]	NA	NA	
tax-6	NA	NA	NA	no[d]	NA	-[i]	-[i]	-[ik]	-[i]	-[b]	-[b]	-[b]	NA	NA	NA	NA	-[g]	NA	NA	
ttx-1	normal[d]	normal[d]	microvilli missing from AFD[d]	no[d]	NA	NA	NA	+[e]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	other sensory structures normal[d]	thermosensory defective[e]

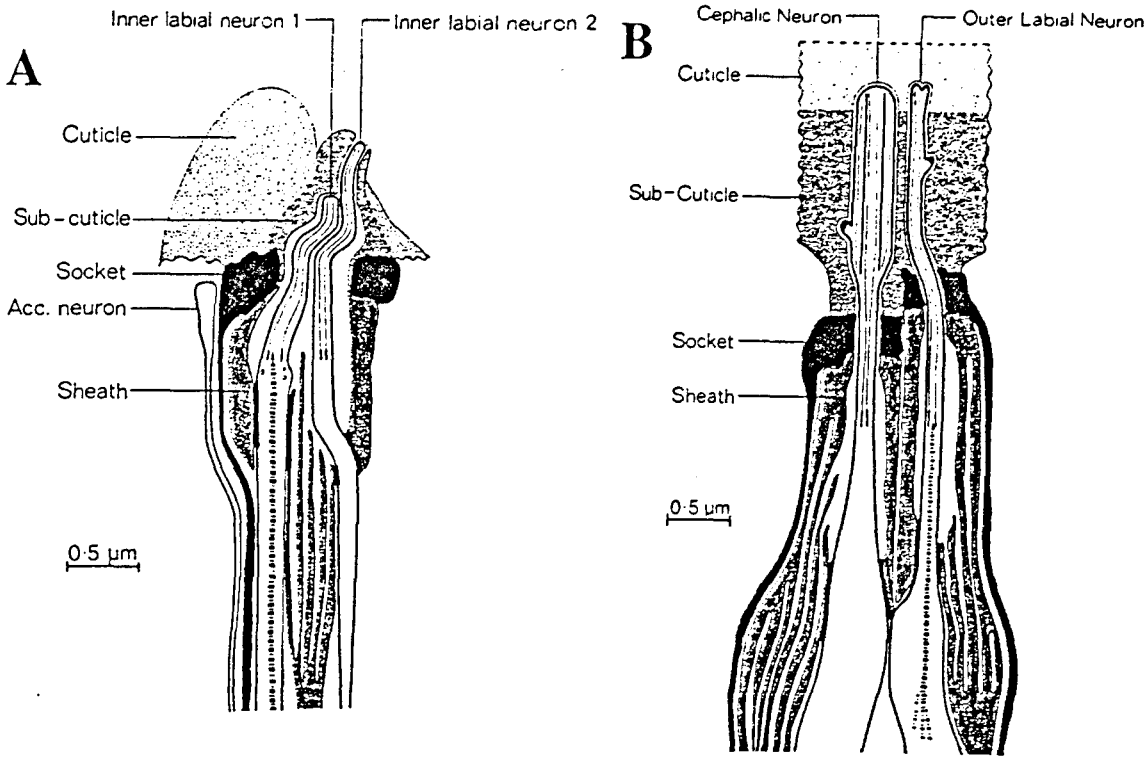
References:
[a] - (Lewis and Hodgkin, 1977) [b] - (Bargmann, Hartwieg *et. al.*, 1993) [c] - (Sengupta, Colbert *et. al.*, 1994) [d] - (Perkins, Hedgecock *et. al.*, 1986)
[e] - (Hedgecock and Russell, 1975) [f] - (Starich, Herman *et. al.*, 1995) [g] - (Dusenbery, 1980) [h] - (Dusenbery, Sheridan *et. al.*, 1975)
[i] - (Dusenbery, 1976) [j] - (DeRiso, Ristoratore *et. al.*, 1994) [k] - (Culotti and Russell, 1978) [l] - (Albert, Brown *et. al.*, 1981) [m] - This study
[n] - (Shakir, Miwa *et. al.*, 1993)

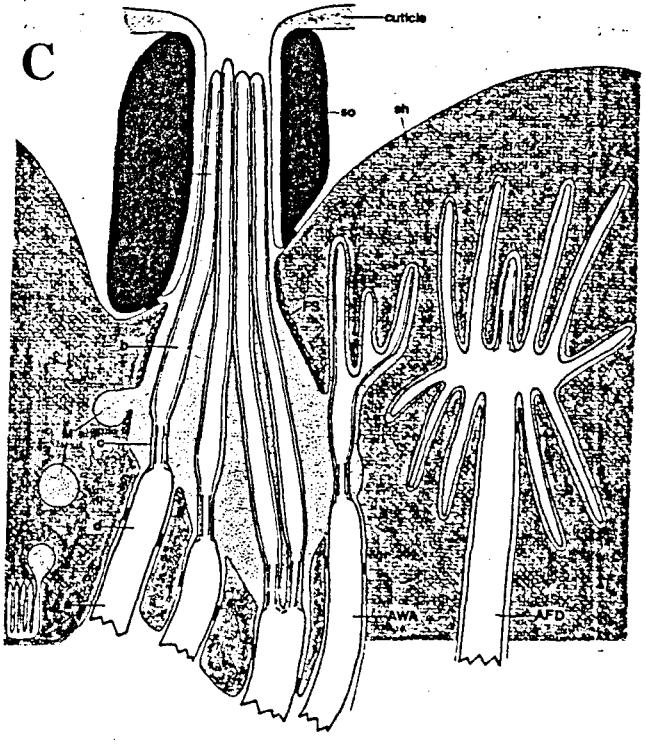
Dauer Larvae Formation: + - wild type with respect to dauer formation, **sd** - slightly dauer formation defective, **d** - dauer formation defective, **r** - defective in recovery from dauer larvae, **c** - constitutive dauer larvae formation.

