CHAPTER FIVE

DISCUSSION

5.1. Dominant ivermectin resistant mutations.

The incidence of dominant IVM resistant mutations in *C elegans* is much lower than the incidence of recessive mutations (Chapter 3) and probably involves two loci. Of four dominant mutations isolated, three occurred at one locus *dyf-12*, indicating that the screen for dominant IVM resistant mutations at 5 ng/mL may be saturated or that *dyf-12* is a better target for this type of mutation. If *dyf-12* is a good target for dominant mutations, a much larger mutagenesis screen would be required to estimate the total number of loci for which dominant IVM resistant mutations can occur. Both *dyf-12* and *dyf-10* have recessive IVM resistance alleles which are also isolated with low frequency, indicating that null alleles of these loci may be lethal, or phenotypically silent. All *dyf-10* and *dyf-12* IVM resistance alleles may be neomorphic since they are isolated at low frequency (Starich, Herman et *al.*, 1995) and the recessive mutations at these loci are distinctive. For example, both *dyf-12*(sa127) and *dyf-10*(e1383) strains are wild type for dauer formation and neither are greatly defective in male mating capability whereas the majority of other Dyf mutants are defective for both or one of these characteristics.

The four dominant alleles can be distinguished from each other by the degree of dominance and sex influence of IVM resistance. *dyf-10*(nr2389) and *dyf-12*(nr272) heterozygotes carrying paternally inherited IVM resistance genes are more resistant than heterozygotes which have inherited the mutations from the hermaphrodite parent. *dyf-12*(nr2477) heterozygotes are more resistant when the IVM resistance mutation is inherited maternally, and there appears to be no effect of mode of inheritance in *dyf-12*(nr2344) heterozygotes.

All four dominant mutations are Dyf as homozygotes. Dyf is recessive in *dyf-10*(nr2389) but incompletely dominant for the three *dyf-12* alleles. There are two aspects of the incomplete dominance, some worms fail to stain with DiO and the remainder do stain but with less intensity. The number of cells staining is the same in faintly staining (nonDyf) heterozygotes and wild type worms.

A unique characteristic of the *dyf-10*(nr2389) allelle is that only hermaphrodite heterozygotes are resistant to 5 ng/mL IVM. Male heterozygotes are not resistant even though they carry the same set of *dyf-10* alleles as heterozygote hermaphrodites. The sex-influenced nature of IVM resistance for *dyf-10*(nr2389) is difficult to explain. There are differences in the anterior sensory anatomy of male and hermaphrodite worms however (Perkins,

Hedgecock et *al.*, 1986), and perhaps the sex influenced nature of IVM resistance in *dyf-10*(nr2389) is another manifestation of differing sensory biology between the two sexes.

The dominant *dyf-10* allele fails to complement dominant *dyf-12* alleles for the Dyf phenotype, even though these loci are unlinked. These Dyf mutations also partially fail to complement other Dyf mutations. A common feature of mutations in genes affecting microtubule associated proteins is intergenic noncomplementation (James, Ranum et *al.*, 1988; Regan and Fuller, 1988; Stearns and Botstein, 1988; Hays, Deuring et *al.*, 1989; Knowles and Hawley, 1991; Endow and Titus, 1992; Interthal, Bellocq et *al.*, 1995). Two cloned Dyf genes have homology to microtubule motor protein sequences ((Tabish, Siddiqui et *al.*, 1995); Grant Pers. Comm., 1994). Microtubules are an integral part of the chemosensory cilia of the amphid (Ward, Thomson et *al.*, 1975; Perkins, Hedgecock et *al.*, 1986). Other Dyf mutants have reduced amphid cilia (Perkins, Hedgecock et *al.*, 1986) so it is possible that *dyf-10* and *dyf-12* encode proteins that interact with each other and/or other microtubule components in amphid cilia. Molecular cloning and sequencing of *dyf-12* and *dyf-10* may reveal homologies between these genes and the sequences of genes encoding proteins associated with microtubules.

Ultrastructural analysis of heterozygotes and homozygotes may reveal cell types important for IVM resistance and not dye filling, as heterozygotes are not Dyf (nr2389) or are not always Dyf (*dyf-12* alleles). The dominant mutations described here may also be neomorphic, this could be tested by observing the phenotypes of these mutations in combination with duplications or deficiencies in the region of these loci. If a dominant mutation were neomorphic, then it might be expected that an individual heterozygous for the mutation and a deletion (*dyf/0*) which spans the locus would have a different phenotype to the homozygote (*dyf/dyf*). A null mutation however might be expected to behave similarly in *dyf/dyf* and *dyf/0* worms. Also by comparing *dyf/0*, *dyf/+* and *dyf/+/+* (ie. a heterozygote with a duplication), the effect of different ratios of mutant and wild type alleles could be observed. If none of the known *dyf-12* alleles are null (the recessive Dyf allele is not likely to be null - (Starich, Herman et *al.*, 1995)), then it would be interesting to create null mutations and observe their phenotype(s). The comparison of recessive loss of function and dominant gain of function alleles has been instrumental in the study of vulval differentiation (Sternberg and Horvitz, 1989; Sternberg and Horvitz, 1991). The generation of these two types of alleles at the *dyf-12* locus might provide an opportunity to learn more about the genetics and biology of IVM resistance and the amphid.

5.2. A model for ivermectin resistance in Dyf mutants.

5.2.1. PROPOSAL:

Dyf mutations act to increase IVM resistance by modulating endogenous signals which influence pharynx function.

This proposal is described schematically in figure 5.1. In the figure wild type worms respond to two factors which contribute to lethal inhibition of pharyngeal function:

1. Nervous inhibition (or lack of stimulation) of the pharynx in response to external stimuli detected by the amphids. ('A' in figure 5.2.) The response of the pharynx to these signals may be potentiated by IVM binding to chloride ion channels on pharyngeal muscle or nerve cells.
2. Inhibition of the pharynx mediated by IVM binding to receptors on pharyngeal muscle or nerve cells.

In Dyf mutants inhibitory 'A' responses are turned off (and/or excitatory 'A' responses are produced constitutively) so that lethal effects of IVM are seen only at higher concentrations. The effect of nervous inhibition or excitation on IVM response may have the following attributes:

1. IVM might act to potentiate glutamate mediated inhibition by binding to the receptor.
2. IVM and the inhibitory signals might act additively; the two responses can combine to decrease pharynx activity below a threshold which is essential for survival of the worm.
3. IVM and excitatory signals might act antagonistically, with excitatory signals negating some of the inhibitory effects of the drug.

Central to the proposal is the idea that signals from the amphid might stimulate excitatory pathways in the pharyngeal nervous system and/or block inhibitory pathways within the pharyngeal nervous system. In Dyf worms this amphidial input via the pathway 'A' is locked on. This proposal leads to the question: What is the basal state of the pharynx in the absence of 'A'?

The existence of endogenous signals which can stimulate the pharynx is possible (this will be discussed below). Also inhibitory pathways exist within the pharynx which might be influenced by input from the extrapharyngeal nervous system. Pharynx activity in worms which are constitutively producing 'A' would be expected to be increased and/or to be more responsive to

stimulatory signals under the model proposed here. Under this proposal, it is the summation of the effects of IVM dose and the prevailing activity state of the pharynx which determines the response to IVM. If the pharynx is in a higher "activity state", then more IVM is needed to cause lethal pharyngeal inhibition.

5.2.2. EVIDENCE FOR THE MODEL.

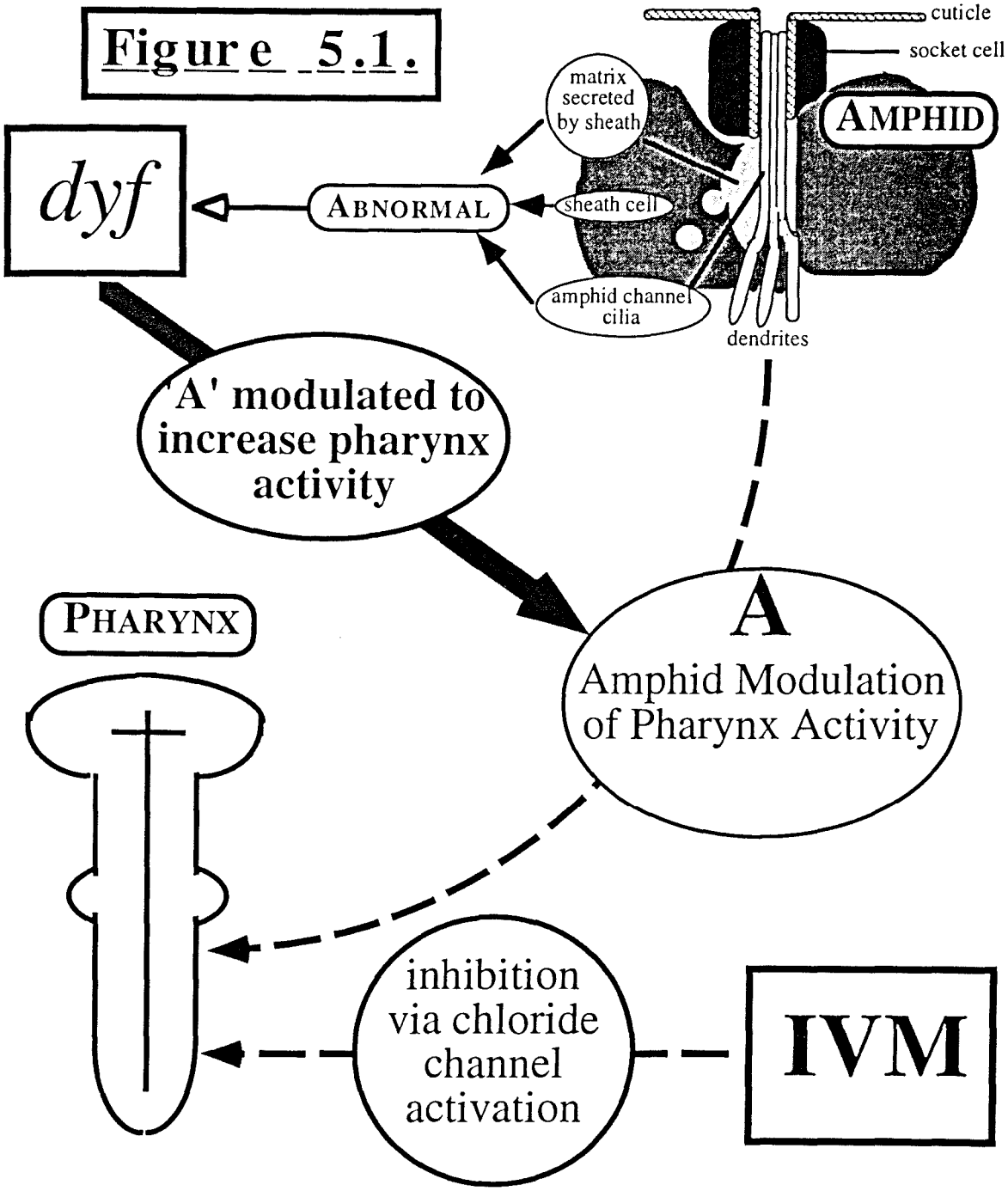
5.2.2.1. The most important site of IVM action for *C. elegans* in laboratory culture is probably the pharynx.

