STUDIES ON THE DEVELOPMENT AND ORGANISATION OF THE NERVOUS SYSTEM OF CAENORHABDITIS ELEGANS

by

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SUMMARY

The nematode Caenorhabditis elegans is a small invertebrate whose nervous system, general anatomy, and normal development are all (comparatively) extremely simple and reproducible, and have all been well characterised. This dissertation describes work based on two different approaches to the study of the control of neural development in C. elegans.

In the first part the course of neural outgrowth in the region of the ventral nerve cord was followed from electron microscope reconstructions of a series of fixed embryos. Following this, neurons whose processes grew out early were removed by laser ablation of their parent cells and the effect on subsequent nerve outgrowth was assayed by electron microscope reconstruction. The first process to grow along the ventral cord is that of AVG, and its presence is required for the normal, highly asymmetrical structure of the cord. Two more examples of dependancy on particular nerve processes for correct guidance can be deduced from experiments in which cells at the back of the animal were removed. The observations of normal development and the ablation experiments can in some cases be related to defects seen in uncoordinated mutants with defective nerve process organisation.

The second approach was to establish and analyse a computer data base containing all the synaptic connectivity data obtained by White et al. (1986), who reconstructed at an electron microscope level the entire central nervous system regions of two C. elegans. specimens. Since the circuitry is highly reproducible, comparisons of connections between equivalent pairs of cells can be used to infer properties of synapse formation. Overall, the C. elegans circuitry is anatomically highly directional, and what little chemical synaptic feedback that is seen is mostly part of reciprocal synaptic connections. There is also evidence for physical organisation of the nerve processes in subbundles of the main process tract in the central nervous system.

PREFACE

The work described in this dissertation was carried out between January 1984 and March 1987 at the MRC Laboratory of Molecular Biology, Cambridge. As described in the summary, two different approaches were used in this work, and the main body of the dissertation is split into two parts, each with its own introduction. However the introduction to the first part provides much of the general background. There is a final conclusion which considers both parts in a broader setting.

It is customary to list a long series of acknowledgements somewhere in the preface to a dissertation. I have derived enormous personal and scientific benefit from my time spent at the Laboratory of Molecular Biology, both from the people who work here and the environment that they have created. I am only going to thank personally two people, my supervisor John White, to whom I owe so much that it would be pointless to try to encapsulate it, and Nichol Thomson, who does all the serial sectioning of C. elegans at the MRC with remarkable consistency, and ultimately without whom none of this work would have been possible. I would also like to thank the Medical Research Council for a Training Award, and King's College for a Research Fellowship.

With the exception of the technical serial sectioning for the first part, this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration. No part of this dissertation has been or is being submitted to any other University.

CONTENTS

PART I THE OUTGROWTH OF NERVE PROCESSES IN THE EMBRYO

CHAPTER 1 Introduction

The first part of this dissertation describes an investigation into the outgrowth of nerve processes in the region of the ventral nerve cord of C. elegans during embryonic development. The course of normal development was deduced from serial section reconstructions of a set of embryos fixed at different stages. Then laser ablation experiments were performed to remove specific neurons whose processes grew out during these early stages, in order to test whether the presence of these processes was necessary for correct subsequent development of the nervous system. Chapter 2 gives materials and methods. The observations from the wild type reconstructions are given in Chapter 3 and the results of the ablation experiments are described in Chapter 4. The two sets of results are discussed together in Chapter 5.

There are no previous direct results on the course of neural outgrowth in C. elegans, although disruption of the final arrangement of nerve processes has been observed in mutants (Hedgecock et al., 1985) and animals in which laser damage has prevented nerve cell migration (Chalfie et al., 1983). Below I first review previous work in other systems on neural guidance, and then give an introduction to C. elegans and its nervous system.

Review of neural guidance

1.1 A review of neural guidance

The building of a nervous system during development can be divided into three phases: the generation of the correct cells in the correct places, the outgrowth of nerve processes, and the formation of synapses. All of these phases show a high degree of specificity, which means that a large amount of information must be expressed by mechanisms that on the whole we do not yet understand, but would like to. In some ways the second phase, that of process outgrowth is the most clearly defined. This is because all neural branching structures are a consequence of a single phenomenon, the migration of growth cones during development, a truth which Cajal saw early and fought hard for (Hamburger, 1981), and which led Harrison to develop the first tissue culture techniques in order to follow outgrowing neurites directly (Harrison, 1910).

A growth cone is a specialised structure at the tip of any growing neurite that migrates through the animal, spinning out the nerve process behind it. This is not the only means by which nerve processes can be lengthened, since change in size and shape of the animal is matched by addition of new material to already existing processes. In many cases most of the length of nerve fibres is created in this way, but it is almost entirely passive, having at most a very small effect on the layout of the neurons axonal structure. For instance, most nerve processes grow along the ventral cord of C. elegans when it is only around 100 microns long, a tenth of its final length. However some changes in overall structure do occur by intercalary insertion; an example is the conversion of an initially bipolar cell to one that is pseudo-monopolar, by retraction of the cell body away from the branch (Kuwada, 1986 and with ventral nerve cord motor neurons in C. elegans). Such small alterations during subsequent development emphasise the importance of looking at outgrowth as it takes place, rather than making inferences from the finished pattern.

Growth cones in vitro

Growth cones are generally spread out, lamellar structures, which often extend fine microspikes, or filopodia (Letourneau, 1983). They mover over surfaces and as they move thevarious lamellar and filopodial extensions are retracted and new ones are extended out, so that the overall shape is continually changing (Bray and Chapman, 1985). It is easy to study growth cone migration in vitro using cultured neurons and a wide range of factors that affect migration have been observed. In order for motion to take place it appears that fairly tight adhesion to the substrate is necessary (Bray, 1979), and this leads immediately to the idea that differential adhesion may be important for growth cone guidance. Letourneau (1980) has shown that growth cones do indeed tend to grow along regions of higher adversity when faced with a choice in vitro. Although this may support the common suggestion that growth cones may in many cases be guided up an adhesive gradient in vivo (Nardi, 1983, Berlot and Goodman, 1984), it does not directly address that proposal, and there are several severe problems with the idea. Growth cones show different morphologies when migrating on artificial surfaces of different adhesivities, but even though the range of morphologies seen on different neurites in vivo is vast, any one growth cone does not change shape as it migrates over a uniform surface. In addition the strength of adhesivity would have to increase

exponentially, which would require an excessive magnitude range of adhesivity for a gradient of any substantial length. In fact growth cones in culture tend to grow in straight lines anyway, only changing directions when they branch. Based n an elegant combination of observations and experiments Bray has suggested that the neurite leaving the back of the growth cone exerts a tension, and that the growth cone always grows away from the source of tension (Bray, 1979). If the angle of the neurite is altered then the direction of growth coordinately changes, and if the tension is relaxed, by for example cutting the neurite, then the growth cones divides in two, the two halves growing off in opposite directions and exerting tension against each other. Together these results suggest that direction changes and branches may occur in vivo either where a path of higher adversity is crossed, or possibly at a point where the growth cone becomes tethered, so that growth in the new direction can pull against something.

Although there is a strong tendency to think of attractive forces on growth cones as being the principle tools of guidance control, it is equally possible for repulsive forces to be influential, and there are several examples that are known. There is a highly selective inhibitory effect when the neurotransmitter 5-HT is released from a micropipette near the advancing growth cone of an identified cell from the mollusc, Helisoma (Haydon et al., 1984). This has been proposed to have developmental significance in the detailed development of the Helisoma buccal ganglion (meinertxhagen, 1985). A retraction of the growth cone in vitro is also seen when rtinal and sympathetic axons meet each other in culture (Bray et al., 1980, Kampfhammer et al., 1986). Although retinal growth cones will cross retinal axons, and sympathetic growth cones will cross sympathetic axons, when one meets the other it shrinks back and withdraws its filopodial and lamellar extensions. Similar avoidance behaviour between different neurites of the same neuron could possibly explain the marvellous space filling, non-overlapping properties of many neurons' dendritic or axonal arborisations. Experimental evidence for such avoidance has been provided by studies of single sensory neurons in the leech, which fill a planar surface from several points in an apparently self-competitive fashion (Kramer and Stent, 1985). As yet there is no experimental evidence of such mechanisms acting between different neurons in vivo, but there are several cases in C. elegans where neurons abut against but do not overlap other members of their own classes; often there is a gap junction between the two abutting processes (see Chaper 7).

Studies in vivo

It is convenient to make a distinction between directional, tropic influences on neural guidance and spatially restricted, contact mediated influences. Both appear to play an important part. To oversimplify the situation, tropic influences are directionally constraining, while differential adhesivities are spatially constraining. There is also a division between specific and nonspecific factors. By nonspecific factors I mean those that would influence any of a large range of different neurons. Neither of these divisions is totally sharp, and in particular specificity is clearly a graded phenomenon.

The classical example of a non-specific factor would be a gradient of positional information (Wolpert, 1971), probably some chemical or surface marker, and the classical experimental system where there is evidence for such a gradient in neural development is in the establishment of a topographical mapping from the retina onto the

optic tectum of lower vertebrates. A series of experiments in which an ordered mapping reformed after parts of the retina and/or tectum were removed or grafted back in abnormal orientations suggested that the original chemo-affinity hypothesis of Sperry (1963), which proposed specific matching between corresponding sectors of the retina and tectum, was incorrect (see Gaze, 1970). More recently Bonhoeffer and Huf (1982) have shown using an in vitro axon growth choice assay that there is a gradient of affinity for temporal axons across the surface of the tectum, with highest affinity for the rostral part of the tectum, which is their normal target. Progressively more nasal axons show less specificity. The overall effect of these affinities would then be established by competition. There are many other proposed sources of information for the retino-tectal system, some also driven by competition (e.g. Willshaw and von der Malsburg, 1979).

However the situation during creation of the retino-tectal map on the surface of the tectum is different from the early outgrowth of processes that concerns the study of embryonic C. elegans outgrowth in this dissertation, since the axons have already reached their target tissue and are finding the correct place on it amongst a group of equivalent cells. For the rest of this review I will focus on the pathfinding properties of growth cones necessary to find their targets from the cell bodies, rather than the final stage as discussed here.

Directional and tropic effects

A very different function of a gradient is to specify a direction up which axons can travel. There are several examples where a general attraction that is not path specific has been indicated experimentally. Harris (1980) has shown this type of effect using the same retino-tectal projection in Xenopus menioned above as an experimental system, but at the earlier stage of development where the optic tract must be formed. Before axon outgrowth he implanted whole eye primordia into abnormal places in the brain, after which in most cases the retinal axons grew out and took a nearly direct route to the tectum, usually via a pathway totally different to the one they normally follow. If the implant was sufficiently caudal then the retinal processes ran instead down the spinal cord, in a particular dorsolateral tract, reproducing previous observations that this part of the spinal cord attracted displaced retinal axons (Constantine-Paton and Capranica, 1976). These results suggest that there is a general attraction of retinal axons to their target, and that this acts over a fairly wide zone, but that the mechanism may not be uniquely used for retino-tectal pathfinding; in the spinal cord, outside the normal range of retinal axons the same attraction system may be used for another set of processes.

A more specific attraction of neurons to their targets has been observed in the vertebrate peripheral nervous system (PNS). Lance-Jones and Landmesser (1981) showed that after a short piece of chick neural tube was reversed the motor neurons till largely found a way to the correct target muscles, crossing over each other on the way. However if the displacement is too great then they often grow to inappropriate muscles (ibid. and Summerbell and Stirling, 1981). Again this influence appears to be over a longer range than the reach of the filopodia, though still reasonably localised (Landmesser, 1984). There are also indications in the insect PNS that after the more specific cues are removed there is still a tendency for sensory neurons to grow proximally towards the central

nervous system (CNS), even along abnormal routes (Berlot and Goodman, 1984, Nardi, 1983).

One suggestion of a possible agent involved in the general attraction of a whole class of nerve fibres is nerve growth factor (NGF). Sympathetic fibres grow over abnormal territory towards a site of NGF injection in vivo (Gunderson and Barrett, 1980). However in both cases the amounts applied are much larger than the ovserved natural levels; NGF is much better known as a trophic agent necessary for neuron survival and a general promoter of neuron outgrowth, and the directional effect may be a subsidiary non-physiological consequence of an overdose of these other behaviours. In a careful set of experiments with explants from embryonic mouse trigeminal ganglia and their target tissue, maxiliary epithelium, Lumsden and Davies (1983, 1986) have shown a clear directional tropic attraction of trigeminal fibres to their target. This is diffusible through the colagen matrix in which the explants sit and the axons grow, and is separable from NGF, which appears to act later in development to preserve the connection. It also has no effect on axons from comparable neighbouring ganglia. Lumsden and Davies argue that NGF is active on too many cell types to be sensible as a tropic agent. However it might be countered that a general tendency for sympathetic axons to grow towards the periphery could be useful.

All these results suggest that there may be general directional (often homing) guidance mechanisms that are not restricted to specific pathways, and apply to fairly broad classes of neurons. Interestingly the range of all the attractions is approximately the same, of the order of a few hundred microns. In cases that are more specific, such as the chick motor neuron guidance, the absolute size of the embryo is larger. Such distances correspond to a fairly small number of growth cone extensions, suggesting that a growth cone could detect a gradient on this scale. Since some specificity is involved and the directions of different sets of fibres can cross (as in the chick limb motor neuron experiments), it seems unlikely that a single gradient, such as a general adhesive gradient, provides the best explanation for them. In at least one case (Lumsden and Davies) the substrate if artificial and the factor is diffusible.

Before automatically explaining any experiment indicating a directional effect by a gradient, it should be born in mind, however, that there are at least two other ways in which a polarity or directionality could be specified. The first is intrinsic to the neuron, simply by the orientation in which it was created by its final cell division. This may often be important for initiating process outgrowth in the correct direction (Jan et al., 1985). The second is by a repeated sequence of more than two signals, in which case the direction can be determined by inspecting neighbouring sequence elements, or equivalently by a moving wave of some signal. This type of signal can operate over very long distances if it is actively maintained, and is the method is slime mould aggregation (Gerisch, 1982).

Fasciculation of nerve processes

A different sort of nonspecific influence that is important for neuronal outgrowth is the strong tendency of growth cones to grow along other neurons, which leads to the fasciculation of nerve processes. This is clearly one of the most important factors

determining the structure of the peripheral nervous system, which is made of nerve bundles, and where closely studied it has also been seen to be important in the early developing central nervous system at stages where processes are not dense (e.g. the insect CNS, Bate and Grunewald, 1981, Goodman et al., 1982). This has been seen by immunofluorescence to be expressed on many neuronal cell surfaces, and also on various epithelial and glial cells (Silver and Rutishauser, 1984). It has been claimed that the modulation of a single molecule such as NCAM could account for a very large proportion of the control of neural outgrowth (Edelman, 1983), but this appears unlikely because of the degree of specificity seen in many different but often adjacent and simultaneous interactions. However there is a large part to be played by fairly non-specific adhesion.

Almost a direct consequence of general neuronal fasciculation is the concept of the preservation of order within nerve bundles by a process tending to stay stuck to its neighbours. Many nerve projections show a general topographic order preservation, both in the central and peripheral nervous system (e.g. the retinal-tectal and spinal cord projections) and a simple method of correct guidance may be to place neurons in positions corresponding to a topological map of their targets and then to preserve the relative spatial arrangement in the outgoing bundle of fibres and rely on non-specific cues to spread the projection onto the target tissue(s). In fish retino-tectal projections Scholes (1979) has shown that order is in general maintained, but that there is a zone of active reorganisation near the tectum, and in other cases where ordering has been observed an active mechanism for correcting the final projection has also been detected (e.g. Landmesser, 1984).

Pioneers and specific fasciculation

The observation that fasciculation is a significant factor led to a realisation of the importance of the first nerve pioneers to grow out, called “pioneers” by Harrison (1910) and to the suggestion that they may be specialised in order to be able to lay down new paths. The pioneers in a various part of different insect peripheral nervous systems have been studied first by Bate (1976a), and subsequently by many others (e.g. Ho and Goodman, 1982, Bentley and Keshishian, 1982, Blari and Palka, 1985, Jan et al. 1985). Although in certain cases outgrowing central neurons grow out over new territory (Ho and Goodman, 1982), the majority of nerve bundles are pioneered by peripheral sensory neurons that essentially always follow a series of other neuronal cell bodies spaced out at intervals on the way to the CNS. This observation led to the “guidepost” hypothesis, that there are a class of specified cells in the periphery that are guideposts (maybe all neurons) and that pioneer growth cones search for and grow towards the nearest guidepost cell within reach at each stage (Bentley and Keshishian, 1982). In this case it appears that no single pioneer is essential, since various cell removal experiments resulted in satisfactory correction or adaptation (Keshishian and Bentley), 1983, Blair and Palka, 1985).

Ho and Goodman (1982) argue for a certain degree of specificity of fasciculation in the grasshopper PNS, particularly for outward growing CNS axons which must choose branches at points where afferent fibres have converged. There appears to be a much greater amount of specificity in the grasshopper CNS. Here again the earliest pioneer fibres have been identified (Bate and Grunewald, 1981), and the subsequent outgrowth of certain identified neurons has been followed (Goodman et al., 1982). A large number of

closely adjacent fascicles are established and growth cones often cross a number of them before fasciculating with a particular one. This has lead to the "labelled pathways" hypothesis (Ghysen and Jansen, 1979, Goodman et al., 1982), that the fascicles are differentially labelled by surface molecules and that growth cones are programmed to recognise a sequence of these labels and grow along them, thus defining a route through the developing nervous system. Ablations of neurons that generate the pathways for identified cells in this system have resulted in the stalling of growth cones (Raper et al., 1984, Bastiani et al., 1986). This contrasts with what has been seen in the PNS, and provides a genuine example of a specialised pioneer, whose presence is necessary for later axons to follow.

The chick PNS experiments described earlier provide another example of the requirement for a preexisting fascicle along which a subsequent neuron type will follow. In the experiments in which sections of neural tube, or limb buds, are displaced, sensory neurons that innervate muscle only follow the correct pathways to their muscles if the corresponding motor neurons do so (Honig et al., 1986). Furthermore, if instead of displacing motor neurons the whole motor neuron pool is removed before axon outgrowth, so that later there is no motor innervation of muscle, then there is effectively no sensory innervation of muscle either, and instead cutaneous sensory innervation is increased (Landmesser and Honig, 1986).

Therefore, in addition tot he nonspecific general tropism and fasciculation that were discussed earlier, there is substantial evidence for specific interactions between neurons and bundles of other neurons with which they will fasciculate. In the case of the insect CNS the specificity appears to be almost certainly mediated by contact; not only are the differing choices too tightly packed for a longer range influence to be sufficiently selective, but there have also been seen in the electron microscope direct interactions of growth cone filopodia inserting themselves deep into the surfaces of cells they wille ventually fasciculate with (Bastiani and Goodman, 1984). Monoclonal antibodies have recently been made that appear to recognise specific fascicles in the grasshopper CNS, and the growth cones that will join them (harrelson et al., 1986). Interestingly in each case several different bundles stain with the same antibody. If the antigens are involved in determining fasciculation then this would be reminiscent of the observation with ectopic retinal implants that there seems to be an affinity of retinal axons for an abnormal target in the spinal cord, as well as the tectum.

Interactions with non-neuronal surfaces

Up until now the interactions between growth cones and their targets, or other neurons, have been stressed, but clearly their relationship to non-neuronal substrates may also be important, particularly for pioneer neurons. In various different situations growth cones have been proposed to migrate over basement membrane, glial cells, epithelial cells, and mesenchyme. One of the strong reasons for proposing basement membrane as a possible neuronal substrate is that both raw basement membrane and several purified basement membrane components, such as fibronectin and laminin, have been shown to provide good surfaces for outgrowth in vitro (Varon-van Evercooren et al., 1982). Also in vitro processes are often found growing in spaces adjacent ot a limiting basement membrane (e.g. the CNS pioneers in the grasshopped, Bate and Grunewald, 1981, or the first fibres

in the fish spinal cord, Kuwada et al., 1986). However this region almost always also contains a large number of glial processes, and at least in the case of retinal axons, the nerve fibres seem to be particularly strongly attached to these glial endfeet (Krayanek and Goldberg, 1981), which have been shown to stain early on for NCAM (Silver and Rutishauser, 1984). The ordered outgrowth of retinal axons can be disrupted by injection of anti-NCAM antibodies (ibid.). In addition Silver and Ogawa (1981) have shown that a preformed glial bridge is ncessary and sufficient for growth of neocortial fibres across the corpus callosum.

On the basis of this type of observation, Singer et al. (1979) proposed the blueprint hypothesis, suggesting that there was a preformed meshwork of favoured pathways established on the glial and neuroepithelial external surface, which would channel growth cones in the same sort of way as Letourneau's adhesive grid in vitro (Letourneau, 1980). As with fasciculation, to which this type of concept is clearly related, non-neuronal blueprints could come in a complete range of specificities, from generally available for all axons to completely specific for a particular growth cone. In the case of the grasshopper CNS it has been possible to implicate a particular glial cell, the segment border cell, as determining the exit site for one of the main connectives to the periphery (Bastiani and Goodman, 1986). It effectively acts as a specific labelled pathway itself.

Summary

There is no case where the underlying mechanisms that control a nontrivial outgrowth pattern for a particular neuron or type of neuron have been determined in detail. One of the reasons for this is that we still know too little about the molecular and cellular basis of growth cone movement and guidance (Letourneau, 1983). On a larger scale, there are a number of experiments suggesting various sources of influence for process outgrowth. These experiments normally involve perturbation of particular factors in vivo and the results can sometimes be open to variable interpretation, depending on the hypotheses being addressed by the interpreter. One certain conclusion, however, is that a large range of different mechanisms can be used to influence neural guidance, usually in various combinations, and often in a redundant fashion. The information necessay for determining the outgrowth of any particular neuron will be expressed via a subset of these factors, the relevant subset probably differing in different stages of outgrowth.

Therefore the best that can be done at the general level is to identify the basic forms of the different types of relevant influence and interaction, and provide a list of tools that are available to whatever program controls development. In generating such a list I again restrict myself to outgrowth from the cell to the target, rather than interactions on the target tissue in which competition and neural activity may well play a part. With this restriction there currently seems to be evidence for the following list:

1. Much of the necessary organisation can be achieved by the initial positioning and orienting of the neurons.

2. There is a general tendency for axons to extend in straight lines unless otherwise influenced.

3. There can be local inhibitory influences on growth cone extension, either humoral or contact mediated.

4. Adhesion is clearly important for growth cone migration, and it seems likely that preformed generally adhesive pathways provide a set of preferred highways for processes to grow along.

5. Also in the realm of general adhesivity, there is a strong tendency for extending neurites to fasciculate together.

6. Both these last two influences can also act in a specific, as well as a non-specific, fashion, for example when a growth cone joins one particular fascicle out of several.

7. There can be a directional attraction of axons, normally from some fairly broad class of neurons, to some target or region, and this can function when a normal route is unavailable. At least in some cases this attraction is mediated by diffusible factors.

For those elements of the list where there is specificity, as in the last two cases, it seems that the same specificity mechanism may be used in more than one place.

Even if this list were complete, it would only provide a framework for two further lines of inquiry. The first is to search for the molecular and cellular mechanisms involved in each type of interaction, and the nature of their possible diversity and specificity. The second is to investigate how the consequent repertoire of available influences intricate outgrowth patterns for the huge variety of different neurons. One way to attack these problems is to choose an organism where the types of interaction involved and the different levels of specificity can be made as clear as possible, and then use the experimental power of molecular genetics as a technique to probe both the nature of the molecules concerned and the internal control structure of the genome. A good candidate for that organism is C. elegans.

So far in this introduction I have mixed examples from invertebrate and vertebrate model systems fairly freely, since many of the results can be directly compared, and it seems likely that factors which control growth cone guidance at the cellular level may well be analogous, if not identical, between even very widely diverged species. The significant difference between invertebrate and invertebrate nervous systems for the purposes of experimentation on axon guidance is that, in addition to in general containing orders of magnitude fewer cells than vertebrate ganglia, many and in some cases all, neurons in an invertebrate ganglion are reproducibly identifiable from one animal to the next. Often there will be only one or a small reproducible number of cells with any particular set of characteristics. Therefore repeatable experiments can be undertaken concerning a known individual neuron and the specific factors involved in controlling the outgrowth of its processes. C. elegans contains only 302 neurons altogether, all of which are identifiable, and for all of which the complete audit anatomy is known at the electron microscope level (White et al., 1986).

Finally, but not least importantly, we turn to the use of genetic techniques to study neural outgrowth. The primary reason for choosing C. elegans as a model organism for the

study of neural development was not the simplicity of its nervous system, but that it is well suited to genetic analysis (Brenner, 1974). The reason that genetics has not been mentioned before this point is that, although it can provide an extremely powerful tool for studying biological function and control and has been extensively used to study neuronal cell determination (e.g. Lehmann et al., 1983, Hedgecock, 1985), it has as yet provided very little insight into neural guidance. In vertebrates a few known mutations affect neuronal branching patterns and guidance, such as mouse mutants weaver, staggerer and reeler, which affect the structure of various cell types in the cerebellum (Caviness and Rakic, 1978). In Drosophila there are several mutations that have been used as experimental tools to remove neurons, or produce them in abnormal places (e.g. the homeotic mutants, Palka, 1982) but the only published mutation that seems to directly affect neuronal guidance is bendless, in which one of the neuron types involved in the escape jump response fails to reach its target (Thomas and Wyman, 1982). However it is not known whether other processes are affected, nor is the wild type development of the particular neuron known. In fact the organism in which the greatest number of neural guidance specific mutants are known is C. elegans (Hedgecock et al., 1985, S. McIntire, J. White, E. Hedgecock, personal communications, discussed further in the next section). In addition to any intrinsic interest and possible significance, it was in order to provide the developmental framework for further characterisation of the molecular mechanisms involved in guidance via this genetic approach that the study described in this thesis was undertaken.

1.2 The C. elegans nervous system

C. elegans is a small nematode, or roundworm, approximately 1mm long in the adult form. It has a simple body structure and a small number of cells: 959 somatic cells including 302 neuons. Development from egg to fertile adult takes only three and a half days at room temperature. Wild type animals used in this study are isogenic, since the egglaying sex is a self-fertilising hermaphrodite, rather than a female, with the consequence that strains are normally propogated asexually, forming clones. Males occur naturally at low frequencies. Ther hermaphroditism also facilitates genetic analysis, and many mutants have been studied. Together these facts make C. elegans a favourable model organism for the detailed study of development at the level of single cells, using both anatomical and genetic techniques, and it was chosen as such by Sydney Brenner (1974).

The life cycle consists of an embryonic stage, inside the egg, which takes about 16 hours, followed by four larval stages, named L1 to L4. The course of development is extremely reproducible. The pattern of cell divisions from the fertilised egg to the adult has been determined completely (Sulston and Horvitz, 1979, Kimbe and Hirsh, 1979, Sulston et al., 1983) and is essentially invariant.

Not only are the pattern of cell division and the general body plan of C. elegans simple and reproducible at a cellular level, but so is its nervous system. The complete nervous system of the adult hermaphrodite has been reconstructed by White et al. (1986) from electron micrographs of serial thin sections. The neurons have simple branching structures, and both the dispositions of cell processes, and the connections they make, appear to be largely invariant between animals. They can be assigned to 118 different

neuronal classes on the basis of morphology and synaptic connectivity (the system of nomenclature is described in Chapter 2). An overview of the nervous system of an L1 larva is shown in Figure 1.2. Its central processing region is a loop of neuropil around the pharynx, called the nerve ring, containing around 175 nerve processes. Running from this is a set of longitudinal process bundles that connect the ring to sensory receptors, the body motor nervous system, and several small ganglia in the tail. There are also circumferential commissures carrying processes from one lonitudinal bundle to another. The most important of the longitudinal bundles is the ventral nerve cord, which runs from the retrovesicular ganglion (RVG) just behind the nerve ring to the preanal ganglion (PAG) at the beginning of the tail, and containing the motor neuron cell bodies for the body motor circuitry.

Nerve cells in C. elegans are small (less than 5 microns in diameter) and it is not currently practical to impale them with microelectrodes. However intracellular recording from selected neurons has been possible in the larger nematode, Ascaris lumbricoides. Attention has been focussed on the ventral cord motor circuitry (reviewed in Stretton et al., 1985), and the distribution of cell types seen there corresponds anatomically very closely to that in C. elegans.

Figure 1.1

Transverse section of a 515 minute embryo (the C reconstruction of Chapter 3). The gut, muscle quadrants (M) and outer hypodermis (h) are all labelled. There are two nerve processes in the ventral nerve cord (AVG and DD3), and one motor neuron cell body (DB4). A left handed commissure is growing out from the DB4 cell body towards the dorsal hypodermis. In its growth cone can be seen a number of small vesicles. Scale bar is 2 microns

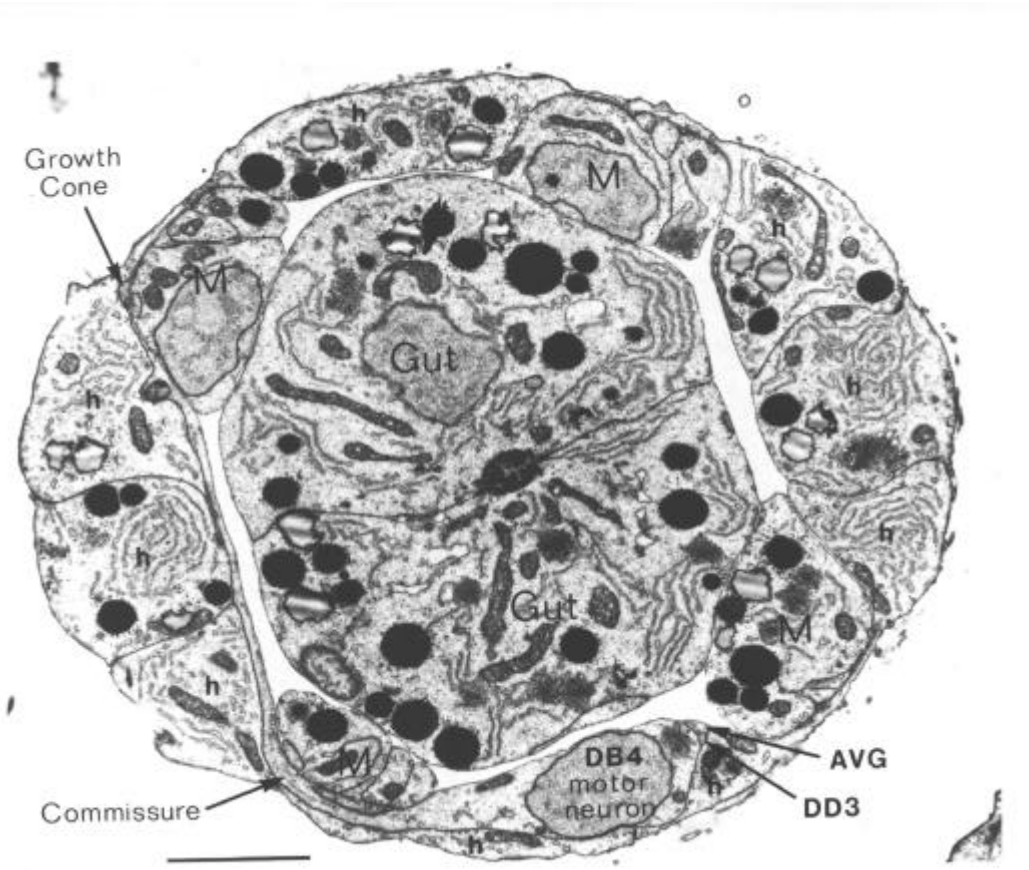
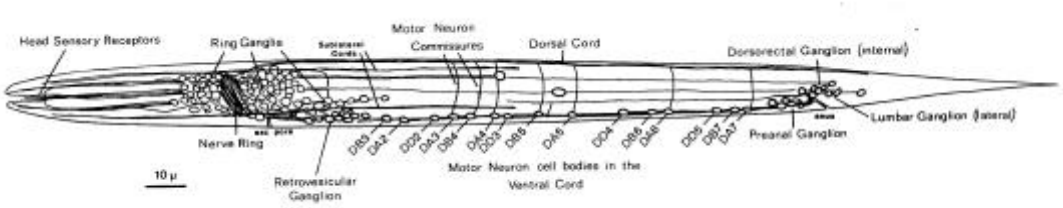


Figure 1.2

A general view of the L1 larva and its nervous system. All the neuronal cell bodies and process tracts behind the retrovesicular ganglion on the midline or the left side are shown. The main region of neuropil is the nerve ring, which is a loop around the pharynx. The ventral cord runs back from this and contains motor neuron cell bodies in addition to processes. Those ventral cord motor neurons that do not send a commissure around the left side of the body to the dorsal cord send one to the right side. There are four small tail ganglia: the preanal ganglion, the dorsorectal ganglion, and two lumbar ganglia, one on each side.