Chemotaxis and avoidance assays: + - wild type response, - - defective response, ± - intermediate response. **Benz.** = Benzaldehyde, **2-But.** = 2-Butanone, **D-trp** = D-tryptophan

Male Mating (MM): **4** - 30-100% of wild type mating efficiency, **3** - 10-30% of wild type mating efficiency, **2** - 1-10% of wild type mating efficiency, **1** - less than 1% of wild type mating efficiency, **0** - no matings recorded. Where a range of mating efficiencies is given, the range reflects the allelic variation in mating efficiency score (there is only one score for each strain).

Figure 1.3. - Appearance of amphid (Perkins *et. al.*, 1986), inner labial, outer labial and cephalic sensilla (Ward *et. al.*, 1975) from *C. elegans*.





Legend for figure 1.3.

Idealised diagrams showing the appearance of Amphid (Perkins *et. al.*, 1986), inner labial, outer labial and cephalic sensilla from *C. elegans*.

A Right sub-ventral inner labial sensillum; the six inner labial sensilla are identical except that only the subventral pair have the accessory neuron (From Ward *et. al.*, 1975).

B Outer labial and cephalic sensilla (From Ward *et. al.*, 1975).

C Amphid: **so** - socket cell, **sh** - sheath cell, **M** - matrix material excreted by the sheath cell into the amphid channel, **FS** - filamentous scaffold underlying the dark-staining non-cuticular lining of the anterior part of the sheath channel. Only three of the eight channel neurons are shown, along with AFD and AWA. AWB and AWC run along the amphid channel and invaginate the sheath cell similarly to AWA. (figure from Perkins *et. al.*, 1986).

sensory transduction pathways. Also a large group of mutants defective in amphid dye-filling (*dyf* mutants) have been identified without ultrastructural reconstruction being done (Starich, Herman et *al.*, 1995). Mutations at many of these loci are defective in attraction to Cl^-, with defects ranging from severe (almost completely abolished for alleles of *dyf-3* and *dyf-7*) to mild (slight reduction in chemotaxis in *dyf-13*). These most likely are additional mutations which result in abnormal amphid cilia, abolishing dye filling and impeding chemotaxis.

Defective amphid ultrastructure is the most common morphological phenotype observed in a large set of mutants with abnormal chemosensory behaviour (*che-1*, *che-2*, *che-3*, *che-5*,*che-6*, *che-7*,*che-10*, *che-11*, *che-12*, *che-13*, *che-14*, *daf-6*, *daf-10*, *daf-19*, *osm-1*, *osm-3*, *osm-5*, *osm-6*, *mec-8*, *vab-3*, *mec-1*, *mec-2*, *mor-1*, *mor-2*, *odr-1*, *odr-2*, *odr-3*, *odr-4*, *odr-5*, *odr-7* and *dyf-1* - see table 1.2.). This set can be subdivided using several additional criteria, the most useful of which is dye-filling (Dyf). For example nonDyf mutants include *odr-1*, *odr-2*, *odr-3*, *odr-4*, *odr-5*, *odr-7*, *che-1*, *mec-1* and *vab-3* ; *odr-1*, *odr-2*, *odr-4*, *odr-5*, and *odr-7*, which are defective only in chemotaxis to volatile compounds and have no ultrastructural abnormalities (Bargmann, Hartwieg et *al.*, 1993; Sengupta, Colbert et *al.*, 1994).

- *odr-3* affects the morphology of the AWC neurons and is only defective in chemotaxis toward volatile compounds (Bargmann, Hartwieg et *al.*, 1993)
- *che-1* affects the branching of AWA, AWB, and AWC, morphology of AFD and the bundling pattern of the channel cilia neurons (Lewis and Hodgkin, 1977) but is deficient in chemotaxis to aqueous compounds only (Bargmann, Hartwieg et *al.*, 1993).
- *mec-1* affects the bundling of amphid channel cilia neurons and results in some being abnormally long (these mutations also have hypertrophied sheath cells in the sensilla of the head)
- *vab-3* mutants have a distorted head shape so that the amphid structures are squashed and pushed out of shape (Lewis and Hodgkin, 1977)
- and *che-14* mutants have misaligned channel cilia such that they sometimes miss the amphid channel (Perkins, Hedgecock et *al.*, 1986).

The remaining mutants are Dyf and can be further divided into those that affect the amphid neuron cilia and those that affect the sheath cell or the matrix produced by it. Thirty loci have been identified to date which can be mutated to impart a Dyf phenotype (Hedgecock, Culotti et *al.*, 1985; Perkins, Hedgecock et *al.*, 1986; Hall and Hedgecock, 1989; Starich, Herman et *al.*, 1995) three of these also affect body movement (*unc-33*, *unc-44* and *unc-101*). *daf-6* mutants have hypertrophied sheath cells and

che-12 mutants have unusual matrix surrounding the amphid channel cilia (Albert, Brown et *al.*, 1981; Perkins, Hedgecock et *al.*, 1986). The remaining mutations affect cilia morphology and/or length and some (eg *dyf-1*) also affect the sheath cell (Lewis and Hodgkin, 1977; Perkins, Hedgecock et *al.*, 1986; DeRiso, Ristoratore et *al.*, 1994). Mutants that are Dyf therefore represent the major class of chemotaxis deficient mutants, and have ultrastructural abnormalities of the amphid cilia. The remainder are mutations in genes which have unknown cellular specificity and genes which specifically affect the olfactory amphid neurons AWA or AWC. Although Dyf is indicated in table 1.2. as either "yes" or "no", actual findings are more complex. For example, mutants in the *osm-3* gene stain variably with DiO, and different alleles vary in the number of neurons which stain; the number of neurons which stain also varies between individuals such that different alleles have differing penetrance of the Dyf phenotype (Shakir, Miwa et *al.*, 1993). Similarly, some of the Dyf mutants described by Starich et *al.* (1995) and Perkins et *al.* (1986) stain for subsets of amphid neurons with varying penetrance and intensity.