The action of IVM at the molecular level is probably to open glutamate gated chloride ion channels and so hyperpolarise cells by increasing intracellular Cl^- concentration (Arena, Liu et *al.*, 1991; Arena, Liu et *al.*, 1992; Cully, Vassilatis et *al.*, 1994; Arena, Liu et *al.*, 1995). IVM therefore is likely to decrease the excitability of neuron and muscle cells. A number of factors implicate the pharynx as the principal site of IVM action *C elegans*.

IVM greatly reduces the efficiency of feeding in *C. elegans* at concentrations of the drug (5-50 ng/mL) (Avery and Horvitz, 1990) comparable to levels at which IVM is lethal to animals in culture (Kim and Johnson, 1991). IVM irreversibly paralyses *C elegans* however only at far higher concentrations (100-1000 ng/mL) (Kass, Wang et *al.*, 1980), so this effect cannot be seen in culture concentrations of 5 ng/mL which are lethal to worms. Also, IVM does not kill sensitive *C. elegans* in culture on contact, but rather arrests development at the L1 (or rarely L2) stage and if removed within two to three days, worms can recover and continue development when transferred to non-drug media (see chapter 4). This observation is consistent with death due to starvation rather than irreversible paralysis. Furthermore, this study has revealed that a number of mutants which have both decreased pharyngeal activity and abnormally high sensitivity to IVM (Chapter 2). It has been reported that glutamate (the natural ligand of the IVM receptor) inhibits pharyngeal pumping via a neuromuscular junction between the M3 neuron and pharynx muscle cells (Avery, Davis et *al.*, 1994).

Although IVM can penetrate the cuticle of some parasitic nematodes (Ho, Geary et *al.*, 1990), but it has also been reported that IVM cannot penetrate the lipid bilayer and is limited to the outer

Figure 5.1.



dyf	(A constitutive) + 5-20 ng/mL IVM	= no lethal effect
Wt	(A variable) + 5-20 ng/mL IVM	= lethal pharynx inhibition
Either genotype	+ >30 ng/mL IVM	= lethal pharynx inhibition

Figure 5.1.

A Mechanism for IVM resistance in Dyf worms.

The mechanism for IVM resistance by Dyf mutations suggested in this figure depends on a putative pharynx modulating pathway that can be influenced by input via the amphids. When the pathway 'A' is acting to excite (or reduce inhibition of) pharyngeal activity, the pharynx is blocked by higher concentrations of IVM than when A is inactive. The effect of Dyf mutations is to block pharyngeal inhibition or to increase pharynx stimulation via the pathway 'A', thus allowing the pharynx to be less susceptible to IVM. The net effect therefore is that the pharynx changes to a more activated state, decreasing the effect of IVM on its function. At higher concentrations of IVM, this type of mechanism cannot negate the effects of the drug. In this diagram 'Pharynx' refers to the entire pharyngeal neuronal and muscle system, and the pathway 'A' modulates the activity of pharyngeal excitatory and/or inhibitory neurons which affect pharynx function.

layer of the cell membrane (Martin, Kusel et *al.*, 1992). The nervous and muscle cells of *C. elegans* are surrounded in most instances by a layer of cuticle and a layer of hypodermal cells (White, 1988), making it difficult for IVM to gain access to their surfaces. The pharynx muscle however, is not isolated from the pharyngeal lumen by a layer of hypodermal cells; the cuticle directly overlies the muscle cells (Albertson and Thomson, 1976). Therefore IVM probably has greater access to the pharyngeal muscle than to other neurons and muscle cells of the nematode.

Thus there is behavioural, pharmacological and biochemical evidence that suggests that IVM acts on the pharynx to kill *C elegans* in laboratory culture conditions. It is unlikely however that the pharynx is the only site of action of the drug in *C. elegans*. It has been shown that IVM can interfere with chemotaxis (Chapter 2, Grant Pers. Comm., 1994) egg laying (Grant Pers. Comm., 1994) and motility (Kass, Wang et *al.*, 1980). Pharynx muscle cells are not the only excitable cells that are situated directly beneath the cuticle in *C. elegans*. The endings of many chemosensory cells are embedded in cuticle or exposed through pores in the cuticle (Ward, Thomson et *al.*, 1975) (also reviewed in chapter 1). The effect of IVM on chemotaxis reported in chapter two might be explained by the accessibility of chemosensory

neurons to IVM. Kass et *al.* 1980 reported on the effects of IVM on a number of neuron types in *Ascaris suum* and muscle cell vesicles prepared from *A. suum* also respond to IVM (Martin and Pennington, 1989). It is probable that many neuron and muscle cells from *C. elegans* and other nematodes are sensitive to IVM, but only a subset of these are exposed to significantly high concentrations of the drug in living animals. In the laboratory, *C elegans* can survive severe disruption to chemoreception, egg laying and motility mediated by a variety of genetic mutations; IVM-mediated effects on these behaviours therefore are unlikely to be lethal in this situation. For parasitic nematodes *in vivo* however, the principal site of action of IVM is still unclear. Some non-feeding parasitic stages, e.g. *O. volvulus* microfilariae are sensitive to IVM (Campbell, 1989) and it is difficult to equate inhibition of the pharynx with lethal effects of the drug in these worms.

5.2.2.2. Amphid defects in *C. elegans* confer resistance to low levels of IVM included in the growth media.

About thirty loci have been identified which when mutated impart low levels of IVM resistance in *C. elegans* (Johnson, Pers. Comm., 1990). Also there are thirty loci in *C. elegans* that when mutated abolish or reduce the uptake of FITC and DiO (FITC and DiO) into the amphid neurons (Hedgecock, Culotti et *al.*, 1985; Perkins, Hedgecock et *al.*, 1986; Hall and Hedgecock, 1989; Starich, Herman et *al.*, 1995). Through testing Dyf mutants for IVM resistance (Chapter 2), analysing the Dyf, Daf and Che phenotypes of mutants selected for resistance to 5 ng/mL IVM (Chapter 2), and complementation testing IVM resistant mutants with Dyf and *che* mutants (Chapter 2 and 3, Johnson Pers. Comm., 1995), it has been shown that the two classes of mutants are identical. Mutant strains carrying *unc-33* or *unc-44* mutations share a number of pleiotropic effects on neuroconnectivity (Hedgecock, Culotti et *al.*, 1985; Siddiqui, 1990; Siddiqui and Culotti, 1991), but other mutants which share these pleiotropic characteristics, and are not Dyf, are also not IVM resistant (Chapter 2). In addition, some mutations which alter head morphology or chemotaxis ability (Chapter 1 & 2) are also slightly resistant to IVM (Chapter 2). IVM resistance to low levels of drug therefore is linked to defects in the structure of chemosensory sensilla. More particularly, IVM resistance is dependent on amphid defects, as mutations which only disrupt non-amphid sensory structures (e.g. *cat-6*, - (Perkins, Hedgecock et *al.*, 1986)) are not resistant to IVM. Also, mutations which only have slight effects on amphid channel neurons, but have more severe effects on other sensory neuron endings (e.g.

che-1, *mec-1* - (Lewis and Hodgkin, 1977)) are not resistant to IVM.

Effects on amphid morphology or function are the only aspects common to all the mutations identified as conferring resistance to IVM in Chapter 2. Some of the collection of mutations isolated for low level resistance by Johnson (Pers. Comm., 1990) have been analysed with respect to binding of IVM to membrane preparations; none of these mutations showed decreased binding of the drug. It is unlikely therefore that any of these mutations are involved in molecular changes to the IVM receptor. Other Avr mutations, which are not considered in this thesis, and exhibit far higher levels of drug resistance, do change the affinity of the receptor for the drug (Johnson Pers. Comm., 1990).

The amphids are a pair of large sensilla which has a pore reaching through the cuticle to the exterior. IVM could possibly penetrate *C. elegans* via the amphid pore in the same way as the FITC and DiO. If this occurred, then mutations which block the amphid channel might block drug entry. A number of observations however conflict with a resistance mechanism model implicating the amphid as a port of entry for IVM. Firstly, the pharynx appears to be the important target for IVM action in *C elegans*, and IVM has direct access to this organ through the cuticle (see previous section). Any role the amphid might play in IVM penetration of the animal would be minor in comparison to the direct access, especially as IVM penetrating the animal via the amphid pore would need to pass through a number of cell layers before reaching the pharynx; IVM will not pass through cell membranes but can only diffuse into the outer layer of the cell membrane lipid bilayer (Martin, Kusel et *al.*, 1992). Also a number of mutations which might be expected to increase the exposure of the amphid (or other sensilla pores) to the drug are not super sensitive to the drug for example *mec-1* and *che-14*, (see chapter 2) (Lewis and Hodgkin, 1977; Perkins, Hedgecock et *al.*, 1986). In addition, a number of Dyf resistance alleles affect the amphid without affecting the inner labial sensilla (e.g. *osm-3* - (Perkins, Hedgecock et *al.*, 1986)), these sensilla also have a pore through which a neuron ending is exposed to the external environment. It might be expected that if access through the amphid pore were important for resistance, that the inner labial pore would be equally accessible to the drug.