Previous studies on neural process guidance in C. elegans have been restricted to examining the structure of the adult nervous system in both wild type animals and mutants in which processes go astray. White (1983) discusses some possible factors that may be important in neural guidance on the basis of the adult electron microscope reconstructions. Chapter 9 of this thesis also considers process placement in the nerve ring using data from the adult reconstructions. Several techniques (mostly unpublished) have been developed to visualise processes by light microscopy, and these have been used to screen mutants that have possible neural defects, such as uncoordinated mutants that do not move well. Hedgecock et al. (1985) filled certain classes of sensory neurons with fluorescein by simple immersion of animals in the dye. Mutants in five unc genes showed guidance defects in these neurons, with processes either growing erratically in abnormal locations, or stopping prematurely. Several mutants are also known in which the outgrowth of the touch neurons is defective (Chalfie and Suslton, 1983). Further studies have been undertaken using monoclonal antibodies (S. McIntire, S. Siddiqui and J. Culotti, unpublished) and by electron microscopereconstruction of mutants (J. White, unpublished).

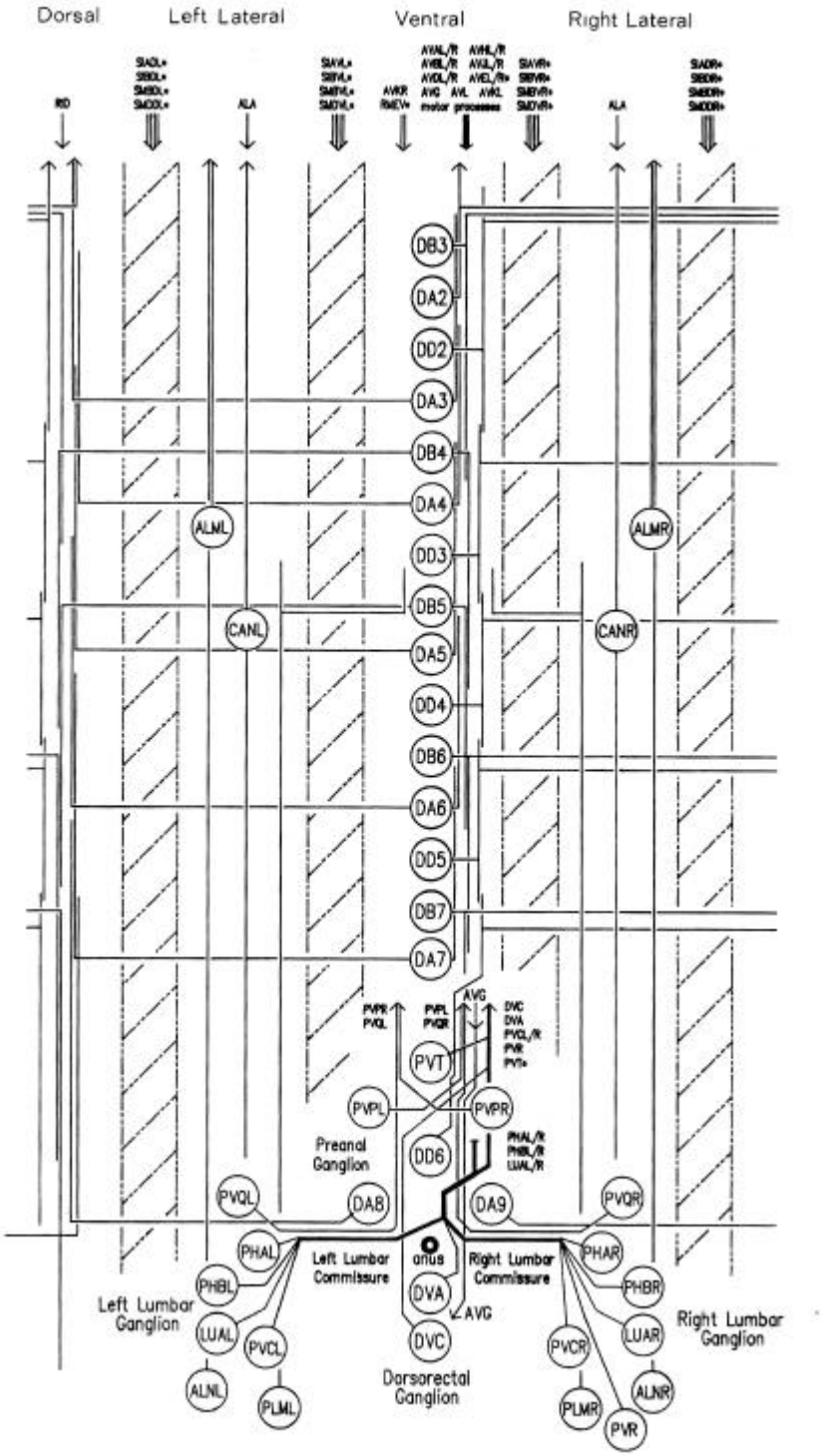
The study of neural outgrowth undertaken here has concentrated on the ventral cord, and to a lesser extent the ganglia at either end (RVG and PAG). Figure 1.3 shows in schematic form all the neurons and nerve processes behind the RVG in a newly hatched L1 larva. The ventral cord contains the motor neurons that innervate body muscles as well as interneuron processes that run to and from the nerve ring. There are two groups of processes in the ventral cord, one on each side of the hypodermal ridge. They are very asymetrical. The right hand cord contains 25 to 30 processes, including the motor neuron processes and many pairs of interneurons which are bilaterally symmetric in the nerve ring, while the left hand cord contains only 3 or 4 processes. The other main longitudinal bundle is the dorsal cord, which contains motor neurons processes and just one interneuron, RID.

The ventral and dorsal cords contain the motor circuitry controlling body movement. There are three classes of motor neuron at the L1 stage, DA, DB and DD (five more classes are added during postembryonic development). In addition to having their cell body and a process in the ventral cord, all these motor neurons send a commissure round the body of the animal to the dorsal cord, where they have another process. Muscle arms from ventral muscles extend to the ventral cord, while those from dorsal muscles extend to the dorsal cord. Movement of the body is limited to the dorsal-ventral plane. The head has more freedom of movment, owing ot more complex innervation of the muscles in the head directly from the nerve ring, but motion of the whole animal is caused by propogating dorsal-ventral waves along the body. DA and DB neurons both have their neuromuscular output in the dorsal cord, and receive input from (different) interneurons in the ventral cord. However they have different polarities: both ventral and dorsal DA processes grow forward, while DB processes grow backward. DD motor neurons receive input in the dorsal cord from the DA and DB neurons, by “intercepting” their neuromuscular junctions, and have output in the ventral cord, which is thought to be inhibitory, ensuring relaxation of the ventral musculature while the dorsal musculature is contracted.

Figure 1.3

Figure 1.3

All the nerve processes and cell bodies behind the RVG. This diagram is a schematic cylindrical projection of the inner surface of the hypodermis and nervous system, obtained by conceptually cutting along the dorsal midline and unfolding flat. The dorsal cord is shown at the left hand edge, anterior is at the top, and posterior at the bottom. The positions of the four longitudinal muscle quadrants are shown by hatched regions. Nerve processes in C. elegans branch only rarely and reproducibly and all the branches in this region are shown. Processes entering the ventral, lateral or dorsal cords from the front are indicated at the top. Those with asterisk after the neuron's name only run part way back along the body. Posterior interneuron processes running forward along the ventral cord are indicated at the top. Those with an asterisk after the neuron's name only run part way back along the body. Posterior interneuron processes running forward along the ventral cord are indicated similarly at the front of the preanal ganglion. All the anterior axons in the ventral cord without an asterisk terminate in the preanal ganglion, except for that of AVG, which is shown ascending into the dorsorectal ganglion. The PHA and PHB neurons from the lumbar ganglia also have posteriorly directed processes that terminate in the phasmid sensilla. Note the different directionalities of outgrowth of the different ventral cord motor neuron classes.


Dorsal
Left Lateral
Ventral
Right Lateral
RID
ALA
AVG
motor processes
DB3
DA2
DD2
DA3
DB4
DA4
DD3
DB5
DA5
DD4
DB6
DA6
DD5
DB7
DA7
ALML
ALMR
CANL
CANR
PVT
PVPL
PVPR
DD6
Preanal Ganglion
DA8
DA9
PVQL
PVQR
PHAL
PHAR
PHBL
PHBR
LUAL
LUAR
PVCL
PVCR
ALNL
ALNR
PLML
PLMR
PVR
anus
DVA
DVC
Left Lumbar Commissure
Right Lumbar Commissure
Left Lumbar Ganglion
Right Lumbar Ganglion
Dorsorectal Ganglion

In addition to those in the ventral and dorsal cords there are a few neuronal cells and processes on the lateral hypodermal ridge and four small ganglia at the back of the animal (figure 1.3). The lateral neurons ALM and PLM are touch receptor classes (Chalfie and Sulston, 1980), while CAN and ALA are associated with the excretory canals, which run through the lateral ridges. In the front half of the animal there are four processes running back under each muscle quadrant from the nerve ring. These sublateral processes are possibly proprioceptive, involved in controlling head movement, since the neurons they belong to are closely associated with the head motor circuitry, SMBD and SMDD being motor neuron classes themselves. The preanal ganglion contains three interneuron cell bodies, DD6, DA8 and DA9. The lumbar ganglia on the sides at the back contain the cell bodies of the ALN and PLM neurons, which have lateral processes, and of the phasmid chemoreceptors PHA and PHB and the ventral cord interneurons PVQ, PVC, LUA and PVR, all of which send anterior processes down to the preanal ganglion and the ventral cord via the lumbar commissures. Finally there are two neurons in the dorsorectal ganglion on the top surface of the rectal epithelium behind the anus, DVA and DVC.

There are both practical and strategic reasons for choosing the ventral cord as the target for study. First, although the final anatomy of the nerve ring has been reconstructed, it is too complex a structure to be able to easily study its development. Its final structure is, however, discussed with respect to developmental considerations in the second part of this thesis. Second, the method of observation used has been reconstructed from electron micrographs, and it is relatively easy to reconstruct the ventral cord region from transverse sections, since processes are mostly lonitudinal, any commissures containing only a few processes. Third, and perhaps most importantly, it is possible to at least some extent to examine functionality defects in ventral cord structure, which allows the combining of work on structure and function. A reasonable functional model of the ventral cord motor circuitry has been proposed, both by analogy to the results in Ascaris and as a result of ablation experiments in which components of the circuitry were removed (Chalfie et al., 1985). Movement is very easily observed, and a large number of uncoordinated mutants have been ovtained that have various defects in movement (Brenner, 1974).

As mentioned previously, some of these mutants have been seen to have defects in nerve process morphology (Hedgecock et al., 1985 S. McIntire, J. White unpublished observations). Particular examples are that some or all circumferential commissures go astray in unc-5, unc-6 and unc-33 mutants, and the PHA and PHB processes get stuck at the bottom of the lumbar commissures in unc-33, unc-44, unc-51 and unc-76 mutants. These defects suggest that the affected genes may be involved in the processes of neural outgrowth that have been studied here. Genes defined in this way provide a possible link between the anatomical experiments and observations described here and the molecular mechanisms involved. The defects they induce are compared with the wild type development and the effects of cell ablations in Chapter 5.

CHAPTER 2 Materials and Methods

C. elegans (var. Bristol, N2 strain) was propogated on lawns of E. Coli grown on agar Petri plates, as described by Brenner (1974).

2.1 C. elegans neuronal nomenclature

The 302 neurons are divided into 118 classes on the basis of morphology and synaptic connectivity. Each neuron's name consists of two or three capital letters denoting the class and a suffix denoting which member of that class it is. The motor neurons in the nerve cord have two letter roots and a number as a suffix, so DA2 is the second member of the DA class (counting from the front). Interneurons have three letter roots and use the suffix letters L, R, D, and V to distinguish left, right, dorsal, and ventral members. Thus PVPL is the left member of the PVP class. Unique neurons, such as AVG, have no suffix. When referring to a class rather than one of its members merely the two or three letter route name is used.

2.2 Electron microscopy

Embryos were isolated by dissolving gravid adults with 1% hypochlorite, 0.5 M KOH for 5 mins, collecting the eggs through a 52 micron filter (Nitex) then rinsing the eggs three times in M9 buffer. The eggshells were digested with chitinase following the the procedure of Wolf et al. (1983), and the remaining viteline membrane was broken mechanically by pipetting the chitinased eggs through a drawn pasteur pipette. After removal of the eggshell the embryos were fixed in 1% OsO_4, 0.8% Kfe $(CN)_6$ (0.1M cacodylate buffer, pH 6.0) for 45 minutes at room temperature. They were then rinsed in 0.05 M cacodylate buffer, pH 7.0, and treated for 15 mins with 0.2% tannic acid (Malinckrodt) in the same buffer. Finally they were rinsed in dH_20 and straight embyos of approximately the right age were embedded, sectioned, and stained as in White et al. (1986). Adults were simply fixed for one hour in 1% OsO_4 in 0.1 M $NaPO_4$, pH 7.4, and cut in half before embedding to ensure proper infiltration. The sections were viewed on an AEI 6B electron microscope and photographs were taken every 2 to 3 sections (nominal section thickness, 50nm) at a magnification of 3 to 10 thousand. I am very grateful to J N Thomson, who did all the serial sectioning with uncanny consistency and reliability. J Priess developed the fixation protocol used here.

2.3 Ablations

In order to remove specific cells from the developing nervous system the parent of the desired cell was ablated using a laser microbeam. To obtain embryos, gravid adults were cut open in a watch glass of distilled water (dH_20). About 30 embryos of approximately the right age were transferred to a 3% agar pad and grouped together. The surrounding agar was cut away to leave a 3mm x 10mm strip, and a cover slip was placed on top, held in place with dabs of hot vaseline on the corners. Extra water was added to prevent dessication. Under the slight pressure of the cover slip approximately half the embryos lie ventral side up, as desired, and the pattern of individual cell nuclei around the desired

Figure 2.1

A line drawing of the position of the cell nuclei on the ventral surface of the embryo at 270 mins, the approximate time when the laser ablations were performed. Anterior is at the top of the page. Nuclei are clearly visible by Nomarski microscopy. Most neural precursor cells at this stage will divide one more time. Cells that were ablated are shaded and their normal daughters are shown. An X represents a cell that dies soon after birth. The smaller crosshatched cells are cells that die around the time of this picture; they are very distinctive and provide useful landmarks. (Adapted from Sulston et al., 1983).

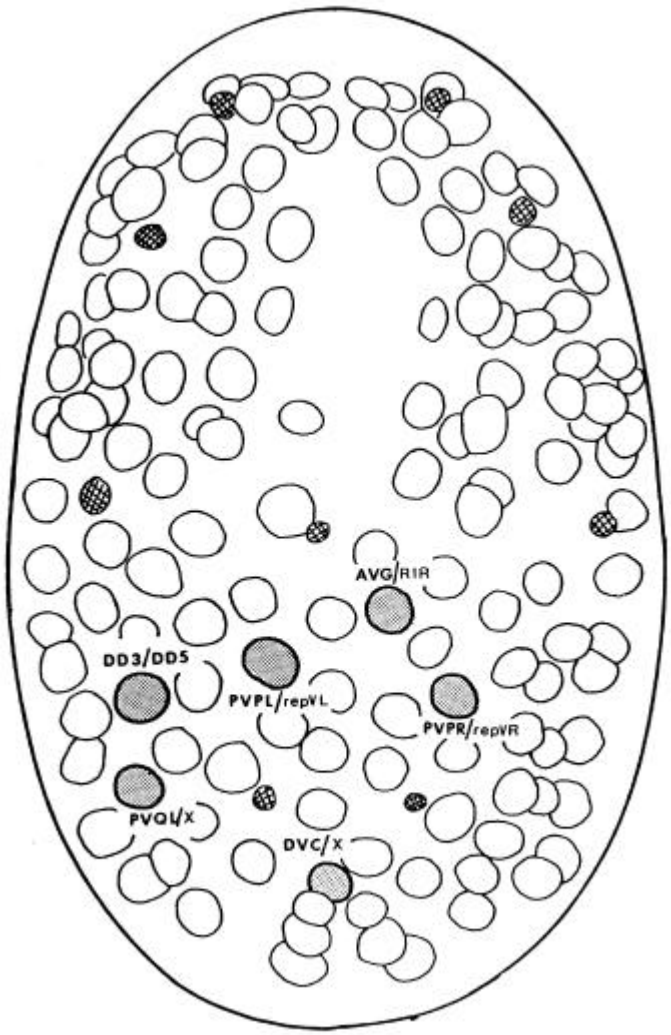
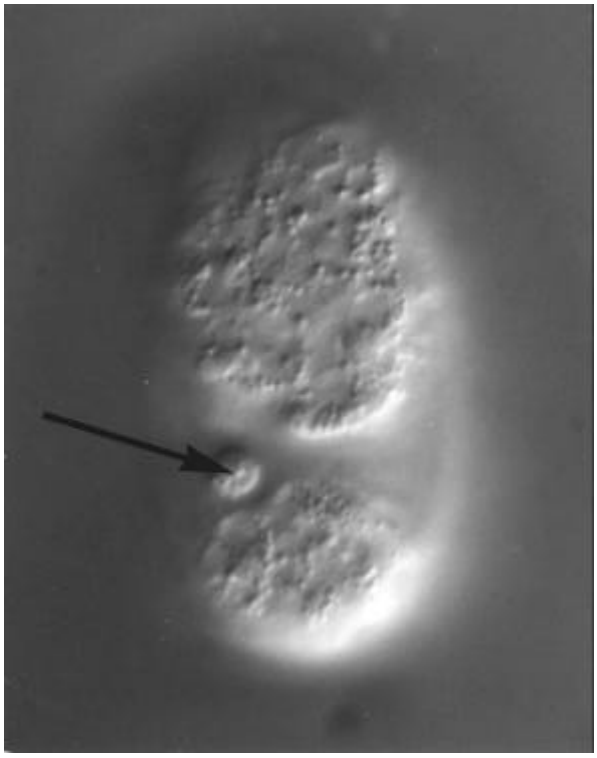


Figure 2.2

After ventral surface cells are killed the remains are excluded from the embryo when the ventral hypodermis seals up at around 320 mins. Here the ablated parent of PVPL is shown at approximately 350 mins (arrowed). The total length of the egg is 60 microns.



time can be reliably recognised using Nomarski optics (pattern shown in figure 2.1). Ablations were executed with a pulsed laer (PRA LA1000/LN102 used with Courmarin 450 dye), whose beam is focussed down the microscope objective as in Sulston and white (1980). The chosen cell was killed with repeated low energy laser pulses (20-100 hits). After 15-20 mins the dead cell shrinks into a condensed refractile ball. If it is on the ventral surface, as with all but one (DVC) of this set of experiments, then it is excluded from the embryo when the hypodermis, which starts as a patch on the dorsal side, closes over about 45 mins after the ablation (figure 2.2). After monitoring exclusion of the dead cell in the experimental embryos, they were transferred either to petri dishes with bacteria if they were to hatch, or, if they were to be fixed as older embryos, to a8 well multi test well slides (Flow Labs) subbed with 0.1% polylysine. The fixation protocolw as above, all the fixation steps being carried out with the embryos inside their eggshells attached to the test well slides. In order to allow access of fixatives etc. to the embryos, the laser wa sued to make a small hole in the eggshell in the presence of the first (OsO_4) fixative at the chosen stage of development. Fixed embryos were dislodged from their slides and embdeed and sectioned as above.

2.4 Reconstruction

Prints were made from each negative and the reconstruction was carried out directly from the prints by writing a label inside each profile with a drafting pen and following the labels from one photograph to the next. In many cases nerve cells and processes were immediately identifiable, but when this was not so an arbitrary label was used and the cell was identified later if possible. The criteria used for neuron identification are given below. Aside from the problem of identification of a correctly reconstructed cell there may be problems in forming a continuous reconstruction itself. Usually these problems are generated either by a number of consecutive sections being unphotographable because of grid bars or dirt on the grid covering the sections, or by the neuropil being cut tangentially to some nerve processes so that the membrane boundaries become indistinct. In all the cases considered here these problems were satisfactorily resolved, when necessary by checking internal consistency (e.g. a process with two attached cell bodies is no good, nor is an unattached process) or consistency with the equivalent cells in other reconstructions. One embryonic AVG ablation reconstruction was abandoned because processes could not be definitely linked across a break. Generally these difficulties are less severe in embryonic than in adult reconstructions, since there are many fewer processes in each bundle and the processes have smoother trajectories; they are not so tightly constrained by other tissues, particularly since the muscle is till not fully developed.

Altogether 19 reconstructions of varying regions of different embyos were undertaken, using around 3000 photographs.

2.5 Staging of reconstructed embryos

None of the reconstructions described in this dissertation came from timed embryos. Stages were assigned by placing them in a developmental sequence and comparing them with short serial reconstructions from less ideal embryos of known age at fixation, and with previously known developmental events that were detectable in the reconstructions (e.g. cell divisions and movements). The timed embryos were obtained by cutting open gravid adults and selecting embryos at the two cell stage. These were incubated at 25 C and then fixed by the same method as the ablation experimental embryos (above). Development times at 25 C were converted to times at 20 C from standard growth curves (Schierenberg, 1978).

2.6 Identification of neurons

There are several factors that make cell and process identification from electron microscope reconstructions relatively straightforward in C. elegans embryos. To begin with, there is simply not very much there. Figure 3.7 show typical ventral cord and preanal ganglion sections. What cells there are are sufficiently different from one another to be easily and reproducibly distinguishable. The positions and identities of all the cell bodies are known throughout embryonic development from the remarkable work of Sulston et al. (1983) obtained by light microscopy with Nomarski optics. All the cells under consideration here have an invariant lineage, and their relative cell body positions are extremely reproducible. Second, the nerve process morphologies are simple enough to be fully traceable in the reconstructions. They are also highly reproducible and all their adult forms are known from the equally enclylopaedic work of white et al. (1986). Figure 1.3 shows the approximate positions of all the neurons behind the RVG (see also Sulston et al. 1983 for camera lucida drawings at different stages). In general all cell bodies and processes were identified in all reconstructions. I give below the specific criteria used to identify the various cells, followed by a discussion of the remaining cases where complete identification was not possible.

AVG: the ventral cord process was followed back to a cell body in the RVG in the A and B wild type reconstructions. AVG is the only neuron in the RVG to send a process back along the whole length of the ventral cord, and the position of the cell body was as expected in each case. In other reconstructions AVG was identified by the fact that it was the only continuous process in the ventral cord (if the series was early enough) or because it is the only process to grow into the DRG (DVA and DVC have cell bodies in the dRG and grow down out of it). Ablation of ABprpapppa, the parent of AVG, removed the ventral cord process that had been identified as AVG.

Ventral cord motor neurons: These were identifiable by cell body order and the direction of outgrowth of processes and commissures, which were known to be invariant from larval and adult reconstructions. In all cases unique identifications could be made which were entirely compatible to the known data (except in the AVG parent ablations commissure direction was altered though the order of cell classes remained as normal). In the early series, before commissures grow out, the cell bodies overlap and there is a

vertical order, with DA cells overlapping dorsally to DD cells, which in turn are dorsal to DB cells (consistent with Normarski observations of Sulston et al., 1983).

PAG cells: The relative positions of PAG cell bodies are shown in figure 1.3. The only variability that was found in reconstructions was that the body of DD6 was sometimes more anterior, underneath PVPL, PVPR and PVT. The following diagnostic criteria confirmed assignments: PVT never sent out a process in any embryonic reconstruction and always was the most anterior ventral ectodermal cell to contact the rectal epithelium (repVL and repVR). PVPL and PVPR have a unique process morphology in the PAG since their processes cross over when they leave their cell bodies, and then grow forward along opposite sides of the cord. DD6 has a standard DD type process; also the PVQL process and, to a lesser extent, PVQR and DVC processes tend to flatten out on the surface of DD6. DA8 and DA9 are the only cells to send processes up the lumbar commissures (left and right respectively). Ablation of ABprpppppaa or ABplpppppaa, the parents of PVPR and PVPL respectively, resulted in the correct PVP cell being missing and an accommodation in position by the other cells in the PAG (Chapter 4).

Note that I have named the PVP cells by the position of their cell bodies and lineage, in accordance with the general practice for C. elegans neuronal nomenclature and with Sulston et al. (1983). The ablations confirm that the cells do not exchange positions after being born. Since their processes cross over this means that the PVPR process is on the left. This is reversed from the nomenclature of White et al. (1986), in which PVPR has its process on the right. The reason for this inconsistency is that the PVP cells are squashed into a line in the adult and the crossover is not apparent. The same holds for the RIF, RIG and SABV cell pairs in the RVG, whose processes also cross over, and which I have also named in accordance with Sulston et al. (1983), rather than White et al. (1986).

DRG cells: DVA and DVC are the only embryonic cells in the DRG. Whenever their processes were seen, except in the anterior D reconstruction, they were followed back to the PAG. In cases where they were not followed back to their cell bodies they were distinguishable because of very different behaviour in the PAG (see below), and because the DVA process descends into the PAG around the right side of the rectum, whilst the DVC process descends around the left side.

Lumbar ganglia cells: The relative positions of cells in the lumbar ganglia are shown in figure 1.3. This region was only reconstructed once, in the wild type C reconstruction. In other cases the PVQ processes in the ventral cord were identified by (I) their characteristic behaviour in the PAG, and strong association with PVP processes (figure 3.7), (ii) the fact that they were by far the most advanced processes coming out of the lumbar commissures. PVQL is the only lumbar commissure process that runs on the left side of the ventral cord (White et al., 1986). The ablation of Abplapppaa, the parent of PVQL, removed the PVQL ventral cord process (Chapter 4). The process of other lumbar ganglia cells were only separately identified in the C reconstruction. In other cases they were identified as a group.

Lateral cells: The few neurons with cell bodies lying on the lateral hypodermis (figure 1.3) are well spaced out and can easily be identified on the basis of cell position.

RVG Cells: The RVG was only reconstructed in the A and B wild type reconstructions. AVG was identifiable by its posterior process. The three bilateral sets of cells (RIF, RIG, SABV) could be paired off according to position, size and process growth. Other cell identifications were made on the basis of position, and are not completely definite. However the only cells that I discuss below are AVG, and the RIF and SABV neurons and their identifications are certain.

The only cases apart from the lumbar commissure processes and the RVG in which definite identifications were not made are in the anterior D reconstruction. Here the majority of interneurons cannot be individually identified. On the left side of the cord only two processes are present at the posterior end of the reconstruction, so they must be PVQL and PVPR, since they grow forward together from the back. There is also one anterior process running part way back. This could either be AVKR or RMEV. On the right side there are 4 processes present at the front of the reconstruction that terminate at some point before the back. These are presumably interneurons with cell bodies around the ring, but to identify them individually would require reconstructing the entire nerve ring region. There are also 7 processes running through the entire reconstruction, which probably include PVPL and PVQR since the left hand versions of these have grown right through the reconstruction. It is not possible to identify the others.

CHAPTER 3 The Pattern of Outgrowth in Normal Embryos

The organisation of processes in the ventral nervous system is established during a short period of little more than an hour, at the same time as the animal is elongating in the eggshell from a stubby 'tadpole' to a worm. Electron microscope reconstructions of varying lengths were made from a series of four embryos at different developmental stages during this period (figure 3.1). Figure 3.4 shows a schematic picture of the state of the ventral nervous system in each of the reconstructions, which will be referred to by the letters A to D. During the period covered by these reconstructions the embryo increases by a factor of about two in length, being about one and a half fold in the egg (100 microns) at the time of the A reconstruction, and three and a half fold (220 microns) in the D reconstruction. At the beginning of the period under consideration here the nerve ring contains the majority of the final number of processes. Uncoordinated muscle activity has already started before the time of the A reconstruction (the onset of twitching is at about 430 mins). Movement becomes more organised around the stage of the final, D reconstruction, although since the embryo is restricted inside the egg shell it is not possible to assess fully the degree of coordination.

3.1 Morphology of growth cones

Growth cones are generally extended flattened lamellar structures that also have long thin filopodial extensions. In C. elegans the most extensive growth cones are seen on the growing tips of the motor neuron commissures. Typically they are a flattened sheet a few tenths of a micron thick and of variable shape and size in the plane of the sheet (figures 3.2, 3.5). The absence of normal looking filopodia may be due to the small scale (2-5 microns across); a vertebrate tissue culture growth cone could extend right round the C. elegans embryo. However stubby finger-like extensions are seen in many cases, and these may perform an equivalent function. Figure 3.2 shows a three dimensional reconstruction of the complete cell DB4 from the B reconstruction, in which the thin sheet-like nature of the growth cone can be clearly seen.

Extended growth cones like those seen on commissures were not seen on processes growing along the ventral or dorsal cords, although some tips do have swollen or spread out endings (e.g. PVCL in figure 3.7). This corresponds to observations made in other organisms that process growing along pre-existing nerve bundles do not have such extended growth cones as those growing over virgin territory (Lopresti et al., 1973).

The quality of the cytoplasmic fixation in the embryos used for reconstruction was poor, since primary fixation is with OsO_4 follwed by tannic acid, which fixes membranes well but leaves little cytoplasmic structure. Therefore neither actin microfiliaments nor microtubules are preserved. However in some cases it is possible to see vesicles in growth cones, as for example in a commissural growth cone in the C reconstruction (figure 1.1). Studies by de Cino (1981) have indicated that transmitter is sometimes released by growth cones. An alternative explanation for the vesicles is simply that they may be a source of new membrane for insertion at the leading edge of the growth cone.

Figure 3.1

This shows the approximate ages of the embryos used in this study from which long series were reconstructed completely, and the parts of them that were reconstructed. Ages were determined as described in Chapter 2.5. The bracket below the embryo indicates the part of the ventral cord shown in figure 3.4.

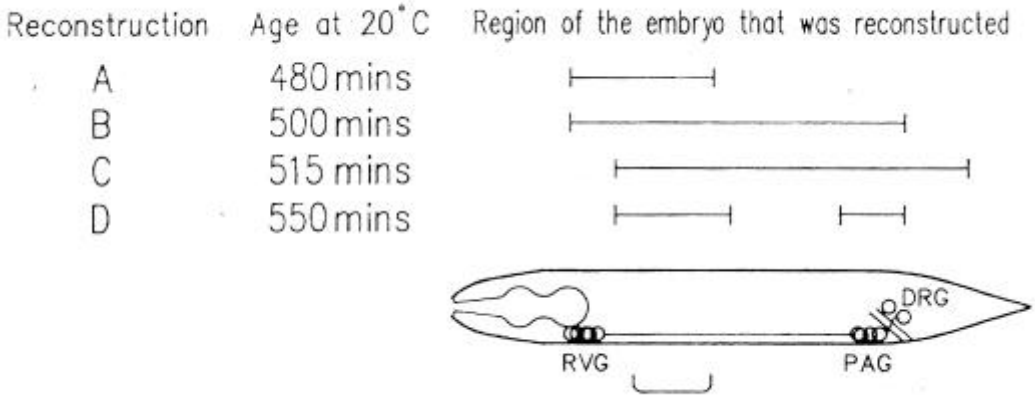


Figure 3.2

A three-dimensional reconstruction of the motor neuron DB4 from the B series. The cell body is on the right. Out of this extends a growing commissure, terminating in the flattened extended structure at the left, which is the growth cone. This diagram was made with the aid of a 3-D reconstruction program written by J.G. White. The growth cone is approximately 5 microns across.

