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.

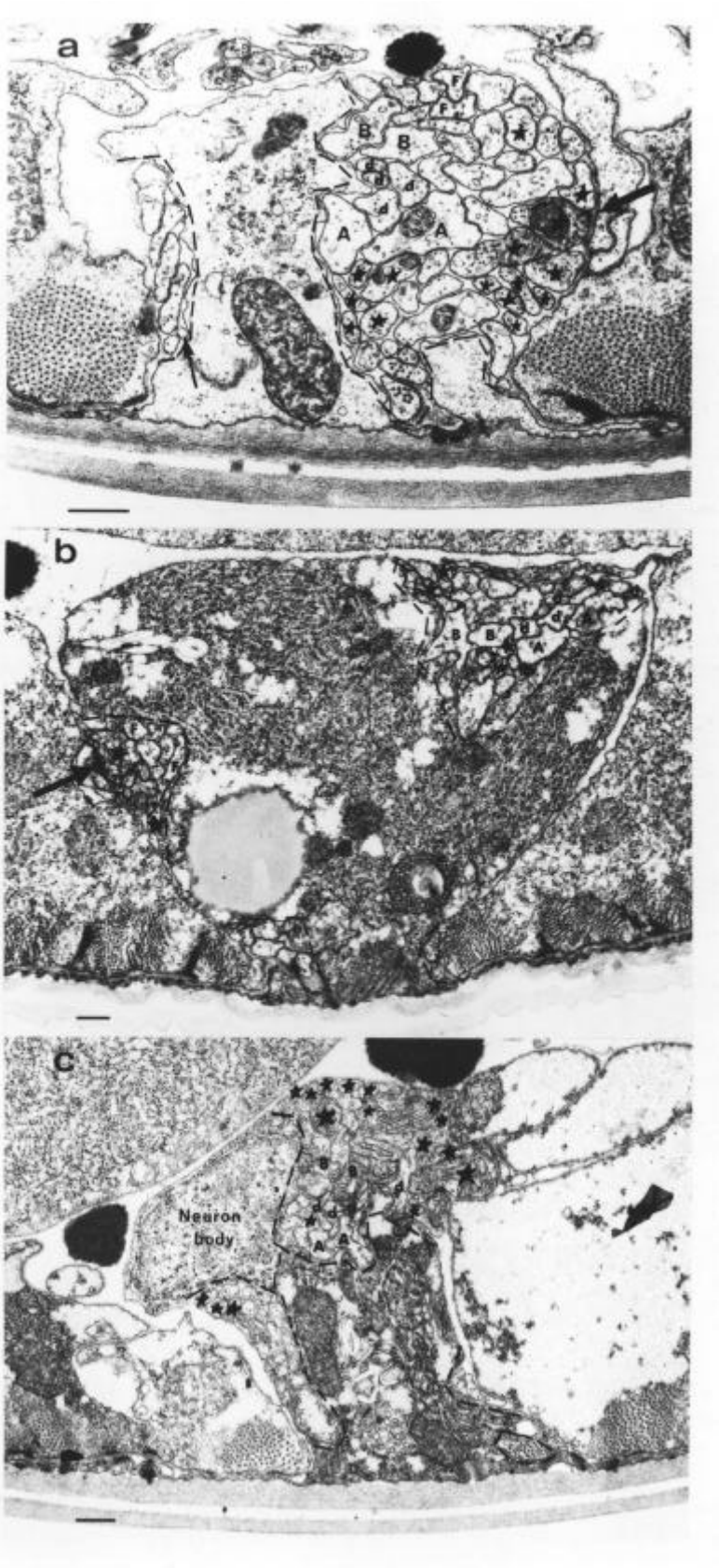

a
b
c
Neuron body

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.

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).

