Frontal eye circuitry, rostral sensory pathways and brain organization in amphioxus larvae: evidence from 3D reconstructions

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CONTENTS

SUMMARY

The cells comprising the frontal eye of a 12.5 day amphioxus larva are described based on 3D reconstructions from serial electron micrographs, along with the fibre tracts and more caudal groupings of cells in the nerve cord to which the frontal eye appears to be linked. The frontal eye consists of a pigment cup, two transverse rows of receptor cells, and clusters of neurons whose close association with the medial receptor cells suggests they may function as an integral part of the eye complex. Neurites from both the receptor cells and neurons supply the ventrolateral nerve tracts, which consist mainly of axons arising from sensory cells located at the rostral tip of the larva. A core group of 3–4 rostral fibres on each side innervate two ventral giant cells located just behind the cerebral vesicle in the primary motor centre (PMC). The circuitry suggests these cells may be responsible for triggering the larval startle response. The ventrolateral tracts also include two types of axial dendrite-like fibres: (i) a single unpaired fibre, a forward continuation of the principal dendrite of the left giant cell, which is the main target for synapses from neurons in the frontal eye; and (ii) sets of paired fibres from cells in the tectum, a dorsal cortex-like structure located at the back of the cerebral vesicle through which the dorsal sensory nerves pass in transit to the PMC.

Recent behavioural studies show that larvae feed in a hovering posture that maximally shades the frontal eye. They also orient to light in this position. The shape and orientation of the frontal eye suggests it could be responsible for this response. The existence of separate pathways from lateral and medial receptor cells, both directly and indirectly to the PMC, suggests the frontal eye may also be involved in modulating locomotory behaviour during hovering.

The visual 'system' described here for amphioxus larvae is more like that of vertebrates than has previously been recognized. Specifically: (*i*) the medial nerve cells of the frontal eye appear to form local circuits with relay and integrative functions similar to those of the retina, involving cell types that resemble specific retinal interneurons; and (ii) output is directed to a region at the back of the posterior c.v. that resembles the vertebrate midbrain, and which may be its homologue. This region has a dorsal tectum and, like the midbrain, includes the anterior part of a ventral zone of motoneurons and reticulospinal interneurons. The morphological evidence supports the idea that the 'brain' of amphioxus is sufficiently like that of vertebrates to provide important clues concerning the basic organization and subdivision of the vertebrate brain.

1. INTRODUCTION

Amphioxus is a key organism from an phylogenetic perspective. Of living chordates, it is probably the closest to the ancestral form from which vertebrates arose and, as such, is a key source of information concerning the nature of the earliest vertebrates (Bone 1960*a*, Jollie 1973, Gans 1989). Its nervous system is of special interest, especially to those using molecular techniques to study chordate relationships and evolution, because of the number and variety of evolutionarily conserved genes that are expressed specifically in nervous tissue (e.g. Holland 1992). Our knowledge of the basic organization and cell types of the amphioxus nervous system is still very limited, however. In a recent report, based on a serial electron microscopical (EM) study, Lacalli *et al.* (1994) describe some of the main anatomical landmarks and distinctive cell types in the cerebral vesicle, a specialized anterior part of the cord that shares a number of features with the vertebrate diencephalon. Among these is the frontal eye, a putative photoreceptor lying at the anterior tip of the cord. Based on its position, which corresponds with that of the eye rudiment in vertebrate embryos, and its structure, the frontal eye could be homologous with the lateral paired eyes of vertebrates, representing either a primitive undivided stage in eye evolution or a medial structure that has arisen secondarily by fusion. Lacalli *et al.* (1994) described the frontal eye in general terms, but lacked detailed information on its cells' neurites, their patterns of connectivity and ultimate targets. This paper remedies this with a more thorough analysis, using higher power micrographs, of both the frontal eye and the fibre tracts and cell clusters to which it appears to be linked. The results (§3 and 4*a*) provide a much clearer understanding of the overall organization and functional pathways of the cerebral vesicle and the region located immediately behind it, the primary motor centre, which is a distinctive zone of motor neurons and interneurons that probably has a major role in controlling larval locomotion. The data provide additional support for the idea that the frontal eye and the vertebrate retina are related by homology, and further, that amphioxus possesses a rudimentary midbrain with functions and organization related to that of vertebrates. The role the various cells and pathways may play in larval behaviour is discussed in §4*b*; the question of homologies between amphioxus 'brain' and that of vertebrates is discussed in §4*c*.

