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).

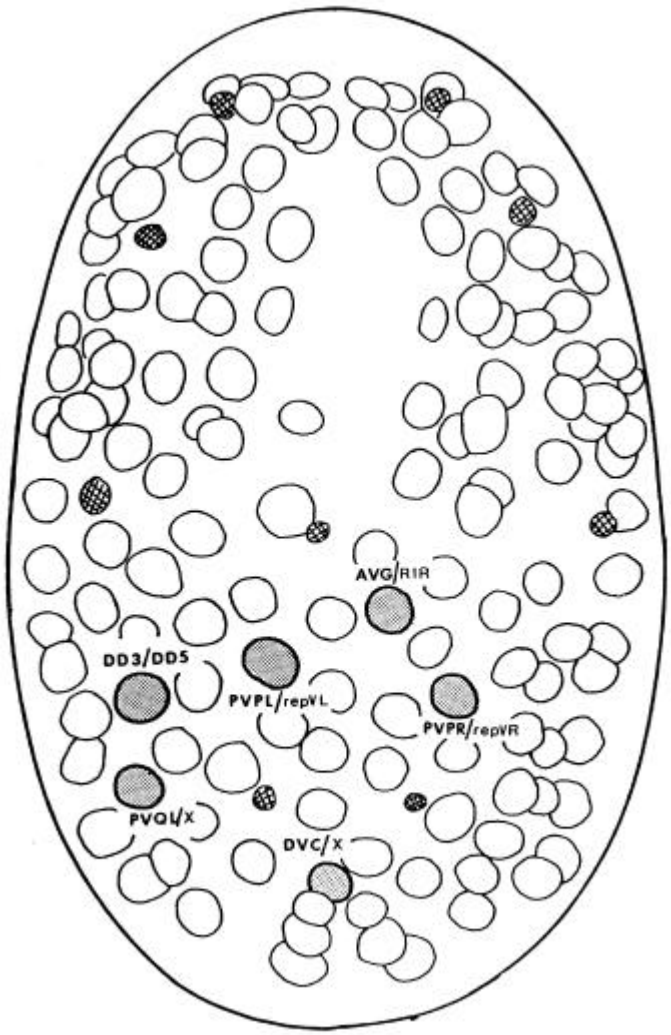
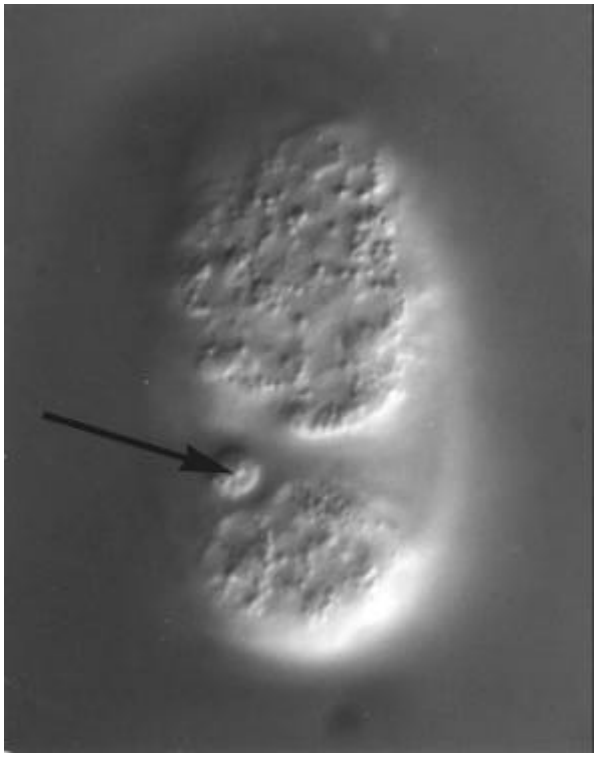


Figure 2.2

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.