The lethal effect of IVM may be due to direct inhibition of amphid function, so in Dyf mutants resistance is due to isolation of target amphid neurons from IVM. These amphid neurons may exert a direct influence over the pharynx so drug action on these neurons would be translated into inhibition of pharyngeal

function. This model also is unlikely for a number of reasons. Firstly, as in a penetrance model (last paragraph), the pharynx is likely to be directly accessible to IVM and direct effects would be expected to outweigh any action of the drug mediated via the amphids. Also, IVM can penetrate the cuticle of the worm, so Dyf mutations would not necessarily prevent the drug reaching the amphid neurons. The amphid sheath cell excretes a matrix substance which surrounds the amphid channel neuron endings (Ward, Thomson et *al.*, 1975) and this substance might prevent IVM reaching shortened neuron endings. A mutation at the *che-12* locus however, in which the matrix is not excreted by the sheath cell (Perkins, Hedgecock et *al.*, 1986), is resistant to IVM. So a direct effect on the amphid neurons is not likely to be involved in the effects of IVM on the pharynx and the amphidial defects associated with Dyf mutations are not likely to prevent IVM reaching the amphid neuron endings.

As they depend on the amphid neurons being isolated from the exterior, models relating IVM resistance in Dyf mutants to penetration of the drug or a direct effect of IVM on amphid neurons are also unable to account for mutations which are not Dyf but are resistant. Certain alleles of *che-3* (Johnson and Clover, 1995) and heterozygotes for dominant alleles of *dyf-10* and *dyf-12* (Chapter 3) do take up DiO into amphid neurons and are resistant to IVM, also nr2389/+ heterozygotes are not Dyf but are IVM resistant (Chapter 3). Another model is needed to explain IVM resistance by these mutations.

Studies on IVM resistance in arthropods (Clark, Scott et *al.*, 1994), have implicated detoxification of the drug, penetrance of the cuticle and disruption of the drug binding site as possible mechanisms of resistance. It is unlikely that any of these mechanisms is involved in IVM resistance as mediated by Dyf mutants in *C. elegans* or in other *C. elegans* mutants disrupting amphid morphology or function. So what is the link between IVM resistance and amphid defects? To address this issue it is necessary to explore the physical or functional links between the amphids and the pharynx (see below).

5.2.2.3. In wild type worms the amphid may participate in pharyngeal regulation, but the pharynx is also self regulatory.

A neuronal link between the amphids and the pharynx exists. This link is presented diagrammatically by the "wiring diagram" presented in figure 5.2. (constructed from information contained in (White, Southgate et *al.*, 1986)). The diagram shows connectivity between one amphid neuron, ADF, and the interneuron RIP which connects the pharyngeal nervous system with the nervous system of the rest of the worm. Also shown are the interneurons AIA, AIB, AIY AIZ, RMG and the putative sensory neuron AUA, all of which potentially receive input from the amphidial neurons (some of the links in the diagram are gap junctions in which the direction of signalling is unknown). The connections between amphid neurons and these neurons are as follows (from (White, 1985; White, Southgate et *al.*, 1986)):

AIA	is postsynaptic with AWC, ASE, ASI, ASH, ASK, ASG and has gap junctions with AWA, ADF.
AIB	is postsynaptic with ASE, AWC, ASI, ASH, ASK, ADL, ASG and has gap junctions with ADF.
AIY	is postsynaptic with AWC, ASE, AWA, AFD
AIZ	is postsynaptic with AWA, AWB, ADF and has gap junctions with ASG and ASH
AUA	is postsynaptic with ADF and has gap junctions with AWB
RMG	has gap junctions with AWB, ASK, ASH, ADL.

The amphid neurons ASE, ASI, ASH, ASG, ASK, ADL, ADF all have free ciliated endings in the amphid channel. The only amphid channel neuron not connected to neurons in figure 5.2. is ASJ. ASJ is however presynaptic with ASK and so is connected with the interneurons in the figure via this route. The amphid neurons AWA, and AWC detect volatile substances (Bargmann, Hartwieg et *al.*, 1993)) and AFD is probably thermosensory (Perkins, Hedgecock et *al.*, 1986). All three have endings embedded in the sheath cell. AWB also has an ending embedded in the sheath cell but has no known function (see chapter 1). AWB stains with DiO (Starich, Herman et *al.*, 1995).

A number of important points arise from the wiring diagram. Firstly, signals from the amphid can arrive at the pharynx via a

Figure 5.2.

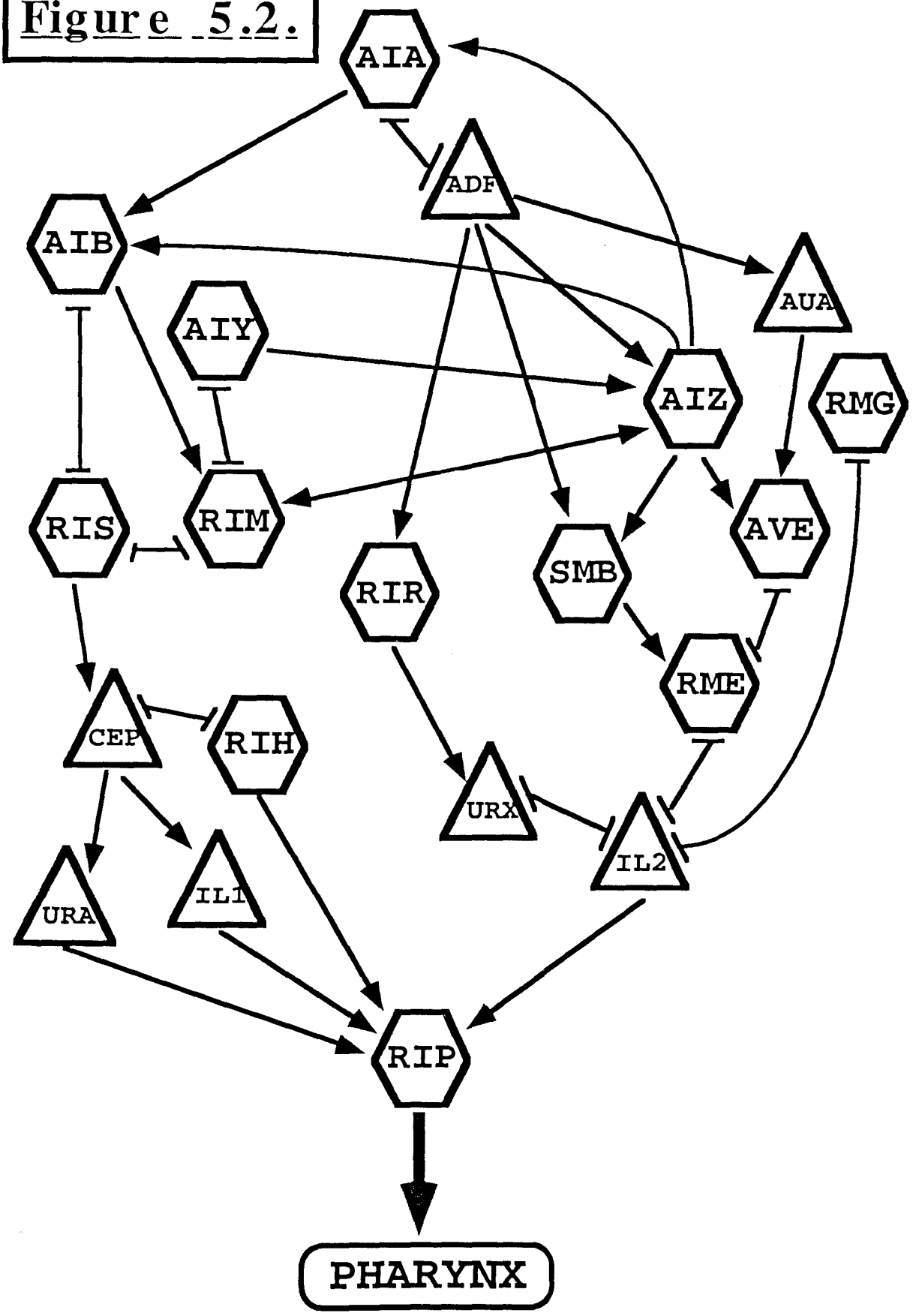

AIA
ADF
AIB
AUA
AIY
AIZ
RMG
RIS
RIM
AVE
RIR
SMB
RME
CEP
RIH
URX
IL2
URA
IL1
RIP
PHARYNX

Figure 5.2.

Neuronal Connections between an amphid sensory neuron (ADF), amphid interneurons and the RIP neuron.

Neurons are represented by hexagons (interneurons) or triangles (sensory neurons). Synapses are represented by arrows, the arrow points toward the postsynaptic partner. RIM and AIZ synapse onto one another, so there is an arrow on either end of the line joining them. Gap junctions are represented by blunt ended lines. ADF is an amphid channel neuron which has different connectivity to the amphid interneurons than other amphid neurons. Other amphid neurons synapse onto (or have gap junctions with) AIA, AIB, AIY, AIZ, RMG and/or AUA (see text). RIP is the only neuron connecting the pharyngeal nervous system from the non-pharyngeal nervous system.

number of routes, some of which may not share common neurons (e.g. amphid'AIB—RIS'CEP'IL1'RIP is distinct from amphid—RMG—IL2'RIP). This may provide functional redundancy between pathways. A second aspect is the occurrence of feedback triangles, for example AIY'AIZ÷RIM—AIY or AIB'RIM—RIS—AIB; these type of feedback loops have been noted previously in *C. elegans* neuroconnectivity (White, 1985). White (1985) suggested that the patterns of triangular connectivity could act to quickly inhibit signals transduced from one neuron to a second neuron via a third. An example is illustrated by the connections ADF'AIZ'SMB, and ADF'SMB in figure 5.2. Sensory input from ADF might stimulate SMB and AIZ simultaneously, AIZ might have inhibitory input onto SMB, so that SMB is activated by ADF and then inhibited by AIZ in quick succession (as in (White, 1985)). This process allows the nematode to monitor pulses of sensory input and changes in the lapse time between pulses allow the nematode to detect its proximity to the source of attractant molecules and so track toward a chemical attractant (White, 1985).

Another interesting aspect of the neuronal connection between the amphids and pharynx is the number and position of other sensory neurons in the pathway. A signal from the amphid must act on RIP via CEP or IL2, and the sensory neurons AUA, URX, IL1 or URA are also involved in a number of amphid'pharynx

pathways. The nature of the input from these neurons is largely unknown.