2. METHODS

This paper deals with *Branchiostoma floridae* larvae at the 3–4 gill slit stage, from a 12.5 day culture, and extends the results reported by Lacalli *et al.* (1994). Methods for collection, culture and microscopy are as previously described. The earlier study was based on four larvae, and featured reconstructions of the cerebral vesicle from a complete serial series obtained from one of these (specimen 4), extending from just behind the anterior tip of the nerve cord through most of somite (myotome) 2. This same region was examined in section series taken at intervals through two larvae collected from the plankton, equivalent to 6 and 8d stages in culture (specimens 2 and 3, sectioned at 2.5 and 4 μm intervals, respectively). For this study, the frontal eye regions of two additional 12.5d specimens were sectioned to about the midpoint of the anterior c.v., one at an interval of 0.5 μm (specimen 5), and one serially (specimen 6), including selected parts of the rostrum. The three specimens from culture were equivalent in age, but differed slightly in stage, specimen 6 being the most advanced; it was 0.05 mm longer than the other two and it alone had a visible rudiment of gill slit 5.

Detailed 3D analysis and reconstruction was carried out only on specimen 4. The data reported are derived principally from this specimen, with the main features and landmarks being checked against other specimens wherever possible. In the frontal eye region, most features could be checked directly between specimens 4, 5 and 6. The medial neurons were the only features showing substantial variability both within and between specimens. The tectum and PMC data was checked against the equivalent region of 6 and 8d specimens. In the case of the PMC, the latter have comparable clusters of large cells, but cell identity could not be established with certainty without complete series. The whole region is highly structured, however, with cells arranged in symmetrical pairs, so left and right sides could be compared in each specimen, which provides a useful internal check.

Reconstructions were prepared using Skandha, a software package developed at the University of Washington by Dr J. W. Prothero and collegues, and a Silicon Graphics Indigo workstation. The images previously published by Lacalli *et al.* (1994) derive from a micrograph series taken at 3800–4200x. This is sufficient for tracing cell outlines and large fibres, but not the small neurites encountered throughout the frontal eye region. For the present study, the original micrograph series was augmented with a series taken at 5200–7000x, which is significantly better for tracing small fibres. The computer images were generated using selected sections, typically 1 in 5, which reduces digitizing and computing time at the cost of some detail.

3. RESULTS

(*a*) *Overview: cerebral vesicle organization*

The cerebral vesicle (c.v.) in amphioxus is the slightly enlarged anterior region of the nerve cord, extending ca. 80 μm from the tip of the cord in young larvae (figure 1). Figure 2 shows a survey view of this region at 12.5 days. Briefly, the cerebral vesicle consists of an anterior tubular portion with a cylindrical central canal, and a posterior portion more like the rest of the cord, with a narrow central canal shaped like an inverted keyhole, and a floorplate. A ventral cluster of secretory infundibular cells marks the junction between these two zones. The only feature of the anterior c.v. visible externally is the cup-shaped pigment spot, located at its anterior tip, which is part of the frontal eye complex. The central canal of the anterior c.v. is