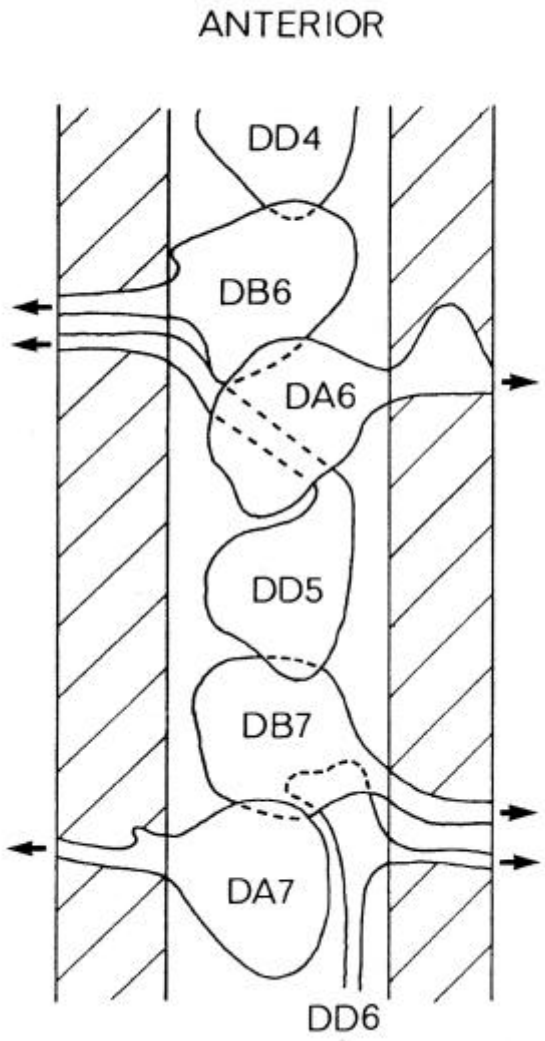
Several of the motor neurons are disrupted. These are motor neuron cell bodies associated with both sides of the cord (VD2 and DB3 are to the left), and also motor neuron processes on both sides (all of VD3 and parts of DB3, VD2 and VB2 processes are on the left). In addition DB3 is lacking a commissure, 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).

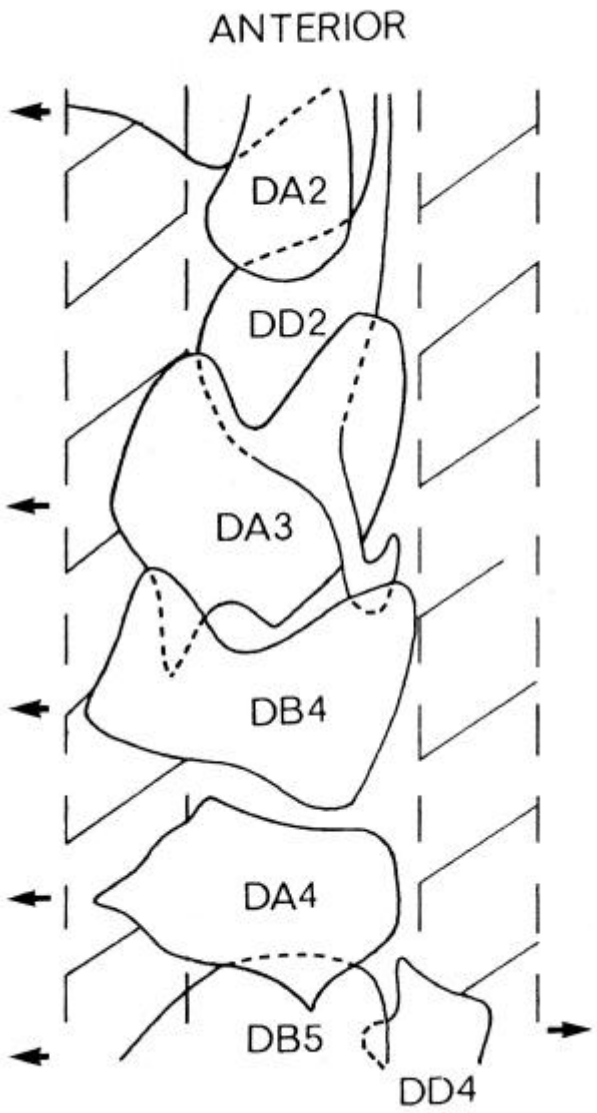


Although the AVG process was clearly missing, there was no other alteration to the organisation of the PAG and the early posterior interneurons that grow forward along the cord from it (the PVP's, PVQ's DVA and DVC), every process following its normal trajectory. However, as in the adult, the ventral 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 Abprpppa) 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.