So a neuronal link between the amphids and pharynx exists: but what is its function?

Avery and Horvitz (1990) (Avery and Horvitz, 1990) showed that *C. elegans* responds to the presence of bacteria in its environment by increasing pharyngeal pumping rate. The amphids are the most likely sensory organs involved in detection of bacteria. Amphid neurons are involved in detection of a number of aqueous and volatile compounds, some of which may be bacterial metabolites (e.g. Biotin) (Bargmann and Horvitz, 1991; Bargmann, Hartwieg et *al.*, 1993; Bargmann, 1993). Amphid neurons are also involved in directing the formation of dauer larvae, a process which proceeds in the absence of bacteria and in the presence of high concentrations of dauer pheromone (Bargmann and Horvitz, 1991). Therefore the amphids most likely detect bacteria and the pharynx responds to bacteria by increasing pumping rate. The pharynx may however detect bacteria via proprioceptors within the pharynx; although no function has been proven for these receptor neurons, their morphology suggests a role in mechanoreception in the pharynx (Albertson and Thomson, 1976).

Serotonin can also stimulate pharyngeal pumping in *C elegans* (Avery and Horvitz, 1990), and there are two serotonergic neuron types in the head of the worm which might affect this activity, RIH (an extrapharyngeal interneuron) and NSM (a pair of pharyngeal neurosecretory and motor neurons) (Raizen, Rosenfeldt et *al.*, 1992). It has been suggested that the pharynx has a basal rate of pumping needed to ensure that bacteria enter the lumen of the pharynx when the animal comes into contact with its food source (Raizen, Rosenfeldt et *al.*, 1992). After some bacteria have been detected by pharyngeal mechanoreceptors, these receptors can take over regulation of pumping. Although the results are unpublished, laser ablation experiments in combination with application of imipramine and serotonin have led to a proposal that the basal rate of pumping is regulated by input from outside the pharyngeal nervous system via RIH, whereas pumping in response to the presence of bacteria in the lumen is mediated by a circuit involving the pharyngeal neurons I1, MC and NSM (Raizen, Rosenfeldt et *al.*, 1992). As *C. elegans* can be grown in axenic medium which contains no bacteria, the presence of bacteria cannot be an absolute requirement for pharynx activity. There is most likely a basal level of pharynx activity which is independent of environmental stimuli.

So there is evidence that a link between the amphids and pharynx exists, and that it might be important to the regulation of pharyngeal activity in response to environmental stimuli.

5.2.2.4. The production of a constitutive signal from the amphid may be involved in IVM resistance mediated by Dyf mutations.

There is evidence therefore that input from outside the pharyngeal nervous system can influence pharynx function (Raizen, Rosenfeldt et *al.*, 1992), even though the pharynx will continue to function in the absence of extrapharyngeal neuronal input (Avery and Horvitz, 1989). It is feasible therefore that sensory information collected by the amphids could be used by the worm to influence pharynx function, and it seems likely that the effect would be to adjust the basal level of pharynx activity rather than mediating pharynx activity during feeding (Raizen, Rosenfeldt et *al.*, 1992). The serotonergic neuron RIH is shown in figure 5.2., in the neural pathway between the amphids and pharynx, supporting the idea that sensory input received by the amphids can potentially influence pharyngeal activity.

By modulating the input of sensory information to the pharynx, Dyf mutations might overcome the negative effect of IVM on pharyngeal pumping. For example, in starved worms, the amphid neurons might produce a signal, when the worms sense food, that is transducted via RIH and RIP to increase the basal rate of pumping in the pharynx. Dyf worms are less able to detect bacteria in their environment and may produce a pharynx activating pathway constitutively. The idea of a constitutive signal being produced by the amphids in Dyf worms has been raised previously (Bargmann and Horvitz, 1991). When amphid neurons are ablated, dauer larvae are formed constitutively, however in Dyf animals where the amphid neuron cilia are shortened or malformed, dauer formation is suppressed. So Bargmann and Horvitz (1991) suggested that Dyf animals produce a constitutive signal preventing dauer larvae formation. This suggests that amphid neurons can be "turned off" by environmental cues rather than "turned on". Both excitatory and inhibitory responses to stimuli have been recorded from chemosensory neurons in vertebrates and arthropods (Dionne and Dubin, 1994). A constitutive amphidial signal in Dyf worms which influences pharynx activity might therefore be central to IVM resistance in these worms. As synaptic input onto excitatory cells is additive, an increase in pharyngeal excitatory input mediated via the amphids and the RIH interneuron might lessen the effect of pharyngeal inhibition mediated by IVM. It is unlikely however that the constitutive signal responsible for IVM resistance and the

signal involved in the Daf-d phenotype are identical, as Avr mutations which affect amphid neuron morphology are sometimes not Daf-d (*dyf-10*, *dyf-12* - (Starich, Herman et *al.*, 1995)) and one is Daf-c (*daf-19* - (Perkins, Hedgecock et *al.*, 1986)).

An alternative mechanism might act via amphid mediated modulation of neurons suppressing pharynx function. The pharyngeal motoneuron M3 has been proposed to be inhibitory (Avery, Davis et *al.*, 1994). This neuron may be a glutamatergic neuron which inhibits pharynx muscle cell excitability via a glutamate activated chloride ion channel on the muscle cell surface (Avery, Davis et *al.*, 1994). As IVM acts at very low concentrations to potentiate responses to glutamate on glutamate gated chloride ion channels (Arena, Liu et *al.*, 1992), the combination of effects of M3 and IVM might be greater than effects of either acting alone. So if extrapharyngeal neuronal input down regulated M3 such that glutamate release was decreased, the effect of low concentrations of IVM might be reduced. At higher IVM concentrations, IVM can directly open glutamate gated chloride ion channels and so synergistic effects of IVM and glutamate might be less important. A model explaining IVM resistance in Dyf worms which includes possible extrapharyngeal effects on M3 is an attractive proposition, as Dyf mutants are resistant only to low levels of IVM (5-30 ng/mL), comparable to the concentrations reported to potentiate glutamate action at chloride ion channels (Kim and Johnson, 1991; Arena, Liu et *al.*, 1992).

Irrespective of whether sensory input from the amphid stimulates excitatory pharyngeal neurons such as NSM or inhibits inhibitory neurons such as M3, an IVM resistance model which places amphid mediated effects on the pharynx in a central role explains a number of the observations reported in this thesis.

Firstly, a large number of mutations which affect amphid structure are resistant to low levels of IVM, but no mutations tested which affect the interconnection of neurons from the amphids are resistant (Chapter 2). This is consistent with IVM resistance involving an active signal from the amphid: ie. mutations disconnecting a circuit that is inoperative do nothing. Also supporting this idea is the observation that mutations (such as *unc-104*, *unc-116* and *snt-1*) which inhibit synaptic transmission can suppress IVM resistance in Dyf animals (Chapter 4). It has been proposed that a constitutive amphid signal is involved in the Daf-d phenotype of Dyf mutants (Bargmann and Horvitz, 1991) as ablation of the neurons causes constitutive dauer formation. *unc-104 dyf* worms are dauer formation constitutive rather than dauer formation deficient, suggesting that *unc-104* blocks the constitutive signal (Vowels and Thomas, 1992; Vowels

and Thomas, 1992). Therefore *unc-104* mediated suppression of the Daf-d and Avr phenotypes of Dyf mutants might act via the same mechanism. If IVM acted to block the amphid-pharynx pathway then mutants such as *unc-104* would be resistant to the drug, and this is not the case (Chapter 2). If IVM resistance were due to the absence of a signal from the amphid then mutations which blocked this signal should impart resistance and should not suppress resistance in Dyf animals.

A second aspect of increased IVM resistance via this mechanism is that the process is a complex one involving a number of multi-neuron pathways. This allows different levels of IVM resistance to be achieved through slight differences in the effect of the Dyf mutations. Consistent with this idea is the large amount of variation in response to IVM exhibited by different mutations; even allelic mutations vary widely in their level of resistance (see data on *dyf-12* in Chapter 3 for example). Also subtle effects on amphid morphology which do not affect dye filling, such as in some alleles of *che-3* (Johnson and Clover, 1995) and in *dyf-10*(d) and *dyf-12*(d) heterozygotes (Chapter 3) are resistant to the drug under this model because of their effects on chemosensory signalling.

A large number of mutations might be expected to influence neuroconnectivity or function of neurons with processes linking the amphid and the pharynx. Many of these mutations might also be expected to suppress IVM resistance in Dyf worms under the model proposed here. In support of this idea, extragenic suppressors of *dyf-12* mediated IVM resistance were isolated at a relatively high rate (Chapter 4). Also some mutations which impede synaptic transmission have been identified which are suppressors of IVM resistance in Dyf mutants (Chapter 4, Grant Pers. Comm., 1994).

Finally, the model explains why some mutations which decrease pharyngeal activity are super sensitive to IVM (Chapter 2). The additive effect of these mutations and IVM might reduce pharynx function below some lethal threshold at concentrations of IVM below that which are lethal to wild type worms.

The model for low level IVM resistance by Dyf worms presented here is hypothetical at this stage. The complexity of the model is necessary to explain the data, however it also poses a large number of questions which need to be addressed in testing the validity of the model.

5.2.5. EXPERIMENTS TO BE DONE

The proposed model for IVM resistance in Dyf worms can be tested experimentally. Some investigations which have been initiated during the work described in this thesis can be used to test the model.

The link between the amphids and pharynx is central to the model of IVM resistance in Dyf worms proposed here. A number of experimental approaches could be adopted which could test for the existence of a functional link and investigate its relevance to IVM resistance.