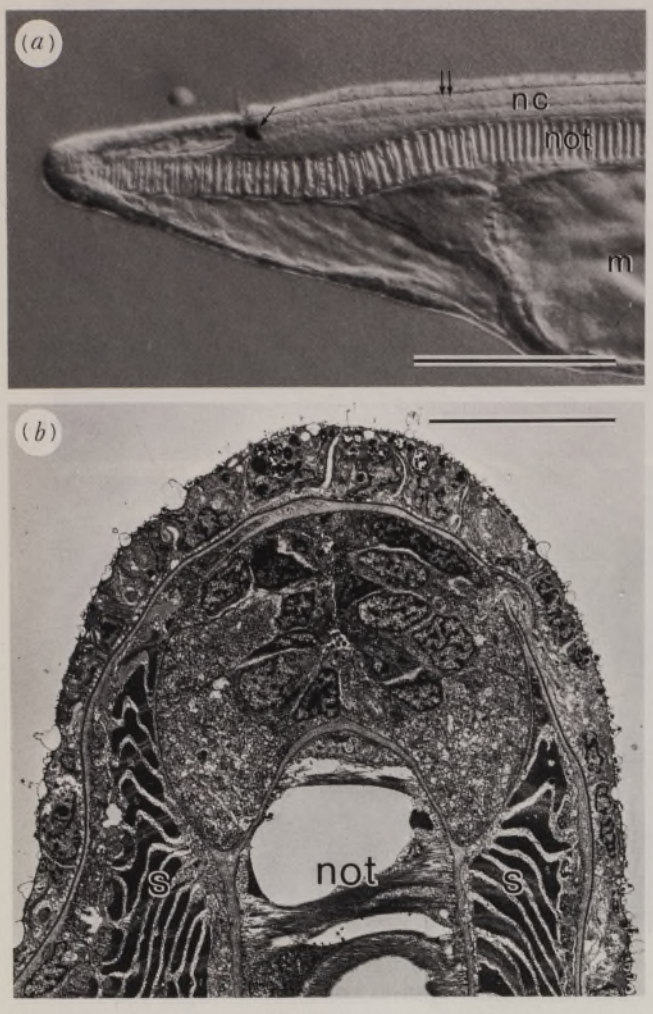


Figure 1. *Branchiostoma floridae* larvae. (*a*) Anterior end of a 14d larva. The notochord (not) extends to the tip of the rostrum. The pigment cup belonging to the frontal eye, indicated by the single arrow, marks the anterior tip of the nerve cord (nc); the mouth (m) lies on the left side of the larva out of the plane of focus. Somite boundaries are not visible in this preparation; double arrows indicate the approximate region of overlap between the first and second somite (i.e. myotomes 1 and 2), which is where the PMC giant cells are located. Scale bar = 100 μm. (*b*) Transverse section thought the nerve cord of a 12.5d larva at approximately the middle of somite 2, showing the relative positions of the nerve cord, notochord (not) and somites (s). Scale bar = 10 μm.

filled with cilia (figure 3), and opens anteriorly through a neuropore. Its floor is thickened, and cells there are arranged in a close-packed columnar array. Its roof is thinner and considerably less developed. The posterior c.v. is dominated by the dorsal lamellar body, a large photoreceptor consisting of lamellae arising from the cilia of cells ranged along its sides, and a ventral commissure, the only large commissure in the anterior nerve cord at this stage.

Based on the results of work now in progress, three further features should be added to those just described: a structure provisionally interpreted here as a balance organ, a rudimentary tectum, and the primary motor centre (PMC).

(*i*) *Balance organ*

The main feature that identifies this structure is a distinctive array of cells with unusually large, swollen cilia (figures 3 and 4). These form a transverse row at the very back of the anterior c.v. just in front of the infundibular cells (figure 5). The ciliary axoneme is replaced by a uniformly dense matrix. The cells themselves are dense, elongate, and have delicate lateral folds that form contacts with adjacent neurons. The basal portion of the entire complex is embedded in the anterior face of the ventral commissure. The unusual appearance of the cilia suggests that the cells may be involved in either motion or gravity detection. Both could be detected in principle by monitoring relative displacement of structures of different density, and cilia could be modified to achieve this by altering their contents. Tunicate larvae have an otolith that functions as a balance organ. It would not be surprising if amphioxus had a similar organ for detecting motion or gravity, and this would help explain some features of amphioxus behaviour, including its hovering posture (Stokes & Holland 1995*b*) and the diurnal migrations observed in some species (Wickstead 1975).