Of all the mutant studies completed, perhaps the simplest has been elucidation of the function of AFD. The AFD neuron of the amphid has a thermosensory role; it is the only anterior sensory neuron which is malformed in the thermosensory mutant *ttx-1* (Perkins, Hedgecock et *al.*, 1986). Microvilli on the ending of AFD are entirely missing in this mutant whereas the ciliated portion of the neuron posterior to them is normal, indicating that the *ttx-1* gene is required only for proper microvilli formation and thermotaxis and cilia probably do not play a role in thermotaxis, since the cilia in *ttx-1* mutants are apparently normal. In contrast, the majority of other mutations which affect the ultrastructure of a range of amphid sensory neurons do not affect AFD and these mutants are normal with respect to thermotaxis (Perkins, Hedgecock et *al.*, 1986). The role of AFD in thermosensation has since been confirmed by laser ablation (Mori and Ohshima, 1994).

Responses of *C. elegans* to environmental stimuli have also been studied by laser ablation of amphid and inner labial neurons (Bargmann, Thomas et *al.*, 1990; Bargmann and Horvitz, 1991; Bargmann and Horvitz, 1991; Bargmann, Hartwieg et *al.*, 1993; Kaplan and Horvitz, 1993). Although the study of mutants clearly points to the amphids as the major chemosensory organs of the

Table 1.1. Neurons with dendritic endings in *C. elegans* amphids.

Neuron	No. of cilia	ending embedded in sheath cell	Runs in amphid channel	Cilia end in amphid channel	Takes up FITC	Takes up DiO	Function
ADF	2	-	3	3	3	-	DF,mn
ADL	2	-	3	3	3	3	AG
ASE	1	-	3	3	-	-	C
ASG	1	-	3	3	-	-	DF,mn
ASH	1	-	3	3	3	3	AO,AG,M
ASI	1	-	3	3	3	3	DF,mn
ASJ	1	-	3	3	3	3	DR
ASK	1	-	3	3	3	3	ml
AWA	-	3	3	-	-	-	O,Ob
AWB	-	3	3	-	-	3	NFA
AWC	-	3	3	-	-	-	O,Od
AFD	-	3	-	-	-	-	T

Key - Table 1.1.:

DF - Dauer formation (Bargmann and Horvitz, 1991), (Shakir, Miwa et *al.*, 1993); **DR** - Recovery from dauer larvae (ASJ is necessary for dauer recovery before moulting to the L4 stage) (Bargmann and Horvitz, 1991); **C** - Major role in detection of aqueous attractants Na+, Cl-, cAMP, biotin, lysine (Bargmann and Horvitz, 1991); **mn** - Minor chemoreceptory role in detection of Na+, Cl-, cAMP, biotin (Bargmann and Horvitz, 1991); **ml** - Minor chemoreceptory role in detection of lysine (Bargmann and Horvitz, 1991); **AO** - Chemosensory role in avoidance of concentrations with high osmotic strength (Bargmann, Thomas et *al.*, 1990); **AG** - Chemosensory role in avoidance of garlic extract (Bargmann, Thomas et *al.*, 1990); **T** - Thermosensory role (Perkins, Hedgecock et *al.*, 1986; Mori and Ohshima, 1994); **M** - Mechanosensory role in response to touch on the head (Kaplan and Horvitz, 1993); **O** - Chemosensory response to volatile compounds isoamyl alcohol and 2,4,5-trimethylthiazole (Bargmann, Hartwieg et *al.*, 1993); **Ob** - Chemosensory response to volatile compounds butanone and benzaldehyde (Bargmann, Hartwieg et *al.*, 1993); **Od** - Chemosensory response to volatile compounds diacetyl and pyrazine (Bargmann, Hartwieg et *al.*, 1993), (Sengupta, Colbert et *al.*, 1994); **NFA** - No function assigned to date.

nematode, the exact effect of each mutation and the cell types affected are often difficult to interpret. Laser ablation provides a simpler approach to elucidation of the functions of various neurons and neuron types (see table 1.1. for a summary of the function of amphid neurons). Chemotaxis toward aqueous attractants such as sodium has been analysed (Bargmann and Horvitz, 1991) with the major neuron type implicated was ASE, with ASH, ASJ, ASK, ADL ASI, ADF and ASG also contributing to chemotaxis ability. The ASH neuron also has a mechanosensory role, ablation of this neuron reduces the response of worms to touch on the nose by 55% (Kaplan and Horvitz, 1993). The response of *C. elegans* to volatile compounds such as benzaldehyde, 2-butanone, isoamyl alcohol, thiazole, pyrazine and diacetyl, has been found to be mediated by the AWA and AWC neurons (Bargmann, Hartwieg et *al.*, 1993). Finally, avoidance of high osmotic strength solutions, garlic extract and extract of dead *C. elegans* appears to be mediated by ASH, with ADL also being involved in the response to garlic (Bargmann, Thomas et *al.*, 1990).

A major emphasis of the investigation of the role of the amphid in chemoreception has been the study of dauer (resting stage) larvae formation. This process is dependent on temperature, density of worms and an associated pheromone and the availability of the bacterial food supply (Golden and Riddle, 1984). Mutants which do not form dauer larvae (Daf-d) have been identified which have shortened or malformed amphid channel cilia (Lewis and Hodgkin, 1977; Perkins, Hedgecock et *al.*, 1986) and/or which have other amphid defects which prevent the uptake of DiO or FITC into amphid channel neurons (Starich, Herman et *al.*, 1995). In *daf-6* mutants hypertrophy of the sheath cell blocks the amphid and phasmid channels preventing the entry of amphid and phasmid channel cilia (Albert, Brown et *al.*, 1981) and consequently the entry of DiO or FITC (Perkins, Hedgecock et *al.*, 1986). Using mosaic analysis, in which a free duplication covering the *daf-6* mutation is somatically lost creating animals mosaic for the *daf-6* mutation, Herman (1984) showed that loss of *daf-6*(+) caused only a subset of the four amphid and phasmid sensilla to fail to take up FITC. As the lineages of the left and right amphid sheath cells and the left and right phasmid sheath cells are symmetrical, this observation suggests that the mutation does not behave cell autonomously. So the DAF-6 protein product is likely to be expressed in each of the sheath cells and not in their precursors. Another mutant, *daf-19*, completely lacks amphid channel cilia and forms dauer larvae constitutively (it is Daf-c) in the absence of appropriate stimuli (Perkins, Hedgecock et *al.*, 1986). Study of the variation between alleles of *osm-3* in dye filling, has revealed a relationship between the penetrance of

staining of the ADF neuron and dauer formation efficiency (Shakir, Miwa et *al.*, 1993) such that the lower the proportion of animals which took up DiO into ADF, the lower the number of dauer larvae which formed.