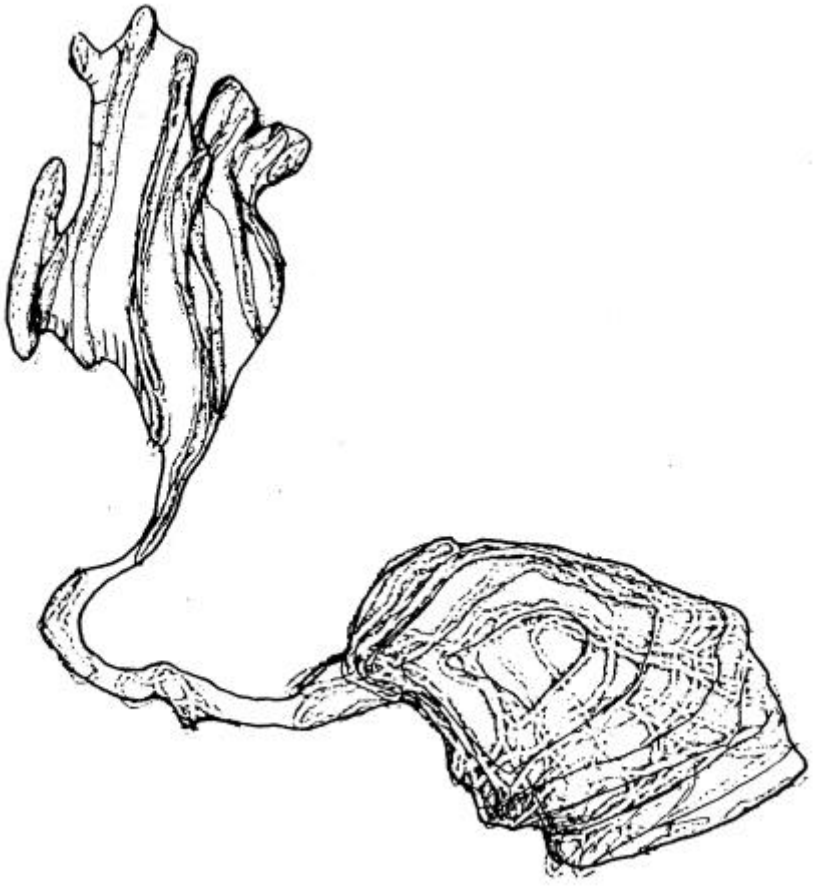
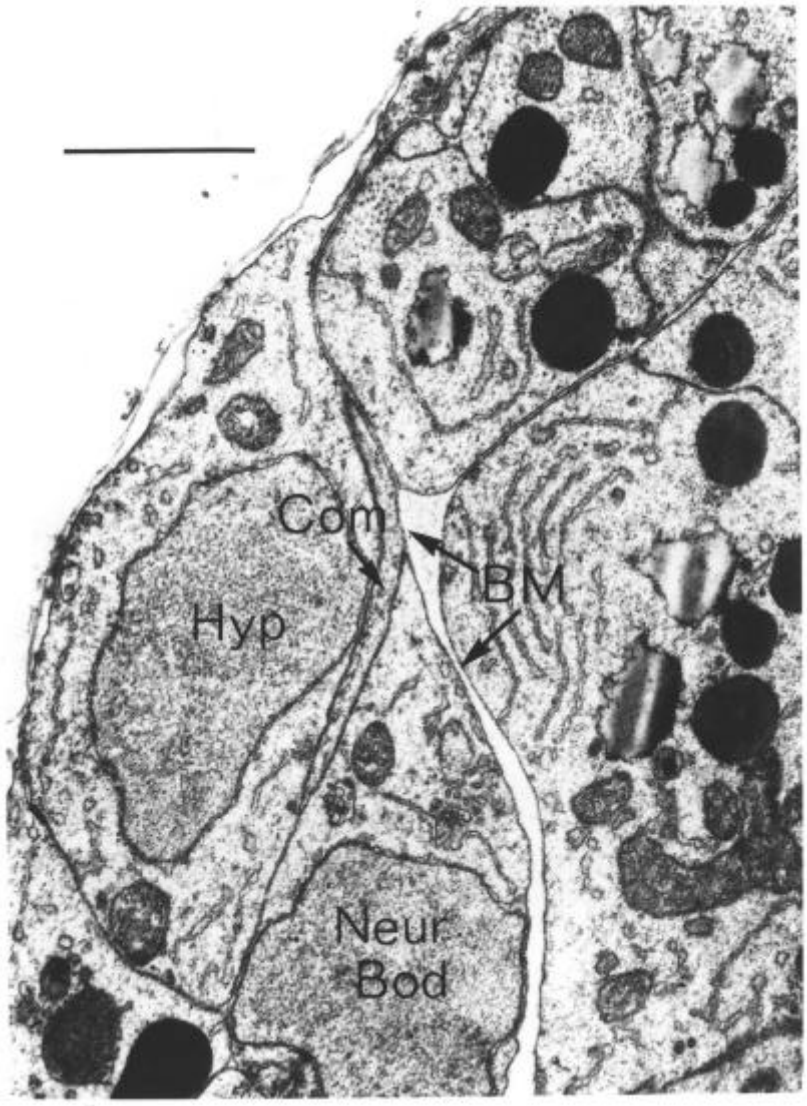


Figure 3.3

The growing DB5 commissure (Com) is forced to choose whether to pass the lateral neuron body of CANL (Neur Bod) on the side of the hypodermis (Hyp) or on the side of the basement membrane (BM). It passes on the hypodermal side, as do all motor neuron commissures in similar situations. This suggests that commissural growth cones attach to and move over cell surfaces rather than basement membrane. From the C reconstruction. Scale is 1 micron.



3.2 The attachment substrate for growth cones

Growth cones have been seen in vitro to extend very well over artifical substrates made of basement membrane components, such as fibronectin and laminin (Baron van Evercooren et al., 1982). This has led to the suggestion that basement membrane may provide a favoured substrate for growth cones to grow over. It is possible in at least one case in C. elegans to determine the substrate on which the growth cone moves. The motor neuron commissures grow out sandwiched between hypodermal cells and the basement membrane. There are several lateral neuronal cells that also lie between the hypodermis and the basement membrane, in the way of the growing commissures. Whenever a commissural growth cones reaches a lateral cell body it leaves the basement membrane and passes between the hypodermis and the lateral neuron (figure 3.3). There has never been observed an exception to this rule. Thus it seems that the growth substrate for these commissures is the surface of hypodermal cells, not the basement membrane.

A couple of similar results are provided by ablation experiments (Chapter 4) in which in one case DD5 moves from the right side of the cord to the left (after removing AVG), and in another case PVQL moves from the left to the right (after removing PVPR). In each case the process changing sides passes under a motor neuron cell body, rather than over it, again maintaining contact with the hypodermis rather than the basement membrane. There are many other examples where processes grow between cell bodies and other processes, well removed from the ectodermal basement membrane. During later development several posteembryonic processes grow the length of the ventral cord in the middle of the main bundle of embryonic processes. The embryonic reconstructions presented here show processes from the lumbar ganglia growing forward through the middle of the cluster of cell bodies in the preanal ganglion. In general wherever there is evidence on the subject of neuronal growth cone guidance in C. elegans it suggests that the substrate for growth is the surface of other cells, rather than a basement membrane. However this does not rule out the possibility that the basement membrane is important in certain cases.

3.3 AVG pioneers the ventral cord

The first nerve process to grow along the ventral cord belongs to the interneuron AVG. AVG has its cell body in the retro-vesicular ganglion at the front of the ventral cord; it is an unpaired cell, and is the most posterior interneuron in the front of the animal to send a process back along the cord. The cell body and process were identified in both the A and and B reconstructions. By the A reconstruction the process has already grown back along the cord. At this stage the DD ventral cord processeshave also grown out on the right side of the cord (figure 3.4). However inspection of a younger embryo revealed that there was a single continual process in the ventral cord at a stage at which DD processes had not grown out (not shown). In the B reconstruction the AVG process grows the full length of the cord and up out of the pre-anal ganglion into the dorso-rectal ganglion, where it stops by the DVC cell body. IT reaches no further than the DRG in all the latter embryonic reconstructions (B to D), although in the adult it is seen to extend right back into the tail spike (White et al., 1986). Therefore there must be a second period of extension postembryonically or during late embryogenesis.

3.4 Motor neurons

The next event after the appearance of AVG is the growth of processes from the DD motor neurons forward alongside the AVG process on the right side of the ventral cord. In the A reconstruction these extend until they nearly touch the next DD cell body (figure 3.4). In the adult, adjacent DD neurons overlap for a short stretch and are linked by gap junctions, but in all the embryonic reconstructions there are small spaces between them of the order of a micron in length (figures 3.4, 3.5). It is of course possible that contact has been made and the processes subsequently withdrawn. Another possible correlate of DD extension in the ventral cord, discussed further later, is that all the DD ends of DD processes are by DB cell bodies. Ventral cord processes from the DA and DB motor neurons do not grow out until later, after the commissures and dorsal cord processes are made.

The first signs of commissure outgrowth can also be seen in the A reconstruction. All the motor neurons have lamellar extensions poking laterally under the ventral musculature at the site where their commissural growth cones will leave the ventral cord (figure 3.4). These nascent growth cones leave from the DA and DB cell bodies, and from near the anterior end of DD axons. Outgrowth of commissures from the motor neurons of all classes is synchronous; in the B series, only about 20 minutes older than the A series, they have all reached the lateral hypodermis, and in the C series they are just about to reach the dorsal hypodermal ridge (figure 3.4). RID, the only process to grow along the full length of the dorsal cord, is not present at this time, and the commissures apparently turn of their own accord, DA ones anteriorly, DB posteriorly, and DD in both directions, and link up to form the dorsal cord. Although they are reproducible, there is no regular anterior/posterior order to the positions of the commissures from the different classes of cells (figures 1.3, 3.5), so the direction in which they turn cannot be simply determined on the basis of the classes of neighbouring processes (e.g. DA's and DB's towards each other). Since the dorsal hypodermis is syncytial and does not contain apparent landmarks it seems that the direction must be intrinsically determined. In addition, if we assume that the direction is determined in the same fashion for all the members of a class, then it must be specified in terms of the anterior and posterior of the animal, rather than whether to turn left or right, since some of the DB axons go round the left hand side of the body and turn right when they reach the dorsal midline, while others go round the right side and turn left.

Figure 3.4

Schematic diagrams of the part of the body indicated in figure 3.1 from the A, B, C and D reconstructions (1), b), c) and d) respectively). The diagrams are cylindrical projections in the same basic form as figure 1.3, with the positions of the muscle bands being shown as hatched areas. The DA3, DB4 and DD2 cell bodies and the DA3, DB4 and DD3 commissures are shown in each case. Initially only the AVG and DD processes in the ventral cord are present (a). Then the commissures grow out simultaneously from all three motor neuron classes (b, c), followed eventually by the ventral cord processes of DA and DB motor neurons and other ventral cord interneurons (d). ALM and CAN are lateral neurons, which migrate back from the front and then send processes forward.

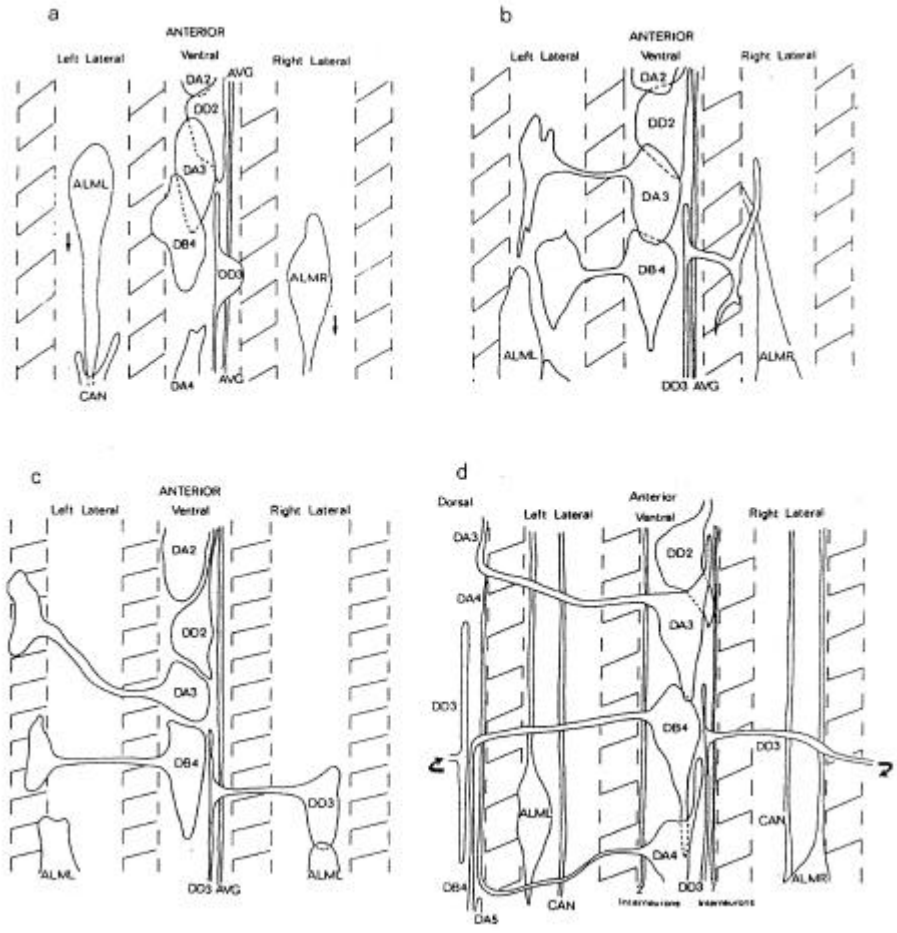
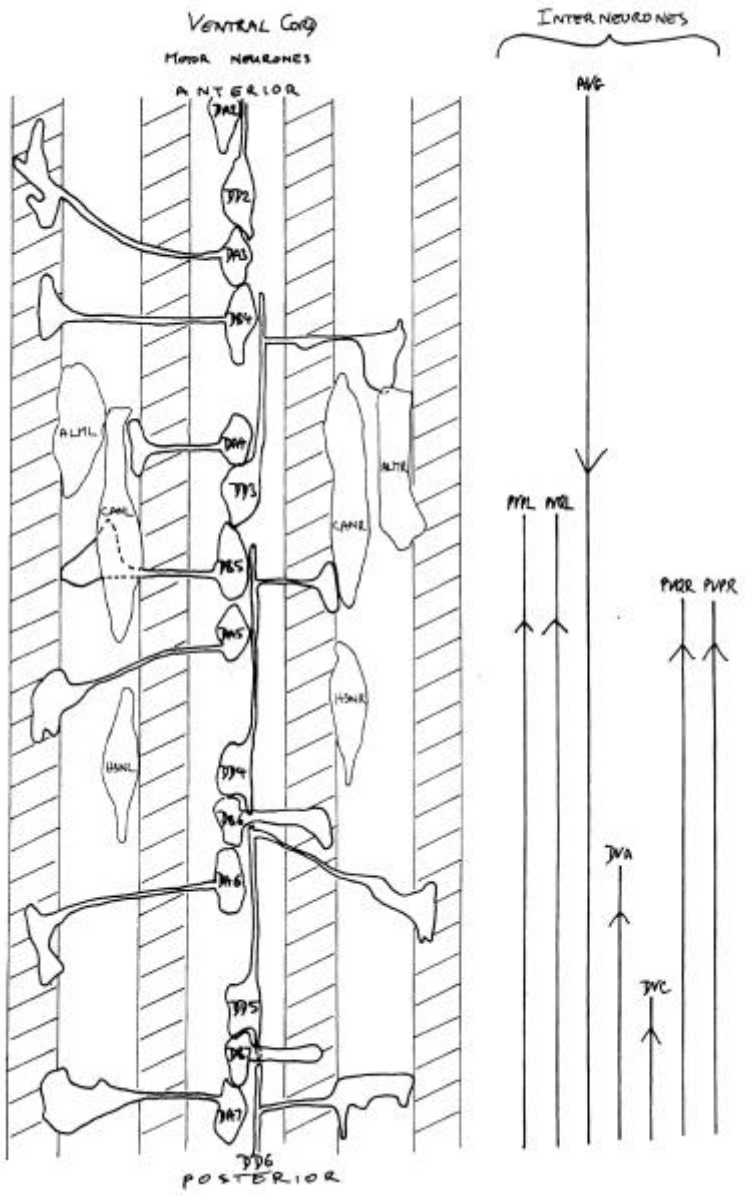


Figure 3.5

A schematic diagram of the entire ventral cord region from the C reconstruction. The motor neurons and lateral neurons are shown on the left in the same form as in figure 3.4. (HSNL/R are postembryonic neurons that grow out processes in the L4 larva to innervate vulval muscles). The positions reached by all the interneurons that have grown substantially into the ventral cord are shown on the right. In addition to the PVT, PVCL and PVCR processes have also grown just past the front of the preanal ganglion in this reconstruction.



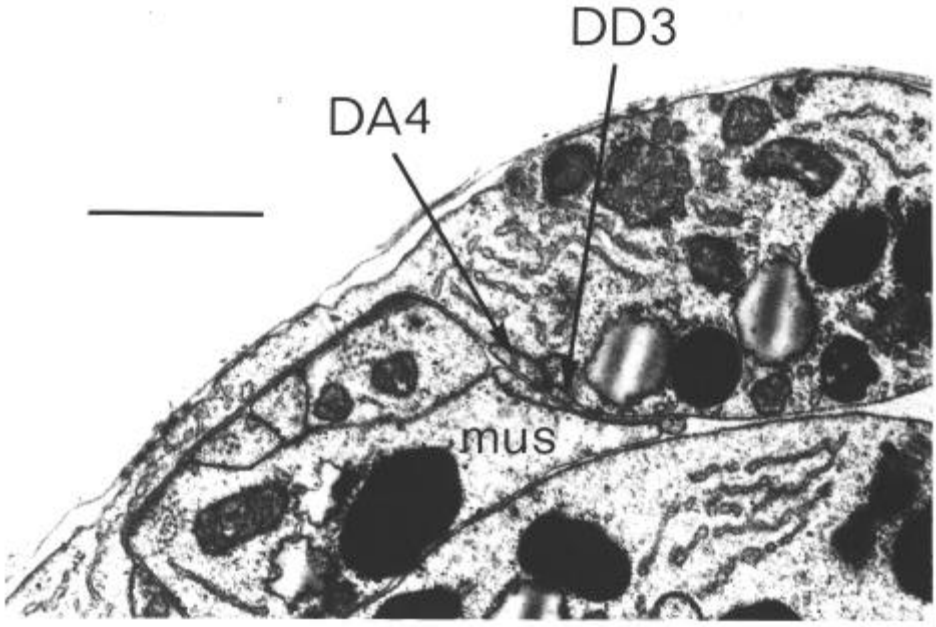
There appears to be a possible correlation between the position of DA commissures and the location of hypodermal cell boundaries. The embryonic ventral hypodermis consists of 6 left/right pairs of cells, known as P cells, which are joined at front and back to the main body hypodermal syncytium, called hyp7. DA3 to DA7 lie on the boundaries between adjacent P cells, and they send solitary commissures to the left directly from their cell bodies out along the P/P cell boundary. In contrast DA2, which is on the boundary between the most anterior P cells and hyp7, sends its commissure forward and out to the right together with those of DB3 and DD2. Similarly DA1, DA8 and DA9, none of which are near a P/P cell boundary, send their commissures together with processes from other cells (DB2 and DD1 in the case of DA1, the lumbar commissures for DA8 and DA9).
There are no corresponding visible cues for DB commissure guidance, and only weak ones for DD commissures. One possibility is that DB cells are involved; DD commissures all leave the cord from approximately opposite DB cells (figure 3.5, note especially DD3, whose commissure exit point is quite a long way behind the DD2 cell body, but opposite DB4). It is hard to tell whether a DD commissure is created by diversion of the growth cone that generated the ventral cord process, or by a genuine branching. The commissure always comes from near the anterior tip of the ventral cord process, the extension of which is essentially complete when the commissure starts growing, suggesting that only a single growth cone is used. However there is a definite T junction in the final structure, and the DD process can make branches, since one is certainly made when the commissure reaches the dorsal cord (figure 1.3). The side of the animal that the DD commissures go round is easier to explain. They all go round the right side of the animal, which is consistent with the position on the right side of the cord of their ventral cord processes, from which the commissures diverge or branch.

It is only after the dorsal cord processes have extended for some distance that we begin to see growth of the ventral cord dendrites from DA and DB neurons, (D reconsruction, figure 3.4). This coincides with the growth back along the cord of some of the ring interneurons, possibley including the motor circuitry interneurons that innervate the DA and DB ventral cord processes. However there are no visible synaptic connections between the interneurons and the growing DA and DB dendrites.

The dorsal cord, in the other hand, does show signs of synaptic activity in the D reconstruction. The DA and DB neurons are already making small, but clear, neuromuscular junctions from their dorsal cord axons (figure 3.6). As in the final adult version these involve a joint synapse onto muscle and a DD process. No corresponding DD neuromuscular junctions in the ventral cord have been seen in this reconstruction. The dorsal cord processes have not reached their final length in the D reconstruction, and in fact are at a rather interesting stage: each DA axon stops where the next one arrives at the dorsal cord and turns. Later the axons overlap considerably, but the regions of neuromuscular output do not; instead they correspond closely to the regions where the axons are present at this stage. It is possible to speculate that there is a pause in axon extension while the zones of neuromuscular activity are being set up, but more data would be required to provide respectable evidence!

Figure 3.6

A neuromuscular junction from the DD reconstruction. Although small this shows all the characteristics of normal neuromuscular junctions in the developed nervous system. The dorsal cord process of the motor neuron DA4 is synapsing jointly onto muscle (mus) and the DD3 process. There is also a DB4 process present in this section. Scale bar is 1 micron.



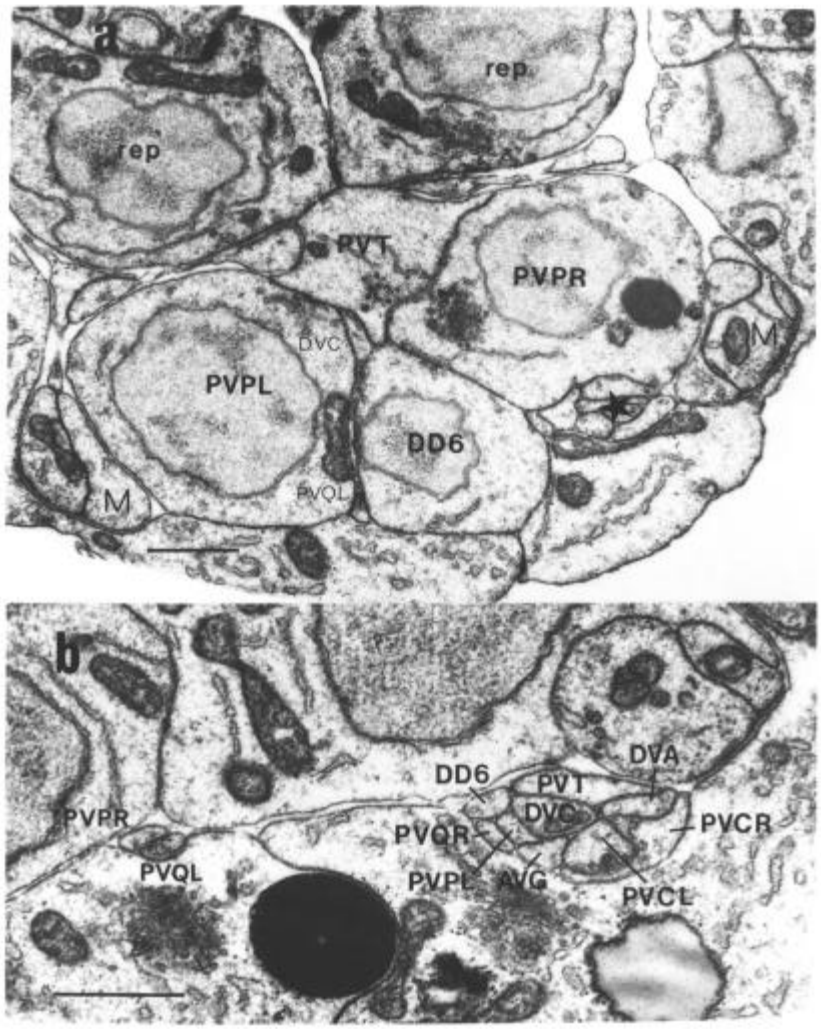
3.5 Later ventral cord interneurons

While the motor neuron commissures are growing out, a set of interneurons are growing forward along the ventral cord from the pre-anal ganglion (PAG) at the back (figure 3.5). The most advanced of these are two pairs of processes, PVPR and PVQL on the left side of the ventral cord and PVPL and PVQR on the right side. These are followed by DVA and DVC, which are both unpaired neurons that run on the right. Figure 3.7 shows a cross section of the posterior cord from the C reconstruction, after these posterior neurons have reached the front of the cord, that we see other anterior interneurons growing back along the cord (figure 3.4).
PVP and PVQ: The PVQ neurons are the most anterior cells in the lumbar ganglia (PVQL in the left lumbar ganglion, PVQR in the right, figure 1.3); their processes descend the lumbar commissures and then run forward through the PAG and along the cord. PVPR and PVPL have cell bodies in the PAG, where they are the only bilateral pair of interneurons. Their processes cross over when they leave the bodies, joining up with the PVQ process on the opposite side, and then run forward along the cord. The structure of PVP decussation is discussd below together with that of three pairs of neurons in the retrovesicular ganglion.

PVQL and PVPR pioneer the left hand ventral cord; this appears to be a joint action, since their anterior tips are never more than a few tenths of a micron apart in any of the reconstructions (e.g. figure 3.5). The tips of the PVQR and PVPL are similarly close to each other, but there is no such tight relationship between the left hand pair and the right hand pair (figure 3.5). The fact that the left hand pair are often more advanced than the right hand pair suggests that the prior presence of AVG and DD processes on the right side of the cord has little effect on PVPL and PVQR outgrowth. The two processes in each pair are tightly associated all the way along the cord back to the point where the PVP processes cross over in the PAG. This association is also seen in the adult reconstructions of the ventral cord; the processes diverge only when they reach the nerve ring (White et al., 1986, unpublished data). Together these observations suggest that PVP and PVQ growth in the ventral cord might be cooperative, and a number of ablation experiments were performed to test this hypothesis (Chapter 4). In general the PVP process is on top of the PVQ process, i.e. there is a ventral to dorsal order of: hypodermis, PVQ, PVP, basement membrane (figure 3.7).
DVA and DVC: DVA and DVC are the two embryonic neurons with cell bodies in the dorso-rectal ganglion, above the rectum (1.3). They both grow forward along the right hand side of the cord behind PVPL and PVQR, but their tips are not close together like those of a PVP/PVQ pair. DVA grows down around the right side of the rectum back along the track of AVG, and in all cases keeps to the outside of the main right hand bundle of processes in the ventral part of the PAG (figure 3.7). DVC, on the other hand, grows down the left side of the rectum and crosses from dorsal left to ventral right, in the same place that two PVP processes cross over (3.8). Neither DVA nor DVC appear to be particularly tightly associated with any other process in the ventral cord. In the adult cord DVA is always at the ventral right hand exremity of the main right hand bundle, whilst DVC runs in the middle of the bundle, in association with DVB, which is the postembryonic dorso-rectal ganglion neuron (White et al., 1986). Other lumbar

Figure 3.7

Typical transverse sections through (a) the preanal ganglion and (b) the back of the ventral cord (from the C reconstruction). In (a) the star indicates the main group of processes that will become the right hand ventral cord. The other cells and processes are individually labelled. This section comes from just posterior to the point where the PVP processes and DVC will cross over (shown in figure 3.8). In (b) the independent growth of PVPR and PVQL along the left hand cord is clear. This section comes from very close the the preanal ganglion, and the PVCL/R and PVT processes are near their front tips, and are a little swollen, particularly PVCR, which is showing signs of wrapping around other processes. Scale bar is 1 micron in each case.



commissure processes: In addition to the DA8, DA9 and PVQ processes the lumbar commissures contain processes descending into the pre-anal ganglion from the following lumbar ganglion cells: PHAL/R, PHBL/R, LUAL/R, PVCL/R and PVR. Of these the PVC cells and PVR eventually grow the full length of the cord; the others stop at the front of the pre-anal ganglion. There is a very characteristic pattern at the back of the pre-anal ganglion where the lumbar processes from the two sides meet, which has been seen whenever the region has been reconstructed (figure 3.9). The processes from each side meet slightly to the right of the midline and "zip up", each contacting its contralateral analogue. The exceptions to this rule are the PVQ processes, which are at the top of the row one each side but stay at opposite corners of the structure and do not make contact. The dorsal to bentral order of this structure is PVQ, PHA, PHB, PVC, with the unpaired process of PVR wrapping around the ventral side of the whole group. This suggests that the contralateral pairs other than the PVQ's have a strong affinity for each other, and it is probably significant that the PVQ's are the only lumbar processes to run up the cord with one process on each side of the cord, as opposed to the more normal pattern of both processes being on the right. Anterior interneurons: Anterior interneurons other than the AVG are only seen growing back along the cord in the D reconstruction. As described in the section on cell identification in Chapter 2 it is not possible to identify these processes. However it is clear that there are at least PVPR and PVQL on the left side. There is one is one process growing part way back along the cord on the left, which may be RMEV or AVKR, and there are 11 interneurons on the right, some of which probably come from the back (e.g. PVPL and PVQR), but others of which are from the front; in particular 4 from the front stop within the anterior D reconstruction. The anterior interneurons seen her may include (some of) the major interneurons that innervate the ventral cord motor neurons.

3.6 Descussation in the preanal and retrovesicular ganglia

The PVP processes cross over in the pre-anal ganglion where they leave their cell bodies and then grow forward on the opposite side of the cord. This crossing over is at the back of PVT and above DD6, at the same place that the DVC process crosses from left to right. PVQL, DVC and, to a lesser extent, PVQR flatten out on the surface of DD6; DVC is always between the PVP crossover and DD6 (figure 3.8). The PVP cross over was observed in the adult reconstruction (White et al, 1986), but it is much less clear there since there are extra cells in the adult preanal ganglion (6 postembryonic motor neurons) and the arrangement of cells and processes is much less well organised. This loss of symmetry and order is already visible in the difference between the B and D reconsructions. In the B reconstruction the cells in the left/right pairs (PVPL/R and DA8/9) are nearly opposite one another, while in the D recosntruction there is a definite tilt to each pair and the whole preanal ganglion is becoming more linear. This is probably caused by a lateral constriction due to elongation of the embryo and an increase in the amount of space taken up by the muscle cells as they mature. In athe adult the symmetry and order present in the early embryo when processes first grow out is almost entirely lost.

Figure 3.8

The crossover of PVP processes in the preanal ganglion (from the C reconstruction). (a) to (d) are a posterior to anterior series, each being separated from the next by about 0.5 microns. (e) to (h) are tracings of (a) to (d) showing the positions of significant processes. The sequence of events is as follows: at the front of its cell body (a) PVPR sends a process across to the left side (b), in front of which DVC and PVPL cross from left to right (c, d). The PVPL process invariably crosses in front of the PVPR process. Scale bar is 1 micron.

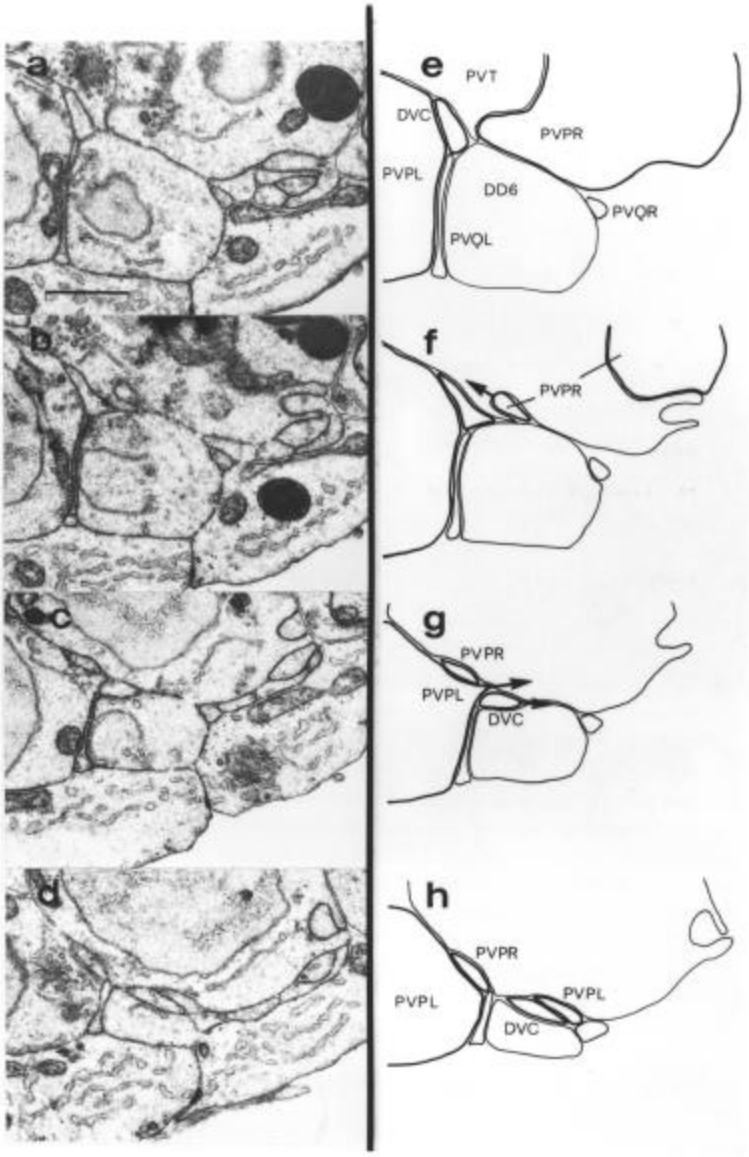


Figure 3.9

The place at the back of the preanal ganglion where the two lumbar commissures meet (from the C reconstruction). All the processes are labelled: QL/R are PVQL/R, AL/R are PHAL/R, BL/R are PHBL/R, CL/R are PVCL/R, and PVR, DA8 and DA9 are all the correct full names. The PHA, PHB and PVC processes all line up against each other, to some extent wrapping around their partners and thus increasing the area of contact (particularly the PHA and thus increasing the area of contact (particularly the PHA processes). In contrast the PVQ processes appear to have no mutual affinity. More anterior to this, the PHA, PHB and PVC processes all grow along the right side together, while the PVQ processes split, one growing on he left and one on the right. Scale bar is 1 micron.