(*ii*) *Tectum*

This is a dorsal region at the back of the posterior c.v. that features, on either side, a row of distinctive small nerve cells referred to here as tectal cells. Fibres from the dorsal sensory nerves enter the tectum and form a series of glomerular synaptic zones within it. The term tectum is used in part as a positional reference, because the tectal zone essentially forms the roof of the cord on either side, and also in recognition of the possible relation between this region and the optic tectum in vertebrates (§4c).

(*iii*) *The primary motor centre*

The PMC is a zone of large ventral neurons, arranged mostly in pairs, that begins just behind the lamellar body and extends for about 30 μm. Although the presence of equally large cells elsewhere in the cord at 12.5 days cannot be ruled out, the data from 6 and 8d larvae suggests this is unlikely. In the latter, the PMC was the only site in the first seven somites where symmetrical pairs of large neurons were encountered in such quantity. The PMC at 12.5 days includes both interneurons, of which the six largest are described in §3f, and the anterior-most sets of motor neurons. Among the latter are the cells that innervate somites 1 and 2, as well blocks of more posterior somites. The PMC appears therefore to be a locomotory control centre of some importance; it is probably responsible for initiating the larval startle response. It is not clear whether it is best considered a part of the cerebral vesicle, or the anterior-most part of the nerve cord proper.

This study began as an examination of the frontal eye region. As it became clear that output from the frontal eye was directed to the ventrolateral tracts, the study was extended to an examination of the source of the main fibres in these tracts and their targets. The results are discussed in detail in the sections that follow, and summarized in figure 31. In brief, most of the fibres in the ventrolateral tracts derive from the rostral nerves (§3b), which enter the front of the cord. Among