If wild type worms can regulate pharynx activity via signals from sensory organs, then environmental stimuli might also be expected to influence IVM resistance. Starved *C. elegans* respond to exposure to bacteria with greater increases in pharynx pumping than well fed worms (Avery and Horvitz, 1990). The effects of IVM on pharyngeal activity from starved and well fed worms might differ. If the basal level of activity of the pharynx is increased by starvation, then the response of starved worms to IVM might be expected to be less than that of non-starved worms. Electrical recordings from the pharynx in whole worms can be taken (Raizen and Avery, 1994). It would be interesting to monitor the response of the pharynx to differing levels of attractant compounds (such as cAMP or lysine) or repellent compounds (such as D-tryptophan or garlic extract) both in the presence and absence of IVM. In addition, the response of the pharynx to known chemicals which influence pharynx function such as imipramine, octopamine, phentolamine, muscimol, GABA and serotonin (Horvitz, Chalfie et *al.*, 1982; Avery and Horvitz, 1990) might be analysed with respect to the exposure of individual worms to attractant and repellent compounds. The response of dissected pharynxes to IVM and other neurological compounds might be considered as a control for these experiments, as excised pharynxes cannot be influenced in any way by the extrapharyngeal nervous system of the worm. These preparations would only be appropriate controls however if the buffer solutions were identical for whole worm and excised pharynx recordings.

Single-cell recordings from *C. elegans* neurons are now possible (Lockery, 1995). Electrophysiological recordings from amphid neurons and interneurons in Dyf worms would be helpful in establishing the existence of constitutive signals from the amphid neurons. Similar recordings from wild type worms could be used to establish the types of environmental cues which influence the pharynx. Initial experiments should focus on the amphid neurons, RIP interneuron and the pharynx muscle cells in

whole worms. Later experiments might help elucidate which pathways in figure 5.2. are most important to amphid mediated responses in the pharynx.

An alternative approach to intracellular recording might be laser ablation. This approach has been used to predict functions of a variety of sensory neurons (Chalfie, Sulston et *al.*, 1985; Bargmann and Horvitz, 1991; Bargmann and Horvitz, 1991; Bargmann, Hartwieg et *al.*, 1993), and has recently been used to investigate the role of some interneurons in *C. elegans* (Bargmann, 1993; Mori and Ohshima, 1994). Pharyngeal responses to IVM in worms which have had selected interneurons ablated, could establish which interneurons are important to signal pathways between the amphid and pharynx. Also IVM resistance in Dyf worms should be abolished by ablation of RIP under the model presented here. Experiments using worms which have had RIP ablated would be important in the establishment of the role of an amphid-pharynx neural pathway in IVM resistance.

An important aspect of the wiring diagram in figure 5.2. is the number of non-amphid sensory neurons which are involved. As the majority of these form links between the amphids and pharynx, it would be difficult to eliminate the role of these neurons in pharynx regulation without influencing the role of the amphids. By eliminating subsets of them however, it might be possible to investigate the role of some of the sensory structures in the head. For example, if normal responses to IVM were observed after ablation of the cephalic neurons (CEP), then responses to IVM might be mediated by the amphids, URX or the inner labial sensilla. Also if ablation of amphid neurons completely suppressed IVM resistance in Dyf worms then the amphids (as expected) would be implicated in the response. However, ablation of CEP and IL2 neurons would be expected to have identical effects to ablation of all amphid neurons, so it would be impossible to distinguish between an additive response mediated by the cephalic and inner labial neurons collectively and a response mediated by the amphids alone. Additive functions of sensory neurons have been previously observed (Kaplan and Horvitz, 1993). The response to touch on the head is mediated by the neurons OLQ, ASH and FLP, and the effect of removing all these neurons is greater than the effect of removing any subset of these.

The interpretation of laser ablation experiments might also be affected by the presence of humoral signals influencing the pharynx from extrapharyngeal sources. Humoral influences have been predicted to influence pharynx function (Avery, 1995). *snt-1* mutations can suppress pharyngeal pumping in worms in which the pharynx nervous system has been ablated. As *snt-1* is only

expressed in neurons, and there is no neuronal link to the pharynx in these worms, the effect of this mutation must be to impede a humoral signal which is necessary for normal pharynx function (Avery, 1995). In the choline acyltransferase mutant *cha-1*(m324) mutant, pumping virtually ceases. However, when the entire pharyngeal nervous system is ablated, there is still pumping (Avery and Horvitz, 1989). This suggests that some extrapharyngeal cholinergic neuron(s) can influence the pharynx (Avery, Pers. Comm., 1995). Laser ablation of neurons would not necessarily block signals between the amphids and pharynx if there were a humoral component to the signal pathway. The identification of neurons or gland cells which released humoral factors would be necessary before this possibility could be investigated by laser ablation. The use of mutants such as *snt-1* and *cha-1* to block humoral responses would not be useful as these mutations also effect synapse function.

The nature of signalling between the amphids and pharynx may be neuronal or humoral or both. Irrespective of the type of pathway, mutants which affect neuronal function can suppress IVM resistance (Chapter 4). Identification of the cells which express these genes might reveal cells important to IVM resistance. In much the same way as the amphid was identified as being important in IVM resistance (Chapter 2), the role of interneurons or neurosecretory cells might be discovered by comparing mutants which abolish or reduce IVM resistance in Dyf animals. The patterns of gene expression, or particular ultrastructural changes in these mutants might reveal a subset of cells affected in all the mutants. The process of identifying suppressors of IVM resistance has begun (Chapter 4). Analysis of the suppressors isolated from the mutagenesis screen described in chapter 4 along with construction of a range of Dyf/Unc double mutants will be useful in the further elucidation of the nature of IVM resistance in Dyf worms. The information revealed by each mutation suppressing IVM resistance will be greater if the mutation is in a cloned gene for which sequence and expression data are available. As a large number of genes are currently being cloned in a variety of *C. elegans* laboratories, and as the entire genomic sequence of *C. elegans* will soon be available, interactions of mutations with Dyf genes may be a powerful aid to understanding IVM resistance.

Construction of mutant strains which contain Dyf and *eat* mutations might also be valuable in investigating IVM resistance. The model presented here predicts that reduced pharynx function in *eat* mutants is additive with IVM responses in the pharynx such that IVM sensitivity is greater in Eat animals than for wild type worms. If this is the case, then *eat* mutations would also be

expected to reduce IVM resistance in Dyf worms. Also, Unc mutations which affect structural components of muscle cells in *C elegans* have been observed to reduce IVM resistance in *dyf/unc* strains (Chapter 4). These mutations might weaken the pharyngeal muscle and act to suppress IVM resistance in a similar manner to *eat* mutants. At least one of these mutations (*unc-54*) however is in a gene expressed only in body muscle (Waterston, 1988). An explanation of the effects of this muscle mutant might be revealed by localisation of DYF protein or transcripts, and by analysing more combinations of *unc-54* alleles with Dyf mutations. This type of suppression may be locus specific and mediated by pleiotropic effects of a subset of Dyf mutants. Some Dyf mutants (e.g. *mec-8* - (Lundquist, Shaw et *al.*, 1993)) have a wide range of pleiotropic defects.

Another aspect of IVM resistance that is worthy of investigation is the apparent temperature sensitivity of IVM resistance in Dyf mutants (Chapter 2 & 3). Although these experiments represent only a preliminary investigation into this phenomenon, it is tempting to speculate on the mechanism behind this effect. The amphid has also been implicated in thermoreception (Hedgecock and Russell, 1975; Dusenbery, 1980; Perkins, Hedgecock et *al.*, 1986; Mori and Ohshima, 1994), and if the model presented here for IVM resistance proves to be correct, then perhaps stimulation of AFD (thermosensory neuron) by high temperature provides antagonistic input into the amphid-pharynx signalling pathway. Formation of dauer larvae is increased at higher temperature (Golden and Riddle, 1984) so perhaps IVM resistance is affected by temperature in an analogous manner. This could be tested by observing the phenotypes of mutants which suppress temperature sensitivity in Dyf strains, or by constructing double mutants carrying thermotaxis defective and Dyf mutations. The expectation might be that some of the thermotaxis mutants (those that are thermotaxis deficient but not Che) might suppress temperature sensitivity of Dyf worms.

Suppression of IVM resistance by *dpy* mutations might be due to an accumulation of mutant gene products, causing a response similar to heat shock. Such an interaction might then make worms which are IVM sensitive at 27°C, also sensitive at 20°C. This model has been suggested for *dpy*-mediated suppression of other temperature sensitive mutations (Levy, Yang et *al.*, 1993). An alternative explanation for suppression of IVM resistance by *dpy* mutations, might be that IVM can gain access to the interior of the worm more easily in these mutants. The cuticle is composed of collagens, and a number of *dpy* genes encode collagen proteins (Johnstone, Shaffi et *al.*, 1992; Levy, Yang et *al.*, 1993; Johnstone, 1994; Kramer, 1994). Although IVM can

penetrate the cuticle of *A. suum*, a number of chemical treatments can increase permeability of the cuticle to IVM (Ho, Geary et *al.*, 1990). Perhaps *dpy* genes also act to increase cuticular penetration of IVM and so increase the rate at which the drug accumulates on pharyngeal cell muscles. An alternative might be that the drug can gain access to other essential neuronal or muscle tissues in *dpy* worms. These ideas might be tested by observing the penetration and distribution of fluorescent IVM analogues in wild type and Dpy *C. elegans*. By injecting IVM, the distribution of the drug in various tissues of *A. suum* has been analysed (Martin, Kusel et *al.*, 1992).

CORRIGENDUM

1: On page 74 it states that "...no mutants are available which have normal phasmids and abnormal amphids or vice versa,...". The mutants *lin-44* and *lin-17* are however defective in phasmid but not amphid filling (See Herman and Horvitz, (1994); and Sternberg and Horvitz (1988))

2. On page 147 there is a discussion which suggests that a possible explanation for unusual mapping data for the *sav* mutants au4 and au17 might be that these mutations are informational suppressors. This is only one explanation of these results.

3. On page 161 it is suggested that as *mec-8* mutants have pleiotropic effects on body wall muscle phenotype, perhaps other Dyf mutations might also have similar defects. This argument was used to attempt an explanation for observed interactions between mutations affecting muscle structure and *dyf* mutations with respect to ivermectin resistance. It appears however that *mec-8* may be a special case as the gene involved encodes a *trans*-acting RNA splicing factor that seems to affect different target loci.