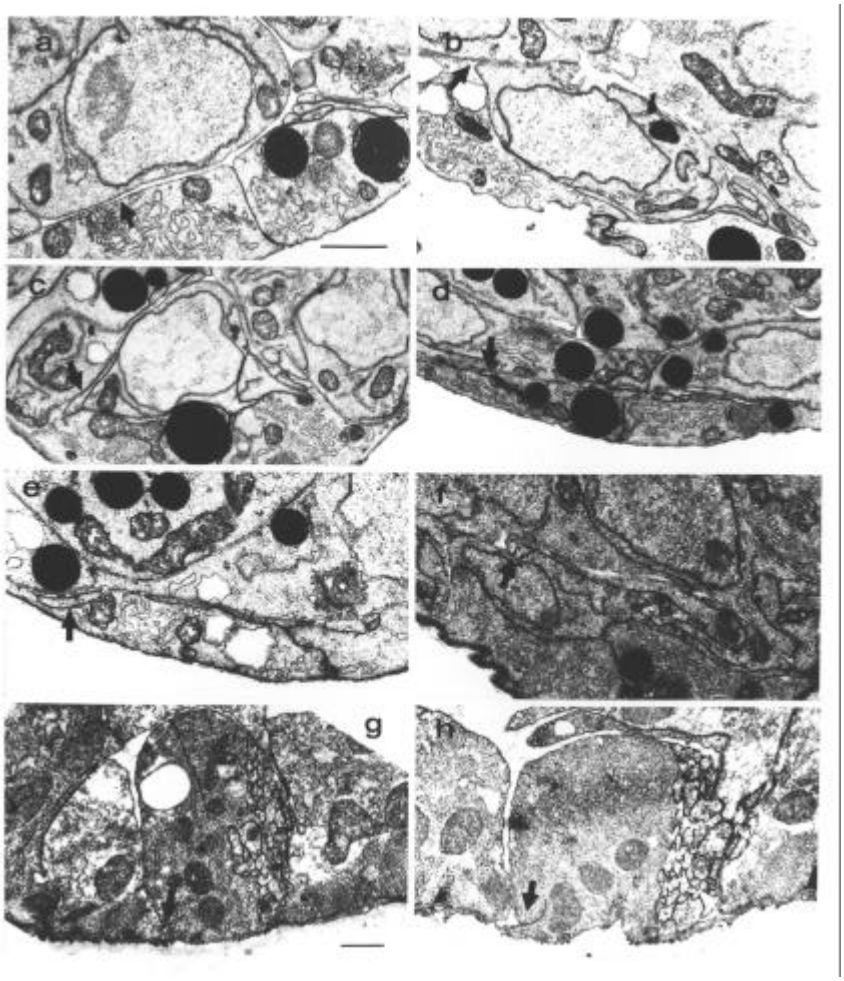
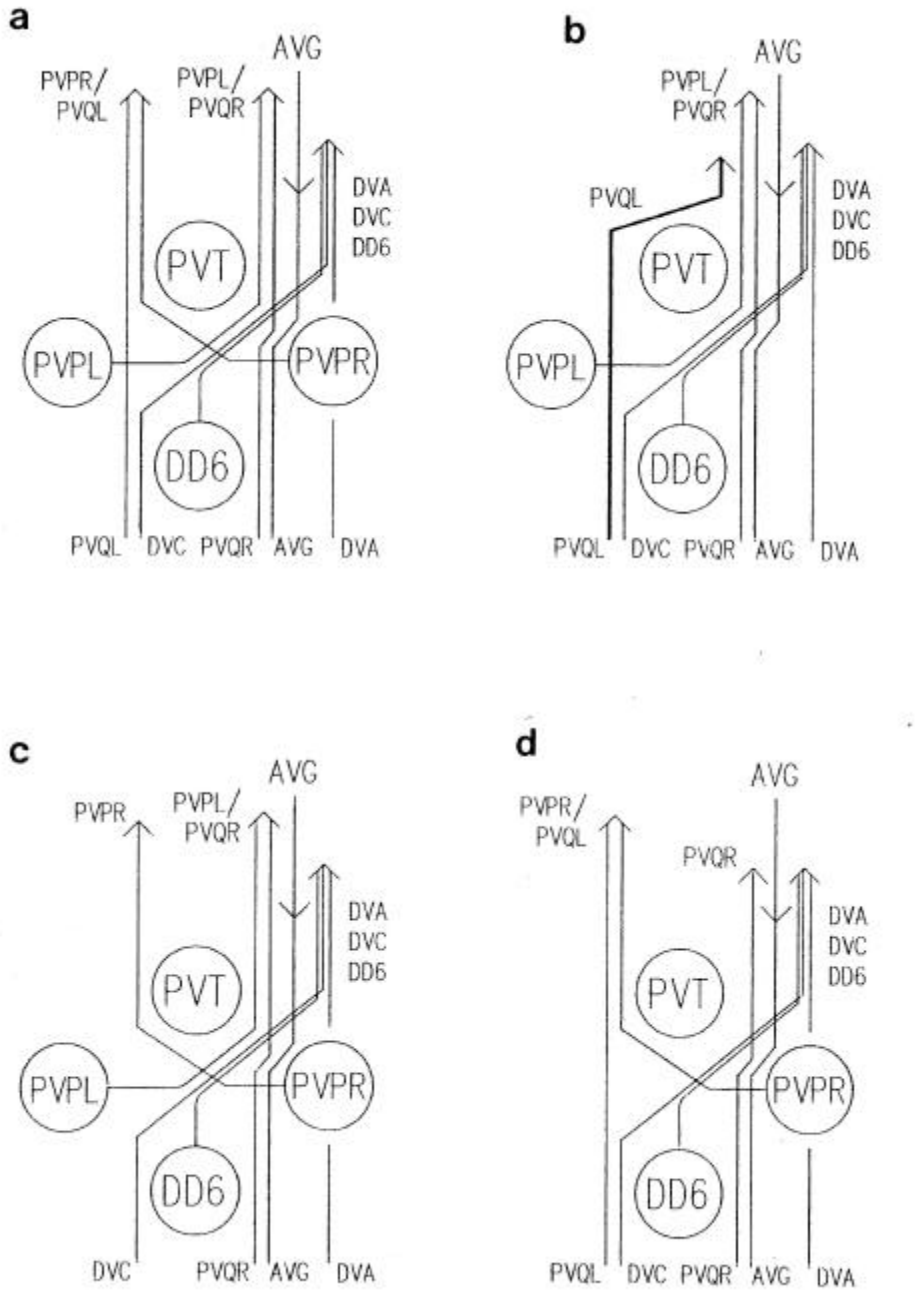