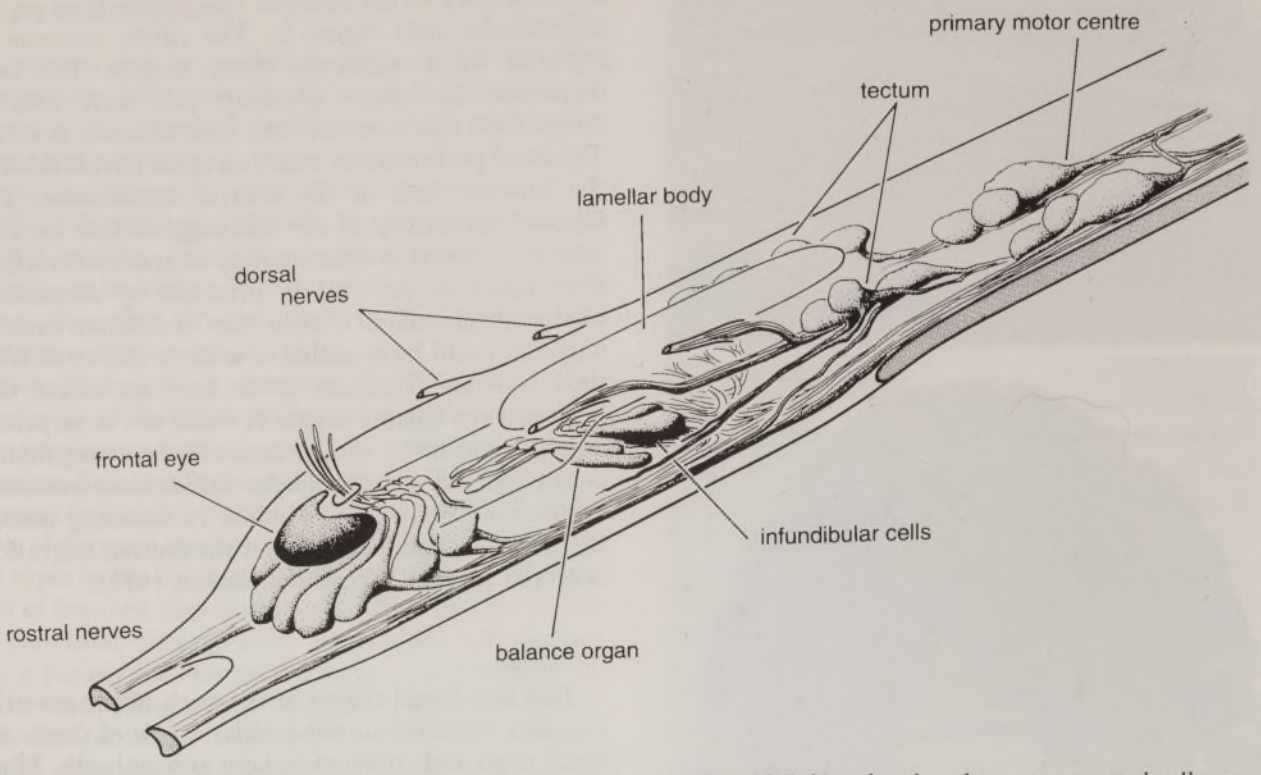


Figure 2. Survey view of the anterior end of the larval nerve cord at 12.5 days showing the structures and cell types dealt with in this account. The slightly swollen region at the front, traditionally referred to as the cerebral vesicle, should probably be thought of as extending at least to the back of the lamellar body. Behind this lies the primary motor centre, which is clearly an important control centre. These two regions together appear to represent what is effectively the 'brain' of the larva at this stage.

the rostral fibres there are, on each side, 3–4 core fibres that provide a direct link between the rostrum and the ventral giant cells located in the PMC. In addition, a small number of axial dendrite-like fibres were encountered in the ventrolateral tracts, providing a pathway via which output from the frontal eye could be transmitted to cells positioned more caudally, in the tectum (§3e) and the PMC (§3f). The connection to the PMC is asymmetric, occurring on the left side only. This is one of a number of consistent but minor asymmetries encountered in the nerve cord, which is nevertheless more symmetrical than the larval head as a whole. The latter is markedly asymmetrical, e.g. in the positioning of the mouth and gill slits, which are lateral and on opposite sides of the body.