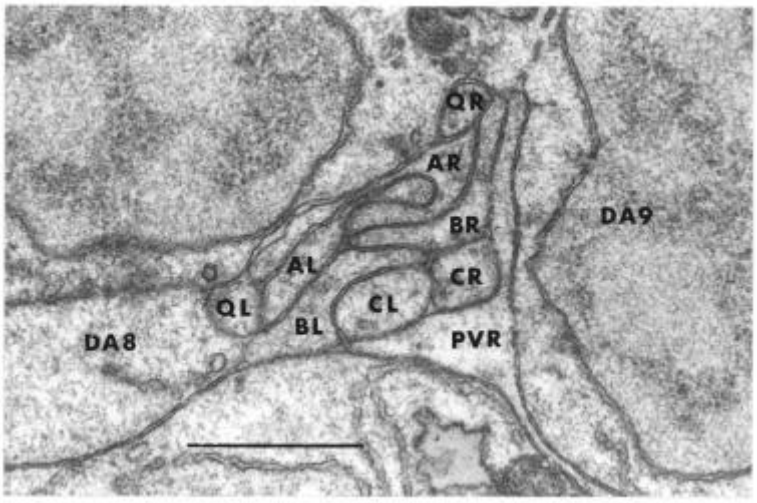
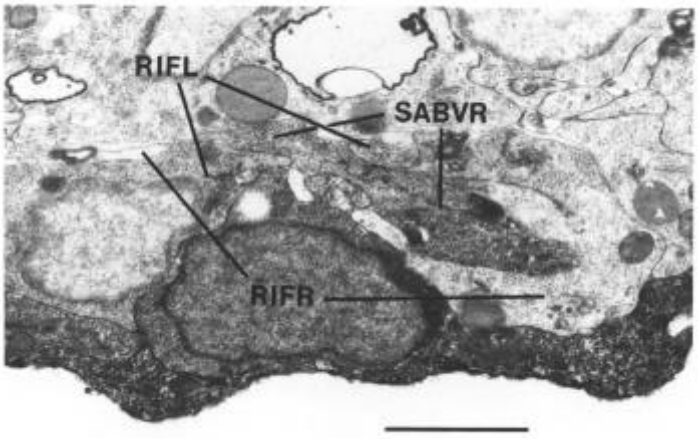


Figure 3.10

The decussation in the retrovesicular ganglion (from the B series). The RIF processes have both just crossed over posterior to this section, and we can see the back of the SABVR cell body and its process also thrusting across the midline. In contrast to the preanal ganglion decussation here processes cross on the surface of the neuropil. Scale bar is 1 micron.



A phenomenon equivalent to the PVP crossover is seen in the retrovesicular ganglion at the front of the ventral cord, which is once again more symmetrical in the embryo than in the adult. There are three bilateral pairs of neurons in the embryonic retrovesicular ganglion: RIGL/R, RIFL/R and SABVL/R, all of whose processes run forward. The RIF processes have grown out in the A and B reconstructions and once again they are seen to cross over by their cell bodies (figure 3.10). Following this observation, careful comparison of the positions of cell bodies in embryonic reconstructions and the embryonic lineage study (Sulston et al., 1983) with those in the adult reconstructions (White et al., 1986) confirmed that the RIF processes did indeed cross over in the adult reconstructions, and that the RIG and SABV processes do the same. The Processes from all three classes cross in nearly the same place in the adult reconstruction, by the SABV cell bodies. The SABV processes are just beginning to grow out and cross in the B reconstruction (figure 3.10), but the RIG processes have not yet grown out.

Therefore crossing over is seen in all the embryonic left/right pairs of interneurons in what might be termed the extended ventral cord, i.e. everything on the ventral hypodermal ridge between the excretory duct and the anus. During postembryonic development another interneuron pair, AVFL/R, is added in the retrovesicular ganglion, but their processes are not bilaterally symmetrical; they are bipolar, running back together down the right side of the ventral cord and forward also together to the left of the excretory duct and round the left side of the nerve ring.

The crossing over, or decussation, of processes to the opposite side of the body from the soma is a property of many nerve types in higher animals, and the cases observed here may provide extremely simple examples of the same event that are susceptible to experimental manipulation of both the cells involved and of their environment. Several cell ablation experiments were performed to investigate factors involved in the PVP cross over in the preanal ganglion (Chapter 4).

3.7 Growth cone insertions into other cells

An observation previously made in other animals is the insertion of thin processes from growth cones into other neuronal processes or target tissues that might be important for guidance (Bastiani and Goodman, 1983). The same phenomeno9n has been observed in the developing C. elegans nervous system, and there are two cases in particular where it is especially noticeable and correlates with possible guidance decision taking.

The first case is when the motor neuron commissures reach the dorsal midline. In one reconstruction (wild type but not on of A to D), a single commissure has just reached the dorsal hypodermal ridge, on emerging from underneath the left dorsal muscle quadrant. The growing tip of this commissure inserts two stubby finger-like processes about 0.1 – 0.2 microns in diameter and 0.4 – 0.8 microns long into the dorsal hypodermal ridge (figure 3.11). It is at this stage that the growth cone must turn through a right angle and grow along the ridge.

Figure 3.11

A finger from the DA6 commissural growth cone inserting into the dorsal hypodermal ridge as it comes out from under the left dorsal muscle quadrant. The particles to the left of the finger are not vesicles (determined by viewing at higher resolution).

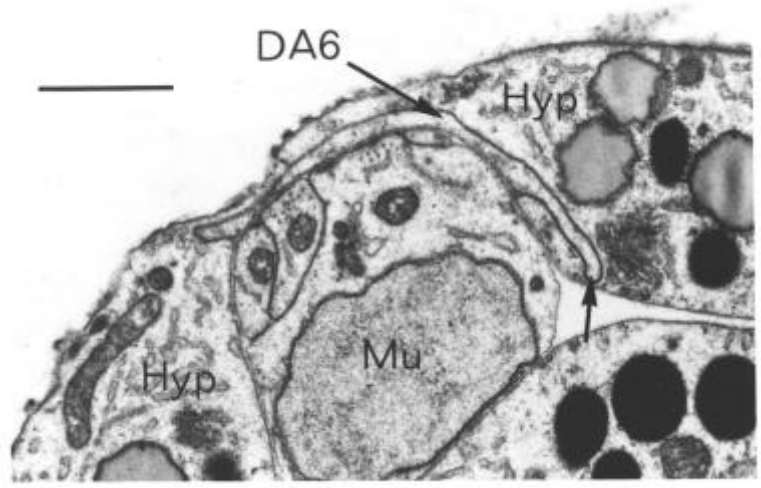
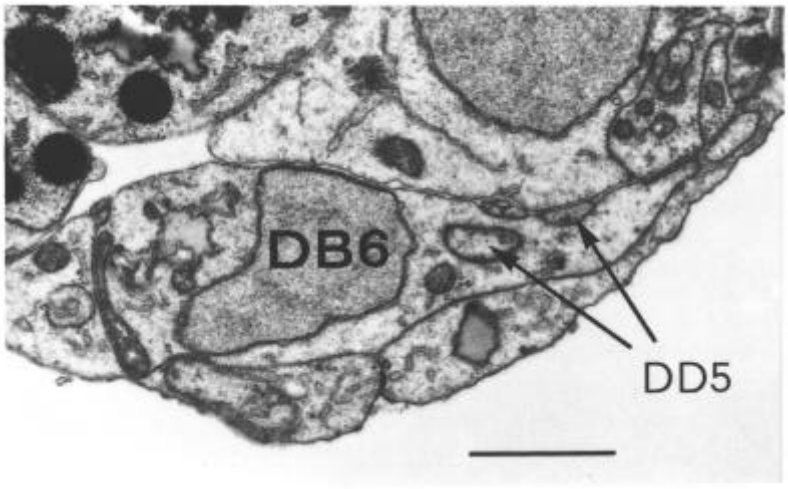


Figure 3.12

An insertion of an extension at the front of the DD5 ventral cord process into the cell body of DB6. DD5 stops growing forward along the cord at about the point that it reaches DB6. Both DD5 and DB6 send commissures out to the right around the same place. A part of the DB6 commissure can be seen. From the C construction. Scale B 1 micron.



The second case concerns what happens when DD processes meet DB cell bodies. In all cases that have been reconstructed the anterior tips of DD6 and DD5 insert into the bodies of DB7 and DB6 respectively. Insertion of DD4, DD3 and DD2 into DB5, DB4 and DB3 also takes place but less frequently and in a less pronounced fashion. An example of DD5 insertion into DB6 is shown in (figure 3.12). These DD insertions into DB cells reinforce the suggestion made above that DB cells might be involved in DD morphology. Where the insertions are most pronounced (DB6 and DB7), the DB commissures grow to the right next to the DD commissures.

There are occasional other insertions into the ventral hypodermis from growth cones of processes growing along the ventral cord (data not shown), but no particular pattern is discernible. Also I have seen no cytological correlates of the insertions, such as vesicles clustering around the insertion in the cell into which the insertion is made, which have been seen in the corresponding phenomenon in insects (Bastiani and Goodman, 1983).

CHAPTER 4 Laser Ablation Experiments

The previous chapter described a times series of embryonic reconstructions that allowed a picture to be drawn of the course of normal nerve outgrowth in and around the ventral nerve cord. This chapter describes a set of cell ablation experiments in which chosen cells were removed by ablating their parents with a focussed laser beam, using a system developed by J. G. White (Sulston and White, 1980). Chapter 2 describes the protocol used. The advantage of killing the parent cell is not only that it unequivocally prevents production of the cell of interest, but also that the remains of the dead cell are excluded from the embryo when the ventral hypodermis closes up, removing them also from any possible influence. The chapter is organised with a section for each set of experiments.

4.1 AVG

AVG is the first process to grow along the ventral cord (Chapter 3). It grows back along the right hand side, and later the adult cord is remarkably asymmetrical, with over 90% of its processes on the right hand side (there are 3 to 5 on the left, depending on anterior/posterior position, as against about 50 on the right). To what degree is AVG involved in establishing this asymmetry, and which cells, if any, depend directly on AVG to correctly determine the positioning of their processes? To answer these questions I removed AVG by ablating its parent cell, Abprpapppa. The sister of AVG, which is also removed by this ablation, is RIR, a ring interneuron whose cell body and processes all lie some distance anterior to those of AVG, and whose synaptic connections in the adult are not closely related to ventral cord circuitry (White et al., 1986).

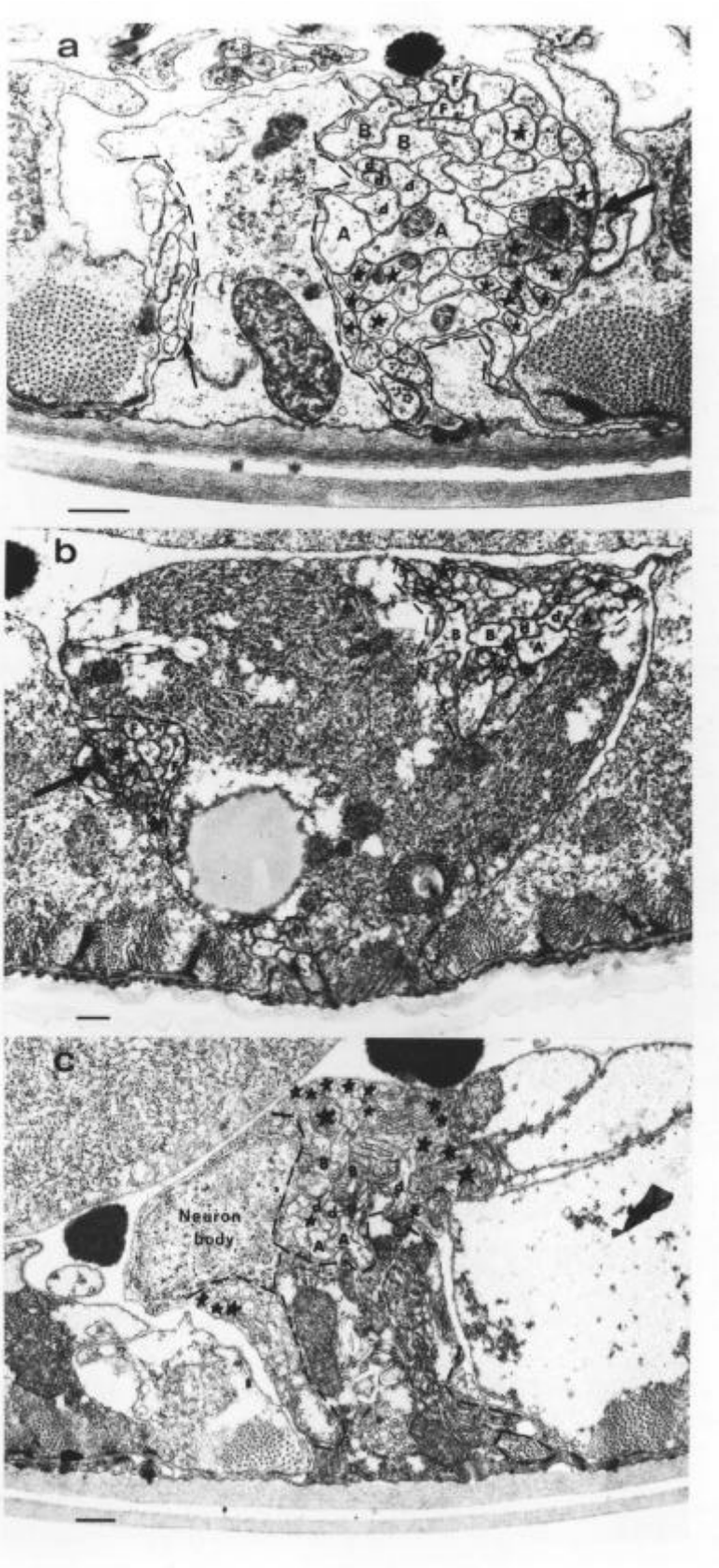
Nine experimental animals were permitted to develop and hatch in order to test whether motor control was affected, a crude test of ventral cord function. Six of these showed a very mild uncoordinated phenotype as newly hatched L1 larvae, in some cases only clearly visible when the worm was made to swim in water, in which case sections of their thrashing bodies looked stiffer than normal. Those worms that were uncoordinated as larvae were similarly uncoordinated as adults.

One of the adults showing an uncoordinated phenotype was sectioned through the front part of the ventral cord and the retrovesicular ganglion. Eileen Southgate reconstructed this series, since she and John White were interested in another question, concerning regulation in the circuitry.

The reconstruction confirmed that AVG was absent sine: (1) there were one too few cells in the RVG, and (ii) there were only two cells in the RVG with posteriorly directed processes that extended back through the complete series (AVFL and AVFR, they and AVG are the only ventral cord interneurons in the RVG). Almost all the cells in the RVG were identifiable, but there were some ambiguities concerning motor neuron identification both here and in the anterior ventral cord. This is because the spatial organisation of many nerve processes, especially those belonging to motor neurons, was abnormal. Cell body positions tended to be slightly displaced from normal, but the general order was preserved.

Figure 4.1

Adult ventral cords from (a) a normal animal, (b) an animal in which AVG had been removed, (c) an unc-3 mutant. The hypodermal ridge is raised in adults compared with embryos. Process bundles are outlined in dashes. There is inly one on each side of (a), but there are four bundles in each of (b), (c), with many more processes (labelled with stars) are on the right, but in (c), (c) there are also motor neurons on the left. There is a neuromuscular junction on the right in (a), and one from DB3 on the left, abnormally, in (b) (thick arrows). The motor circuitry interneuron processes are labelled A for AVAL/R, B for AVBL/R, and d for AVDL/R and AVEL/R, which are indistringuishable in this part of the cord. They are abnormally rotated, with motor neuron processes on the hypodermal side of them in (b). In (a), (b) the two AVF neurons are labelled F. They are on the left in (b), which is not normal. The thin arrow in (a) points to a hypodermal extension, rather than a neuronal process. Scale bars are 1 micron in each case.


a
b
c
Neuron body

The most striking aspect of the process bundle disorganisation can be seen in a random cross section of the cord behind the RVG (figure 4.1): instead of a large bundle of about 50 processes on the right and one of 4 or 5 on the left there are several smaller bundles, including two on the left hand side. There is no fixed arrangement of these small bundles as one progresses along the cord: processes occasionally transfer between bundles, and sometimes bundles fuse to form a larger grouping or split to form two smaller ones. However there are always significantly more processes on the right than on the left. The total number of processes appears normal (this comparison can only be made approximately, since the number of motor neuron processes present at any particular point is variable).

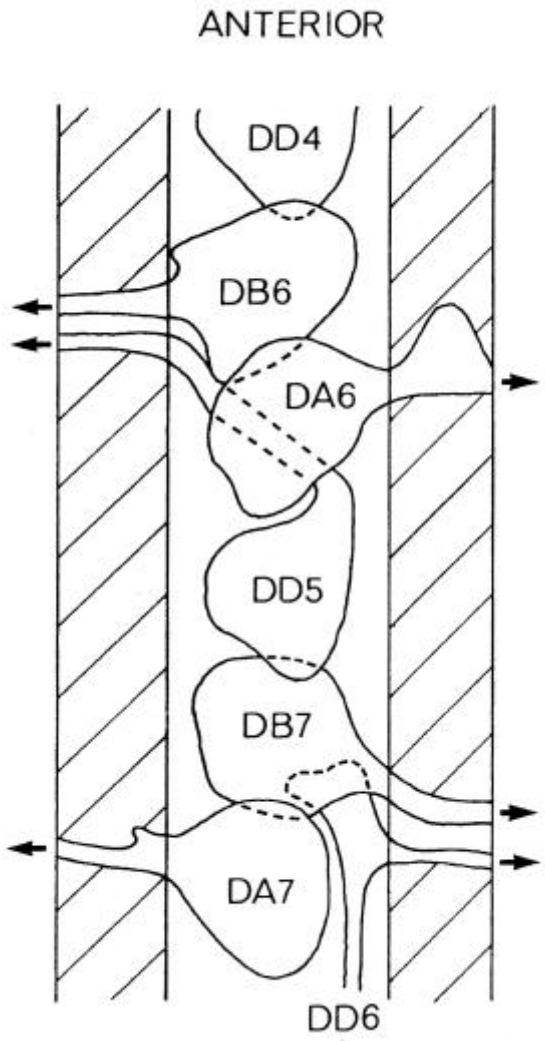
Several of the motor neurons are disrupted. These are motor neuron cell bodies associated with both sides of the cord (VD2 and DB3 are to the left), and also motor neuron processes on both sides (all of VD3 and parts of DB3, VD2 and VB2 processes are on the left). In addition DB3 is lacking a commissure, and DD2's commissure is severley misplaced or missing (the DD2 cell body is off the posterior end of the reconstruction, but its commissure should come out from the cord with that of DA2, some 150 sections anterior to the end of the series). Instead of a commissure, the DB3 process has a branch that crosses to the left hand side and shows some characteristics of the normal dorsal branch, in that first it runs backwards from the crossover point, and second it contains three neuromuscular junctions (figure 4.1). Normally all ventral cord neuromuscular activity is from the right hand cord, and all DB3's neuromuscula routput is from its backward dorsal branch.

Many of the interneuron processes cannot be identified because they make no synapses and their cell bodies are outside the bounds of the reconstruction. Among those that can are the two AVF neurons, which have cell bodies in the RVG, and which both send their processes back down the left hand cord in this recosntruction, as opposed to the right normally (figure 4.1). It is also possible to identify the 8 main motor circuitry interneurons by class (2 each of AVA and AVB, and the 4 VD and AVE neurons, figure 4.1), because of their patterns of synaptic output and gap junction formation with the motor neurons. In most cases where they are accessible to the motor neurons the normal synaptic connections are made. Normally these motor circuitry interneurons run in the central left side of the main (right hand) ventral cord, with a regular internal order: AVB's on top, AVA's on the bottom, and AVD's and AVE's loosely sandwiched in between. In this reconstruction they all keep together in the main right hand bundle, and amongst themselves they roughly preserve their normal order, but the whole group is often displaced from its regular position and orientation (figure 4.1). Thus it appears that, as a group, their internal organisation remains, but that they have lost the external cues that give the group as a whole a fixed position relative to other processes, some of which, indeed, are separated by being in other bundles.

In addition to this adult reconstruction I also looked at an embryo in which the parent of AVG had ablated, fixing it at the stage when the motor neuron commissures are normally just growing out from the ventral cord (around 500 minutes). In this case I recosntructed the back part of the ventral cord and also the preanal ganglion (PAG).

Figure 4.2

A schematic illustration of the reconstruction of the ventral cord from the embryo in which AVG had been removed. The illustration has the same form as the central region of the diagrams in figure 3.4. There is no continuous interneuron process in the cord, indicating that AVG was both correctly identified and correctly removed. The positions where commissures are leaving the ventral cord are indicated by arrows. The DD6, DA7 and DB7 neurons look normal, but the DD5 ventral cord process switches from right to left, and all the commissures from DD5, DB6 and DA6 are leaving the cord from the wrong side (compare with figure 3.5).

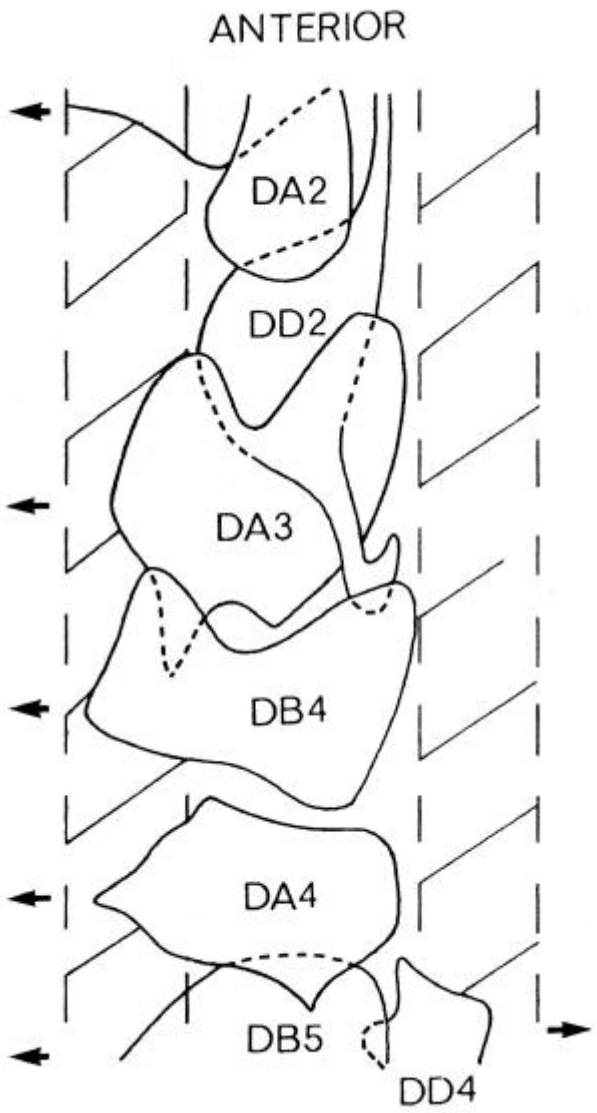


Although the AVG process was clearly missing, there was no other alteration to the organisation of the PAG and the early posterior interneurons that grow forward along the cord from it (the PVP's, PVQ's DVA and DVC), every process following its normal trajectory. However, as in the adult, the ventral cord was disorganised. In this case the most posterior three motor neurons (DD6, DA7 and DB7) looked normal, but DD5's anterior process in the ventral cord, although leaving the cell body on the right side as normal, switched sides from right to left and sent out its commissure on the left. DB6, whose commissure usually goes to the right with that of DD5, sent it s commissure to the left also. DA6's commissure, which is usually on the left, went to the right instead (figure 4.2).

In summary, it seems that, in the absence of AVG, the motor neurons in the ventral cord are variably disorganised in terms of process growth. Some examples look normal, while others send processes on the wrong side of the cord, or fail to form commissures, etc. This applies to both embryonic and postembryonic motor neuron classes, although the postembryonic neurons look much less affected. A second, possibly related, consequence of AVG removal is a splitting of the ventral cord into several bundles, some of which are on the left hand side of the cord. Some interneurons are also split off into these alternative bundles, but in the adult example that was reconstructed the main motor circuitry interneurons look fairly normal. Many of the motor neurons are able to make correct synaptic contact both with their innervating interneurons and with muscle. This probably explains why the observed behavioural phenotype of removing AVG was only minor uncoordination, when a difference was noticeable at all.

Figure 4.3

A schematic illustration of the same form as figure 4.3 of the ventral cord reconstruction of the embryo in which the DD3/5 parent had been ablated. DD3 is missing (see figures 3.4, 3.5 for the comparable region in normal animals). The DD2 process has grown slightly back but has not grown beyond the front of DB4, while DD4 has not grown forward beyond DB5. There is thus a gap of an entire cell between the DD processes. However since this is a young embryo (approx. 175 minutes) one cannot say if the gap will be filled later.



4.2 DD3/5

In the wild type embryo, after the growth of AVG back along the right hand cord, the DD motor neurons grow out processes in the ventral cord next to AVG. These processes grow forward until they meet or almost meet the next DD in the sequence (in the various wild type embryonic series they were often separated by a gap of about 1 micron, Chapter 3). Then commissures grow out to the right from near their front tips. By removing a DD cell and examining whether the processes would extend further along the cord to fill in the gap, I was able to test whether process growth is terminated solely by contact, and if so, whether the position of the commissure also changed. Does it always leave from the front of the ventral cord process?

In fact it was easy to remove DD3 and DD5 together, creating two gaps in a single animal, since they are sisters. As with AVG it was checked that their dead parent (Abplppappp) was excluded from the embryo after laser ablation, and that the relevant DD cell was missing in the subsequent reconstruction.

I have reconstructed the front part of one embryo from the seven that were fixed and sectioned. In this animal, which is the same age as the wild type A series (around 480 minutes), the gap left by removing DD3 has not been filled by DD4. Instead the DD4 process stops at the front of DB5, only very slightly further forward, if at all, than normal (figure 4.3). There is a short posterior extension from DD2, which is not unusual (figure 3.5), but this stops around DB4, leaving a gap with no DD processes along the whole extent of the DA5 cell body.

4.3 PVP and PVQ

The third set of ablation experiments concern the four PVP and PVQ neurons. To summarise briefly: these form the first group of interneurons to grow forward from the back of the ventral cord. The PVQ cell bodies are in the lumbar ganglia; they send processes down the lumbar commissures through the preanal ganglion (PAG), wheter they pick up contact with the PVP processes, and then forward along the ventral cord, one on each side. The PVP bodies lie in the PAG; their processes leave their bodies heading towards the midline, cross over, and then grow forward on the opposite side of the ventral cord. So PVPR runs with PVQL on the left hand side, while PVPL runs with PVQR on the right. The growing tips of each PVP/PVQ pair, either on the left or right, are always very close (within 0.5 microns). The only processes apart from PVPR and PVQL to grow down the left side of the ventral cord in the embryo are AVKR and RMEV, both of which grow back from the front, RMEV stopping part back. In the adult the left vulval motor neuron HSNL also grows forward on the left side of the cord from the vulva half way along the body. In the oldest wild type embryonic series (D series) only one of AVKR and RMEV was seen growing back in the anterior cord (it is not knows which one), and this was only after PVPR and PVQL had reached the front.

The questions that can therefore be asked concerning possible organising roles for PVP and PVQ processes are:

(1) Are one or both of a PVP/PVQ pair needed for the other to grow along the cord?
(2) Are PVPR and PVQL needed for growth of the other processes down the left cord?
(3) Are the PVQ processes, or the other PVP cell, necessary for crossing over of the PVP processes in the preanal ganglion?
(4) Is the growth of a PVQ process down a lumbar commissure necessary for other lumbar ganglion cell processes on the same side to reach the preanal ganglion?

Experiments were carried out in which PVPR, PVPL and PVQL were independently removed. As with AVG and DD3/5, a block of fixed experimental embryos was completely sectioned for each of the sets of ablations (7 embryos for PVPR, 5 for PVPL, and 5 for PVQL). In addition 5 adult PVPR experimental animals were cut at 3 random sites in the posterior half of the cord to help answer the second question. Again the parent cell was ablated in each case and only embryos that excluded the dead cell on closure of the hypodermis were considered further. In the case of PVQL the parent is Abplapppa and the sister cell normally undergoes programmed cell death and engulfment soon after being born; therefore there is no additional cell missing in experimental animals at the time of process outgrowth. The sisters of PVPL and PVPR (parents Abplppppa and Abprpppppa) are left and right ventral rectal epithelial cells (repVR and repVL). Together with repD these form a ring of rectal cells that lie above and forward of the PAG; they are sufficiently distant to be unlikely to be important in nerve process guidance in the PAG. In the reconstructions of PVPR and PVPL experimental embryos the correct repV cell was seen to be missing; in each case the rectum had resealed by extension forward of one of the posterior neighbouring pair of rectal epithelial cells (K and K') rather than circumferential filling in by the other rep cells.

I will consider the four questions posed in above in turn:

1. It is easier to observe the presence or absence of PVP/Q processes on the left side of the cord (PVPR and PVQL) than on the right side, since during the stages under consideration those are the only nerve processes on the left side. I first considered the embryos in which PVPR had been removed. Of the four embroyos in which it was possible to identify a region of the ventral cord anterior to PAG where the PVPL and PVQR processes were visible on the right side of the cord, none had any processes on the left side (figure 4.4). The PAG and posterior cord of one of these embryos was reconstructed; in this case PVQL grew forward as normal through left side of the PAG past the point where it would normally have picked up contact with PVPR and then, at the front of DD6, which was displaced slightly anterior to its normal position, it switched sides from left to right and ran forward for a short distance with PVPL and PVQR (figure 4.5). Its anterior tip, however, was more than 2.5 microns posterior to the tips of PVPL and PVQR (which were off the anterior end of the series, 54 sections from the PVQL tip). Therefore it appears that PVPR is necessary for growth of PVQL along the left side of the cord, and that in its absence PVQL is retarded somewhat, but grows

Figure 4.4

The ventral cords of animals in which a PVP or PVQ cell has been removed. In each case an arrow points to the left hand cord. For embryonic cords compare with figure 3.7 (b) for a control, and for adult cords compare with figure 4.1 (a). Two examples of each experiment are shown. (a), (b) Embryonic cords after removal of PVPR; there are no processes on the left side, but sufficiently many on the right to show that PVQL would normally have been visible in these situations. (c), (d) Embryonic cords after removal of PVQL; there is one process on the left side. (e), (f) Embryonic cords after removal of PVPQ; 2 processes on the left. (g), (h), Posterior adult cords after PVPR removal; there are still no processes on the left side in the posterior half of the animal. Scales bars in (a) for (a) to (f), and in (g) for (g), (h), 1 micron in each case.