The role of the amphid in dauer larvae formation has also been investigated by laser ablation of amphid neurons (Bargman and Horvitz, 1991). The neurons ASI, ASG and ADF are necessary to prevent dauer formation in the absence of appropriate stimuli; when these neurons are ablated, the worms form dauer larvae even in the presence of plentiful bacteria under non-crowded conditions. The formation of dauer larvae in response to ablation of ASI, ASG and ADF is transient, with dauers moulting again to form L4 larvae within a day. When ASJ was ablated as well however, dauer larvae failed to develop further, implicating ASJ in the recovery from the dauer state in response to increased food supply and decreased pheromone concentration. The mutant *daf-19,* in which all the chemosensory cilia of the amphid are entirely missing, is daf-c; presumably the loss of the cilia from ASI, ASG, ADF and ASJ in *daf-19* mutants phenocopies worms in which these neurons have been laser ablated.

Some of the characteristic changes which occur between the L2 and the dauer stage involve the amphid (Albert and Riddle, 1983). The neurons ASG and ASI are shorter in dauer larvae and the AWC and sheath cell endings are substantially larger, with the sheath cells from the two amphids spanning the entire circumference of the head, forming a continuous single cell. The AFD cell also is larger in dauer larvae, with the number and density of microvilli also being greater than in the L2.

Interestingly, not all amphid defective mutants are defective for all four phenotypes. For example all known alleles of *dyf-12*, *dyf-13* and *dyf-10* are Dyf but not Daf-d, some alleles of *dyf-9* and *mec-8* are normal with respect to dauer formation and *daf-19* is Daf-c. Also a subset of Dyf mutants have additional characteristics not shared by the majority of other loci; *mec-8* mutants have mechanosensory defects in addition to chemosensory ones, and *unc-33*, *unc-44* and *unc-101* are defective in locomotion as well as being Dyf. These defects are most probably pleiotropic and not a consequence of amphid defects.

All the amphid neurons therefore, with the exception of AWB, have been assigned a sensory function important to *C elegans* behaviour and the amphid has been implicated in the most diverse range of functions of all the sensilla. No function has been assigned to AWB so it is possible that there are behavioural aspects of amphid function which are yet to be established. Some behaviours regulated by the amphid are modulated by groups of

more than one neuron, providing functional redundancy in responses to stimuli and others are controlled by single cell types, so the way in which the amphid regulates behaviour is complex. The interconnections between amphid neurons and between amphid sensory neurons and interneurons is also complex (White, Southgate et *al.*, 1986), perhaps providing a mechanism for integration of different amphid functions. Mutant characterisation and laser ablation studies have been the major instruments used in the elucidation of amphid function. These experiments have revealed the major role that the amphids play in detection of chemical stimuli in the environment of the worm, and also have implicated the amphid in thermoreception and mechanoreception. Most recently, molecular analysis of genes involved in amphid function has begun (*osm-3* -Tabish, Siddiqui et al., 1995; *odr-7*- Sengupta, Colbert et *al.*, 1994; *unc-33*- Li, Herman et *al.*, 1992). The identification of genes involved in amphid development and/or function and the cells in which they are expressed will eventually lead to an even greater understanding of amphid function and structure in nematodes.

1.3.1.2. Inner Labial Sensilla

The six inner labial sensilla also open to the exterior through cuticular pores. These sensilla open onto the six lips which surround the buccal capsule of the worm (Ward, Thomson et *al.*, 1975). They consist of a sheath and socket cell and two ciliated dendritic neuron endings. The ciliated endings of IL1 and IL2 pass along the channel formed by the sheath and socket cells, but only IL2 is exposed to the exterior with IL1 ending much further back in the channel. Despite being exposed to the exterior of the worm, IL2 (in common with amphid neurons ASE and ASG) does not take up Dio or FITC, indicating that dye filling does not depend solely on exposure to the exterior of the worm.

A number of *dyf* mutants have ultrastructural defects associated with the inner labial sensilla in addition to their amphid phenotypes. The sheath cell of the inner labial sensilla in *daf-6* is enlarged as is the amphid sheath cell (Albert, Brown et *al.*, 1981). In *che-13*, *osm-1* and *osm-5* mutants all the sensory cilia in the head are reduced in length, (Perkins, Hedgecock et *al.*, 1986), including cilia in the inner labial sensilla, and in *daf-19* mutants all cilia in the head are missing. In *che-10* the morphology of the IL1 neurons as well as amphid channel neurons is affected.

Other mutants exist which affect the inner labial sensilla and non-dye filling neurons of the amphid. *che-1* mutants have shortened IL2 neurons although the effect in the two alleles

studied is variable between sensilla on the one animal (Lewis and Hodgkin, 1977). *che-1* mutants also have reduced numbers of microvilli on the AFD cell of the amphid and the microvilli which are present are extremely distorted in shape. *mec-1* mutants have hypertrophied sheath cells in the inner labial sensilla and the amphid sheath cells show increased numbers of vesicles (Lewis and Hodgkin, 1977).

che-6 mutations are unique in that they only affect the inner labial sensilla and result in malformation or shortening of IL2, as for *che-1* mutants. The severity of the effect typically varies between sensilla on the one animal (Lewis and Hodgkin, 1977). The *che-6* gene is also deficient in recovery from dauer larvae (Lewis and Hodgkin, 1977), perhaps implicating the inner labial sensilla in this function. The neuron ablation study of Bargmann and Horvitz (1991) did not analyse the effects of ablation of the IL2 neurons in dauer larvae formation or recovery.