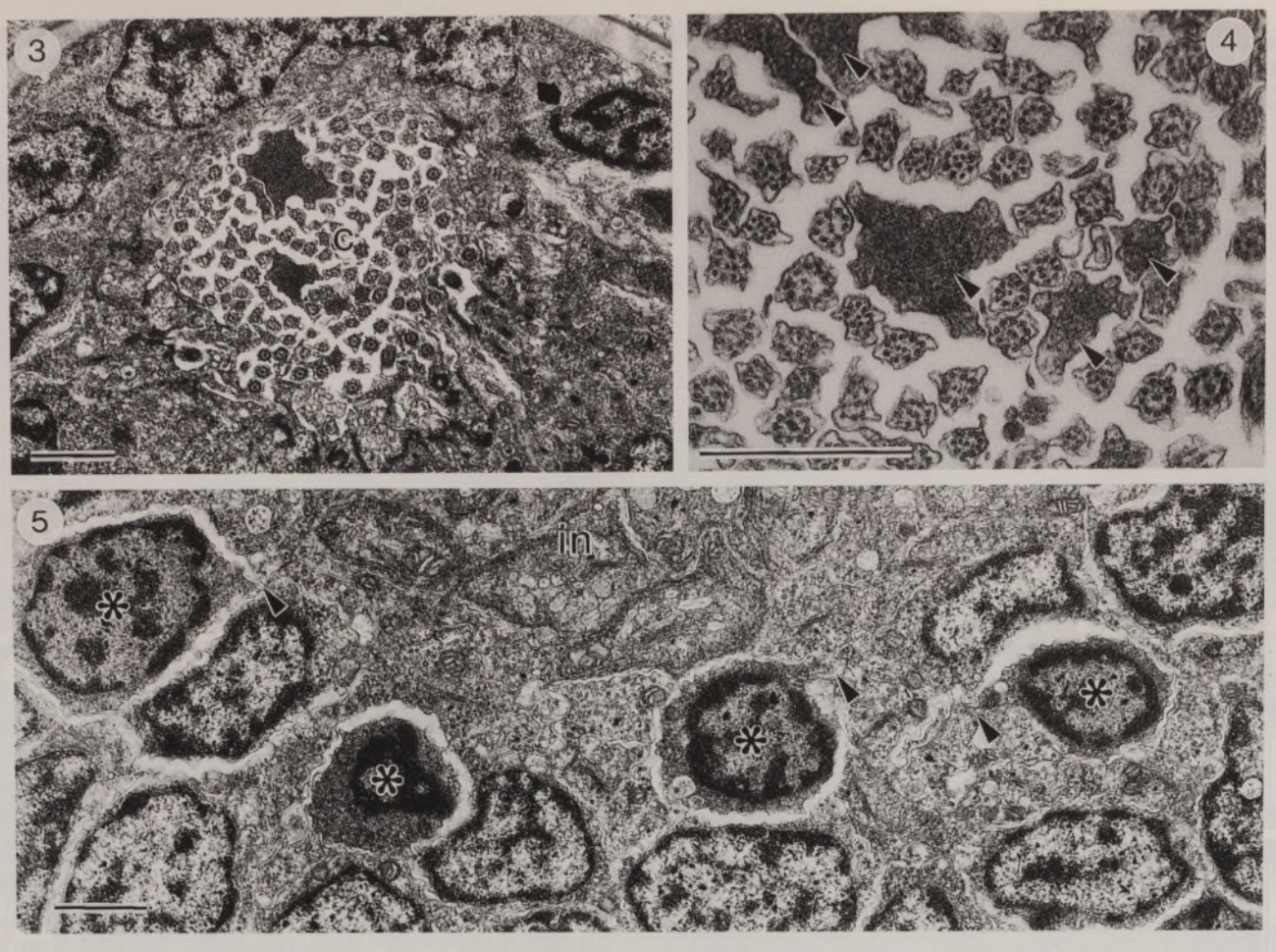
There are obvious limitations to using ultrastructural data and cell morphology alone to distinguish neuronal cell types or make inferences concerning their probable function. This is less of a problem for cells that resemble conventional neurons in having, for example, identifiable dendrites and axons. Many amphioxus neurons lack these, however, especially in the anterior c.v., where the majority of contacts between cells are also morphologically rather unspecialized. This makes interpretation difficult. Neurons in the anterior c.v. also have cilia, which makes it difficult to rule out the possibility that some or all of them act as primary sensory cells. Figures 6–8 show examples of anterior c.v. neurons. There are two notable features.

1. The rootlet complex appears to act as an organizing centre. Rootlets and golgi are closely associated, and the rootlet probably provides structural support to the upright, apical part of the cell. Axons typically arise from a specialized basal endfoot near the base of the rootlet, whereas the nucleus may be displaced some distance by the pressure of surrounding cells.

2. The cells have a variety of neurite-like processes, among which are a considerable number of subapical ones. These arise near the cell apex just below the apical belt of desmosomes (figure 8) and form small plexus-like zones that evidently enable adjacent cells to make reciprocal contacts. This may provide a way of linking cells of similar type as functional units. None of the neurites formed by neurons in the anterior c.v. seem to travel great distances. The majority of cells in this region are thus multipolar, and irregular local plexes are more common than organized fibre tracts.