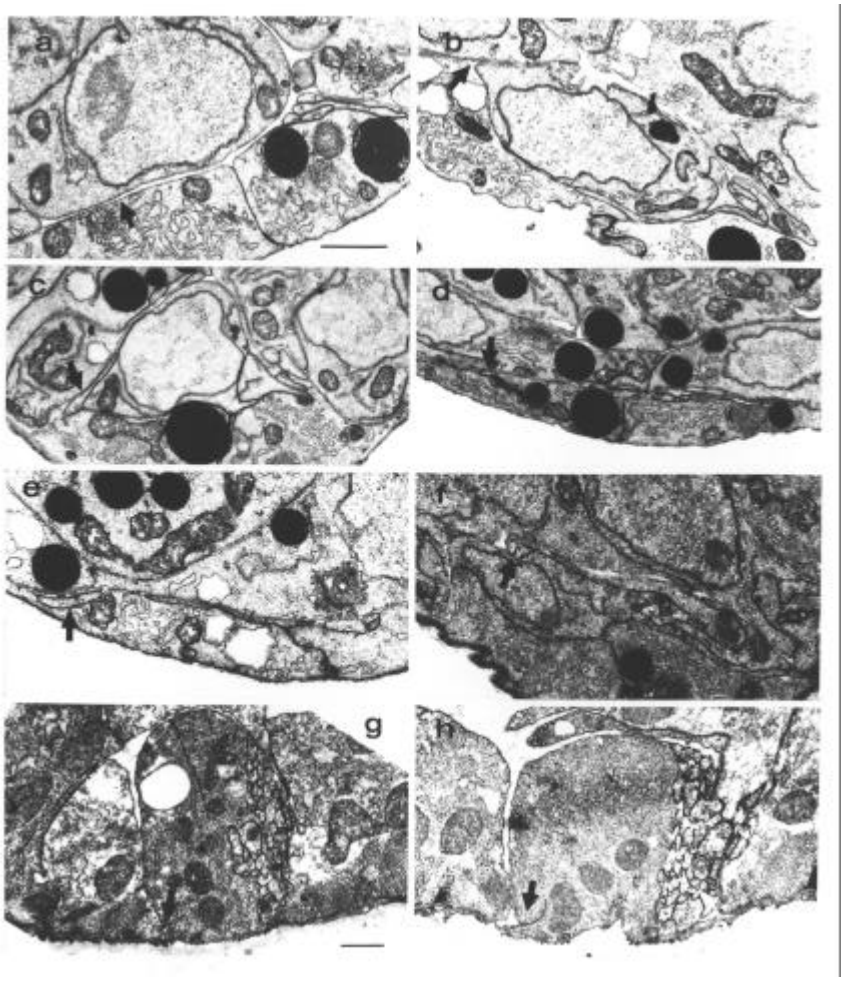
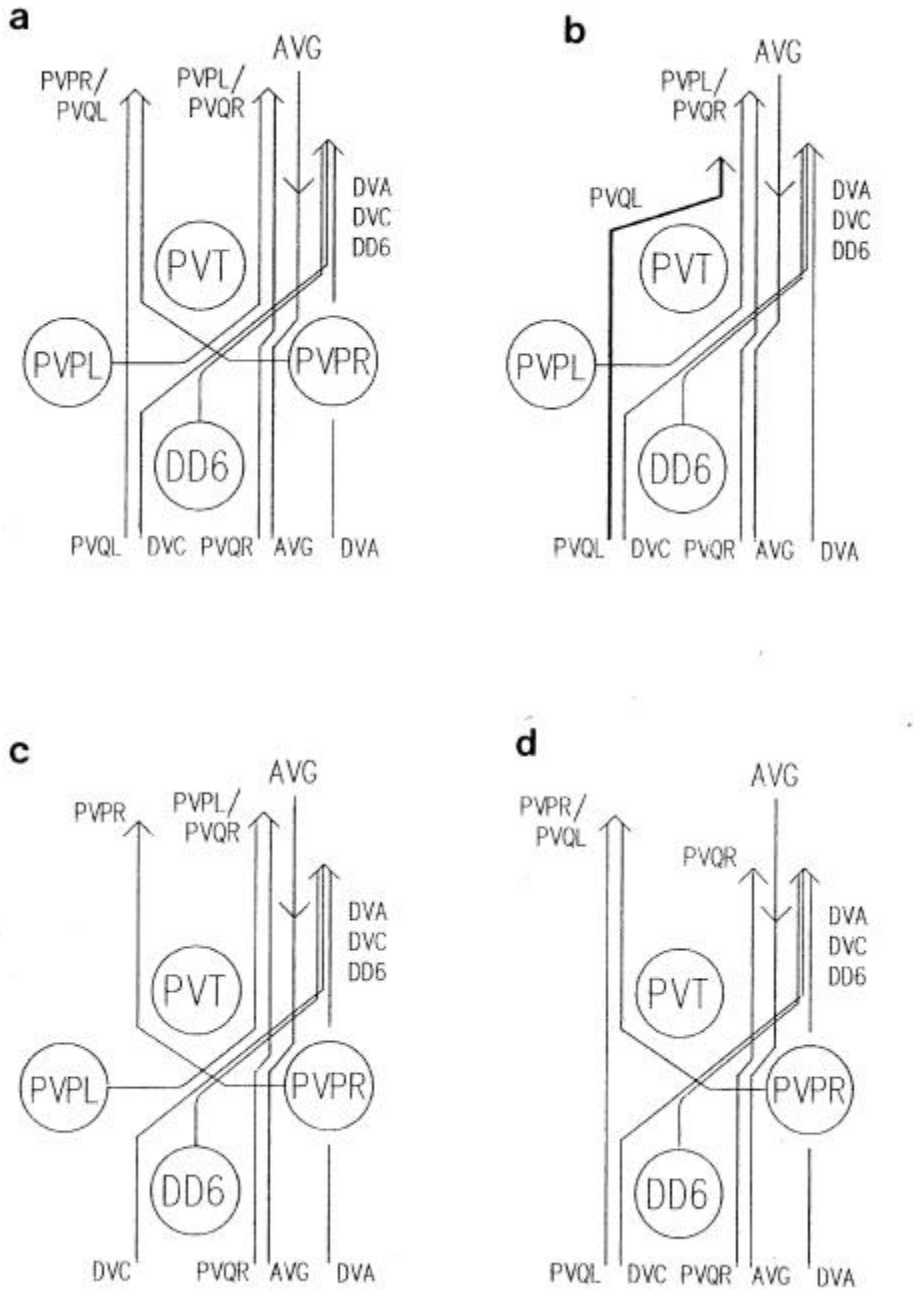


Figure 4.5

Schematic diagrams of the front of the preanal ganglion in normal embryos and ones in which a PVP or PVQ cell has been removed, based on complete reconstructions of the preanal ganglia in these animals. The ages of the reconstruction varied but they were all around 500 minutes. (a) normal, (b) after PVPR removal; (c) after PVQL removal, (d) after PVPL removal. Neither of the last two experiments caused any effect on other processes in this region.



Forward along the established path of PVPL and PVQR.

However, when PVPRL was removed, in each of the three embryos for which the same region anterior to the PAG was identified, a solitary process was seen on the left side (figure 4.4). The PAG region of two of these embryos was reconstructed; in each case PVQL was missing and PVPR grew as normal along the left side of the cord. In the younger of the series (about 470 minutes) it stopped about 1.7 microns (34 sections) posteriorly to the point where the PVPL/PVQR processes on the right stopped; the older series did not contain the anterior tips of any of the processes. Therefore, in contrast to PVQL, it appears that PVPR is competent to grow by itself to the left side of the cord.

Finally, I considered the consequences of removing PVPL, the bilaterally homologous experiment to that of removing PVPR. In this case PVQR stayed on the right side, rather than crossing to join PVPR and PVQL (two animals: one was reconstructed completely and one animal had two long processes but no third process in the left cord, even near the PAG, figures 4.4, 4.5). However it must be remembered that, in the absence of PVQL when PVQR was removed, since event though the PVPL process is absent on the right side of the cord there are still AVG and DD processes there, whilst when PVPR was removed there was nothing on the left side. The corresponding reciprocal experiment of removing PVQR was not attempted, since it seemed unlikely that there would be an effect in the more populated right hand side of the cord, where there had been none when PVQL was removed on the left side.

In summary, PVPR is necessary for growth of PVQL on the left side of the cord. In its absence PVQL grows on the right side. However PVPL is not necessary for PVQR to grow on the right side, presumably because PVQR can follow the preexisting AVG and DD processes there. In contrast, the removal of PVQL has no significant effect on PVPR.

2. To answer the question of whether PVPR and PVQL are needed for growth of other processes down the left cord, I ablated the patent of PVPR in six animals and looked at the left side of the adult, rather than the embryonic, so that all processes would have had the opportunity to complete growth. The fixed animals were cut at three random sites in the posterior half of the body, where AVKR is normally present on the left side together with PVPR and PVQL. One of the five animals was rejected because of poor fixation. None of the remaining five had any consistent process showing on the left side of the cord (figure 4.4). In several cases there appeared to be a process visible at one of the sites. This was probably a fold or finger of hypodermis; such hypodermal extensions are common around the adult ventral cord (for example there are two in the section from the control reconstruction in figure 4.1). Therefore both PVPL, as expected from the previous result, and AVKR were missing from the left side in all five cases, implying that the PVPR/PVQL pair is necessary for AVKR to grow down the left hand ventral cord. It is not possible to ascertain whether AVKR had switched to the right side of the cord in the experimental animals, or had failed to grow back at all, without reconstruction of the complete nerve ring.

3. The removal of neither a PVP nor a PVQ cell affected the crossing over the opposite side of the PVP processes when they leave their cell bodies in the centre of the preanal ganglion (figure 4.5). The embryonic PAG reconstructions after removing eithe rPVPR or PVPL show that the remaining PVP cell sent its process across the midline in exactly the same location as usual. This rules out an explanation of the chiasm being caused by mutual attraction of PVP processes. The reconstructions after PVQL parent ablations also showed no change.

4. However there did appear to be an effect on the left lumbar commissure when PVQL was removed. In neither of the two experimental animals that were reconstructed did any other processes come down the left lumbar commissure into the PAG, although in each case the DA8 process had already grown dorsally via the same path out of the PAG. One of the reconstructed animals was young enough that the following processes on the right side had only just passed through the commissure; however in the second four of the right hand lumbar processes other than PVQR had grown half way through the PAG (figure 4.6).

4.4 DVC

One further ablation experiment was tried in an attempt to understand why the PVP processes cross over in the preanal ganglion. As described in Chapter 3, at the point where the crossover takes place the process of DVC spreads out into a thin sheet that separates the cell bodies of PVT and DD6; the PVP processes actually cross between PVT and the DVC sheet. It therefore seemed possible that DVC was essential for the crossover. Therefore five embryos were fixed in which the parent of DVC had been ablated, two of which were later reconstructed in the region of the PAG.

The sister of DVC (parent Caapa) normally undergoes programmed cell death before differentiating and so is unlikely to be required for the development of the PAG. Since the DVC parent lies underneath the tail hypodermal cells at the time of its ablation it is not excluded from the embryo as in all other cases. However I checked that condensed nuclear debris was visible about 20 minutes after the ablations, and the absence of the DVC cell body was confirmed in the two reconstructed embryos.

No effect was seen on the PVP crossover in either reconstruction. Instead PVQR and PVQL flattened out somewhat and met in the centre, partially replacing DVC's role in separating PVT and the crossing over PVP's from DD6 (figure 4.7). This possibly suggests that PVQL, PVQR and DD6 have somewhat interchangeable or redundant functions at this point in organising the PAG. However the multiple ablations which might test this suggestions have not yet been attempted.

Figure 4.6

The complete posterior nervous system in 500 minute embryos, shown as in figure 1.3. (a) Normal, (b) after PVQL removal. Although there was no effect on the PVP processes in (b) (see figure 4.4), the other processes from the left lumbar ganglion have failed to grow down the left lumbar commissure. However the DA8 process, which also grows in the lumbar commissure, but in the opposite direction looks normal.

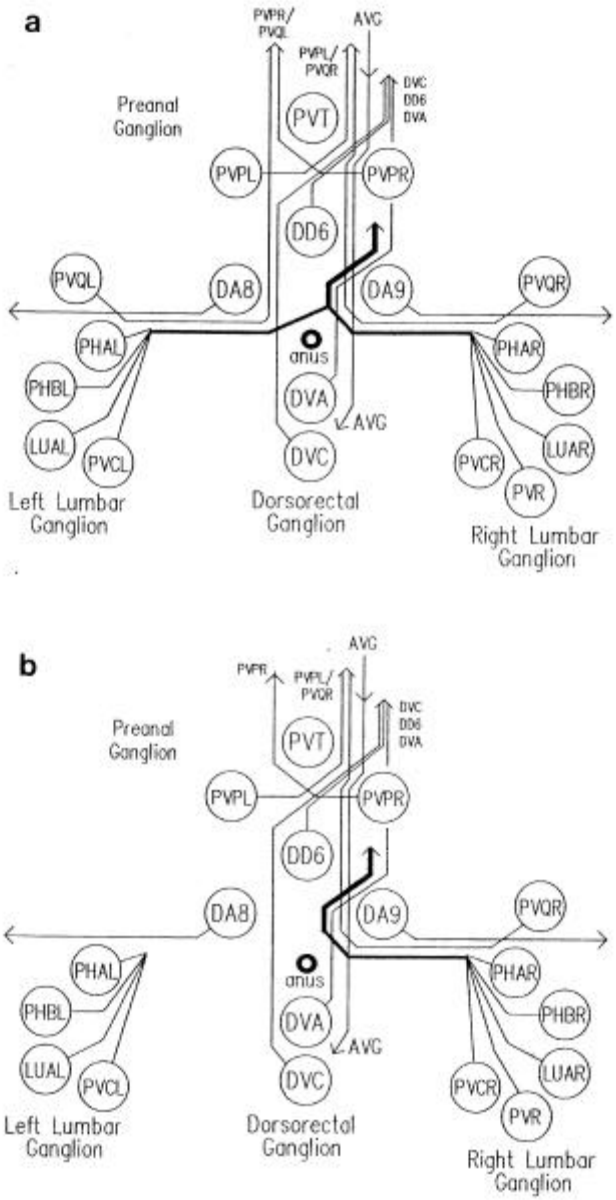
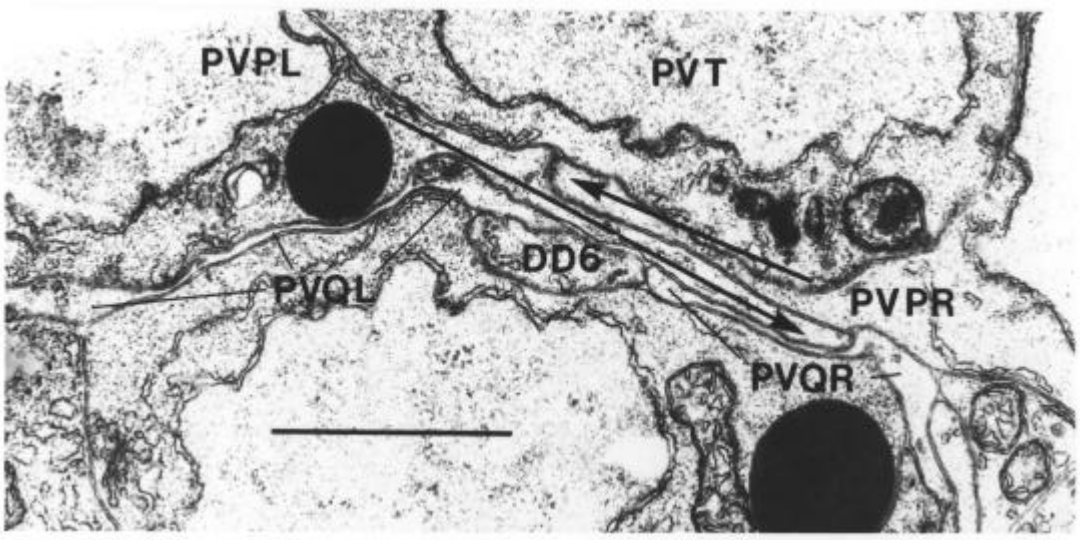


Figure 4.7

The site of PVP process crossover in an embryo in which DVC has been removed. The PVP processes still cross over (thick arrows). However the situation is a little abnormal because PVQR flattens out much more than normal (compare with figure 3.8). Scale bar is 1 micron.



CHAPTER 5 Discussion

The last two chapters have described the results of a number of electron microscope reconstructions of the developing ventral nervous system in both normal and experimental C. elegans embryos. In a short space of around an hour the first nerve process grows back along the ventral cord from the front, the motor neurons in the ventral cord grow commissures around the body of the animal to form the dorsal cord, and a number of additional processes grow forward from the preanal ganglion at the back of the animal. The short time taken in laying down the skeleton of the ventral nerve cord and preanal ganglion reflects the rapid development of C. elegans embryos (13 hours total). The small number of cells present, and the simple morphologies of the nerve cells, allow precise suggestions to be made about the roles of individual cells during process outgrowth. Several possible intercellular interactions were investigated by killing the parents of specific cells with a focussed laser beam. Before discussing the pattern of process outgrowth in the ventral nervous system, and how it might be controlled, I will first consider the reliability of the observations on which the work is based.

5.1 Reliability

The approach of reconstruction from serial electron micrographs precludes the examination of a large number of individual animals, either in the wild type time series or in any particular experiment. It is reasonable to ask whether reliable conclusions can be drawn from the necessarily small number of reconstructed animals that have been presented: there are two possible sources of error or variation: experimental "noise" created by variability in the observational and experimental techniques, and natural variability of the phenomena themselves.

As far as the determination of process disposition is concerned the technique is very reliable; each individual reconstruction provides a large amount of information at a very fine level of detail, so that essentially all the nerve processes present can be positively identified and a complete picture of the relevant parts of each neuron determined (the exceptions are discussed in Chapter 2). The technique of laser ablation of individual identified cells is also very specific. It is unlikely that the killed cell has any residual influence, because, except for the DVC parent ablation, when no subsequent change was seen in any other cells anyway, the dead cell was observed to be excluded from the embryo when the hypodermis closed up (figure 2.2). It is in principle possible to damage neighbouring cells at the time of ablation, and in fact one of the embryos sectioned in the DD3/DD5 set showed signs of general morphological disorganisation, presumably due to such damage. However in all the cases discussed, except the AVG experiments, any changes that were observed were confined to a small number of neurons normally associated with the particular missing cell. Although control experiments in which random neighbouring cells were ablated were not performed, altogether six different cells all near together on the ventral surface of the 270 minute embryo (figure 2.1) were ablated without there being any overlap in the observed consequences.

As regards intrinsic variation, all the reconstructions are consistent with a fixed time sequence of normal axonal outgrowth. When a change in this pattern was seen in laser ablation experiments then, again excepting the AVG experiments, it was clean and restricted in its extent, was generally observed in at least two cases, and was consistent,

never being seen in one case but not another. The situation with respect to the removal of AVG appeared to show variability and is discussed more fully in the next section. However, taking all the results together and in conjunction with the known fixed adult anatomy, there is sufficient evidence to indicate that the developing C. elegans nervous system is simple and reproducible enough for the techniques used here to provide an accurate picture of events.

The high level of reproducibility and the generally restricted, fixed effect of removal of individual cells are typical of C. elegans development and anatomy. The cell lineage and the disposition of somatic cells at all stages of development are known to be nearly invariant (Sulston and Horvitz, 1979, Kimble and Hirsh, 1979, Sulston et al., 1983) the final antomy is equally stereotyped (White et al., 1986). Although a number of cases of adjustment in cell lineage after individual cell ablations are known (e.g. Sulston and White, 1980, Sulston et al., 1983) they are the exception rather than the rule, and in no case do they result in complete regulation back to the native form. All the results of ablation experiments performed here are consistent with only the daughters of the ablated cell being missing, and with there being no change of identity of any other cell. In addition they confirm the relevant cell assignments in the embryonic lineage, since in each case only the expected cell or cells was or were missing.

5.2 The asymmetry of the ventral cord

One of the striking features of the C. elegans ventral nervous system is the almost, but not quite, complete asymmetry of the ventral nervous cord, which has around 55 neurons on the right side and only 4 or so on the left. If all the processes were together on the right hand side then it could be regarded as a single fused nerve that was displaced to one side for steric reasons, but since a small number of left/right pairs of processes are arranged symmetrically (PVQ, PVP, AVK and in the adult, HSN) the question arises of why not all the others? In fact the arrangement is essentially symmetrical anterior to the RVG; the cord splits into two to pass the excretory duct on both sides, with each bilateral pair of processes being split so there is one member on each side, an stays symmetrical throughout the ventral ganglion and into the bottom of the nerve ring.

Most animals with a symmetrical body plan have a symmetrical ventral nervous system, often consisting of a chain of ganglia linked by paired nerves, which are sometimes fused but clearly retain their symmetrical character. There are in fact some nematodes that have symmetrical paired ventral cords (Martini, 1916). Chitwood and Chitwood (1974), in discussing the differences amongst nematode species, state (p. 162):

Differences in the central nervous system lie chiefly in the degree of subdivision of the lateral ganglia, the form of the ventral ganglia, and the degree of fusion of the ventral nerves.

They go on to state that in many species both around the RVG and for some distance anterior to the PAG there are symmetrical paired nerves, though in most cases these are fused for the main part of the length of the body. They continue (p. 163):

The apparent doubleness in both anterior and posterior ends of the ventral nerve caused Meissner and many later authors to conclude that the entire nerve was at one time double. … (we) subscribe to the primitive double ventral nerve hypothesis.

Several observations that have been made in this study are relevant to the origin of cord asymmetry. Perhaps I should start with AVG. AVG is a unique neuron with its body in the RVG, it is the first neuron to send a process out along the ventral cord, and it sends it along the right hand side. When AVG was removed by ablating its parent the cord was seen to be disrupted in two ways.

First, as seen most clearly in the embryonic AVG reconstruction, the organisation of the embryonic ventral cord motor neurons was disturbed. In particular a DD process was seen to switch across to the left side of the cord and send its commissure round to the left rather than the right, and the DA and DB cells near this point also sent their commissures round the opposite side to normal. The switch of the DD process to the left cord confirms that AVG must normally grow out before the DD ventral cord processes. In the adult AVG reconstruction the DB3 and DD2 cells show abnormal process organisation. These effects would seem to be a direct consequence of the absence of AVG, because the outgrowth of DD processes and motor neuron commissures follow directly after the outgrowth of AVG. The postembryonic motor neurons do not seem so badly affected as the embryonic neurons, although the VD3 process in the adult reconstruction is switched from being on the right side to the left.

The second effect of removal of AVG, seen in the adult reconstruction, is a general disorganisation of the cord in which instead of a large ordered bundle on the right side and a very small one on the left there are several intermediate sized bundles at various positions on the left and right sides (figure 4.1). This indicates that AVG is ultimately necessary for correct organisation of the interneurons as well as motor neurons, whose disarray appears earlier. However AVG does not seem to be necessary for outgrowth of processes, since the total number of processes in a cross section of the experimental adult cord is within the expected range, and all the fully reconstructed cells send out processes in the correct direction, if not on the correct side.

It is also clear that AVG is not the sole determining influence for the left/right organisation of the ventral cord, because in the embryonic experimental reconstruction all the early interneurons from the back were growing forward correctly (PVQR, PVPL, DVA and DVC on the right, and PVQL and PVPR on the left). Also in the adult AVG reconstruction the majority of processes was at all times on the right, including the motor control interneurons (AVAL/R, AVBL/R, AVDL/R and AVEL/R). In the only positively identified case of interneurons growing on the wrong side, both AVF's were seen to grow on the left (they are normally both on the right, figure 4.1).

The fact that removal of AVG leads to no major behavioural defect suggests that it has no critical function of its own. In the adult reconstruction, although it is a fairly large cell, it has been seen to make very few connections to other neurons, the only consistent ones being large gap junctions to the two RIF interneurons and a small amount of synaptic input from the PHA phasmid neurons (probably chemosensory) (White et al., 1986). It has been postulated to be a sensory receptor itself on the basis of its adult extension beyond the dorsorectal ganglion into the tail, although no ultrastructural specialisation is seen there (ibid.). One might instead speculate that its main function is developmental. If one considers that it is just as important for a nervous system to be able to build itself as to function correctly in the end, it makes sense that there be selective pressure for neurons important in development even if they serve little or no purpose in the final circuitry. Another candidate for such a cell in the C. elegans nervous system is PVT. This is a large cell demarcating the front of the preanal ganglion and forming the most anterior link between the rectal epithelium and the ventral ectoderm, which has no observed synaptic output and only a couple of possible inputs. However no experiments have been performed to test the suggestion that it too may be primarily involved in developmental organisation. Of course one should beware of suggesting that every neuron must have a major function; it is quite likely that there are also redundant cells present that are not particularly important at any time.

The disarray seen in the ventral cord of the adult AVG reconstruction is very reminiscent of that seen in a reconstruction of a mutant in the gene unc-3 (e151) (figure 4.1, J G White, E Southgate and N Thomson, unpublished results). In that case too there were several subbundles, looking very similar to those of the AVG reconstruction; the majority of processes were on the right, including the identifidable cluster of major motor interneurons which again retained their internal organisation, but not their relative position in the bundle. The defect appears to be restricted to the ventral cord since the nerve ring was correctly organised according to several electron mircoscopic criteria, bu the phenotype of unc-3 mutants is much more severe than that after ablation of the AVG parent, and indeed in the reconstruction of the mutant it appeared that some postembryonic motor neurons might be missing or not properly made.

There are two other uncoordianted genes for which mutants show relevant defects. The DD and VD commissures can be visualised by immunocytochemical staining with antibodies against the neurotransmitter GABA (helping to confirm that the DD and VD classes are probably GABAergic and inhibitory); they normally all grow to the right. However in mutants for unc-71(e451) and unc-73(e936) a significant proportion of the commissures grow round the left side of the animal (25% and 35% respectively; S McIntire, pers. Comm.). The ventral cord is also seen to be disorganised, in that in some places in the cord the VD and DD processes, which normally run so close together that they are inseparable by light microscopy, are clearly separated. It would be interesting to see what happens in early ventral cord development, particularly to AVG, in all of these mutants.

The suggestion derived from the reconstructions of the adult AVG animal and the unc-3 mutant that left/right pairs of processes tend to stick together may be significant. When the lumbar neuronal processes meet in the PAG at the bottom of the lumbar commissures they “zip” together, each process in contact with its homologue, except for PVQL/R

which remain apart (figure 3.9). Eventually PVQL/R end up on separate sides of the cord, while the others all stay together on the right side. This affinity of a process for its opposite homologue provides a simple mechanism to ensure that processes stay together. Then perhaps only a slight bias is needed to send the pair to one side rather than the other. The experiments in which the parents of PVP cells were ablated reveal an underlying preference for the right side in at least one case. When PVPR was removed PVQL crossed to the right side rather than grew along the left side of the cord by itself, but when PVPL was removed PVQR still grew along the right side. It may be that the presence of preexisting fibres on the right rather than the left was the determining factor in this particular case, but after AVG, the DD axons, DVA and DVC have grown out on the right side, which might prove sufficient to continue to attract later arrivals.

To return to Meissner's suggestion the the primitive ventral nerve was double, it may be worth discussing the advantages and disadvantages of a fused cord over paired nerves. The obvious disadvantage of a single cord like that of C. elegans is the loss of possible left/right control over body movement. Although there are four bands of muscle in C. elegans both ventral quadrants receive the same input from the right hand ventral cord, as do both dorsal quadrants from the single dorsal cord. Therefore the body of the animal moves only in the dorsal/ventral plane, although the head can and does move freely in all directions. However there are extra cross connecting motor neuron and interneuronal classes in the head, and it is likely that in order to obtain reasonable left/right coordination, something similar would be needed in the body. There is no sign of this, even in vestigial form. On the other hand, if, as seems likely, the putative primitive twin-nerved ancestor did not have the capability for left/right body control (I have found no mention of any nematode that does), then there is a strong case for bringing the motor circuitry elements together in one nerve. First it allows an effective halving of the number of motor neurons; with the system as it is in C. elegans there is only one active motor neuron of each class at each cross section of the body. Second it removes at source any loss of synchrony between wave generation on the left and right sides of the body. Third it provides back up in an extremely important part of the animal's nervous system by having twofold redundancy of each motor circuitry driving interneuron. However there is no obvious reason why the interneurons not involved in the motor circuitry should join together or not, since they serve no function in the cord but merely use it as a route from one end of the cord to the other. Indeed this view is supported by the fact that a minority of three apparently unrelated classes (AVK, PVP and PVQ) are still bilateral in C. elegans.

In conclusion I would like to speculate that the primitive nematode ventral cord was double and symmetric, and that the selection pressure for the currently more common asymmetric cord came from the motor circuitry. It appears that AVG plays a critical role in organising the left/right asymmetry of the motor neurons. An important factor for the interneurons appears to be the mutual affinity of left/right pairs (and of the motor circuitry interneuron classes for each other, since they preserve their approximate relative structure under perturbation by AVG parent ablation and unc-3 mutation). The interneuron pairs of groups may then tend to go to the right side either directly or under the influence of AVG, the motor neurons, or other previously determined processes, such as that of DVA. If this picture is correct then the fact that so many left/right pairs of non-motor circuitry interneurons also join up and grow together on the right would suggest

that, even in situations like this where all the cells are individually distringuishable, neural guidance may be often controlled by non-specific factors that affect a large number of neurons.

5.3 Motor neuron outgrowth and formation of the dorsal cord

The preceding section described how the presence of AVG appears to help determine the side of the cord that the DD processes grow along. A second question concerns how the DD processes growing along the ventral cord know where to stop and send out their commissures. Although they have short posterior processes, the main DD ventral cord processes extend forward from the cell bodies, eventually making contact with the next DD cell along. However there is a certain amount of evidence to suggest that the determining factor for DD ventral cord growth may not be the next DD cell, but the position of the next DB cell body. First the DD commissures always exit from next to DB cell bodies, even when these are not immediately behind the next DD cell (e.g. DD3/DB4 in figure 3.4). Second there often seems to be some sort of recognition event involving DD process tips inserting themselves into DB cells at the time of and soon after process outgrowth, particularly at the back of the cord (figure 3.12). Third, in Acaris, where distances are much greater, all the DD commissures exit opposite DB cell bodies together with DB commissures, which are all on the right hand side behind the RBG (Johnson and Stretton, 1987). VD and AS commissures also grow out together in Ascaris (ibid.). Neighbouring VD and AS cells are sisters, but there is no lineal relationship whatsoever between DB and DD cells (in C. elegans, and presumably also in Ascaris, whose early lineage is identical to that of C. elegans, Sulston et al., 1983). Fourth, after DD3 and DD5 were removed by ablating their parent, DD4 did not extend to fill the whole space left by DD3, but instead stopped and began sending out a commissure at an only very slightly anterior position to normal (figure 4.3). This experiment does not prove DB involvement, however, because it remains possible that the normal growth length is intrinsically determined, as appears to be the case with the postembryonic touch cells AVM and PVM (Chalfie et al., 1983). A more conclusive, but unperformed, experiment would be to remove a DB cell.

The next event after DD process outgrowth is the growth of the motor neuron commissures. All the commissures grow out synchonously and reach the dorsal midline at the same time, well before any other longitudinal process has grown along the dorsal cord (RID will do so eventually). There is therefore a problem of recognising the correct point at which to turn, and a subsequent problem of deciding the direction in which to turn. Although adjacent to the basement membrance, the commissural growth cones appear to grow on the surface of the hypodermis, rather than the basement membrane (section 3.2). Similar behaviour was inferred from experiments on early optic nerve outgrowth (Krayanek and Goldberg, 1981). When the growth cones reach the dorsal ridge they have been seen to insert finger-like extensions into the hypodermis, indicating that some cell recognition event may have taken place (figure 3.11). Therefore it seems that the best candidate for the source of the required information is the dorsal hypodermal ridge itself, and that the growth cone “tastes” the hypodermis as it advances, eventually recognising the dorsal ridge.

The suggestion that there is a specific property of the dorsal hypodermal ridge that is recognised, while simplifying the explanation of how the dorsal cord is formed, creates problems of its own. The dorsal hypodermis is a syncytium containing many nuclei and covering the dorsal side of the animal from head to tail and from one lateral ridge to ther other (the lateral boundaries can be seen in the section in figure 1.1). The commissure therefore grows on the surface of this syncytium for some time before it recognises a specific part of it. In so doing it crosses the path of some later longitudinal nerves, such as the ALM process, and the sublateral bundle (SAAD, SABD, SIBD, SMDD, see figure 1.2). Hence it appears that some property of the membrane must be localised to only that part of the cell surface covering the dorsal ridge. The syncytium is formed in the embryo in a curious fashion by two rows of cells passing between each other and then fusing. Mutations in two genes, unc-83 and unc-84, are known to affect this process (Sulston and Horvitz, 1982). Although mutant L1 larvae move well, they have been seen in electron microscope reconstructions to contain defects in the structure of the dorsal cord (J. G. White, unpublished observation), which might be due to the failure in the correct localisation of recognition components in the dorsal hypodermal ridge.

Once the motor neurons have turned onto the dorsal cord, they seem to grow out rapidly along it and, if they are DA or DB neurons, start making neuromuscular junctions (D reconstruction, figure 3.6). It is only at around this time or later that their dendrites grow out in the ventral cord, so they start neuromuscular activity receiving organised synaptic input. A system in which neurons generate synaptic activity before they receive their controlling input would be expected to generate a lot of random signals, but would allow the whole nervous system to be built simultaneously instead of sequentially, starting with sensory neurons and progressing along the processing pathway.

5.4 Discussion

Decussation of nerve processes, in which an entire group of cell processes cross the midline, is a standard phenomenon in most animal nervous systems, and a scaled down version of the same type of behaviour can be seen in C. elegans in the crossing over of processes from paired interneurons in the preanal and retrovesicular ganglia. The PVP processes cross in the PAG (figure 3.8) and the RIF, RIG and SABV processes cross in the RVG (figure 3.10). Since the general property of decussation appears to be functionally unnecessary, it may give some insight into general constraints on developmental organisation.

It is very clear in C. elegans that there is no ultimate functional advantage to be gained from the decussation. The crossovers are not used to facilitate transfer of information from one side of the nervous system to the other by receiving input on one side and having output on the other, since in almost every case all the synapses and gap junctions observed in the adult wild type reconstructions are on the parts of the processes beyond the cross over point. The exception is that the RIF cells both make gap junctions to AVG on their cell bodies, but this also would not be logically different if the cell body positions were reversed. It is not even the case that the symmetrical body positions of the neurons involved are preserved into later development; in fact the cell bodies in both the PAG and the RVG get squashed into a single row as the muscles mature.

This situation is different from that in most vertebrate decussations, in which the cells remain on the opposite side from their axonal termini, and have some functionality on both sides. However, even there it is clear that, considering the whole organism, there is more crossing over than is necessary. An engineer would have the right side of the brain receive information from, and control, the right side of the body. Some communication between the two dies is certainly necessary, and this is seen for example in the corpus callosum between the two hemispheres of the cerebral cortex (and in the C. elegans nerve ring). However such connections are inherently different from the general sensory and motor decussations, for which the argument can still be made that they are functionally necessary, and are more likely to reflect developmental than functional constraints.

One common factor between the four miniature examples of decussation in the PAG and RVC of C. elegans is that they are all between pairs of neurons touching across the ventral midline. It might be suggested that their mutual affinity causes their processes to grow towards the opposite cell, and therefore cross over. However after either PVPL or PVPR was removed by ablating its parent the other stayed in position and still sent its process across the midline and along the opposite side of the cord as normal. In addition there are three pairs of cells in the ventral ganglion in front of the excretory duct which are also adjacent across the midline (AIA, SMBV and SAAD) and none of them cross over. Instead the simplest unifying property of the decussating pairs is regional: they comprise all the left/right pairs of interneurons associated with the ventral hypodermal ridge between the excretory duct and the anus. This, however, suggests neither a mechanism nor a reason for the crossing over.