The inner labial sensilla might have a dual chemosensory and mechanosensory or thermosensory role, however laser ablation of the IL1 and IL2 neurons does not change response to touch on the nose of the worm (Kaplan and Horvitz, 1993), so mechanosensory reception by IL1 must be distinct from that mediated by ASH, FLP or OLQ.

1.3.1.3. Outer Labial and Cephalic sensilla.

The two other classes of sensilla of the head of *C. elegans* are the six outer labial sensilla and four cephalic sensilla. These sensilla all have associated sheath and socket cells which form a channel through which one ciliated neuron ending (CEP - cephalic, OLQ or OLL - outer labials) passes, to end embedded in the cuticle (Ward, Thomson et *al.*, 1975). By analysing the response in laser-ablated animals, the OLQ neurons have been shown to be involved in response to touch on the nose of the worm (Kaplan and Horvitz, 1993). In the same set of experiments, the OLL and CEP neurons were not found to be involved in this behavioural response. There are no cuticular pores associated with these sensilla in the hermaphrodite and the sensilla had previously been presumed to have a mechanosensory function (Ward, Thomson et *al.*, 1975). In males, the cephalic sensilla contain an additional neuron (CEM) which does penetrate the cuticle and is believed to be chemosensory (Perkins, Hedgecock et *al.*, 1986).

Only one mutant has been described which affects the outer labial and cephalic sensilla but which does not affect other sensilla of the head (Perkins, Hedgecock et *al.*, 1986). *cat-6* mutants have altered morphology in the CEP and OLQ cilia.

Many mutants which affect other sensilla also change the morphology of the outer labial and cephalic sensilla. In *mec-1* mutants hypertrophy of the sheath cells of outer labial and cephalic sensilla is seen in addition to the effects on the inner labial and amphid sheath cells. *che-2*, *che-3*, *che-13*, *osm-1*, *osm-5* and *daf-19* mutations affect all sensilla of the head (Lewis and Hodgkin, 1977; Perkins, Hedgecock et *al.*, 1986). In *che-11* mutants the CEP cilia are shortened and amphid cilia morphology is changed. The mutation *che-14* also affects the cuticle of the head of *C. elegans* so that CEP neurons which normally do not stain with FITC do take up FITC (Perkins, Hedgecock et *al.*, 1986).

1.3.1.4. Phasmids and deirids.

The phasmids are a pair of sensilla situated just anterior to the tail of *C. elegans*. They consist of a channel, formed by sheath and socket cells, which opens to the exterior. Two ciliated neuron endings, PHA and PHB, pass along the channel and are presumed to be chemosensory (Perkins, Hedgecock et *al.*, 1986). Both PHA and PHB accumulate FITC and DiO in wild type worms. The position of the phasmids is shown in figure 1.4.

There are two pairs of deirids which each have socket and sheath cells and a single ciliated neuron ending which is embedded in the cuticle. The deirids are positioned laterally; the anterior pair are about level with the base of the pharynx (figure 1.4.) and the posterior pair are positioned about three quarters of the way between the tip of the head and the tail (figure 1.4.) (White, Southgate et *al.*, 1986), deirid neurons do not fill with DiO or FITC.

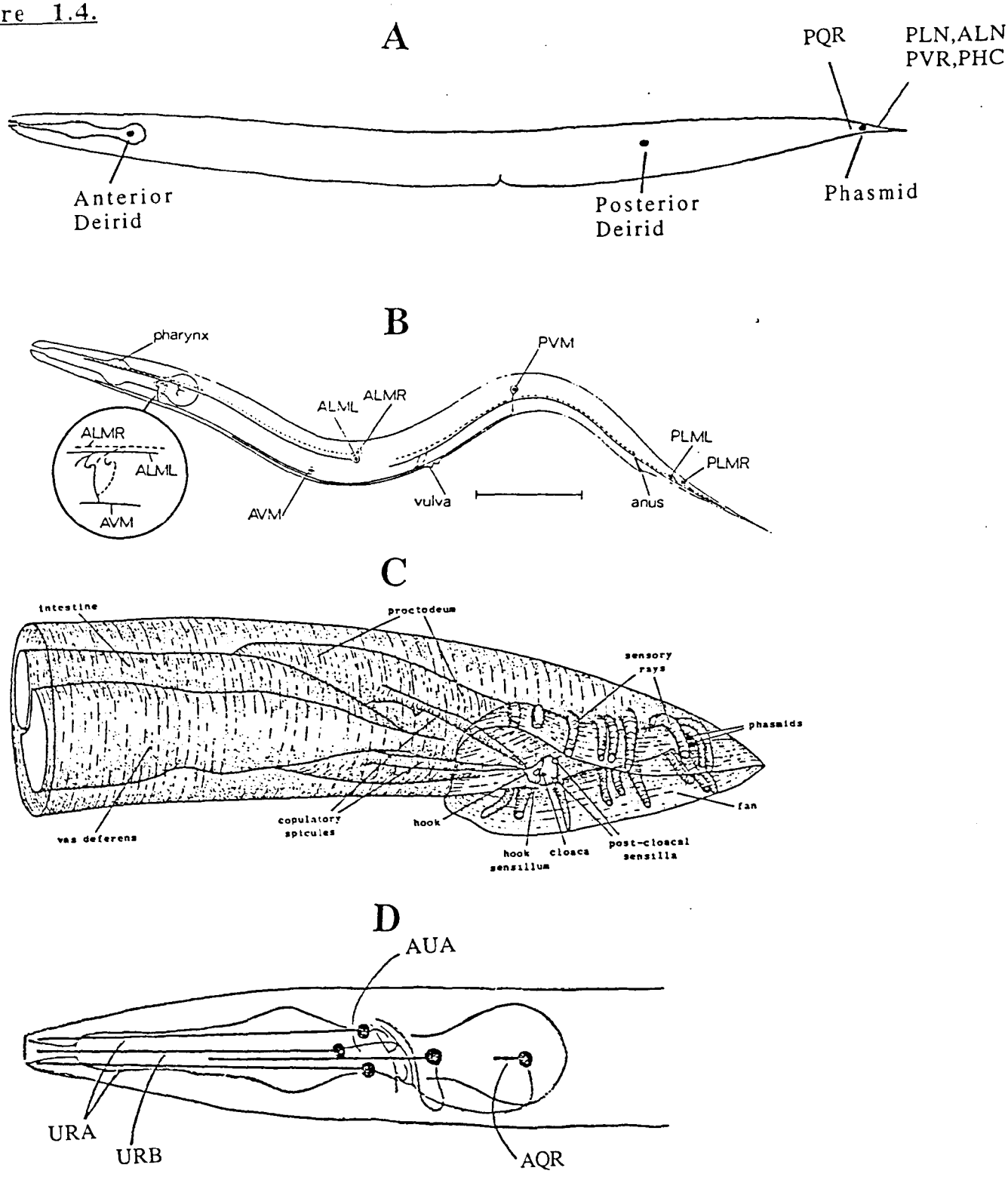
All nonUnc mutations which abolish or reduce amphid neuron staining also affect phasmid staining (Perkins, Hedgecock et *al.*, 1986; Starich, Herman et *al.*, 1995). This suggests some compositional similarities between the two sensilla types. In *cat-6* and *che-14* mutants, the deirid neurons ADE and PDE, which normally do not stain with FITC, frequently (but not always) take up the dye (Perkins, Hedgecock et *al.*, 1986). A similar phenotype is exhibited by the CEP neurons in these mutants suggesting similarities between CEP, ADE and PDE and perhaps correspondingly between cephalic and deirid sensilla. Perhaps *che-14* and *cat-6* are homeotic transformation mutants which can transform CEP, ADE and PDE into neurons homologous to amphid or phasmid neurons which do normally accumulate FITC.