(*b*) *Rostral nerves*

The rostral nerves (figure 11) originate at the tip of the rostrum in clusters of sensory cells. The majority of these form two clusters, each of 8–10 cells (figure 9). Both clusters lie on the left side, near the tip of the rostrum, one behind the other. The front cluster connects to the right rostral nerve, which crosses from right to left in front of notochord; the back cluster connects to the left rostral nerve. The cells in these clusters are uniciliate, and their cilia emerge together. Individual examples of a second type of sensory cell were found nearby, with cilia sunken in a distinct pit surrounded by a ring of microvilli (figure 10). There is some disagreement as to how epithelial sensory cells in



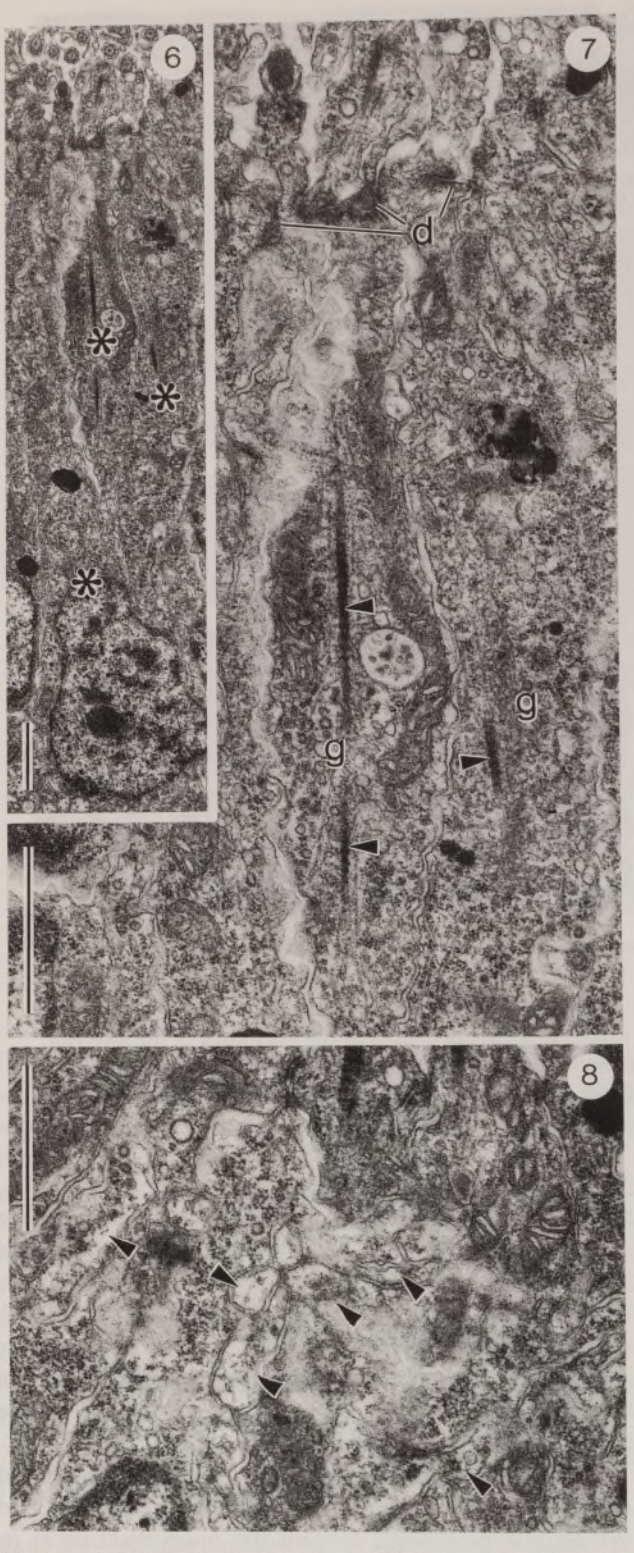
Figures 3-5. Cells with specialized cilia, provisionally interpreted here as belonging to a balance organ. Scale bars = 1 μm. Figure 3. A section through the central canal (c) of the nerve cord at about the midpoint of the anterior c.v. Enlarged, matrix-filled cilia are visible among more typical ciliary profiles. Figure 4. Detail of figure 3; arrows indicate examples of matrix-filled cilia. The six largest can be traced to an array of cells at the very back of the anterior c.v. Figure 5. A section near the back of the anterior c.v., at the level of the infundibular cells (in), showing four of the six dense cells (*) to which the enlarged cilia in figure 4 belong. Arrows indicate examples of the lateral folds by which the dense cells contact the subapical surfaces of surrounding nerve-like cells.