One possibility is that the crossing is ballistic: both processes are attracted to some point or region on the midline and once they get there they keep on growing in the same direction and thus cross over. Nerve processes in vitro tend to grow in straight lines (Bray, 1979). The attraction of the ballistic hypothesis is that it permits there to be no intrinsic distinction between the two cells. The fact that all the decussating pairs in the RVG cross in the same place supports the hypothesis. Also the PVP crossing point in the PAG seems to be special, since the DVC process crosses from top left to bottom right in the same place, on its way forward through the preanal ganglion. The change in position of DVC does not define the site, because removal of DVC by ablating its parent had no effect on the PVP processes and their crossover. Neither did removal of PVQL, which normally contacts PVPR as soon as it crosses to the left and grows forward with it.

If we accept the ballistic hypothesis then it seems likely that PVT defines the site in the preanal ganglion, since the PVP processes cross between PVT and the processes of DVC and PVQ neurons, which are flattend out over the surface of DD6, partially separating the PVP cells from DD6 (figure 3.8). Alternatively it may be that the site is defined by the DVC and PVQ processes in a redundant manner, so that removal of any one of them makes no difference. The affinity of these three processes for the DD6 cell body is striking; they spread over its surface whereever it is available, and when DVC was removed the PVQ processes spread further to mostly fill the gap (figure 4.7). Further experimentation removing either PVT or DD6 might prove illuminating.

A variant of the ballistic hypothesis is that the initial directions of outgrowth of the processes are both intrinsically towards the midline, and so the processes simply cross over before turning forward. All the cells involved migrate ventrally from lateral positions as the hypodermis closes over the ventral surface of the embryo. It might be that the growth cones start out continuing the direction of migration of the cell and thus cross the ventral midline. This argument would apply equally well to the ventral ganglion cell pairs that do not cross, and it is certainly not necessary for an axon to leave a cell body in the same direction that the cell has been migrating. For example the ALM cell bodies are seen migrating backward along the lateral hypodermis in the C and D reconstructions, and in the E reconstruction they are sending axons forward along the same path they have just followed but in the opposite direction (figure 3.4). However, even if this does not provide a complete explanation, it does suggest how intrinsic opposite polarities of the two cells in each pair may be established.

5.5 Selective fasciculation

I have already suggested that AVG helps organise the ventral cord by providing a preferential parth for growth of, at the least, the DD axons. The wildtype outgrowth of PVP and PVQ processes from the back of the cord, in which their tips always were found very close together along the cord (section 3.5), suggested that there might be some interaction involved. Therefore a series of ablation experiments were performed to investigate PVP and PVQ outgrowth (section 4.3).

PVP and PVQ processes grow on both sides of the ventral cord. The left hand cord contains only three processes at hatching, PVPR, PVQL, and AVKR (plus RMEV at the front, see fig. 1.3). The normal sequence of events is that PVPR and PVQL grow forward together, and AVKR was only seen to be growing back after they had reached the front. It appears that PVPR is needed for the other two to grow on the left side, because when it is removed no processes are seen n either the embryonic or adult left hand cords (figure 4.4). If PVQL is removed then PVPR still grows forward along the cord by itself. Therefore, although PVQR is not a unique pioneer in normal development because the PVQL growing tip is parallel with its own, it does appear to have a primary role in establishing the left hand cord. When VPR is removed the PVQL process still grows forward along the cord, but on the right side rather than the left, and apparently somewhat delayed compared to PVQR and PVPL which normally grow on the right. In this case therefore the ability to grow and the basic directionality of growth are preserved, although the actual path taken was altered, as when AVG was removed. This corresponds to what is seen when guideposts are removed in the insect PNS (Berlot and Goodman, 1984), or motor neurons in the chinck embryo (Landmesser and Honig, 1986). It is not known whether the AVKR process also extended along the right hand cord in the absence of PVPR and PVQL on the left side.

These results are not symmetrically reproducible on the other side of the ventral cord, since PVQR still grows forward along the right side in the absence of PVPL. However, as discussed above, the cord is not symmetrical. While PVQR and PVQL are the first processes to grow along the left side of the cord, there are other preexisting processes on the right at the time when PVQR grows forward (AVG and DD axons) which might

provide some degree of non-specific affinity that assisted PVQR in growing along the right side. This could in principle be tested by removing PVPL, AVG and DD6.

Although the removal of PVQL had no effect on the outgrowth of PVPR along the left hand ventral cord, it did appear to affect the growth of other processes down the lumbar commissure from the left lumbar ganglion to the preanal ganglion (see figure 1.3 for a schematic plan of the normal situation). In neither of the reconstructed embryos in which PVQL had been removed did any of the left lumbar processes grow down lumbar commissure, although they had done so on the right side. As well as containing processes descending from the lumbar ganglion, the lumbar commissures contian a DA motor neuron process ascending from the preanal ganglion. This was present in both the $PVQL^{-}$ reconstructions.

These results suggest that there is a specific need for PVQL in order for the other lumbar ganglion cells to grow correctly in the right direction. Similar behaviour is seen in the developing grasshopper CNS, where in several cases it has been shown that an identified neuronal growth cone normally fasciculates with a specific preexisting fascicle, in the absence of which it fails to grow in any organised fashion (Raper et al., 1984, Bastiani et al., 1986, duLac et al., 1986). In one case it was shown that a specific subset of the processes in the preexisting fascicle is required (Raper et al., 1984). This corresponds to the observation that the DA process in the lumbar commissure is not sufficient to promote growth of other processes down the commissure.

If PVQL provides guidance for the left lumbar processes by some process of selective fasciculation, then this fasciculation does not last for long. When processes from the two lumbar commissures meet in the preanal ganglion all the cell types other than PVQ immediately form contact with their bilateral homologues, “zipping up” with each other (figure 3.8). The other left lumbar processes then leave PVQL to join their right hand homologues and PVQR on the right hand side. Therefore it seems that there is a hierarchy of affinities that applies the left lumbar processes other than PVQL; first they follow, and in fact require, PVQL, then they leave PVQL in order to join their right hand homologues.

These observations all fit the “labelled pathways” hypothesis (Ghysen and Jansen, 1979, Goodman et al., 1982), that growth cones are programmed to recognise a sequence of surface labels on fascicles, possibly in some adhesive hierarchy, and that this determines their path through the developing nervous system. The situation when the left and right lumbar processes meet is somewhat novel, in that then two equivalent sets of processes fasciculate together, and must decide which of the two PVQ neurons to follow. There is no good clue as to what determines this (discussed earlier in the section on cord asymmetry.

The observations about lumbar commissure formation contrast with those made in the ventral cord that, even if normal cues are missing, processes tend to keep on growing in the correct direction. A plausible explanation of this difference is tat there is a non specific property of the ventral cord which permits or promotes neuron growth along it. Apart from the presence of other processes, at the relevant time there is a continuous line of motor neuron cell bodies along the ventral midline, which may act as general

guideposts in the same way as neuronal cell bodies that have been proposed to facilitate neuron outgrowth in the insect PNS (Bentley and Keshishian, 1982).

There are a number of uncoordinated mutants that are known to be defective in outgrowth of processes from the lumbar ganglion cells, on the basis of fluorescent staining of the PHA and PHB phasmid sensory neurons by direct uptake of fluorescein isothiocyanate (Hedgecock et al., 1985). Mutants in unc-33, unc-44 and unc-76 all show the same phenotype. Rather than growing forward into the preanal ganglion the phasmid axons stop abruptly where they meet at the bottom of the lumbar commissures, often with swollen endings. This is at the point where the resorting of the fibres takes place, with the majority of the left lumbar processes leaving PVQL to grow forward with their contralateral homologues. The fact that there are several genes with both this phenotype and also defects in movement is interesting in relation to a suggestion made earlier (in the discussion of ventral cord asymmetry). This proposed that the mutual affinity of ventral cord bilateral homologues may be a basic general mechanism whose biological purpose is to bring together the motor circuitry interneurons, and which affects other neurons incidentally. A prediction of this hypothesis would be that the anterior motor circuitry interneurons would also be afected by the mutations. In mutants for unc-6 (referred to as unc-106 in Hedgecock et al.), the PHA and PHB axons normally fail to grow down the lumbar commissures, but instead wander forward along the lateral hypodermis. This is reminiscent of the defect seen in the left lumbar commissure when PVQL was removed. However the defect in unc-6 mutants is more general than that following PVQL removal, since axons from the postembryonic PVD neurons on the lateral hypodermis also fail to reach the ventral cord, and motor neuron commissures are also disrupted (S McIntire, personal communication).

5.6 Conclusion

In the introduction to this part of the dissertation it was proposed that a number of different mechanisms could be used to influence neuronal guidance, often concurrently, and a list of possible types and sources of influence was provided. The behaviour of outgrowing neurites in both normal and experimental C. elegans embryos that has been described here has suggested new examples of several different types of influence.

The formation of the dorsal cord could be explained by the presence of a preexisting preferred pathway along the dorsal hypodermal ridge. This would essentially be an epidermal blueprint, as proposed by Singer et al. (1979). DD growth along the ventral cord may be limited by some inhibitory effect of DB cells, although from the observations that are available the inhibition seems more likely to be caused by selective recognition accompanied by membrane insertion than by the retraction of growth cones as seen by Kampfhammer et al. (1986) in vitro. The decussation of processes in the preanal and retrovesicular ganglia may be due to the tendency of growth cones to grow in straight lines, as discussed by Bray (1979). In the lumbar commissures and the determination of which processes grow along the left and right nerve cords there appear to be several examples of selective fasciculation, similar to that proposed in the labelled pathways hypothesis (Ghysen and Jansen, 1979). There also appeared to be a general directionally or premissive property of the ventral cord region that meant that, even when specific cues were removed, processes still grew out along the cord.

All these proposed interactions fall broadly into some class of interaction that has been suggested previously. Further experiments of the same type as described here, some of which I have mentioned in the discussion, could be carried out to define more precisely the characteristics of particular interactions. The other possible approach to furthe rwork is to use the existing picture as a basis for an investigation of the genetic factors controlling neural outgrowth, eventually uncovering the critical molecular mechanisms involved using molecular genetic techniques (Greenwald, 1985). In this discussion I have mentioned a number of mutants that affect neural guidance in the ventral nervous system, in some cases in ways that are partially interpretable in terms of the mechanisms proposed here. The genetic approach is discussed further in the final conclusion after part II.

PART II The Organisation of the Adult Nerve Ring

CHAPTER 6 Introduction and Methods

6.1 Introduction

Around the beginning of this century several attempts were made to map all the nerve processes in a nematode nervous system, using light microscopy of methylene blue stained animals, most notably by Goldschmidt (1908), who argued erroneously that a nervous system was a syncytial network of anastomatosed cells. That view was soon disproved, but it was not until recently that the goal was realised of determining the anatomical structure of a complete nervous system at the level of individual processes and synaptic connections (White et al., 19860. As part of that achievement the entire central nervous system of two C. elegans specimens was reconstructed from electron micrographs of serial sections. The purpose of the investigation reported here was to extract information about the organisational structure of the C. elegans nervous system from the resulting anatomical data, concentrating in particular on the syaptic circuitry.

The approach taken was to construct a computer database containing information about all the synapses and gap junctions between the neurons, and also form one animal an indication of the amount of contact between each pair of neurons. This information was used for three separate lines of investigation. The first was to study the distribution of symapses within the nervous system in order to investigate the variability of the circuitry in different circumstances, and the type of variation seen, and to use that variability to make inferences about possible factors involved in determining whether connections are made. This work is described in Chapter 7. The second line of study, described in Chapter 8, considered the general organisational structure of the synaptic circuitry, and how it might relate to function. The third, described in Chapter 9, used the data on the contact between neurons to investigate the physical organisation of nerve processes in the neuropil.

The date for all these investigations are purely anatomical; there are no physiological studies on the nerve ring neurons in either C. elegans or Ascaris. Some of the functional circuitry involved in the motion response to a touch stimulus in C. elegans has been deduced by a combination of laser ablation experiments and the detailed anatomy (Chalfie et al., 1986). However practically all of the discussion concering possible function of parts of the ring circuitry has to be based on the electron microscope anatomical data showing sensory endings, synapses and gap junctions, and motor output onto muscle. As will be shown in Chapter 8, there are some fairly broad statements that can be made about the organisation of connections at the level of the whole nervous sytem, or groups of neuronal classes, but caution must be exercised in interpreting plausible connectivity patterns in any detail. In particular no attempt is made to predict the inhibitory or excitatory nature of particular synaptic connections, or to stimulate, even conceptually, any piece of circuitry.

The type of study undertaken here is novel because the data available are unique in their completeness at such a fine level of detail. There have been many studies of circuitry at the physiological level in other animals (research on a number of well defined invertebrate systems is reviewed in Selverston, 1985) and it is often possible to dye fill the neurons from which recordings have been made to determine their anatomy at the light microscope level. However the overall distribution of connectivity between all the different identified neurons in even a part of a central nervous system has not previously been analysed at an electron microscopic level, owing largely to the much greater complexity of other animals' nervous tissue. Perhaps the system about which most is known is the vertebrate retina, which has been studied in detail at both a physiological and electron microscope level (Dowling and Boycott, 1966, McGuire et al., 1986, reviewed in Sterling, 1983). Around 50 types of cell falling into a few basic classes have been identified, and much is known about typical connections between these cell types. However particular cells and situations are not reproducible and electron microscope studies have necessarily concentrated on the properties of single cells (McGuire et al., 1986).

6.1 Methods

Data from two electron microscope reconstructions, the H series and the U series of White et al. (1986), were used to construct a computer database. The part of the animal that is represented in the database is the whole of the central processing region, or nerve ring, which consists of a ring of neuropil around the pharynx in the head of the animal that contains about 175 nerve fibres and the bast majority of the synapses in the entire nervous system. A general description of the C. elegans nervous system can be found in Chapter 1. Chapter 2 introduces the nomenclature of C. elegans neurons. The database contains the following information about the nerve processes in the ring: for each pair of neurons it stores the number of gap junctions between them and the number of chemical synapses in each direction. In addition, for the H series, there is a measure of the adjacency or degree of mutual contact between each pair of processes. This adjacency was obtained by looking at every 5^{th} micrograph in the reconstruction series and counting the number of these pictures on which the given pair of neurons were in contact.

The word synapse is reserved for chemical synapses in this discussion; electrical connections are referred to as gap junctions because they are identified as such from the electron micrographs. White et al. (1986) presents the criteria used in identifying synapses and gap junctions in C. elegans electron microscope reconstructions. Synapses are made en passant between adjacent processes. Although synaptic boutons are not seen, synapses can be recognised in electron micrographs by the presence of presynaptic density and the accumulation of vesicles. The chemical synapse count in the database combines data from monadic and dyadic synapses. In dyadic synapses, which are seen frequently in C. elegans, there are two postsynaptic partners. It excludes cases where the only connection seen between two cells is half a dyadic synapse, since such observations have been seen to be unreliable.

In general throughout the presentation and discussion of results a distinction is made between a connection between two neurons, and a synapse between them. There is a connection if there are one or more synapses. All the results that do not concern

comparison between the two different animals represented in the H and U series were obtained with data from the H series alone, because the adjacency information, which is only available for that animal, is often an important factor in the analysis. For a general H series neuron, A, I will refer to its contralateral homologue as A', and to the corresponding in the U series as A^u.

The database program is written in C and implemented on a VAX-8600 minicomputer. The main data is stored in a large array in virtual memory, together with a set of referencing arrays that allow easy access and cross comparison. All the analysis softwre is contained in one program that is modular in design and uses a free format command input system developed previously (Durbin et al., 1986). An analysis requiring a new algorithm is implemented by writing a new subprogram and entering it as an option in the command tree.

This investigation relies on much previous hard work by Nichol Thomson, Eileen Southgate, and John White in performing the original reconstructions, and crosschecking all the data. I would also like to thank Barbara Cross and Mabel Eggo for assisting in typing some of the data into the computer.

Table 7.1 Distributions of connections

	All	Ranges of Adjacency of A to B 1–2	3–5	6–10	>10
a) Chem A->B given A adjacent to B	.09 (8778) [4.2]	.02 (3374) [1.8]	.06 (1832) [2.6]	.11 (1497) [3.6]	.23 (2075) [5.1]
b) Gap junction A–B given A adjacent to B	.07 (4350) [1.3]	.04 (1677) [1.0]	.08 (915) [1.2]	.07 (738) [1.4]	.09 (1020) [1.7]
c) Chem B->A given chem A->B	.14 (819) [4.7]	.00 (56)	.07 (112) [3.0]	.11 (169) [3.2]	.19 (482) [5.2]
d) Gap junction A–B given chem A->B	.06 (819) [1.5]	.02 (56) [1.0]	.06 (112) [1.3]	.05 (169) [1.6]	.07 (482) [1.5]
e) Chem A->B given 1 synapse A'->B'	.55 (84) [1.7]	.65 (20) [1.2]	.56 (18) [2.0]	.64 (14) [1.9]	.61 (23) [1.7]
f) Chem A->B given >2 synapses A'->B'	.95 (523) [5.3]	.79 (14) [2.9]	.96 (53) [3.2]	.98 (100) [4.3]	.97 (351) [6.0]
g) Chem A -> B given A' not adj to B' but A adjacent to B	.01 (1886) [2.2]	.01 (1274) [1.4]	.02 (366) [1.9]	.02 (176) [3.3]	.04 (70) [4.3]

On the left is indicated the type of connection being considered and the range of cell pairs A,B over which to calculate the frequency of the connection being made. Each entry in the table has three figures: the first is a frequency, the second in parentheses is the number of cell pairs over which that frequency was calculated, and the third in brackets is the average number of synapses (gap junctions) made in those cases where a connection is made. The first column gives the overall figures, while columns 2 to 5 break down these numbers according to the adjacency of A to B. A' is the symmetrical homologue of A. Since gap junctions are asymmetrical, unordered rather than ordered pairs A, B are considered for row (b). Also adjacencies to muscle are not considered for row (b) because gap junctions are not made through the basement membrane.

CHAPTER 7 Synaptic Disibutions and Reproducibility

7.1 Synaptic distributions

Altogether there are 3462 chemical synapses and 754 gap junctions between the 183 neurons in the H series database. The two different types of connection, gap junctions and chemical synapses, are distributed rather differently in the nervous system, as can be seen from table 7.1. Table 7.1 rows a) and b) show the proportion of pairs of adjacent processes that are connected by chemical synapses or by gap junctions.

Although there are 4.5 times as many chemical synapses as gap junctions, the proportion of pairs of adjacent cells connected by chemical synapses is similar to that connected by gap junctions (9% and 7% respectively). This is because a pair of cells is often connected by several chemical synapses, but rarely by more than one or two gap junctions. In other organisms there may be hundreds or thousands of synapses between a given pair of neurons (e.g. a cerebellar basket cell make many synapses on a Purkinje cell). The small number of synapses in C. elegans is probably due to the extremely small size of the entire nervous system.

7.2 Adjacency and synapse formation

It is immediately obvious from table 7.1 (rows a, b) that both the probability of being connected by a chemical synapse, and the number of synapses actually formed, are highly correlated with adjacency. There is a 14 fold increase in the proportion of connections formed when pairs of cells that have a high adjacency are compared with pairs that touch only briefly. This correlation is far less marked for gap junctions, for which the corresponding increase is only a factor of two. While the bast majority of chemical synapses are between pairs of cells with adjacency greater than 10, nearly half the gap junctions are between cells with adjacency less than or equal to 5.

It might be thought that the increase in the average number of synapses formed with higher adjacencies provides evidence that synapse formation is dependant on the area of contact. However, for any given pair of neurons it is possible to show that the number of synapses made between them does not substantially change with their adjacency. This proposal can be tested by considering pairs A, B which have contralateral homologues A', B'. In general A', B' will have a different adjacency from that of A, B, and they will often also form a different number of synapses. If we assume that the symaptic formation mechanisms for symmetrical pairs on the two sides are equivalent then we can test statistically whether the number of synapses formed tends to vary proportionally with the adjacency, or remains independent of adjacency. The details of the test are given in the appendix, but the results are as follows. There were 391 pairs of processes which formed synapses on both sides and showed different adjacencies on the two sides, and the value of the test statistic was 7103 with an expected standard error of around 1330. If the number of synapses varied proportionally with adjacency then the value should ideally be 0, while if it was independent the value should be around 7655. It is clearly many standard error values from 0, and only about half a standard error distance from 7655. Therefore there is strong evidence against a proportional system, and the data are consistent with the number of synapses between a given pair of neurons being determined independently of the adjacency. The slightly lower value of the calculated test statistic

than that expected by a wholly independent model can be explained by the fact that there must be some effect at very low adjacencies. If the adjacency is 0 then clearly no synapses can be made, and when it is only 1 or 2 there are spatial limitations preventing a large number of synapses being formed.

7.3 Reciprocal synapses and joint chemical/electrical connections

It is important to consider the correlation between synapse formation and adjacency when looking for statistical interactions between the different types of synapse. For instance, the frequency of reciprocal chemical synapses is consistent with synapses being independently specified in each direction. Although the overall probability of a connection from neuron B to neuron A given one from A to B is high (14% as opposed to 9%, see table 7.1 row c), this is because most of the pairs with a synapse from A to B have high adjacency, and so are more likely than normal to have a reverse synapse from B to A. When the probability of a reverse synapse is shown separately for each adjacency range, as in table 7.1, it can be seen to be essentially the same, or possibly slightly lower, as that for an unconditional synapse.

The same approach can be used to consider whether there is any statistical interaction between chemical synapses and gap junctions. In this case there is a slight negative correlation, since the probability of a gap junction between a pair of cells that are linked by chemical synapses is reduced by 1 or 2 percent from the unconditional probability in each adjacency range (table 7.1 row d). This difference can be partly explained by the fact that a significant proportion (66/284) of gap junctional connections are between members of the same neuronal class, while it is rare for there to be intraclass chemical synapses (discussed below).

Therefore the presence of one type of connection between cells does not provide any indication of whether another type, either gap junctional or chemical in the opposite direction, will also be present. The only exception to this is when the cells are members of the same class, in which case if there are chemical synapses (only 5 examples) then they tend to be reciprocal (4/5), as would be expected. Together with the overall dependence of synapse formation on adjacency these results mean that there is a remarkably high probability of some form of connection between processes that have a high adjacency. If the adjacency is greater than 10 then the proportion of pairs forming either a gap junction or a chemical synapse one way or the other is 47% (478/1020).

7.4 Connections between members of the same neuronal class

Gap junctions are far more frequent than normal when both cells are members of the same neuronal class. The 302 neurons in C. elegans have been put into 118 different classes on the base of similar morphology and connectivity (White et al., 1986). Many of these classes, particularly those with processes in the nerve ring, consist of a pair of bilateral, symmetry related neurons. There are 61 such pairs in which the two cells make physical contact and 35 (57%) of these have gap junctions while only 5 (8%) have chemical synapses.

It is common for there to be a gap junction where the ends of two processes from different members of the same neuronal class abut, such as when left and right symmetry related processes meet at the top of the nerve ring. This abuttal is interesting in itself, since it suggests that the processes might stop growing when they establish contact with the tips of their contralateral homologues. A similar phenomenon is seen in the ventral and dorsal nerve cords, where the processes from consecutive neurons of two classes of motor neuron (VD and DD) abut and make gap junctions but do not overlap; the other classes overlap (except AS), and sometimes make gap junctions. In the ring there are 24 pairs of cells whose processes end where they meet at the dorsal midline, and a gap junction is present in 22 of these cases. Of the 37 other classes of cells that have interclass cell contacts, but which do not abut, only 13 interclass gap junctions. None of the pairs that form internal chemical synapses abut, nor do any of interneurons that send adjacent processes back along the ventral cord; perhaps if they had inhibited each other's growth they could not have grown out together along the cord.

Table 7.2 : Reproducibility of connections

	All	Number of synapses between homologues			Adjacency of A to B		
		1	2	>2	0	1–2	>10
a) Chem A->B	.75	.31	.63	.92		.55	.88
given chem A^U->B^U	(916)	(112)	(296)	(508)	(65)	(78)	(461)
b) Chem A->B	.87	.55	.77	.95		.69	.92
given chem A'->B'	(810)	(84)	(203)	(523)	(24)	(55)	(463)
c) Gap junction A-B	.79	.75	.97	.98		.83	.78
given gap junc A^U-B^U	(260)	(216)	(31)	(13)	(10)	(54)	(93)
d) Gap junction A-B	.89	.87	.96	.91		.89	.91
given gap junc A'-B'	(283)	(216)	(45)	(22)	(8)	(66)	(88)
e) Chem A->B	.67	.42	.58	.74		.32	.89
given chem A'->B and A adjacent to B	(448)	(50)	(108)	(290)		(98)	(171)
f) Gap junction A-B	.57	.56	.62	.57		.42	.76
given gap junc A'-B and A adjacent to B	(216)	(155)	(47)	(14)		(69)	(58)

As in table 7.1, each row gives the proportion of cell pairs in a set chosen by some condition that make a particular type of connection. In this table the condition is always that a similar connection exists between a corresponding pair of cells elsewhere. The first column gives the number of pairs satisfying the condition in parentheses, and the proportion of these that form the matching connection. These figures are broken down in two ways in columns 2 to 7. Columns 2 to 4 give the reproducibility as the number of synapses (gap junctions) between the homologues varies, while columns 5 to 7 give the figures for low and high adjacencies between A and B themselves. If A is not adjacent to B (column 6) then there can be no connection, so the proportion is automatically 0. A' is the contralateral homologue of A, and A^U is the equivalent cell in the U series animal. As in table 7.1 rows (c), (d) consider unordered rather than ordered pairs of neurons.

7.5 Reproducibility of connections

There are two sources of synaptic reproducibility that might be expected in the database. The first is from animal to animal, and the second is due to internal bilateral symmetry within one animal. Most neuronal classes that are associated with the nerve ring, and so represented in the database, consist of one or more bilateral pairs of homologous neurons. One problem with the comparison between animals is that the U series animal was an adult, while the H series animal was an L4 larva. Although almost all neural development in the nerve ring is embryonic the sex-specific circuitry concerned with egg laying is incomplete in the H series, and there may be other less obvious differences due to age.

The probability that a given pair of H series neurons A, B will be connected by chemical synapses if their contralateral homologues !' and B' are connected is 87%, while the probability that they will be connected if their U series homologues A^u and B^u are connected is only 75% (table 7.2 rows a, b). The corresponding figures for gap junctions are 89% and 79% (table 7.2 rows c, d). It therefore appears that gap junctional connections are slightly more reproducible than synaptic ones and that the two sides of the same animal are more similar than the two different animals. Part of the difference in chemical synaptic connectivity between the two animals is that there are more chemical synapse connections in the U series than the H series (916 as opposed to 819), and the extra connections are certain to be unmatched. However the difference between the animals is still probably significant because there are fewer U series gap junction connections than ones in the H series (260 against 284) but a lower proportion of U series gap junctions are matched in the H series than ones of the opposite side of the H series (above).

The difference between the "within animals" (left/right) comparison and the "between animals" (H/U) comparison can be further illustrated by considering the pairs that form connections in only two of the four possible places (each side of each animal). There are six ways that this can happen: both sides of the H series but not at all in the U series, both sides of the U series but not in the H series, and four different ways that there could be one synapse in the U series and one in the H series. If the similarity between animals were the same as that between sides of the same animal then the size of all these classes should be the same: the number of pairs synapsing only in the H series, and the number synapsing only in the U series, should be a quarter of the number that synapse just once in the H series and once in the U series. In fact there are 23 pairs that synapse only in the H series, 51 only in the U series, and 64 that synapse once in each series. For gap junctions these numbers are 41, 22 and 14. In both cases there are more connections than expected that are U series specific or H series specific, indicating that there are significant differences between the two animals.

Similarly, a test can be performed to detect whether there is also a reproducible difference between the right and left sides of the nerve ring. If there was such a difference then one would expect that the connection between a pair of A^u,B^u in the U series would resemble more closely that between their exact equivalents in the H series, A,B, than that between the corresponding cells on the contralateral side, A' and B'. In

fact the figures are 75% reproducibility to A,B, 74% to A'B' for chemical synapses, and 79% to A,B, 77% to A',B' for gap junctions. Therefore if there is consistent difference between the two sides it is very slight (1 or 2%). It would not be possible to identify the source of any consistent difference that may be reflected in this small change of reproducibility, because there is too much noise from the difference between the two animals.

7.6 Reproducibility depends on the number of synapses made, not on adjacency

Overall the observed levels of reproducibility suggest that there is an underlying regular pattern. In particular the two sides of the same animal appear to be sufficiently similar to allow us to assume that they were subject to the same synaptic specification procedure during development. This allows us to consider some aspects of the question of how neural connectivity is determined. By identifying the sources of inaccuracy of synaptic reproducibility it is possible to obtain information about, and thus to make suggestions about, the mechanisms for establishment of specific circuitry. This approach has already been used to indicate the independence of the number of synapses made between a pair of neurons from their adjacency. All the effects we will consider would not be altered significantly by one or two percent change due to possible slight genuine differences between the two sides.

Although it appeared at first sight that there was an unexpectedly high variation in the formation of chemical synapses, almost all the inconsistencies are due to the unreliability of weak connections, i.e. connections with only a small number of synapses. For pairs connected by three or more synapses (which is true for about two thirds of connected pairs) the probability that their homologues are connected is greater than 90%, whereas if there is only a single synapse then the probability that their homologues will be connected is only around a half. This difference exists both between and within animals (for exact numbers see table 7.2).

Clearly one cause of a mismatch in which a connection seen is present on one side but missing on the other might be that the neurons are not in contact in the case where the synapse is missing. However if they do touch each other then the dependence of reproducibility on the number of synapses made is practically independent of adjacency. If we consider A, B such that A' and B' are connected by only one synapse then the probability of connection between A and B is around 60% in all adjacency ranges. At the other extreme if A' and B' are connected by three or more synapses then the probability that A and B are connected is 79% (11/14) if their adjacency is one or two, and more than 95% in any adjacency range greater than two (table 71 rows e, f). It is necessary to consider the data this way, because a direct comparison of reproducibility at different adjacencies would suggest that adjacency is an important factor (table 7.2 rows a, b, final columns). In fact this effect is mostly secondary, caused by the fact that connections with higher adjacency tend to have a larger number of synapses. If we combine this result with the earlier observation that the number of synapses made between a given pair of neurons is essentially independent of adjacency, then it appears that, except at very low adjacencies, the probability that two given processes will form a synapse is also practically independent of their adjacency. The overall correlation between synapse formation and adjacency (Table 1, row a) therefore implies that the physical organisation

of processes is such that neurons that repoducibly synapse tend to have higher adjacencies. This is considered further in the discussion.

The picture that emerges is that pairs of neurons are programmed to make an approximately predetermined number of synapses, and that the connection will be more reliable if this number is greater than one or two. There is no significant similar set of results for gap junctions, because, as far as numbers of junctions are concerned, more than three quarters of gap junctional connections involve only one gap junction, and as regards adjacency, there is very little correlation between the presence of gap junctions and adjacency. It is however true that in the cases where there are two or more gap junctions on one side the chance of there being at least one on the other side is higher than normal (94%).

7.7 Mismatches of chemical synapses are due to extra connections more often than to missing connections

If we assume that there is an underlying "normal" pattern of connectivity then there are essentially two ways that a mismatch can occur. Either an additional abnormal connection can be made, or a normal connection can be missing. For any given connection involving a bilaterally represented neuronal class there are four equivalent possible locations that the connection could occur in the database, one on each side of each animal. If a mismatch exists in one animal then one can look in the other animal to get some idea of whether the connection is normally there or not. For chemical synapses there are over four times as many connections that occur in only one of the four cases as in three out of four cases (137 and 32 respectively). This implies that four times as many mismatches are due to a single extra connection being present as are due to a single connection being missing. The same is not true of gap junctional connections, for which there are 74 present in only one case and 58 in three cases.

These observations concerning unmatched chemical synaptic connections suggest that there are a set of extra synapses in addition to a fairly consistent set of basic connections. We can attempt to estimate the consistent set of synapses by counting only those synapses in addition to a fairly consistent set of basic connections. We can attempt to estimate the consistent set of synapses by counting only those synapses that are seen in three or four of the possible cases (both cases if both members of the pair of neurons are unique). There are 2890 of these synapses per animal, leaving 1184 extra synapses altogether in both animals, associated with 647 different cell pair types. Some of these synapses are between cells that are already connected by consistent synapses. However less than half (498) fall into this class; those that do so add fairly evenly to consistent connections with both more and fewer consistent synapses. The remainder (686) are formed between pairs that are not consistently connected. There is a tendency for these to be between processes that have higher adjacency; 437 are between processes with average adjacency 6 or greater.