1.3.2. Touch receptors.

The touch receptors are specialised mechanosensory neuron endings which are characterised by large microtubules in long lateral processes that lie just beneath the cuticle of the nematode (Chalfie and Thomson, 1979) (Shown in figure 1.4.). These neuron processes are attached near the cuticle by an extracellular mantle material (Chalfie and Thomson, 1979). There are six touch receptor neurons: a pair of ALM neurons, which run along the worm in the anterior half and are positioned dorso-laterally; a pair of PLM neurons which run along the posterior half of the animal sub-laterally; an AVM neuron which runs ventrally along the anterior third of the worm; and a PVM neuron which runs ventrally along the posterior two thirds of the worm with regions overlapping AVM (Chalfie and Thomson, 1979). The microtubules in the touch receptor cells have 15 protofilaments instead of the 11 protofilaments seen in other cells (Chalfie and White, 1988). This difference also reflects the response of *C. elegans* to sublethal concentrations of anti-mitotic drugs. Benzimidazoles affect movement and growth rate of wild type worms, resulting in many neurons producing far fewer processes than in untreated controls. The touch receptor cells however are unaffected by similar concentrations of benzimidazole drugs. In contrast the drug colchicine abolishes touch response in wild type worms but has no effect on coordination of movement (Chalfie, Dean et *al.*, 1986).

By ablating touch receptor cells and the interneurons and motor neurons which receive direct or indirect input from them, much has been deduced regarding the function of touch receptors (Chalfie, Sulston et *al.*, 1985). Laser ablation of the PLM cells abolishes the movement response of worms to touch on the posterior part of the animal. Ablation of ALM cells similarly abolishes anterior touch response. Ablation of AVM however only partially abolishes anterior touch response and ablation of PVM produces no response change in wild type worms. The complete touch response circuitry has been deduced from serial section electron microscopy and laser ablation experiments (Chalfie, Sulston et *al.*, 1985).

Figure 1.4.


A
PQR
PLN,ALN
PVR,PHC
Anterior
Deirid
Posterior
Deirid
Phasmid
B
pharynx
PVM
ALML
ALMR
ALMR
ALML
AVM
AVM
vulva
anus
PLML
PLMR
C
intestine
proctodeum
sensory
rays
phasmids
fan
vas deferens
copulatory
spicules
hook
hook
sensillum
cloaca
post-cloacal
sensilla
D
AUA
URA
URB
AQR

Legend for figure 1.4.

A A diagram of an adult hermaphrodite showing the approximate positions of the Phasmids, Anterior deirids and posterior deirids. Also shown are the approximate positions of the free dendritic ends of neurons PQR, PLN, ALN, PVR and PHC, which are putative sensory neurons. Only the PQR neuron has a ciliated dendritic process. The figure is derived from diagrams in White et *al.*, (1986).

B Diagram of an adult hermaphrodite showing the positions of the touch cell receptor neurons ALM, PLM, AVM and PVM, from Chalfie et *al.* (1985). The inset shows the neural network formed by ALML, ALMR and AVM via gap junctions in the adult worm.

C Left subventral view showing the structures of the *C elegans* male tail (from Sulston et *al.*, 1980). The sensory rays, post-cloacal sensilla, hook sensillum and spicules are sensory structures only found in the male. The phasmids are sensory organs found in both sexes. Other male specific structures shown are the vas deferens, fan, cloaca (the hermaphrodite has a simpler anus) and proctodeum.

D This figure is a composite of diagrams from White et *al.*, (1986). It shows the head end of the *C. elegans* hermaphrodite and the positions of neurons AUA, AQR, URA and URB which have free dendritic endings in the head of the worm and have putative sensory function. An outline of the pharynx is included in order to visualise the relative positions of the neurons. The dendritic ends are shown in black for emphasis and the axonal endings in grey. Only AQR has a ciliated dendritic ending.