4. The generation of *dyf-12* null alleles would be a difficult task if null mutations in this gene are silent or lethal as the argument on page 165 predicts. Three possible strategies might be followed however:

a) - An intense screen for lethal mutants failing to complement the deletion nDf19, might yield null alleles of *dyf-12* which are lethal as homozygotes. The phenotype of these mutations in double heterozygotes with *dyf-12*(nr272), *dyf-12*(nr2344) or *dyf-12*(nr2477) might be predicted to be similar to the phenotype of individuals of the genotype *dyf-12*(nr272)/nDf19, *dyf-12*(nr2344)/nDf19 and *dyf-12*(nr272)/nDf19.

b) - If the mutations nr272, nr2344 and nr2477 are hypermorphic or antimorphic, then null mutations might be expected to lessen the drug resistance and/or the penetrance of the Dyf phenotype in *dyf-12*(0)*dyf-12*(d)/*dyf-12*(0)*dyf-12*(d) homozygotes. A screen for intragenic suppressors of one of the dominant *dyf-12* mutations then might yield *dyf-12*(0) alleles. Although the phenotype of *dyf-12*(0)*dyf-12*(d)/*dyf-12*(0)*dyf-12*(d) homozygotes is not known, the phenotype of *dyf-*

12(sa127)/nDf19 is wild type, therefore double heterozygotes carrying null alleles of *dyf-12* and dominant alleles might also be non-Dyf, the null allele making heterozygotes carrying nr272, nr2344 or nr2477 appear wild type. Even if null alleles are not lethal therefore, it might be possible to generate them in a screen for revertants of the incompletely dominant Dyf phenotype.

c) - As the Dyf phenotype of dominant *dyf-12* mutants is only semi-penetrant, and as the Dyf phenotype of sa127 is recessive, it might be possible to clone the *dyf-12* gene by transformation rescue. Cosmids spanning the *dyf-12* region can be identified by comparing the physical and genetic maps and identifying the region of the physical map which corresponds to the genetic map position for *dyf-12*. Once *dyf-12* has been cloned and the sequence of the gene is known, it would be possible to generate transposon induced deletions of the locus (*dyf-12*(0) alleles). This method and its applications have been reviewed recently in Hodgkin *et al.* (1995).

5. Dominant IVM resistant alleles of *dyf-10*I and *dyf-12*X fail to complement one another for the Dyf phenotype (see table 3.4, page 111). Genetic studies have implicated genes encoding proteins which bind to or are part of intra-cellular structures such as microtubules in instances of intergenic noncomplementation described previously (see discussion, chapter 3). A molecular model for the interaction of *dyf-10* and *dyf-12* would most likely also involve microtubule structures, as these are principal components of the amphid cilia (which are likely to be defective in these mutants). Tubulin genes have already been cloned from *C elegans* and it is unlikely that either *dyf-12* or *dyf-10* encode tubulins. Many microtubule associated proteins (MAPs) exist in the eukaryotic cell however (Cleveland, 1993) and it is possible that *dyf-10* and/or *dyf-12* could encode such proteins. Though this is purely speculative, it is possible to imagine that intergenic noncomplementation could occur between mutations in separate MAP genes. If proper microtubule assembly, for example, depended on a certain concentration of MAP proteins being present in the cell, and two similar proteins could partially substitute for one another in this task, then reducing the level of these proteins by half might cause an observable phenotype. Now, if these proteins are interchangeable, inactivating both copies of one or the other gene would produce a similar phenotype to the inactivation of one copy of each gene. Thus the phenotype of the double heterozygote resembles the phenotype of either

homozygote. Additionally, one could imagine that if the amount of these proteins was reduced still further an even more severe phenotype could result. The *dyf-10, dyf-12* example gives an opportunity to test this idea by creating the double homozygote.

6. *vab-3*(e648) was found to have an increased sensitivity to IVM (page 60). The *vab-3* gene has been cloned and found to be a Pax-6 homologue. Pax-6 genes play a role in anterior development in vertebrates and in *Drosophila*. *vab-3* is expressed in the anterior of *C. elegans* and is expressed in both neuronal and hypodermal cells (Chisholm and Horvitz, 1995). It is possible that *vab-3* might increase sensitivity to IVM by weakening the pharynx in some way. The buccal capsule in *vab-3* mutants is malformed (possibly due to the dependence of buccal hypodermal cells on VAB-3). One might hypothesise therefore, that a malformed buccal opening would reduce the amount of bacteria ingested with each pump of the pharynx. A lower IVM concentration therefore might be needed to reduce food intake to a lethal level.

7. The following is a table of kinesin like proteins and their map positions in *C. elegans*. Apart from *unc-116* and *unc-104*, none of these genes has been investigated by mutagenesis. *unc-116* and *unc-104* can suppress the IVM resistance of some Dyf mutant strains (see chapter 4).

Open Reading Frame Number	Chromosome Number	Gene Name
C05D12.5	II	none
C16C10.2	III	none
C33H5.4	IV	none
C52E12.2	II	*unc-104*
R05D3.7	III	*unc-116*
R144.1	III	none
T09A5.2	II	none
ZK546.1	II	none

8. The *C. elegans* sequencing consortium has now sequenced the region of chromosome X on which *dyf-12* is situated. Analysis of the region has not revealed any putative open reading frames which have homology to MAPs, kinesins, dyneins, tubulin, actin or actin binding proteins. Thus it is unlikely that the *dyf-12* gene has homology to any of these known classes of genes involved in

cytoskeletal structure or function. However, this does not rule out the possibility that *dyf-12* belongs to a presently unknown class of proteins involved in microtubule function. Alternately, *C. elegans* genes with homologous roles to vertebrate MAPs, might not share highly conserved amino acid sequence with their vertebrate counterparts. This has been observed when comparing vertebrate and nematode globin genes for example - reveiwed in Blaxter (1993).

9. The mutation *unc-60*(e723) was observed to decrease the resistance of *dyf-10*(nr2389), *dyf-12*(nr272) and *dyf-12*(nr2477) to IVM in double mutant strains. The *unc-60* gene has been cloned and encodes two alternately transcribed proteins, one of which has homology to cofilin (McKim *et al.*, 1994), an actin binding protein which may act to inhibit actin polymerisation and to inhibit binding of myosin and tropomyosin to actin filaments (Moriyama and Nishida, 1993). Although there is no expression pattern data yet available, it is possible that the cofilin-like A transcript of *unc-60* could be expressed in the pharynx. The pharynx might then be weaker in *unc-60*(e723) mutants and be more susceptible to IVM. Of course, this transcript could also be expressed in other non-muscle cells which might also be important to IVM sensitivity (i.e. marginal cells in the pharynx). The second B transcript is probably not involved in IVM resistance suppression as it was not greatly effected in the e723 mutant used in these experiments.

10. On pages 180-184 a number of suggested further experiments are listed. The proposed order in which these might be done is as follows:

A. Three groups of experiments are perhaps the most important for testing the model proposed on page 165.

a) The model depends on the pharynx responding to external chemical stimuli, at least in some circumstances. Therefore experiments monitoring pharynx activity when the worm is exposed to chemoattractants and repellents, will be important.

b) Laser ablation of interneurons connecting the pharynx with the somatic nervous system in Dyf mutants might be expected to abolish IVM resistance.

c) The model depends on IVM being capable of crossing into the worm through the cuticle. Although some experiments have been done with *Ascaris* which demonstrated that IVM could cross the cuticle in that nematode, it might also be useful to demonstrate that IVM can penetrate the *C. elegans*

cuticle. These experiments could be done with radio labelled IVM, IVM antibodies or fluorescent IVM analogues.

B. In the second group of experiments to be attempted I would include experiments monitoring the sensory and interneurons between the amphids and pharynx in response to chemostimulants and IVM. In this group I would also include experiments using mutants which disrupt neuronal function in combination with *dyf* mutants to further analyse suppression of IVM resistance by genetic disruption of the neuronal link between amphid and pharynx. If expression pattern data was available for some of these, the interpretation of the results would be facilitated.

C Once the first two groups of experiments were complete, I suggest that the remaining experiments in the discussion should be attempted. Of particular interest amongst these might be the creation of *dyf/eat* double mutants. If *eat* mutants can act as suppressors of IVM resistance in *dyf* mutants, this would indicate that down regulation of the pharynx can increase sensitivity to IVM.

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APPENDICES

APPENDIX A

A.1. GENE, PHENOTYPE, ALLELE AND GENE PRODUCT NAMES.

Gene names for *Caenorhabditis elegans* are three letter codes which are given in lower case and italicised. Each locus is also given an italicised number which is attached to the gene type name with a dash. Allele numbers consist of a two or three letter code (lower case) and a number. Allele numbers are given in parentheses after the gene name where relevant. Strain names consist of two upper case letters followed by a number. Where the gene product of a locus is referred to it is given in non-italicised capital letters and numbers. Phenotypes of various mutations are given a three letter code also and in some instances the gene type name and phenotype may have the same code. When a phenotype is refered to it is non-italicised and the first letter is a capital. Some phenotypes however have no three letter code and are referred to using full words.

Listed below are the gene type names and phenotypes referred to in this thesis.

Gene type name	Description
aex	ABoc expulsion defective
avr	avermectin resistant
bli	blistered cuticle
bor	bordering behaviour
caf	abnormal caffeine resistance
cat	abnormal catecholamine distribution
che	abnormal chemotaxis
daf	abnormal dauer formation
deg	degeneration of certain neurons
dpy	dumpy: shorter than wild type
dyf	amphid neuron dye filling defective
eat	eating; abnormal pharyngeal behaviour
egl	egg laying defective
fem	feminisation of XX and X0 animals
glp	abnormal germ line proliferation
him	high incidence of males
lev	levamisole resistant
lin	abnormal cell lineage
mab	male abnormal
mah	ts reversible paralysis
mec	mechanosensory abnormality
mor	morphological: rounded nose
osm	defective avoidance of high concentrations of sugars or salts (osmotic)
pha	defective pharynx development
phm	pharyngeal muscle
ric	resistance to inhibitors of cholinesterase
sav	suppressor of avermectin resistance
sma	small
sog	suppressor of *glp*
sup	suppressor
unc	uncoordinated
vab	variable abnormal

Phenotype name	Description
Avr	Avermectin resistant
Che	Chemotaxis abnormal
Dpy	Dumpy
Dyf	Dye Filling defective
nonDyf	Amphid neurons do fill with FITC or DiO, but staining may be fainter than wild type.
Egl	egg laying defective
Male mating abnormal	male mating abnormal
Notch	Atrophy of one side of the head, leading to a notched (dented) head shape.
Osm	Osmotic avoidance defective
Sav	Avermectin resistance suppressed
Unc	Uncoordinated
Wt	Wild type: can be used with respect to the non-mutant form of any phenotype

A.2. *Caenorhabditis elegans* AND *Haemonchus contortus* STRAINS USED.