White (1983) suggested that mismatches might arise because synaptic connections were only specified between processes that are normally adjacent, so that if a nerve process was misplaced and acquired new unexpected neighbours it might make additional, incorrect connections to some of them. There is quite a large variation in the set of cells

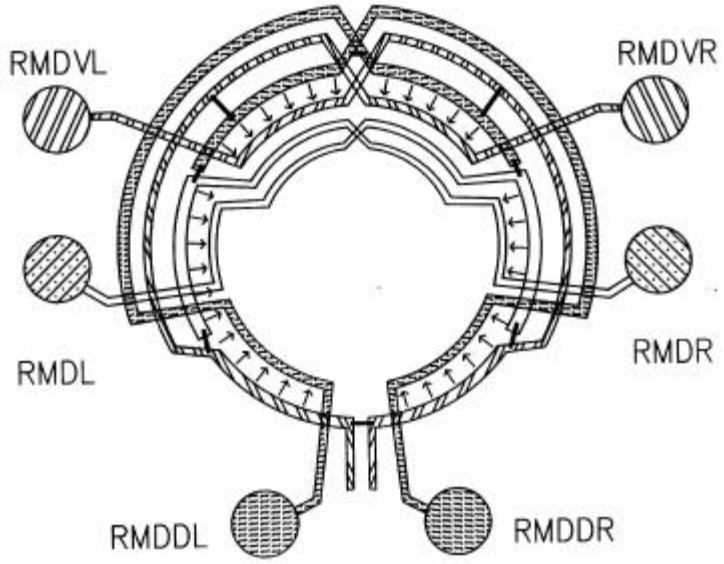
contacted by any neuron, since if A is adjacent to B then the probability of A' being adjacent to B' is only about 75% (7028/9214). However this is not the predominant source of mismatches. If A' is not adjacent to B' the probability of a connection from A to B is far smaller than normal (table 7.1 row g compared to row a). Only about a quarter of all mismatches can be explained in this fashion (24/108 for the bilateral comparison and 65/224 for the H/U comparison – 5th column of table 7.2). The proportions are similar for gap junctions (8/32 and 10/55 respectively). These results suggest first that specificity is probably determined for at least an extension of the normal set of neighbours, covering processes with which a neuron is likely to stray into contact, and second that physical contact is not a major limiting factor in determining synapse formation, since most of the marginal synapses are made with processes with which the neuron is normally in contact. Instead the earlier observations suggest that additional synapses are mostly made between processes that normally have reasonable adjacency.

7.8 Connections are not determined purely by neuronal classes

So far we have compared pairs of neurons that are directly symmetry related, such as A, B to A' B'. There are also many situations where a neuron B contacts both A and its contralateral homologue A'. These contacts are not symmetry related, but if connections are determined purely by the classes of the two neurons, then one would expect a connection between A and B whenever there was one between A' and B. In fact the overall measures of this type of reproducibility are 67% for chemical synapses and 57% for gap junctions (table 7.2 rows e and f respectively), significantly less than the levels of reproducibility for are 67% for chemical synapses and 57% for gap junctions (table 7.2 rows e and f respectively), significantly less than the levels of reproducibility for symmetry related connections (rows a, c). The given frequencies of a matching connection from A to B are conditional on A and B being adjacent, since there is no automatic reason why A and B should be adjacent whenever A' and B are, ecause there is no geometrical symmetry relating to the two pairs. This difference is due neither to the number of connections from A' to B tending to be low nor to the adjacency of A and B being low, since there is still a difference when the frequency is tabulated with respect to either of these categories (columns 2 and 6 of table 2). Indeed the reproducibility is down to 70% even in the cases where there are 3 or more synapses, which are 95%

Figure 7.1

A schematic diagram showing the interconnections between the 6 members of the RMD class of neurons. The circle represents the nerve ring. At each radial position around the ring one of the RMD neurons has synaptic output (arrows), both onto muscle and onto other neurons, including the diametrically opposite RMD neuron, which "intercepts" the neuromuscular junctions (as with the DD process in figure 3.6). In general the RMD neurons are monopolar, with their proximal regions showing this intercepting behaviour, and their distal regions being synaptically active. Gap junctions (thin bars) link neighbouring neuromuscular regions but are not formed in general even where processes are close, sich as for instance where chemical synapses are made. Some variability was seen in this general pattern, since in the U series RMDL had output (both to muscle and other neurons) from the proximal as well as the distal part of its process).



reproducible in the symmetric left/right comparison. The only set of conditions under which there is a respectable degree of reproducibility is when the adjacency of A to B is high (column 7).

What might cause the difference between A and A'' in synaptic specificity with respect to B? If the difference is cell intrinsic, as opposed to being activity related, then either A and A' must be inherently distinguishable or else either they or B must be regionally specialised. Regional specialisation would account for the observations because in general different parts of A and A' contact B, and they contact it in different places (see figure 7.1 for an example concerning cells all of the same class). There are several indications that suggest that this might be a correct explanation. Although few neurons in the C. elegans nerve ring have a classical bipolar morphology, with a presynaptic dendritic structure and a predominantly postsynaptic axonal structure, there are many neurons whose processes show regional differences (White et al., 1983, White et al., 1986), and most of the cases of differential specificity that we are considering, such as that between the RMD neurons shown in figure 7.1, involve these cells. In addition, if the differences were due to regional specialisation, then there would be the highest chance of a mismatch if the cells only touched in one place, and much less chance if they were in contact over a large proportion of their axonal structure. This suggests that the mismatch frequency would be lower when the adjacency of A and B was high, which is indeed the case (table 7.2 column 7). Such an effect would not be expected if the difference between A'B and AB synapses was due to distinct identificatory labelling of A and A'.

Similar results are obtained when comparing synaptic specificity of dorsal and ventral members of the same neuronal class. Once again their interactions with other neurons are not symmetry related, and so the lower levels of reproducibility that are observed (69% for chemical synapses and 52% for gap junctions) can be explained by regional specialisation of some of the neurons.

DISCUSSION

The main conclusions of this set of investigations are:

1. The overall pattern of connectivity between neurons is fairly reproducible. However at a detailed level there are differences. There is a greater difference between the two animals than between the two sides of one animal.

2. Although chemical synapse formation is correlated with adjacency when all pairs of neurons are considered, between any particular pair both the probability of forming a connection, and the number of synapses made, are essentially independent of adjacency.

3. Synaptic reproducibility is very high (>95%) if several synapses are normally made. However there is a substantial number of unmatched single synapses, present in only one of two symmetrical cases. A significant majority of unmatched chemical synapses appear to be due to the formation of abnormal or infrequently made connections, rather than the loss of a normal connection.

4. The formation of a connection between two neurons is not purely dependent on the classes of the neurons involved and whether they are adjacent. A possible additional factor is that in many cases synaptic specificity is regionally localised on neurons.

I shall discuss these points in turn.

7.9 Differences between repeats of equivalent circuitry

There are two significant questions concerning the overall reproducibility of the synaptic connections in the nerve ring. The first is whether the differences seen are systematic and functional, or merely random variation due to looking at an essentially fixed pattern at too fine a level of detail. The second is whether the left and right sides of the nerve ring are developmentally equivalent in terms of synaptic circuitry. Some parts of the nervous system show significant reproducible left/right asymmetry (e.g. the ventral nerve cord, see Chapter 5), but at a gross level the two sides of the ring are symmetrical. These questions could be addressed by considering the four examples of each cell pair interaction present in the database, one for each side of each animal.

To consider the second question first, a comparison was made to search for consistent synaptic differences between the left and right sides across the two animals. This indicated that they were very similar, although there may be a slightly greater similarity overall between the same sides of the two animals than between their opposite sides (approximately 1%, the effect for instance of a single possible asymmetric neuron class). This possible minor difference was ignored for the rest of the analysis in order to consider the two sides as developmentally equivalent when investigating their differences. There was a certain amount of unsystematic variation between the two sides. Clearly it is not easy to assess the functional significance of anatomical changes in synaptic connectivity in a circuit whose function is largely unknown. However there was no obvious observable pattern to the differences that might have suggested a rewiring of any piece of the circuitry, such as loss of one connection but a gain of a compensating connection to a parallel interneuron, and the vast majority of differences involved connections with just one or two synapses, or a single gap junction. It seems reasonable to suggest that the variation seen here is mostly due to random fluctuation in process positioning and synapse formation.

The equivalent comparison between the two difference animals, however, revealed that the two sides of each animal were more similar than the same sides of different animals, suggesting possible real differences between the two animals. The U series is from an older animal but again, except for the introduction of the major egg-laying motor neurons (HSN's) and a generally raised number of connections from the labial receptors (IL1, IL2 classe), the differences appear to be scattered randomly throughout the circuitry, and to consist mostly of only one or two synapses for any particular pair of cells. Since the

animals are isogenic, the systematic differences must be either age related, or a consequence of environmental differences during development. Laboratory specimens of C. elegans are cultured on bacterial lawns grown on agar plates; their environment varies only in the level of food supply and the degree of dessication! There have been no studies to determine whether any of the C. elegans neural circuitry shows variation under different conditions. A possible approach would be to compare the ventral nerve cord circuitry of animals that have been raised swimming freely in liquid culture with the standard nerve cord of animals raised on agar plates, which has been reconstructed many times.

7.10 Connection formation and adjacency

A simple model for formation of synapses between two neurons would be that there is a cell-cell recognition event where the cells contact, and that synapse formation is a local event, so that the probability of forming a synapse, and the number formed, would increase with increasing length of mutual contact. However there is clear evidence from the variation in adjacency and synapse formation in equivalent pairs of cells in the database that this is not the case. Both the probability of forming a synapse, and the number of synapses formed, are essentially independent of the degree of adjacency, providing that some contact is made. This implies that there is internal regulation of synapse formation at a cell-wide level. Such regulation is biologically reasonable, because it would be asking a lot of a process placement mechanism to finely control the exact amount of contact between different neurons, and the circuitry is reasonably fixed. Indeed the adjacency of a particular pair of neurons is much more variable than their connectivity. The next chapter pursues further the question of process placement in the nerve ring.

Although there is no apparent link at the level of individual process pairs, there is a strong general correlation between synapse formation and adjacency. Neuron pairs that are synaptically connected tend to have high adjacencies, and the more synapses they form the higher the adjacency tends to be. The amount of contact is in general much larger than that needed to make the synapses in. In a sense adjacency seems to depend on the likelihood of forming synapses, rather than synapse formation on adjacency. This also makes biological sense, since the goal of positioning nerve processes is to place in contact with each other those neurons that will form connections. However the same correlation is not seen for gap junctions, for which this post hoc biological rationale is just as relevant. This seems to suggest an effect on neuronal placement, and thus possibly process guidance, of either chemical synapses or gap junctions (or something involved in forming them). One might postulate either process attraction linked with synapse formation, or a repulsion, or stopping of growth, associated with gap junctions. When considering connections within the same class it was observed that bilaterally symmetric processes that gap junction with each other often terminate when they meet at the midline, while those few classes that form intraclass synapses all substantially overlap. Elsewhere in C. elegans the muscle arms in the body are clearly attracted to the presynaptic processes, since when motor neuron axons are displaced the muscle arms still go to them and receive neuromuscular input (Chapter 4, and J White, S Brenner, unpublished observations). However, although muscle arms resemble postsynaptic processes, they are phylogenetically anomalous and possibly a special example.

7.11 An underlying pattern of connectivity with additions?

The average number of chemical synapses between a pair of synaptically connected neurons 4.2, and there are many cases with more than 10 synapses, whereas there are rarely more than one or two gap junctions between a pair of neurons. We have seen that in individual cases the level of adjacency between two processes does not appear to be important to synaptic reproducibility. Instead the most significant general factor as an indicator of reproducibility is the number of synapses made. Connections with only one or two synapses are unreliable, while those normally containing many synapses are nearly all present in all cases. This result is statistical; there may be individual pairs of neurons that always connect but only form one synapse (e.g. OLQV and RIC only contact briefly but in each case seen make a single very large synapse full of vesicles). However overall there is evidence that the probability of forming a connection and the number of synapses are linked.

If we consider mismatches in which a connection is present on one side but not the other, there are four times as many cases where the connection is seen on neither side of the other animal as ones in which it is seen on both sides. An explanation of this result would be provided by the hypothesis that there is an underlying pattern of circuitry that is consistently present, but there are also always a number of additional synapses that are selected from a wide range of possibilities, and which therefore do not generate reproducible connections. The distribution of this set of additional synapses was estimated by subtracting away consistently seen synapses. Since this operation was performed with synapses rather than connections it leaves some additional synapses between processes which have a consistent connection, for example where in one of the four cases 10 synapses were made, and in others only 6. However the majority of additional synapses are between processes that are not reproducibly connected, which implies that the variation observed in the circuitry is not simply due to modulation in level of a small restricted set of possible connections. The new connections normally contain only one or two synapses. This would provide an explanation for the low reproducibility of synaptic connections containing only one or two synapses. Both the adjacency distribution of the additional synapses, and a direct count of the proportion of mismatches for which the unconnected pair of neurons are not adjacent, indicate that most of the additional connections are between pairs of neurons that normally have a reasonable adjacency, rather than being due to a process wandering away from its normal circle of connections.

One possible alternative explanation for the extra “random” connections is that they are due to errors in scoring synapses during the reconstructions. It is sometimes hard to unambiguously identify whether or not a process is postsynaptic to a particular synapse. However the fact that the comparison between the U and H series contains many differences with similar characteristics to those described above, but many of which are reproducible from side to side of each animal, suggests that they are real.

7.12 Localisation of synaptic specificity within neurons

There are a significant number of cases where synapses or gap junctions are formed between members of two classes of neurons when they make contact in one place on their processes, but not when equivalent cells touch in other places. It seems more likely that this is due to regional localistion of the potential to form the relevant type of synapse in the same way for all neurons in a class, than to all the different members of each class having different recognition properties. A more direct example of clear localisation is seen in the adult ventral cord, where the VA and VB motor neurons are bipolar, sending out processes in one direction that only receive input, and only have neuromuscular output from their other process, a classical axon/dendrite morphology. The interneurons are closely available to both processes. However many of the ring neurons are not bipolar, and it would appear that many of the specialisations, presumably involving differential localisation of surface components, would have to be between distal and proximal regions of the same nerve process, as in figure 1. The following neuronal classes can be tentatively implicated as candidates for regional specialisation on the grounds that they are involved in more than one mismatch and have a suggestive axonal structure: RMD, SMD, SAA, SIB, SMB, RIC. There are many others with apparently discontinuous axonal structure (White et al., 1986). An example of a different type of change between proximal and distal regions of the same nerve process is given by the single neurite of the major interneuron AIB, which changes relative position in the neuropil, running for the first half of its length with chemical receptor axons, and th distal half with motor neurons and motor circuitry interneurons (White et al., 1983).

7.13 Conclusion

Two types of information can be inferred from the foregoing analysis. First a picture can be drawn of the degree of overall synaptic rigidity in the C. elegans nerve ring, and how the connectivity might vary or be modified. Second there are a number of observations suggesting factors that might be important for the formation of synapses between individual pairs of neurons.

It is apparent that synaptic circuitry in C. elegans is not so rigidly reproducible as the positioning and lineal origin of cells, which are practically identical from animal to animal. However the data support the suggestion that there is a broad core of connections that are constant, including most of the strong synaptic connections containing many synapses. This core is subject to a reasonably low level of variation itself (at the level of one or two synapses per connection), but there are also a number of additional chemical synapses (about 10% of the number in the core pattern) connecting other pairs of processes, which are not reproducible. Gap junctions show less variation, and no indication of a strong additional component.

There is some evidence that the changes and additions might not be random, since there are many consistent ones, each small in itself, between the H series animal and the U series animal. Since these two animals are isogenic they must be due to either environmental or age-related differences. If the additions are functional then there are two possible ways in which they could be used. The first is to “tinker” with a standard pattern, slightly altering the influence of various parts of the nervous system on each

other but not changing the functional roles of cells. The second is to introduce a new behaviour, or to flexibly wire a particular task so that it is performed in a different way in different animals. This is different from the question of functional flexibility in a single neuron taking part in more than one task, for which there is plenty of evidence: the IL1 and URA classes are for instance both sensory and motor neurons. Although no data are available on the variability of function of nerve ring neurons in different animals, I believe that two observations point towards the tinkering rather than the respecification theory. First, in no case is a systematic pattern of connectivity change discernible (other than the introduction of the egg-laying circuitry in the sexually mature U series animal). Second, the great majority of changes consist of only one or two synapses per connection.

There are several observations concerning the formation of connections between pairs of neurons. It seems likely that the potential to form synapses and gap junctions may be localisable to particular parts of a single process. The probability of a chemical synaptic connection being formed, which may depend on environmental as well as genetic factors, and the number of synapses formed, appear to be regulated so as not to depend on the amount of contact between a pair of cells. However the factors involved in synapse formation may influence adjacency, because synapse formation, but not gap junction formation, is correlated with adjacency, suggesting that one or the other of the two processes may have an effect on nerve process placement. The evidence for these properties generated by this study is statistical and indirect, and therefore inappropriate as a basis for a series of direct intrusive testing experiments. However it is not susceptible to the bias that might be generated by studying intensively a single connection. Also, since a very large number of cell pairs were considered, it is statistically significant; the conclusions do almost certainly reflect pervasive properties of one simple nervous system, and they may also be relevant to other organisms.

CHAPTER 8 The Logical Organisation of the Circuitry

As well as looking at typical local interactions between neurons in the nerve ring the completeness of the information in the database allows us to investigate the large scale structure of the neural circuitry. We can ask such questions as: what is the processing depth from sensory input to motor output, i.e. how many intermediary neurons are there?, and to what extent is the circuitry unidirectional, progressing linearly from input to output? It is unlikely to be completely directional; one expects to find a certain amount of feedback, both in control-type circuits, in analogy to electrical engineering, where feedback can be used to adjust gain and bias to optimise the response to stimuli, and in rhythmic pattern generators if they exist. In fact the questions of processing depth and directionality are related, since it is necessary to have ordered the circuit diagram before being able to count the number of intermediary neurons between sensory input at the top and motor output at the bottom. I shall therefore consider first the extent to which the circuit diagram can be directionally ordered.

8.1 Directionality

Let us consider the network of chemical synapses and ignore gap junctions for the time being since, although they can be rectifying and directional, one can not detect any directionality from electron micrographs. The aim is to vertically order the neurons so that as many as possible of the synapses point downwards, with their presynaptic neuron above the postsynatic neuron in the ordering. This was done with a sorting algorithm that is described in the appendix.

In fact the operation was carried out with combined data for neuronal classes, rather than with the individual cells, since all the members of the same class should occupy the same functional position in the circuitry. Since the earlier analysis suggested that there was a core set of connections that were almost always present, to which were added a number of sporadic connections consisting of only one or two synapses, orderings were obtained for two sets of data, one made by adding all the synapses that were seen in at least three out of the four possible places (left and right sides in both H and U series). In the latter case the few synapses between two unpaired cells were only counted if they appeared in both the H and the U series. This more stringent synapse set is the same as the set of consistent synapses discussed in Chapter 7. For ease of comparison the summed data were divided by two and the stringent data multiplied by two so that both sets gave numbers in terms of synapses per animal.

The results suggest that on a large scale the circuitry is very directional, and that almost all the chemical synaptic feedback that does take place is in the form of reciprocal synapses. The more stringent set of data could be arranged so that more than 95% of the connections pointed downward, leaving only 140 out of 2890 (4.8%) pointing upward (figure 8.1). Of these, 116 were involved in reciprocal connections, which have to contribute upward synapses because they have synapses in both directions. This leaves only 24 persistently seen synapses that are involved in upward connections, 12 per side. These 12 synapses are distributed over 11 connections, so there is no case in the whole nerve ring of strong, consistently seen, indirect synaptic feedback. However it appears that direct feedback using reciprocal connections is an important feature of the circuitry,

Figure 8.1

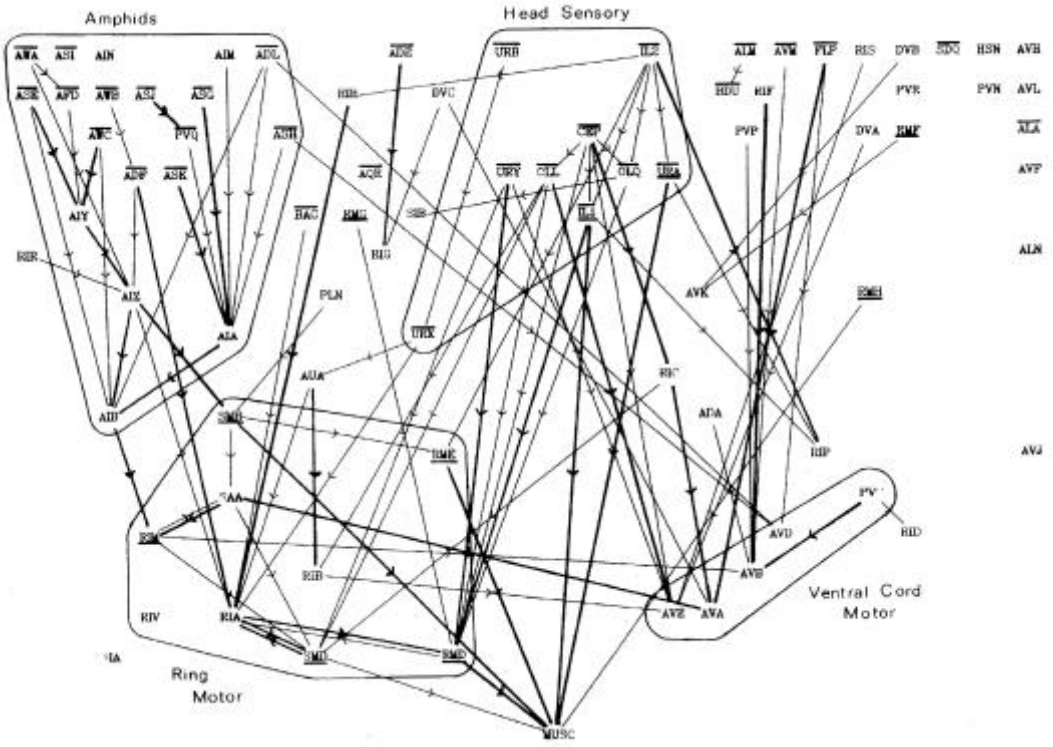
All the neuronal classes in the nerve ring arranged in the optimal vertical ordering to minimise the number of upward chemical synapses. The next three pages contain 8.1 (a), (b) and (c). Each has the classes arranged in the same positions, but shows different sets of connections.

(a) shows all the chemical synaptic connections in the stringent set with 5 or more synapses with fine lines, and those with 10 or more synapses with heavy lines. Sensory neurons are indicated by a bar above the cell name, and motor neurons by a bar underneath the class name. Four subjective groupings of related neurons are encircled. These are discussed further in the text.

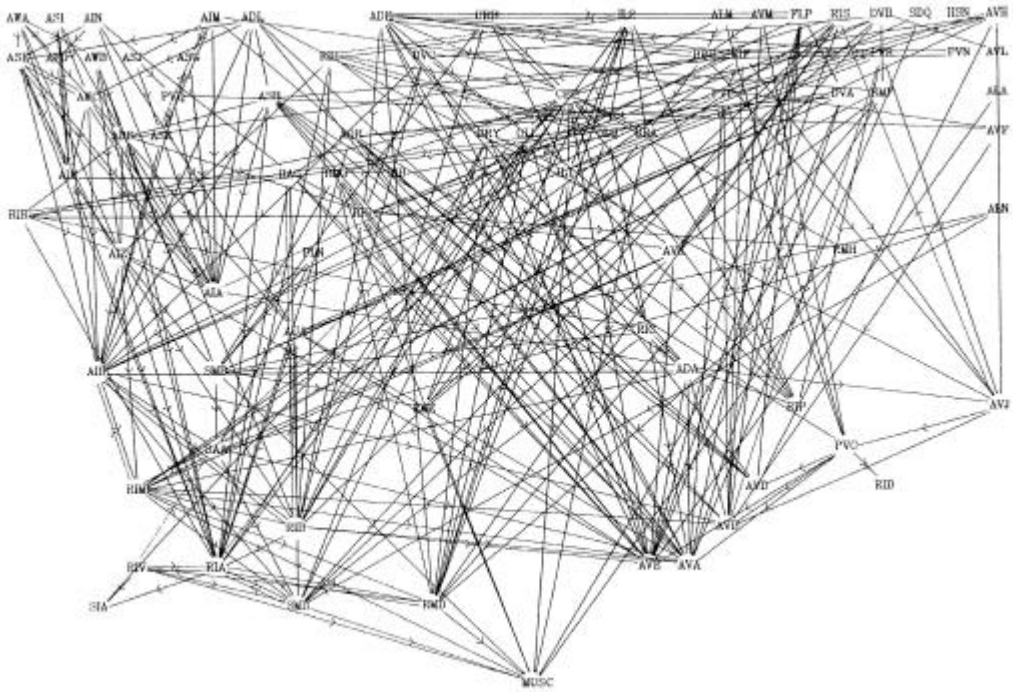
(b) shows all the chemical synaptic connections in the stringent set.

(c) shows all the gap junctional connections in the stringent set.

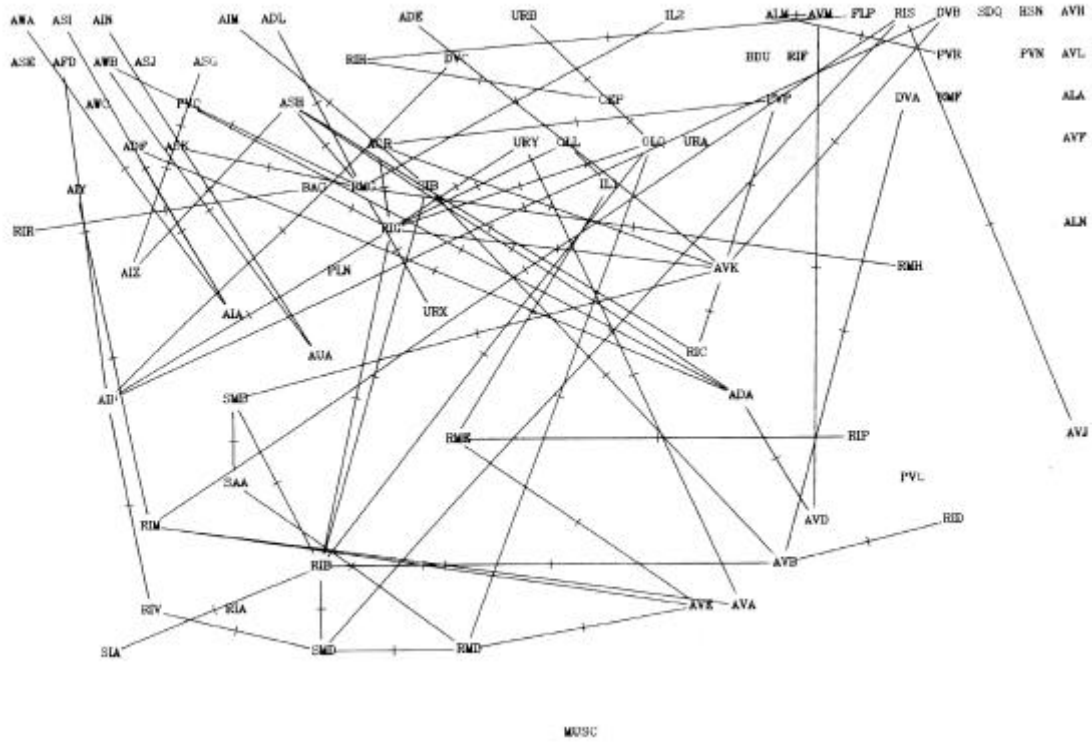
(a)



(b)


AWA ASI AIN AIM ADL ADE URB IL2 ALM AVM FLP RIS DVB SDQ HSN AVH
ASK AFD AWB ASJ ASG RSH DVC BDU RIF PVR PVN AVL
AWC PVC ASH CEP DVA RMF ALA
ADF ADK ALR URY CLL OLQ URA AVF
ADY BAG RMG SIB IL1 ALN
RIR RIG RMH
AIZ PLN AVK
ALA URX
AUA RSC
AD SMB ADA
RMB RIP AVJ
SAA PVL
RIM AVD RID
RIB AVB
RIV RIA AVE AVA
SIA SMD RMD
MUSC

(c)


AWA ASI AIN AIM ADL ADE URB IL2 ALM AVM FLP RIS DVB SDQ HSN AVH
ASK AFD AWB ASJ ASG RSH DVC BDU RIF PVR PVN AVL
AWC PVC ASH CEP DVA RMF ALA
ADF ADK ALR URY CLL OLQ URA AVF
ADY BAG RMG SIB IL1 ALN
RIR RIG RMH
AIZ PLN AVK
ALA URX
AUA RSC
AD SMB ADA
RMB RIP AVJ
SAA PVL
RIM AVD RID
RIB AVB
RIV RIA AVE AVA
SIA SMD RMD
MUSC

since reciprocal connections contained 378 downward synapses as well as the 132 reverse synapses already mentioned, therefore accounting for 20% (494/2890) of the persistently observed synapses.

It was not possible to order the averaged data so clearly. In that case there were 3898 synapses per animal, of which 386 (9.9%) pointed upwards in the arrangement that had been found to be best for the stringent data. When the ordering was specifically optimised for the averaged data the number of upward synapses was only reduced to 328 (8.4%), nearly twice the percentage that was seen with the stringent data. These observations support the suggestion made in Chapter 7 that at least a proportion of the additional synapses are different in nature from the consistently observed synapses, having perhaps a more random distribution.

8.2 The organisation of the feedback

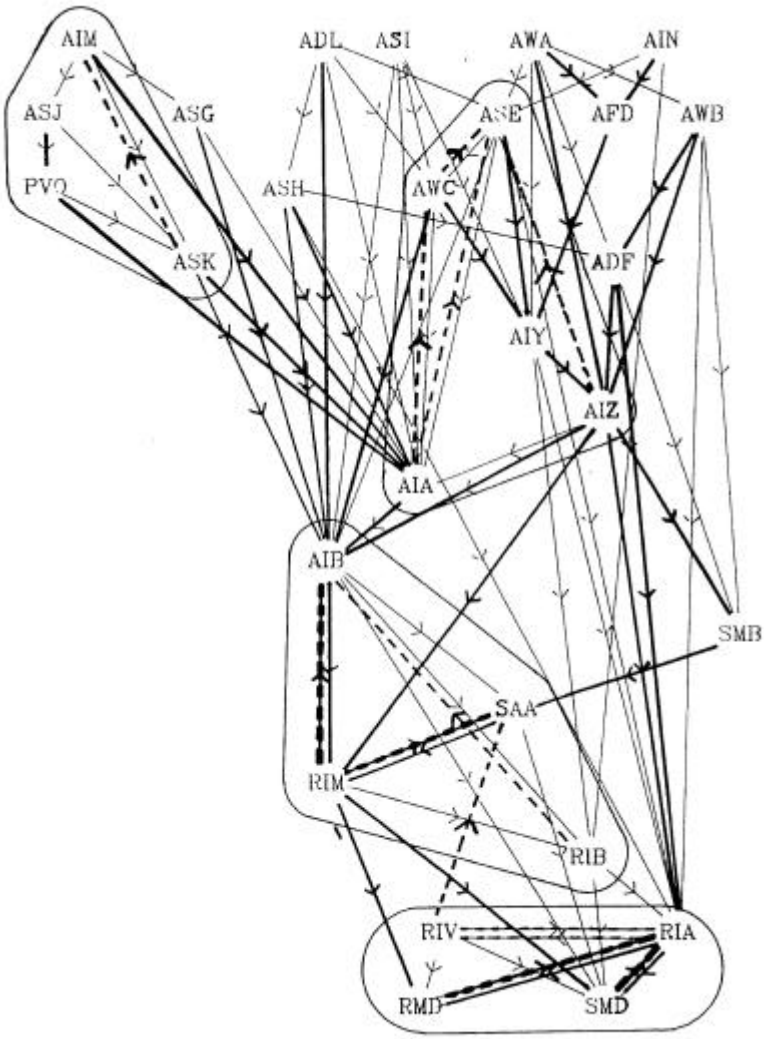
Several groups of neurons have been indicated in figure 8.1 on subjective grounds because they seem to be involved in a particular part of the circuitry. Two sensory and two motor groupings have been defined. One contains neurons associated with the amphid sensilla, whicha re multiply innervated sensilla on either side at the front of the head, probably concerned mainly with chemo- and osmo-detection (Ward et al., 1975). As well as sensory receptor neurons there are a number of interneuron classes that appear to be predominantly concerned with processing information from these receptors. The other sensory grouping contains a second set of neurons with sensory receptors at the front of the head, distinct from the amphids. These all have their cell bodies in front of the nerve ring, and tend to form direct connections to motor circuitry (or directly to muscle in the cases of IL1, URA). There are additional sensory neurons, such as the touch neurons (ALM, AVM, Chalfie et al., 1985) that are not assigned to either of these classes. The two motor circuitry groupings contain the interneurons that innervate ventral cord motor neurons on the one hand, and some of the circuitry that controls neuromuscular activity onto head muscles from the nerve ring on the other hand.

Figure 8.2 shows the amphid circuitry and part of the ring motor circuitry in greater detail, including all the synaptic connections. Although there are several examples of reciprocal feedback they do not interlink the whole circuitry. The groups of neuronal classes that are connected so that each neuron could potentially influence all the others in group are outlined in figure 8.2 (the connection between RIV and SAA breaks this rule, but only contains one consistent synapse). Let us call such groups “modules”. Neurons higher in the directional ordering are isolated from modules that lie below them. Thus all the amphid circuitry apart from AIB is isolated from motor activity. In this sense even the reciprocal feedback that is seen only has a limited effect on the overall directionality of the circuitry.

One important consideration that might invalidate the suggestion of a highly directed flow is that we have ignored gap junctions. Figure 8.1 © shows the distribution of all the gap junctional connections in the nervous system. Some of these are within groupings identified previously. There are also some classes that make a lot of gap junctions, but very few chemical synapses (e.g. RIG, RMG, AVK). It is of course not known whether or not any of these gap junctions are rectifying and thus possibly directional themselves.