Mutations at a minimum of 18 separate loci affect movement of *C. elegans* in response to light touch (Chalfie and Au, 1993). *mec-7* mutants have 11 protofilament microtubules in the place of 15 protofilament microtubules in the touch receptor neurons; and in *mec-12* mutants the anterior branches of the touch receptor neurons are missing. In adult worms, these branches create a neural net in the anterior part of the worm, joining the ALM and AVM cells (Chalfie, Sulston et *al.*, 1985). Clearly this aspect of the neuronal structure of touch receptors is important to touch response as *mec-12* mutants do not respond to light touch. Mutations in the *mec-1* and *mec-5* genes affect the extracellular mantle associated with touch cells, implicating this structure in touch detection (Chalfie and Au, 1993). Bargmann

(1994) (Bargmann, 1994) suggests that touch sensitive ion channels might be attached to the rigid interior microtubules and the exterior mantle, so that displacement of the mantle relative to the interior of the cell can activate or hyperpolarise the cell, thus providing a molecular mechanism for mechanosensation and a connection between the behavioural and ultrastructural defects of *mec-1*, *mec-5*, *mec-7* and *mec-12* mutants.

Two genes which regulate the development of touch cell receptors are *unc-86* and *lin-32* (Chalfie and Au, 1993). These genes affect the fates of a number of different cell lineages, however, so are unlikely to be involved in the final determination of touch receptor development. By contrast, the gene *mec-3* only affects the touch receptor cells. In *mec-3* mutants, the ALM neurons develop similarly to their non-touch receptor lineal sister cells, the BDU neurons. *mec-3* therefore might act to determine steps in touch cell differentiation later than *unc-86* and *lin-32* (Chalfie and Au, 1993). The *unc-86* gene product has been identified as a transcriptional activator, and footprinting using the UNC-86 protein has shown that it binds to controlling regions of the *mec-3* gene (Xue, Finney et *al.*, 1992). The MEC-3 protein also binds to a different regulatory region on the *mec-3* gene. Touch cell differentiation therefore is most likely directed by the actions of MEC-3, which is under the control of UNC-86 and which is also self-regulating.

Touch response to the nose of the worm is not affected by the touch receptor cells. Ablation of both AVM and ALM cells did not abolish this reponse (Kaplan and Horvitz, 1993). The neurons OLQ, FLP and ASH are involved in touch response (sections 1.3.5., 1.3.1.) (Kaplan and Horvitz, 1993). As there are also many more putative mechanoreceptive (or nocioceptive) neuron endings in the worm (sections 1.3.5., 1.3.1.), the complete array of touch responses may not yet have been investigated (ie there may be many more behavioural phenotypes which are yet to be studied in *C. elegans*). Alternatively there may be some neuron endings which are non-functional, having perhaps served some function in an evolutionary precursor to *C. elegans*.

1.3.3. Sensory structures of the male tail.

The male tail contains a number of sensory structures which are not present in the hermaphrodite (a diagram of the position of these is given in figure 1.4.). The neurons innervating the male tail make up the majority of the male-specific neurons in *C elegans*. In *C. elegans* the male tail has eighteen ray sense organs, two spicule sensilla, two postcloacal sensilla and a hook sensillum (Sulston, Albertson et *al.*, 1980).

The male has a copulatory bursa which wraps around the hermaphrodite during mating. The nine bilateral pairs of rays in the male bursa each contain two neuron endings and a single structural cell (distinguishing them from the sensilla elsewhere in the body which have two structural cells - the sheath and socket cells) (Sulston, Albertson et *al.*, 1980). Eight of the sensory rays have an opening through the cuticle, three point dorsally, three ventrally, two laterally and one has no exterior opening (Liu and Sternberg, 1995).

The hook sensillum has two sensory neurons and a sheath and socket cell, and opens just anterior to the male cloaca (which is used for defecation and sperm deposition) (Sulston, Albertson et *al.*, 1980). The two post cloacal sensilla have three sensory neurons and three support cells and open posterior to the cloacal opening (Sulston, Albertson et *al.*, 1980). Lastly there are two spicule sensilla which have two sensory neurons each and a sheath and a socket cell (Sulston, Albertson et *al.*, 1980). The spicules are retracted into the body cavity except whilst mating when they protrude from the cloaca.

The role played by the various male sensory structures in the male tail has been analysed by laser-ablating various sensory neurons and accessory cells and observing mating behaviour (Liu and Sternberg, 1995). The dorsal opening rays were the only ones found to play a major role in mating. If the dorsal side of the male comes into contact with the hermaphrodite first, then the dorsal opening rays mediate the ability of the animal to correctly orient the bursa in preparation for mating. Another aspect of mating behaviour is location of the hermaphrodite vulva. Males do this by swimming backwards along the hermaphrodite with the bursa in contact with the surface of the hermaphrodite. If the male comes to the end of the hermaphrodite before locating the vulva, he must execute a tight turn in order to stay in contact with the hermaphrodite and search the other side for the vulva. This turning activity is divided into two components, the coiling activity, which is mediated by rays 5, 7 (dorsal opening) and 9 (lateral opening) and the timing of the turn, which is mediated by rays 7, 8 and 9 (dorsal, ventral and lateral opening respectively). The hook sensillum is used to locate the general area of the vulva and the post-cloacal and spicule sensilla are then used to precisely locate the opening. If the hook sensillum is ablated, the male can still find the vulval opening by using the post-cloacal and spicule sensilla, though this behaviour is easily distinguishable from wild type mating behaviour (Liu and Sternberg, 1995). The final two steps in mating are spicule insertion and sperm transfer. The SPD spicule sensory neurons mediate spicule insertion via the SPC motor neurons. Once the spicules are inserted, the SPV spicule

sensory neurons act to cease inhibition of sperm release. Ablation of the SPV neurons results in release of sperm outside of the vulva (Liu and Sternberg, 1995).