A.2.1. *C. elegans* strains from the Caenorhabditis Genetics Center.(University of Minnesota, St Paul, USA)

CB769 *bli-1*(e769)II
CB768 *bli-2*(e768)II
CB767 *bli-3*(e767)I
CB937 *bli-4*(e937)I
CB518 *bli-5*(e518)III
BE16 *bli-6*(sc16)IV
RC301 *bor-1*(g320)X
CB1111 *cat-1*(e1111)X
CB1112 *cat-2*(e1112)II
CB1141 *cat-4*(e1141)V
CB3383 *cat-6*(e1861)V
CB1034 *che-1*(e1034)*fer-1*(hc1)I
CB1033 *che-2*(e1033)X
CB1124 *che-3*(e1124)I
CB1073 *che-5*(e1073)IV
CB1126 *che-6*(e1126)IV
CB1128 *che-7*(e1128)V

CB3329 *che-10*(e1809)II
CB3330 *che-11*(e1810)V
CB3332 *che-12*(e1812)V
CB3323 *che-13*(e1805)I
CB3687 *che-14*(e1960)I
CB1377 *daf-6*(e1377)X
CB1387 *daf-10*(e1387)IV
DR47 *daf-11*(m47)V
DR20 *daf-12*(m20)X
CB1375 *daf-18*(e1375)IV
DR86 *daf-19*(m86)II
TU38 *deg-1*(u38)X
CB27 *dpy-3*(e27)X
CB14 *dpy-6*(e14)X
CB88 *dpy-7*(e88)X
CB130 *dpy-8*(e130)X
DA531 *eat-1*(ad427)IV
DA465 *eat-2*(ad465)II
DA631 *eat-3*(ad426)II,*him-8*(e1489)IV
DA572 *eat-4*(ad572)III
DA464 *eat-5*(ad464)I
DA467 *eat-6*(ad467)V
DA521 *eat-7*(ad450)IV
DA599 *eat-8*(ad599)III
DA563 *eat-9*(e2337)I,*him-8*(e1489)IV
MT1079 *egl-15*(n484)X
MT3353 *egl-15*(n484)*sma-5*(n678)X
MT2247 *egl-44*(n1080)II
MT2316 *egl-46*(n1127)V
ZZ15 *lev-8*(x15)X
MT306 *lin-15*(n309)X
TU82 *lin-32*(u282)X
MT1514 *lin-39*(n709)III
mab-5(e1751)
TN110 *mah-2*(cn110)X
CB1066 *mec-1*(e1066)V
CB75 *mec-2*(e75)X
CB3273 *mec-2*(e1084)*lon-2*(e678)X
CB1071 *mor-1*(e1071)III
CB1125 *mor-2*(e1125)IV
PR808 *osm-1*(p808)X
PR802 *osm-3*(p802)IV
PR813 *osm-5*(p813)X
PR811 *osm-6*(p811)V
MT3643 *osm-11*(n1604)X
PR671 *tax-2*(p671)II

PR673	*tax-3*(p673)A
PR678	*tax-4*(p678)
PR672	*tax-5*(p672)V
PR675	*tax-6*(p675)A
CB151	*unc-3*(e151)X
CB120	*unc-4*(e120)II
CB152	*unc-5*(e152)IV
CB78	*unc-6*(e78)X
FH85	*unc-9*(sc27)X
CB101	*unc-9*(e101)X
CB450	*dyf-?*(au9)*unc-13*(e450)I
CB57	*unc-14*(e57)I
CB73	*unc-15*(e73)I
CB81	*unc-18*(e81)X
CB66	*unc-22*(e66)IV
CB138	*unc-24*(e138)IV
CB156	*unc-25*(e156)III
CB845	*unc-30*(e191)IV
CB169	*unc-31*(e169)IV
CB189	*unc-32*(e189)III
CB204	*unc-33*(e204)IV
CB315	*unc-34*(e315)V
CB271	*unc-40*(e271)I
CB408	*unc-43*(e408)IV
CB362	*unc-44*(e362)IV
CB286	*unc-45*(e286)III
CB307	*unc-47*(e307)III
CB369	*unc-51*(e369)V
CB444	*unc-52*(e444)II
CB190	*unc-54*(e190)I
CB723	*unc-60*(e723)V
CB228	*unc-61*(e228)V
CB644	*unc-62*(e644)V
CB541	*unc-71*(e541)III
CB936	*unc-73*(e936)I
DR96	*unc-76*(e911)V
CB1068	*unc-79*(e1068)III
CB1414	*unc-85*(e1414)II
CB1416	*unc-86*(e1416)III
CB1216	*unc-87*(e1216)I
HE130	*unc-98*(su130)X
DR1	*unc-101*(m1)I
CB1265	*unc-104*(e1265)II
FF41	*unc-116*(e2310)III
CB2	*vab-1*(e2)II
CB96	*vab-2*(e96)IV

CB648 *vab-3*(e648)X
CB698 *vab-10*(e698)I

A.2.2. *C. elegans* strains from J. Thomas (University of Washington, Seattle, USA).

JT6117 *osm-3*(p802), *unc-104*(e1265)
JT6234 *osm-5*(p813), *unc-104*(e1265)
JT6067 *osm-1*(p808), *unc-104*(e1265)
JT6119 *daf-6*(e1377), *unc-104*(e1265)
JT6120 *daf-10*(e1387), *unc-104*(e1265)
JT6240 *che-13*(e1805), *unc-104*(e1265)
JT6233 *osm-1*(p808), *unc-116*(e2310)
JT6926 *daf-19*(m86), *unc-116*(e2310)
CB1197 *unc-44*(e1197)

A.2.3. *C. elegans* strains from A. Rose (University of British Columbia, Vancouver, CANADA).

BC128 *dpy-14*(e188)*unc-29*(e403)
KR287 *dpy-24*(s71)*unc-75*(e950)

A.2.4. *C. elegans* strains from C. Johnson (Nemapharm, Madison, USA).

NS5 *che-3*(nr5)
NS109 *dyf-?*(nr109)
NS272 *dyf-12*(nr272)
NS2344 *dyf-12*(nr2344)
NS2389 *dyf-10*(nr2389)
NS2477 *dyf-12*(nr2477)
unc-13(e51) (outcrossed)

A.2.5. Strains obtained from R. Herman (University of Minnesota, USA).

SP1205	*dyf-1*(mn335)I
SP1234	*dyf-2*(m160)III
SP1603	*dyf-3*(m185)IV
SP1708	*dyf-4*(mn332)V
SP1614	*dyf-5*(mn400)I
SP1170	*dyf-6*(mn346)X
SP1180	*dyf-7*(m537)X
SP1196	*dyf-8*(m539)X
MT3559	*dyf-9*(n1513)V
SP1709	*dyf-10*(e1383)I
SP1710	*dyf-11*(mn392)X
SP1711	*dyf-12*(sa127)X
SP1618	*dyf-13*(mn396)II

A.2.6. *C. elegans* strains created as part of the work described herein. (Unless indicated otherwise, Strains are available from Warwick Grant, Flinders University of South Australia, Adelaide).

WG4	*dyf-?*(au4)
WG10	*dyf-?*(au10)
WG11	*che-3*(au11)
WG12	*che-3*(au12)
WG13	*bli-2*(au13)II; *dyf-?*(au4)
WG17	*unc-18*(e81)*dyf-12*(nr272)X
WG18	*dpy-8*(e130)X; *dyf-10*(nr2389)I
WG19	*unc-18*(e81)X; *dyf-10*(nr2389)I
WG20	*dpy-8*(e130)*dyf-12*(nr2477)X
WG21	*dpy-8*(e130)*dyf-12*(nr2344)X
WG26	*unc-9*(ec27)*dpy-7*(e88)X
WG27	*unc-6*(e78)*dpy-7*(e88)X
WG30	*dpy-7*(e88)X; *dyf-10*(nr2389)I
WG31	*sav-?*(au1)A; *dyf-12*(nr2477)X
WG32	*sav-?*(au2)A; *dyf-12*(nr2477)X
WG33	*sav-?*(au3)A; *dyf-12*(nr2477)X
WG34	*sav-?*(au4)A; *dyf-12*(nr2477)X
WG35	*sav-?*(au5)I; *dyf-12*(nr2477)X
WG37	*dpy-7*(au7)*dyf-12*(nr2477)X
WG38	*sav-?*(au8)A; *dyf-12*(nr2477)X
WG39	*sav-?*(au14)IV; *dyf-12*(nr2477)X
WG47	*sav-?*(au15)III; *dyf-12*(nr2477)X
WG48	*sav-?*(au16)A; *dyf-12*(nr2477)X
WG49	*sav-?*(au17)A; *dyf-12*(nr2477)X
WG50	*sav-?*(au18)A; *dyf-12*(nr2477)X
WG51	*dpy-?*(au19)III; *dyf-12*(nr2477)X
WG52	*dpy-?*(au20)II; *dyf-12*(nr2477)X
WG53	*dpy-7*(e88)*dyf-12*(nr272)X
WG55	*unc-9*(ec27)*dyf-12*(nr2344)X
WG56	*unc-9*(ec27)*dyf-12*(nr272)X
WG60	*unc-9*(ec27)*dyf-12*(nr2477)X
WG61	*dpy-7*(e88)*dyf-12*(nr2477)X
WG71	*che-3*(nr5)I; *unc-104*(e1265)II
WG72	*che-3*(nr5)I; *unc-116*(e2310)IV
WG73	che-3(m535)I; *unc-116*(e2310)IV
WG74	*che-3*(nr165)I; *unc-104*(e1265)II
WG75	*che-3*(m535)I; *unc-104*(e1265)II
WG76	*dyf-10*(nr2389)I
WG77	*dyf-10*(nr2389)I; *unc-104*(e1265)II
WG82	*dpy-?*(au20)II; *dyf-12*(nr2477)X
WG86	*dpy-7*(e88)*dyf-12*(nr2344)X
WG87	*mah-2*(cn110)X; *dyf-10*(nr2389)I
WG88	*dyf-12*(nr2477)X; *unc-104*(e1265)II
WG89	*dyf-12*(nr2344)X; *unc-104*(e1265)II
WG90	*dyf-10*(nr2389)I; *unc-3*(e151)X
WG91	*dyf-12*(nr272)X; *unc-104*(e1265)II
WG92	*dyf-6*(mn346)X; *unc-60*(e723)V
WG100	*che-3*(nr165)I; *unc-116*(e2310)IV