Figure 8.2

A more detailed view of a particular part of the synaptic circuitry. This shows the amphid neurons and their main paths of connectivity to the ring motor circuitry. All connections present in the stringent data set are shown; connections with 5 or more synapses have heavier lines. All upward synaptic connections are dashed, again with the dashes being heavier if there are 5 or more upward synapses to the connection. Groups of classes that are potentially connected by feedback are encircled. The only exception is that the upward connection between RIV and SAA was not used to link the AIB, SAA, RIM, RIB module to the RIV, RIA, RMD, SMD module, since it only contains one synapse.



However in at least one case a gap junctional connection has been shown to be functionally important in one direction, by removing the cell involved with a focussed laser beam (Chalfie et al., 1985).

8.3 The processing depth

The ordering of the circuitry allows us to count the number of synapses between sensory input and motor output. The method used calculates a hypothetical flow of information through the synaptic connections down through the nervous system (see Appendix for details). It necessarily treats all the observed synapses as having equal functional effect and so the resulting estimates are probably physiologically very inaccurate. However they provide a reasonable basis for a broad comparison of the flow of information from different sensory modalities.

There are on average 3.5 chemical synapses between sensory neurons and the head muscles, basing the calculation on the more stringent data and the ordering derived for in in section 8.1. As might be expected, there is a lot of variation in the number of intermediary connections. The actual number can vary from a minimum of one in the case of neuromuscular output from the sensory motor neurons IL1 and URA to a maximum of 16 for a particular sequence of synaptic connections starting from the amphid receptor ASI. However this upper band is somewhat misleading; the average distance from muscle of any given neuronal class is never greater than 5.9 synapses (for ASJ). Nevertheless there is clear systematic variation dependent on the type of sensory receptor being considered. Input from the amphid receptors takes the longest time on average to reach muscle (4.4 synapses), reflecting the extra stage of amphid specific interneuron processing shown in figure 8.2. The other head sensory input is rapid, taking only 2.2 synapses on average, and the average value for the remaining sensory neuronal classes is 3.2 synapses.

The same method that calculates depth of processing also generates an estimate of the proportion of “sensory influence” that reaches different final types of output (see the appendix for details). There are three major discernible targets for output from the nerve ring: the head musculature via direct neuromuscular synapses, the ventral cord motor circuitry interneurons, and the RIP class of neurons, which provide the sole connection to the pharyngeal nervous system, which is thought to pump constitutively unless repressed by RIP. In general the number of synapses connecting sensory neurons to the ventral cord circuitry or RIP is about one less than the number needed to reach the head musculature, possibly because there is an extra layer of motor pattern generating circuitry (considered further in the discussion). The “fast” head sensory neurons have proportionally more output onto the head muscles, and provide the majority of output onto RIP. It seems reasonable to suggest that they carry out much of the short range sensing involved in moving the head to feed and searching out a path round obstacles for the body to follow when moving. The amphids generate a balanced number of connections to both the head muscles and ventral cord interneurons, and have no link with RIP. The other sensory neurons provide comparatively more input onto the ventral cord interneurons. This is perhaps reasonable because many of their sensory endings are in various other parts of the body, rather than being at the tip of the head.

8.4 Discussion

The strongest generatl feature of the C. elegans nerve ring circuitry is its extremely high directionality. The neuronal classes can be ordered in such a way that less than 5% of the synapses point backward. This organisation is clearly very different from that of many higher organisms. For example in the mammalian cortex every projection from one area of cortex to another seems to be matched by a reverse projection (van Essen, 1979). However there are also structures that are only a few synapses deep that seem to be fairly directional, such as the vertebrate retina (Sterling, 1983).

Almost all the synapses that do point backward are members of reciprocal connections, which were shown in Chapter 7 to be almost as frequent as would be expected on the basis of the distribution of directed synapses. There are almost no persistently seen synapses involved in indirect feedback. In addition the feedback that is seen appears to be largely restricted to affecting small groups of neuronal classes, or modules. In the case of the circuitry shown in figure 8.2 it is possible to suggest functions for the observed moduels in terms of the different stages of processing needed. However in discussing the possible function of elements of the circuitry one should bear in mind that all the data is anatomical; there is no functional or physiological data.

The outputs of the amphid receptors shown in figure 8.2 appear to be processed fairly independently from the rest of the sensory input. Their output is eventually concentrated onto the interneurons AIZ, AIA and AIB. AIZ and AIB synapse onto RIB, RIM and RIA, the major interneurons that appear to be involved in controlling the head musculature. These RIX neurons then synapse onto the RMD and SMD which are two of the main head motor neuron classes, each containing 6 neurons interconnected in a complex fashion (see figure 7.1 for the RMD connections).

Feedback in the AIM, ASJ, PVQ, ASK module could best be used to modulate their own receptor output. The ASE, AWC, AIY, AIZ, AIA group combines the output of a set of, mainly chemosensory, amphid receptors. The AIB, SAA, RIB, RIM group receives input from other modalities as well as the processes chemosensory data from the neurons seen in figure 8.2, and has output to both the head and body motor circuitry (RIM is ittself a head motor neuron). It may make the basic decision on body movement, which the RIA, RMD, SMD, RIV module then executes. The feedback in this final motor output module, both between the interneurons RIA and RIV and the motor neurons, and within the motor neuron classes themselves, may be involved in the generation of oscillatory head movements that then propagates backward as waves. In several respects the connections seen here resemble those seen in central pattern generators (oscillation generators) in other invertebrate systems (for several examples see Model neural networks and behaviour, ed Selverston, 1985). In these other systems reciprocal connections between neurons also appear to play an important part, and it is often seen that neurons are multifunctional, for instance with both motor neurons (Miller and Selverston, 1985) or command interneurons (Getting and Dekin, 1985) taking part in the pattern generating circuitry.

One of the proposed modules, the one containing AIB, SAA, RIB and RIM, contains a mixture of neurons that subjectively appear to be part of the amphid circuitry (AIB), and the motor control circuitry (RIB and RIM). Much of the amphid receptor circuitry shown at the top of figure 8.2 is concentrated onto AIA and AIB. It appears that one of these two neurons may be involved in feedback within the amphid circuitry, possibly to "tune" its output, while the other is involved in feedback that may determine the relative importance of the amphid output to the movement of the animal. Another suggestion to explain the organisation of AIA and AIB, made initially by J G White, is that they receive joint input and that AIA inhibits AIB, thus causing the combination to act as a differentiating circuit which could be used to detect gradients during side to side head movement.

The principle of a highly directed network containing small processing modules provides for only a very limited use of circuit feedback. This use is to improve the versatility of small scale processing units made out of a very few neurons. One reason that C. elegans does not have longer loop neural feedback may be that, having only a shallow overall processing depth, it uses sensory feedback to perform this role. The advantage of this is that it measures the actual, rather than the intended, outcome of motor activity. Indeed it is thought that proprioceptive feedback may be important in the propagation of locomotory waves down the body. However, even if more complicated nervous systems do have more sophisticated circuit structures, it may still be useful to effectively isolate functional units as much as possible from the internal working of other parts of the nervous system, even if those working are somewhat relevant. If this is done then additional operations can be added easily at any stage without perturbing the rest of the system.

CHAPTER 9 Process Placement in the Nerve Ring

In order for nerve cells to make connections with each other they must be in physical contact. Therefore the physical arrangement of the neuropil is an important part of its design. The C. elegans nerve ring is essentially a large parallel bundle of fibres bent around the pharynx. A typical transverse section through the C. elegans nerve ring shows an apparently homogeneous group of process outlines on each side. Bilaterally symmetric processes occupy approximately symmetrical positions within the bundle, but there is local disorder on the scale of a few process diameters so that in general it is impossible to identify processes on the basis of their positions, even over fairly long stretches of reconstruction (although characteristic diagnostic properties of certain neurons do make them identifiable).

It is presumably unnecessary to specify the exact relative positions of all the processes, but important for there to be reasonably tight control over process position because processes do not branch, so the only way to make contact is to lie next to each other in the bundle. How is position controlled? There are essentially two different possible sources of order, either from contact with other processes or from an external source of information, such as a gradient (e.g. Bonhoeffer and Huf, 1982). The most likely form of contact mediated information would be a mutual adhesivity that kept two or more neurons together and therefore simplified the task of specifying their positions. Such selective fasciculation has been proposed as important in laying down other invertebrate nervous systems during development (see chapter 1 for review) and there are indications that it is important in process outgrowth in C. elegans (PVP/PVQ behaviour, discussed in chapter 5).

Figure 9.1

The distribution of adjacency in the database. The crosses connected by the heavy line indicate the number of cell pairs in the database with a particular adjacency. The fine lines are the corresponding numbers from the outcome of the random mixing stochastic model, using three different values of the parameter, p, and averaged over 10 runs to get smooth results. The best overall fit to the distribution is given by p – 0.08. This leaves two regions of misfit, X and Y, which are discussed in the text. Note that the vertical axis in this graph is nonlinear.

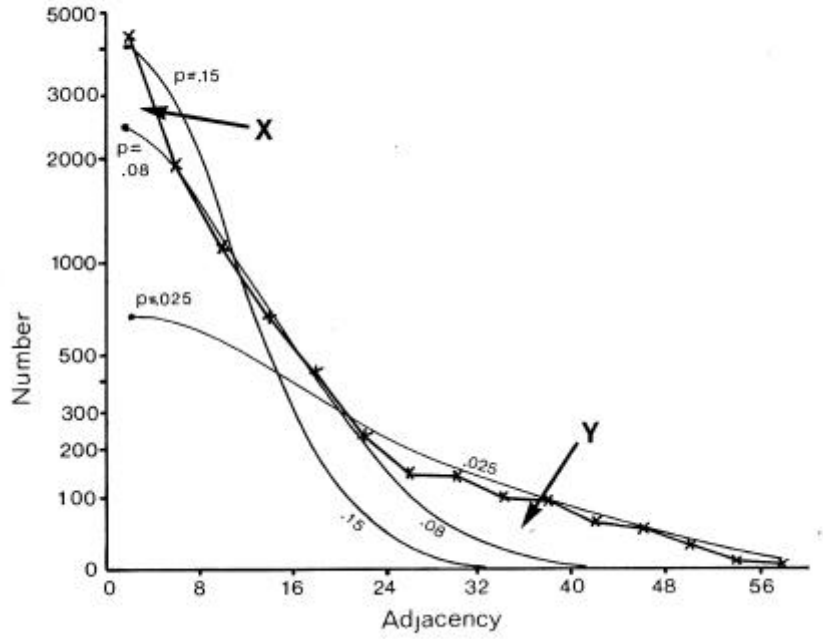
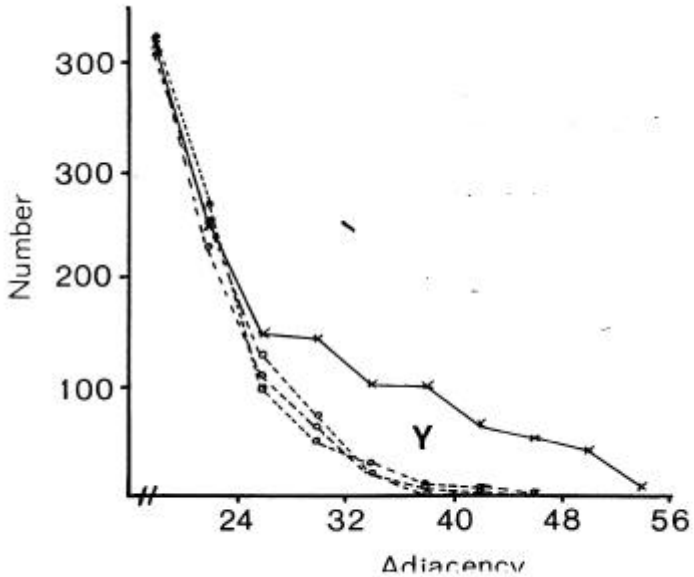


Figure 9.2

An expansion of the region Y with three separate simulations of the random mixing model with p = 0.08. The gap between the true data and the model data is clearly significant. Since the vertical scale is linear in this case the area of region Y corresponds to the number of "extra" high adjacency contacts. This predicts around 400 extra persistent contacts, or 2.3 per neuron (178 neurons).



9.1 Specific persistent contacts

If selective fasciculation were important in organising the nerve ring, and the adhesive forces remained after early development, then one would expect to find pairs of processes with persistent contacts. These should be detectable in the database as pairs of neurons with exceptionally high adjacencies. If one looks at the distribution of all the adjacencies in the database it would be the sum of two components, a random mixing component, and a high adjacency component due to persistent contacts. The distribution of adjacencies is shown in figure 9.1. There is a clear change in slope at the curve at an adjacency of around 30.

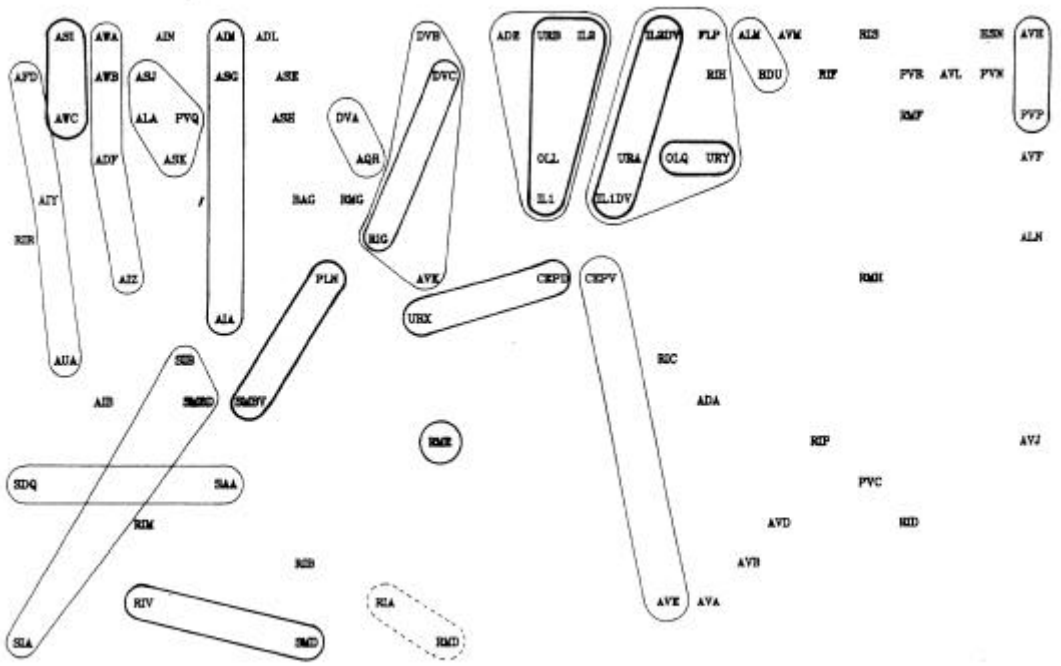
In order to assess the significance of this shoulder, and to estimate its size, and hence the average number of persistent contacts made by a neuron, I produced a stochastic model of a collection of randomly mixing parallel fibres. This operates by recording the positions of the fibres in a hexagonal grid representing a slice through the process tract, and then moving to the next slice and allowing neighbouring processes to exchange positions with a certain probability. The adjacency of a pair of fibres is then taken to be the number of slices in which they are neighbours. The total number of slices was taken to be 75 to make the total adjacency (sum of all its adjacencies) of each fibre the same as the average total adjacency for the processes in the database. The second parameter, the probability of a process switching, p, was chosen so as to best match the model's distribution of adjacencies to that of the database. Thje best fit is given by p = 0.08.

There are two regions of misfit that cannot be eliminated, denoted by X and Y. Region X is due to a very large number of additional contacts of very short duration, which probably arise from processes crossing at an angle in the nerve ring. Such events are known to occur in the nerve ring but are not considered by the computer model. Region Y is the shoulder that includes longer contacts than predicted by the random model. Figure 9.2 shows an expansion of the shoulder region of the database distribution together with data from 3 simulations of the model. The shoulder is clearly significant beyond the variation in the simulations due to randomness in the model. However it is fit quite well by the random model with a low switching probability (p= 0.025, figure 9.1), which is not surprising, because low switching probabilities for a subset of process pairs are an approximation to specific adhesion between the processes, which is the sort of feature that we predicted might give rise to a shoulder beforehand.

It is possible to estimate the number of significantly persistent contacts from the graph in figure 9.2 as about 400, and thus to arrive at a figure of on average 2.3 persistent specific contacts per neuron. This is very crude – there may be many specific contacts of shorter length – but it gives an indication that there may be fascicles or bundles of mutually adhesive processes in the C. elegans nerve ring. However if such bundles are common then they cannot contain very many processes, because the average number for long bundles must be only 3 or 4. A second test suggests the same result. The average number of contacts made by a neuron is 52.1, most of which are short. If we compare the adjacencies of all the contacts with that of the longest contact then we see that on average 12.6 are longer then 25% of the maximum, only 4.8 are longer than 50% of the maximum, but 2.4 are longer than 75$ of the maximum. Thus it seems that a very small number of contacts are comparatively consistent.

Figure 9.3

Clusters of neuronal classes obtained by hierarchical clustering of the adjacency data. There are three thicknesses of line, corresponding to an association measure of 25 or more for the thickest, 15 or more for the intermediate one, and 8 or more for the thinnest one. All these clusters were seen on both sides of the nervous system. In some cases the dorsal and ventral members of the same class ended up reproducibly in different clusters (e.g. CEP, IL1, SMB). The positions of classes were moved as little as possible from those in figure 8.1, in order to show the relationship between possible bundle assignments and circuitry. The RME class is ringed because the four RME neurons form a tight bundle with each other. The RIA and RMD classes are linked in a dashed cluster because there are a number of RIA/RMD pairs that have associations just under 8.



9.2 Identified bundles

It is hard to tell with individual process pairs whether their high adjacency in accidental or not, but if several processes combined in a bundle it should be objectively deducible from the adjacency information in the database. A bundle will consist of a group of processes with the property that all pairs in the group are highly adjacent, but no other process is very adjacent to the group as a whole.

The number of possible groups goes up exponentially with the size of the group, so it is not possible to try every one even with small groups. However there is a branch of multivariate statistics called cluster analysis that is specifically designed to handle this type of problem, and a variant of a standard algorithm from this theory was used to extract clusters of highly mutually adjacent processes that are likely candidates for bundles. The details of this algorithm are given in the appendix, but the final result is a hierarchial set of nested clusters with a measure of the degree of association at each level, which corresponds to an average internal adjacency. Any real clusters, such as the proposed bundles, should stand out as having a high association measure at the level of the group, but not combine well with an external process or group at the next level down. Figure 9.3 shows the bundles detected by the algorithm in the C. elegans database at associations measure cutoffs of 25, 15 and 8. In the case of contralateral homologues, either bundles were seen on both sides, or the same bundle included both homologues.

In order to provide an objective significance criterion for the association measure of a cluster, I used the same algorithm on data from a simulation of the random mixing model described in the last section (with $p = 0.08$). The maximum association measure obtained was 12.75 and less than 10% of the values were greater than 7.5. Thus according to this criterion all the bundles shown in figure 9.3 at an associational level of 15 are likely to be significant, as are most of those at a level of 8, especially when they occur on both sides of the animal.

Since the clustering method is hierarchial and continues to make larger and larger clusters it does generate further amalgamations of the bundles seen in figure 9.3. Although the association measure for such bundles falls below our significance test level there is evidence that some of them are real, primarily because the same groupings are seen for homologous bundles on the two different sides of the same animal. The fact that they have a low association measure implies that they are not true completely mixing bundles, but they may be either super-bundles – bundles of bundles – or cases where processes are shared by several bundles. The suggestion that particular processes might belong to more than one bundle is taken further in the discussion section.

9.3 Discussion

There is some evidence in the database for the presence of reproducible persistent contacts between nerve fibres, both between pairs of neurons and between groups of three or more processes that run together round the ring as sub-bundles within the complete process tract. The largest grouping of neurons which all had fairly high adjacency to each other contained seven cell types (figure 9.3) but most of the likely bundles generated

by cluster analysis of the neighbourhood information contained only two or three cell types. The average number of high adjacency contacts per neuron was also small (2.3).

The analysis presented here suffers from its reliance on identifying specific contacts by unusually high adjacencies. It would therefore miss any important short term contacts, and would also be confused by processes that for half their length are in one part of the neuropil, and for the other half in another part. There is a clear example of such behaviour in the case of the interneuron AIB, which runs near AIA in the proximal part of its trajectory, and near RIB in the distal part (White, 1983). This is consistent with AIB's role as the major linking interneuron between the amphid receptor circuitry and the motor control circuitry (figure 8.2). Such switching of bundles could be used by other processes that carry information between sufficiently different groups of processes.

Another example of the possible presence of sub-bundles in the nervous system is provided by the motor neuron processes in the ventral nerve cord. The VA and VB classes of motor neuron are both bipolar, with an axonal process that produces neuromuscular output for part of its length (the other part neither makes nor receives connections) and also receives some input, and a dendritic process that is purely postsynaptic. All the dendrites run together in one place, under the main motor neurons, while all the axons run in a group against the basement membrane. Although these two groups of processes are adjacent they rarely mix. In addition there are a number of places where a motor neuron commissure cuts across the entire nerve cord; when this happened the commissure usually runs between the dendritic and axonal groups, separating one group from the other, but splitting neither (7/13 cases; in 5/13 a VB dendrite is on the wrong side – in only one case is the axonal bundle split). In this case a general adhesion between like processes may be useful in keeping all the dendrites near their source of inervation, and keeping the axons near the basement membrane, where neuromuscular junctions are made.

There is a strong relationship between the proposed groupings of the neurons into bundles and the circuitry. Figure 9.3 has been organised so as to show the extent to which the bundles are formed from neurons that are near in the processing diagram in figure 8.1, which was obtained purely from connectivity data. However it is by no means true that all persistent pairwise contacts are between neurons that are connected, either by chemical synapses, or by gap junctions (e.g. CEP and URX, or the ventral cord motor neuron bundles). In some cases bundles correspond to parts of the processing modules defined previously on the basis of internal feedback, but they also often contain vertical groupings of neuronal classes from the directional ordering, sometimes with elements from two modules, one of which feeds into the other. Such organisation is to be expected if the main criterion for process placement is to maximise the adjacency of symaptic partners, since the main flow of information is down through the network, across the modules.

The observation that there are a small number of persistent contacts suggests that specific fasciculation mechanisms are significant in the C. elegans nerve ring, and appears to rule out the specification of process position by a general mechanism that acts equivalently on all cells. This is perhaps not surprising in an organism with such a small number of cells, almost all of which are distinct, forming different sets of specific connections. The data

certainly do not allow the prediction of a set of hierarchical forces that could determine position in the nerve ring. There is also the problem present in all the analysis of the database of trying to investigate the underlying mechanisms involved in building a structure (the nerve ring) by looking at the finished product. However, taken together with the evidence for the role of specific fasciculation in embryonic neural outgrowth presented in the first part of this thesis, there are strong grounds for believing that the organisation of the nerve ring may make use of small specific bundles to correctly position processes so that synaptic connections can be made.

APPENDIX

A.1 The statistical test for synapse number correlation with adjacency

All pairs of neurons A,B in the H series were considered for which there was a synaptic connection both from A to B and from A' to B' (A',B' are the contralateral homologues of A,B), but where the adjacency between A and B was different from that between A' and B'. Let S_1 be the number of synapses from A to B, S_2 can be the number from A' to B, a_1 be the adjacency of A and B, and a_2 be the adjacency of A' and B'. Since each set of four is only counted once we can assume that $a_1 > a_2$. The a_i are treated as independent variables (i.e. they do not depend on the s_i), and the s_i are treated as the outcomes of randome variables S_i, which are possibly dependant on the a_i. There are two hypotheses that will be tested: a proportional relationship between S_i and a_i, and independence. More precisely, the proportional model presumes that synapses are made with a certain probability per unit of length of contact. In this case S_i will be Poisson distributed with mean (and variance) proportional to a_i. However the constant of proportionality may differ for different sets of A,B,A',B'. The independent model proposes that the S_i have mean S, independent of a_i, but again possibly different for different sets of neurons.

The test statistic that was used is the sum over all chosen sets of $T = (a_1s_2 – a_2s_1)$.

If S^i is proportional to a^i, then T should have mean value zero. Its variance can be estimated as the sum of the variances of the contributing terms, which are $(a_1^2a_2m + a_2^2a_1m)$ where m is the Poisson rate, best estimated by $(s_1+s_2) / (a_1+a_2)$. This simplifies to being the sum over all the sets of $a_1a_2(s_1+s_2)$.

If S_i is independent of a_i, then T should have mean M, where M is the sum over all the sets of $S.(a_1-a_2)$, where S is the mean number of synapses for each set. The best estimator for S is $(s_1+s_2)/2$. In order to estimate the variance of the differences from the mean, (M-T), we must propose a variance for S_i. (It cannot be estimated because then we would lose all our degrees of freedom). It seems reasonable to assume in this case also that the S_i have a Poisson distribution, or in any case that their variance is approximately the same as their mean, S. Then the estimated variance of (M-T) is the sum over all sets of $S.(a_1+a_2)^2/2$.

To test each hypothesis the difference between T and its expected value under the hypotheses is divided by the standard error (the square root of the estimated variance) to give a normalised error, U. Since we are adding together hundreds of similar terms T should be distributed normally, and so theoretically U has a t-distribution, since we have estimated the variance of T. However, because there are hundreds of degrees of freedom (one for each set), U can be tested as if coming from a standard normal distribution.

In total there were 391 sets. The value of T was 7103. If we assume the proportional hypothesis then the standard error is 1324.3 and U is 5.36 which is very significant. We can therefore reject the proportional model. If we assume the independent model then M is 7655 and the standard error is 1338.0 so U is 0.41, which is not significant. So it is quite possible according to this test that the number of synapses formed is independent of adjacency.

A.2 The sorting algorithm used to order the neural circuitry

The basic method of this algorithm is to start with a random ordered list and repeatedly use a simple rearrangement principle to reduce the overall number of upward synapses. The process stops when this number cannot be improved by a rearrangement of the type under consideration. In general this will not give a true optimum order, because the rearrangement principle is not general enough. However, by repeated application of the algorithm to different starting lists one can get an indication of the distribution of final results. If, as they were in the case under consideration here, the results of these repeated optimisations are very similar, then it is likely that they are near the true minimum. The algorithm was run many times until the lowest value so far had come up repeatedly, at which point it was accepted as the optimum.

The actual rearrangement system chosen in this case is to run through the current list and, for each neuron, determine where in the list it should be placed. If this is different from the current position then it is moved there and the neurons in between are shunted one place back in the list to fill the gap.

A.3 The method used to determine processing depth

This method deals with some notional material (sensory influence) which flows down through the network of connections, moving through a synapse at each time step. Each sensory neuron under consideration is given a unit amount of material at time zero. Then at successive time steps the material is redistributed, all the material in each neuron being divided amongst the neurons that it both synapses to and is above in the ordering. The amount that each postsynaptic cell receives is proportional to the number of synapses made. If there are no postsynaptic partners then the material is lost. Clearly material can reunite that has come via different routes but using the same number of synapses from sensory neurons. The requirement that only downward synapses are permitted prevents problems with cycling.

This method makes the assumptions that the influence of a connection is proportional to the number of synapses it contains, and that influence is neither lost nor amplified, merely passing through neurons and being redistributed at each time step. Both these assumptions are neurobiologically unrealistic, but they are probably the best that can be done with the information available. By keeping track of the distribution of material at each time step one can build up a picture of the distribution of time steps required for influence to reach a specific neuron (muscle can be treated as the final postsynaptic neuron), and also of the proportion of influence from the chosen set of sensory neurons that passes through any particular interneuron, or for instance that reaches head muscle as opposed to body muscle.

A.4 The clustering algorithm used to detect bundles

This is a hierarchical clustering algorithm (see e.g. Seber, 1984). The principle is to identify the two items that are most likely to belong to the same group and to link them together. Then a new distance, or, in our case, adjacency, is defined between this pair and each of the remaining items. One then returns to the first step and looks for the most adjacent pair in the reduced set of items, which will include a combined pseudo-item.

This process of joining the two closest items continues recursively until only one item is left. At each stage a measure of the association of the two items joined together is given by their adjacency, which in general is a combined adjacency.

Different versions of this process vary in the way that the combined adjacency of the merged item to the remaining items is determined. I used a variant of the group average method (Seber, 1984) that was tailored to this particular problem. I kept data on the circumferential zones of the nerve ring in which each process ran (e.g. lower left). This was necessary because it is only possible for two processes to be adjacent to the extent that they run in the same zone. The adjacency between two groups is then defined as the ratio of the total adjacency between their constituent processes to the summed circumferential zone length that they have in common. By keeping the total constituent adjacencies and the summed zonal lengths at each stage these "zonal ratios" can be easily combined when two items are merged. I also prevented the fusion of groups with comparatively small overlaps, because the data for such cases would be correspondingly noisy and if they were to belong to a genuine bundle there would have to be an overlapping intermediate fibre in any case. This zonal ratio system does, however, permit bundles that are longer than some, or even all, of the constituent processes, and this is an important feature of it.

CONCLUSION

The results of the various observations and experiments described in this dissertation have been discussed already in their own sections. Therefore instead of rehashing the same arguments I propose here to consider these results in the light of previous experience with using C. elegans as a model developmental animal, and to speculate in which type of direction future work, particularly on the genetics of neural specification, might take us.

The studies described in both parts of this dissertation have relied on the fact that the C. elegans nervous system is both extremely simple and highly reproducible, so that information can be gained from a comparatively small amount of data. However there is also a possible penalty to be paid in studying an organism with a very small number of cells, all of which are reproducible from individual to individual. These properties potentially permit structures to be put together piecemeal by some form of internal program specific to each part, rather than by general mechanisms.

The initial reason for attempting a computer database analysis of the synapse and connectivity data was to attempt to find internal logical patterns in the connectivity data which might allow rules to be proposed for specifying which cells connected to which, for instance by placing the neurons in possibly overlapping "super-classes" that might have common recognition properties, so that if two cells were in compatible classes and also in contact then they would form a connection. There are examples of pairs or groups of cells that are in different places and make mostly different connections, but which make similar connections to cells that they both contact, and which share other properties in common (White et al., 1983). However an overall search for such grouping reveals nothing that is statistically significant. One possible problem that may be important is that regional specialisation of neurons, as discussed in Chapter 7, would create complications in any search for classes of neurons with equivalent synaptic potential. This does not mean that label receptor matching systems for determining synaptic connectivity do not exist, but merely that there are too few cells and there is too much variation to deduce them from the final connectivity data.

A similar observation was made when the complete cell lineage was determined, which is more reproducible than the nervous system. Although there are a few suggestive repeated motifs, the overall arrangement of which precursors produce which cells is essentially haphazard and mosaic, correlating as much with position as with pattern in the lineage (Sulston, 1983). This could be taken to indicate that external interactions with extracellular environment were important in determining cell fate, but abalation experiments largely revealed no effect on adjacent cells (Sulston and White, 1980, Sulston et al., 1983). Overall this suggests intrinsic programming, but it has an advantage for the study of intercellular determination, which is that those instances where specific cell interaction is important, of which there are a number of clear examples (Sulston and White, 1980), may be comparatively isolated. A number of the cell lineage mutants that have been obtained affect situations where induction or regulation takes place (Sternberg and Horvitz, 1984), and these may provide an excellent tool to study specific determinitive cell interactions during development in vivo. One particular gene of this type has recently been cloned and sequenced, and its protein sequence has homology to a

family of extacellular proteins including growth factors and their receptors (Greenwald, 1985). Indeed there is an argument that clean developmental switch genes, which cause the change of cell fate from one type to another, will often be associated with inductive or regulative situations: a defect in a single component of an extracellular signalling pathway, such as the signal or the receptor, would cause an effective loss of signal, while internal choice determination may be a complex activity requiring many components simultaneously at each stage, and with no clear default behaviour. Having obtained one of the components for an interactive mechanism via a mutant, one then has a genetic handle on the subsequent parts of the mechanism.

The relative positioning of neuronal processes is much more complex than that of most other types of cells, and it must be expected that a large amount of intracellular interaction is required for process positioning and synapse formation. However much of this may be non-specific. As with the lineage ablation studies, the ablation experiments described in Chapter 4 in general had remarkably little effect on other cells. The DD3/5, DVC and PVPL removal experiments showed no immediate effect on guidance of other neurons at all. As discussed in Chapter 5 there are already mutants affecting process guidance in various ways. There are also mutants known that affect synaptic connectivity in the ventral and dorsal nerve cords in a way that can be interpreted as switching the specificity of certain cells from one type to another (J. White, L. Nawrocki, personal communication). It is possible that some of these mutants may also affect comparatively isolated determinative intercellular interactions, which may provide models for similar interactions in more complex animals. Even if not they may still reveal interesting mechanisms involved in specific guidance and synaptic connectivity. However, by itself, genetics can be problematical because it may be hard to determine what one is studying. It is ultimately in combining genetics with the detailed and specific anatomical observations and experiments that are possible in such a simple organism that I believe C. elegans has most to offer development neuroscience. If I were to continue working with C. elegans I would investigate the early anatomical development of some of the guidance mutants and follow up the molecular and genetic opportunities they generate.

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