Figure 4.5

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.

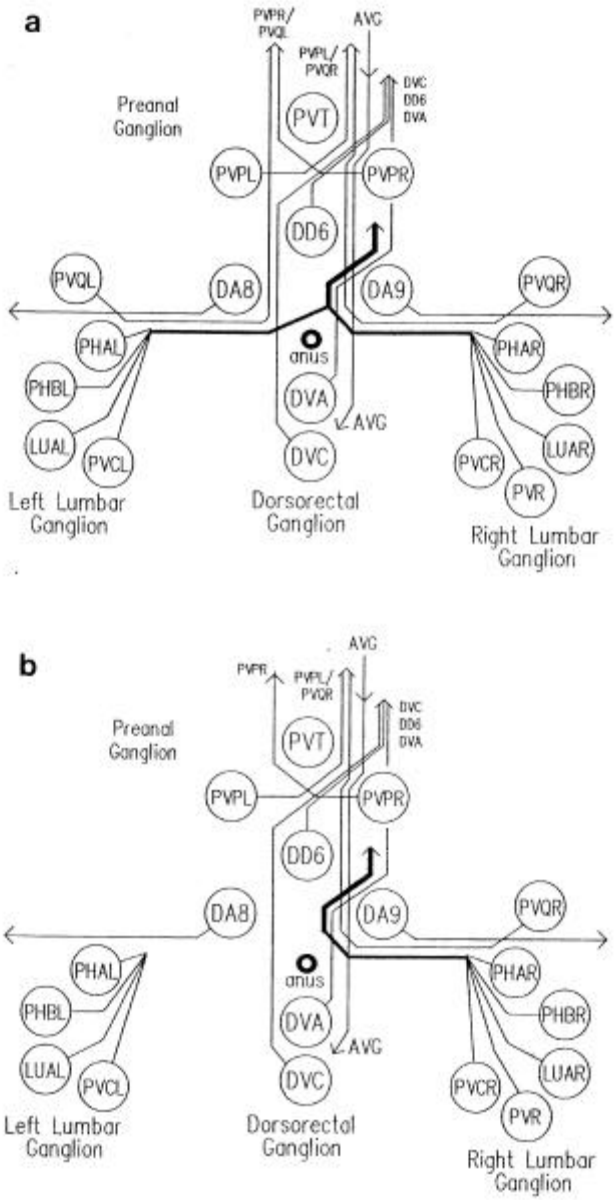


Figure 4.7

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.