WG102 *unc-60*(e723)V; *dyf-7*(m537)X
WG103 *dpy-7*(e88)X; *dyf-10*(nr2389)I
WG104 *unc-60*(e723)V; *dyf-12*(sa127)X
WG106 *unc-60*(e723)V; *daf-10*(e1387)IV
WG107 *unc-60*(e723)V; *dyf-12*(nr2477)X
WG108 *unc-32*(e189)III; *dyf-12*(nr2477)X
WG109 *unc-4*(e120)II; *dyf-12*(nr2477)X
WG110 *unc-24*(e138)IV; *dyf-12*(nr2477)X
WG113 *unc-22*(e66)IV; *dyf-12*(nr2477)X
WG116 *che-3*(nr5)I; *osm-3*(p802)IV
WG117 *dyf-12*(sa127)X; *unc-104*(e1265)II
WG118 *unc-54*(e190)I; *dyf-7*(m537)X
WG119 *unc-52*(e444)II; *dyf-12*(sa127)X
WG120 *unc-54*(e190)I; *dyf-12*(sa127)X
WG121 *unc-52*(e444)II; *che-3*(nr5)I
WG122 *dyf-10*(nr2389)I; *unc-104*(e1265)II
WG123 *che-12*(e1812)V; *unc-104*(e1265)II
WG124 *mec-8*(e398)I; *unc-104*(e1265)II
WG125 *dyf-7*(m537)X; *unc-104*(e1265)II
WG126 *che-11*(e1810)V; *unc-104*(e1265)II
WG127 *osm-6*(p811)V; *unc-104*(e1265)II
WG128 *dyf-12*(nr272)
WG130 *unc-22*(e66)IV; *dyf-10*(nr2389)I
WG131 *unc-116*(e2310)IV; *dyf-10*(nr2389)I
WG132 *dyf-12*(nr2477)X
WG133 *dyf-12*(nr2344)X
WG144 *unc-87*(e1216)I; *dyf-12*(nr2477)X
WG145 *unc-86*(e1416)III; *dyf-12*(nr2477)X
WG146 *dpy-7*(au7)
WG147 *unc-32*(e189)III; *dyf-12*(nr2477)X
WG148 *unc-60*(e723)V; *dyf-12*(nr2344)X
WG150 *unc-60*(e723)V; *dyf-12*(nr2477)X
WG151 *unc-15*(e73)I; *dyf-12*(nr2477)X
WG152 *dpy-7*(au7)*dyf-12*(nr2344)X
WG153 *dpy-?*(au19)III; *dyf-12*(nr272)X
WG154 *dpy-?*(au19)III; *dyf-12*(nr2477)X
WG155 *dpy-?*(au19)III; *dyf-10*(nr2389)I
WG156 *dpy-?*(au20)II
WG157 *dpy-?*(au19)III; *dyf-12*(nr2344)X
WG158 *dpy-?*(au20)II; *dyf-10*(nr2389)I
WG159 *unc-60*(e723)V; *dyf-10*(nr2389)I
WG160 *dpy-7*(au7)X; *dyf-10*(nr2389)I
WG161 *unc-60*(e723)V; *dyf-12*(nr272)X
WG162 *dpy-7*(au7)X; *dyf-12*(nr272)X
WG164 *dpy-7*(au7)X; *dyf-12*(nr2477)X

A.2.7. *H. contortus* strains obtained from L. LeJambre (CSIRO division of Animal Production, Armidale).

VRSG Resistant to benzimidazoles, levamisole and organophosphates, Sensitive to ivermectin.

CAVR Resistant to ivermectin. Sensitive to benzimidazoles, levamisole and organophosphates.

A.2.8. *H. contortus* strain obtained from B. Wagland (CSIRO division of Animal Health, Glebe).

McMaster	Susceptible to all commonly used anthelmintics. Exsheathed L3 larvae were obtained.

APPENDIX I

B.1. ADDITIONAL DATA: C 'TER THREE.

B.1.1 Dose response of homozygotes id heterozygotes of four dominant IVM resistance mut ns - Data obtained using original (non-outcrossed) str s.

Genotype tested	IVM concentration (ng/mL)			
	5	10	15	20
nr272/nr272	+	+	-	-
nr272 +/+ *unc-18*	+	+	-	-
nr2344/nr2344	+	+	+	-
nr2344 +/+ *unc-18*	+	+	+	-
nr2389/nr2389	+	+	-	-
nr2389 +/+ *unc-18*	+	+	-	-
nr2477/nr2477	+	+	+	-
nr2477 +/+ *unc-18*				

The table shows homozygotes of nr272, nr2344, nr2389 and nr2477 for IVM resistance at 5, 10, 15 and 20 ng/mL. A + indicates growth on IVM and a - indicates failure of worms to grow and reproduce on that drug concentration. Results for homozygotes and heterozygotes from non-outcrossed strains are shown here for comparison with data in table 7 (Chapter 3)

B.1.2. Dyf phenotype of heterozygotes carrying the dominant ivermectin resistant *dyf* alleles nr272, nr2344, nr2477 and nr2389 (from non-outcrossed strains).

Genotype	Fraction of nonDyf individuals [fraction] % {95% C.I.}
nr272 +/+ *unc-18*	$[^{12}/_{26}]$ **46%** {28.9-66.0}
nr272 +/+ *unc-18* I	$[^{6}/_{28}]$ **21%** {10.5-40.3}
nr272 *unc-18* /+ +	$[^{28}/_{35}]$ **80%** {60.8-90.0}
nr272 *unc-18* /+ + I	$[^{10}/_{24}]$ **42%** {32.6-60.7}
nr2344 +/+ *unc-18*	$[^{13}/_{35}]$ **37%** {24.9-58.7}
nr2344 +/+ *unc-18* I	$[^{0}/_{14}]$ **0%** {0.0-31.9}
nr2344 *dpy-8* /+ +	$[^{5}/_{30}]$ **17%** {5.6-34.7}
nr2344 *dpy-8* /+ + I	$[^{2}/_{31}]$ **6%** {0.9-21.6}
nr2477 +/+ *unc-18*	$[^{18}/_{33}]$ **55%** {33.3-68.8}
nr2477 +/+ *unc-18* I	$[^{11}/_{28}]$ **39%** {27.3-58.1}
nr2477 *dpy-8* /+ +	$[^{20}/_{34}]$ **59%** {37.2-72.0}
nr2477 *dpy-8* /+ + I	$[^{11}/_{27}]$ **41%** {26.4-60.2}
nr2389 +/+ *unc-18*	$[^{35}/_{35}]$ **100%** {90.0-100}
nr2389 +/+ *unc-18* I	$[^{25}/_{25}]$ **100%** {86.3-100}
nr2389 *dpy-8* /+ +	$[^{34}/_{35}]$ **97%** {84.6-99.8}
nr2389 *dpy-8* /+ + I	$[^{25}/_{27}]$ **93%** {68.9-94.1}

I Grown on 5 ng/mL IVM.

Numbers in each cell represent the percentage of nonDyf animals (ie. animals exhibiting any uptake of the lipophilic dye DiO into their amphid neurons when stained overnight (see methods)). The number of worms exhibiting staining over the total number scored is shown in square brackets and confidence intervals for the percentage of nonDyf worms are given in brackets.

B.2. ADDITIONAL DATA: CHAPTER FOUR.

B.2.1. Raw mapping data generated in linkage mapping of *sav* mutants isolated form two mutagenesis experiments (Chapter 4.).

Sup allele	I *unc-15* (e73)	II *unc-4* (e120)	III *unc-32* (e189)	IV *unc-24* (e138)	IV *unc-22* (e66)	V *unc-60* (e723)
au1	7/23[b]	12/23[bl]	4/20 [l]	4/24 [l]	ND	16/23[cl]
au2	6/19	15/24 [l]	3/15 10/20[bl]	5/20 0/24 [l]	4/23	5/23 [l]
au3	2/24	6/24 [l]	4/23 [l]	5/24 [l]	ND	9/24[bl]
au4	1/23[a]	3/22 [l]	9/22[bl]	0/12[a] 0/17 [l]	10/28	5/24 [l]
au5	1/24[a]	13/24[bl]	6/24 [l]	3/24 [l]	ND	4/24 [l]
au8	3/24	6/23 [l]	0/10 5/19 [l]	4/24 [l]	ND	5/23 [l]
au14	9/22	3/23 [l]	5/20 0/23 [l]	0/20[a] 0/24 [l]	0/24[a]	4/23 [l]
au15	5/23	5/27	1/28[a]	3/27	ND	7/51
au16	4/24	4/10 22/22[cl]	9/22[bl]	5/41	26/28	6/24 [l]
au17	6/22	8/26	3/24	6/32	0/23[a]	0/27[a]
au18	2/24	19/30[b]	18/27[b]	11/31	16/23[b]	7/27

Legend B.2.1.

[a] = Ratio is significantly lower than expected (P=0.05) evidence of linkage.
[b] = Ratio is significantly higher than expected (P=0.05) can be explained by assuming the suppressor has a dominant maternal effect in the *unc* background.
[c] = Ratio is significantly higher than expected (P=0.05) cannot be explained.
[1] = *sup/+,dyf-12* (nr2477) males used in cross
ND = Not Done
Numbers represent the number of F2 Unc animals whose progeny were sensitive to IVM (5ng/mL in agar). Uncs were generated in one of two ways: (protocols A and B: see also methods - Chapter 4.)

Protocol A.
N2 males were crossed with *unc/unc,dyf-12*(nr2477)*/dyf-12*(nr2477) and the male progeny (*unc/+,dyf-12*(nr2477)) were crossed with *sup/sup,dyf-12*(nr2477)*/dyf-12*(nr2477). The F1 progeny were
individually to d
being homozygou
Protocol B. (1)
N2 males were
12(nr2477) and
crossed with *u*
progeny were allowed to self and Unc F2's were picked individually to drug plates. The likelihood of an Unc individual being homozygous for the suppressor in this case is 1/8.