1.3.4. Pharyngeal sensory neurons.

The pharyngeal nervous system can act autonomously to allow pharyngeal pumping in the absence of stimuli from the remainder of the nervous system (Avery and Horvitz, 1989). Seven of the fourteen neuron types in the pharyngeal nervous system have putative mechanoreceptive endings just below the cuticle of the pharynx lumen (Albertson and Thomson, 1976). Structurally these dendrite endings can be divided into two classes: interneurons I1, I2, I3 and I6 are of one type and dendritic endings of M3 (motor neurons), NSM (neurosecretory-motor neurons) and MC (marginal cell neurons) are of another type. Of all these neurons, only the ablation of MC results in severely reduced pharyngeal function: pumping becomes slower and adults have a starved appearance (Avery and Horvitz, 1989). The ablation of M3 causes the elimination of "P-phase transient" peaks from electrical recordings of the pharynx (electropharyngeograms) (Raizen and Avery, 1994) and loss of coordination of posterior and anterior pharynx relaxation, decreasing the efficiency with which bacteria are trapped in the lumen. The results of these experiments are difficult to interpret as all these neurons appear to have multiple functions (Albertson and Thomson, 1976), however it appears that at least MC and M3 play a major role in pharyngeal function and it is likely that sensory input makes up at least part of that function. Raizen and Avery (1994) suggest that M3 is involved in the coordinated timing of pharynx relaxation. The neuron has sensory endings in the posterior part of the pharynx and motor output to the middle and anterior part. The application of exogenous glutamate phenocopies the action of M3, suggesting that M3 is a glutamatergic inhibitory motor neuron (Avery, Davis et *al.*, 1994).

1.3.5. Other putative sensory neurons.

A number of putative sensory neuron endings occur within *C. elegans* which are not closely associated with particular body parts or sensilla. The only one of these neurons for which a function has been assigned is FLP (Kaplan and Horvitz, 1993). The function(s) of the remainder have not been investigated but the position and ultrastructure of some of them allow some speculation as to their possible functions (White, Southgate et *al.*, 1986).

BAG, FLP, URX and URY are neurons with flattened endings in the head of the worm (see figure 1.2.). The four non-ciliated URY neuron endings are positioned between the outer labial and inner labial sensilla and form a large flattened concave shape in cross section. The two non-ciliated URX neuron endings are closely associated with the sheath cell of the dorsal inner labial sensilla and have an irregular flattened cross-section. Two BAG neurons have ciliated endings associated with "elliptical, closed, sheet-like processes" (White, Southgate et *al.*, 1986). From their position and structure it is tempting to propose that these neuron endings serve a thermosensory or mechanosensory role in the head of the worm. Lastly the two FLP neurons have ciliated endings associated with flattened processes between the amphid and sub-lateral inner labial sensilla.

Responses to touch on the nose of the worm are 20-30% reduced by laser ablation of the FLP cells (Kaplan and Horvitz, 1993). Ablation of FLP in combination with OLQ results in 34-49% reduction in response and ablation of ASH, FLP and OLQ results in a 65-85% reduction (Kaplan and Horvitz, 1993). This example illustrates the overlapping and additive function of some sensory neurons in the head of the worm. In the same set of experiments BAG was ablated and this had no affect on response to touch on the nose.

AUA, URA and URB have non-specialised dendritic endings in the process bundles of the amphid neurons (AUA) or labial sensilla process bundles (URA, URB) (their positions are shown in figure 1.4.) (White, Southgate et *al.*, 1986). Their function is unknown, however the neuroconnectivity of these neurons suggests a role in integration of sensory information, as all three receive inputs from other sensory neurons in the head. On the basis of synaptic output, AUA and URB are interneurons and URA is a motoneuron with connections to muscles in the head. One possibility is that these neurons are redundant processes which innervated the amphids and labial sensilla of an evolutionary precursor to *C. elegans*.

AQR and PQR both have small cilia not associated with any sensilla. The AQR cilium is located near the basal bulb of the pharynx. Perhaps this serves as a proprioceptor which fires when the pharynx is in motion. This would be useful as although the somatic nervous system has input onto the pharynx via the RIP interneuron, no neuronal processes emanate from the pharynx to synapse with extrapharyngeal neurons. PQR is located posteriorly with its free ending in the tail of the worm and perhaps is mechanoreceptive.

ALN and PLN are neurons with free dendritic endings in the tail of the worm. ALN is closely associated with ALM and PLN is

associated with PLM; so a mechanosensory function for ALN and PLN seems likely. PVR also has a free dendritic ending in the tail and forms a gap junction with PLM(right). Finally two PHC neurons have free dendritic endings in the tail of the worm and are closely associated with PLN and PLM neurons. It seems likely that all these neuron endings have a mechanosensory role in the tail of the worm

1.3.6. Conclusions.

The nematode *Caenorhabditis elegans* is richly endowed with sensory structures, as are many nematodes (Maggenti, 1981; Nicholas, 1984; Bird and Bird, 1991). Good relationships between structure and function of some of the sensory structures of *C. elegans* have been elucidated (Chalfie, Sulston et *al.*, 1985; Avery and Horvitz, 1989; Bargman and Horvitz, 1991; Bargmann and Horvitz, 1991; Raizen and Avery, 1994; Liu and Sternberg, 1995; Kaplan and Horvitz, 1993). Laser ablation experiments have demonstrated functional redundancy between neuron classes and additive effects of neurons on behavioural responses, with investigations of chemosensation by the amphid (Bargmann and Horvitz, 1991), control of dauer formation by the amphid (Bargmann and Horvitz, 1991) and response to touch on the front end of the worm (Kaplan and Horvitz, 1993), illustrating these points. There still remain however, a variety of sensory structures for which no function has been demonstrated or for which function has only been partly revealed. The use of mutations, cell ablations, and molecular biology in conjunction with recorded wild type ultrastructure and analysis of mutant ultrastructure should continue to increase understanding of the sensory structures of *C. elegans*. In this thesis, a relationship between amphid defects and resistance to IVM is established, similarly some amphid defective mutants are resistant to caffeine (Starich, Herman et *al.*, 1995). The genetics of IVM resistance, information from the literature on IVM mode of action and sensory function in *C. elegans* is used in Chapter 5 to propose a mechanism for amphid defect mediated resistance to IVM.