amphioxus should be classified (e.g. see Stokes & Holland 1995*a*), and examples of both both primary and secondary sensory cells have been identified (Bone & Best 1978; Baatrup 1981). The sunken pit cells in figure 10 resemble Type I cells reported by Stokes & Holland, and judging from their numbers, it is possible they are the source for the rostral core fibres. The terminal portions of the rostral nerves were not traced in detail in this study, and there are irregular blind-ending neurites throughout the rostral region whose origin could not be determined. These could be efferent fibres from cells located in the cord, or they may be part of a peripheral network of fibres arising from one or both types of rostral sensory cell.

The rostral nerves contribute 12–15 small fibres to each of the ventrolateral nerve tracts. Many of these terminate as the tracts pass through the anterior c.v., but a core of 3–4 fibres remains (figure 14) that can be traced to the back of the posterior c.v. (figure 29), where they terminate in synapses with dendrites from the two giant cells (figure 31). Additional fibres from nearby cells are added progressively to the rostral nerve during its transit through the anterior c.v. Most originate from medially positioned cells, including the receptor cells and neurons of the frontal eye, and accumulate near the top of the nerve and along its sides. Fibres from the more laterally positioned receptor cells course along the sides of the cord and enter laterally. As more processes are added, a series of layers are formed around the nerve (cf. figures 12, 13). A sizable plexus is eventually built up, but it has little internal structure. Irregularly organized plexes of unspecialized fibres seem to be characteristic of the anterior c.v. at this stage.

The largest vesicle-containing terminals associated with the nerve belong to the receptor cells in row 1, and are usually positioned around the main tract or at its sides. The terminals are spacious, and contain scattered vesicles of diverse types (figures 12, 13).

Three other distinctive fibres were encountered in the ventrolateral tracts. They are clearly identifiable in both specimens 4 and 6, tentatively so in specimen 5, and could be traced, in specimen 4, to more caudal cells. The largest of the three is an anterior prolongation of the principal dendrite of the left giant cell (figure 17*a*, 25). It lies on the medial margin of the rostral nerve on the left side and is recognizable by its large size, and the large number of specific contacts and synapses it receives (figures 18e, f and 30). In both specimens 4 and 6, the fibre crosses to the right side before terminating. In specimen 4 this occurs beneath row 3 in the first of the three small commissures



Figures 6–8. Typical ventral midline neurons from the anterior c.v. Scale bars = 1 μm. Figure 6. A survey view; three neurons (*) are cut in oblique section, two through the rootlet complex. Figure 7. Detail of the cells in figure 6; arrows indicate ciliary rootlets with which the golgi complex (g) is invariably associated. A zone of desmosomes (d) links the cells near their apices; the cilia are typically supported on apical knobs that extend somewhat beyond this zone into the central canal. Figure 8. A second view of the subapical region of the cells shown in figures 6 and 7, taken several sections away. This section passes through clusters of subapical processes that make local contacts between cells. Arrows indicate examples; many contain vesicles.

reported by Lacalli *et al.* (1994) from this specimen. In specimen 6, the fibre crosses at the very front of the cord.

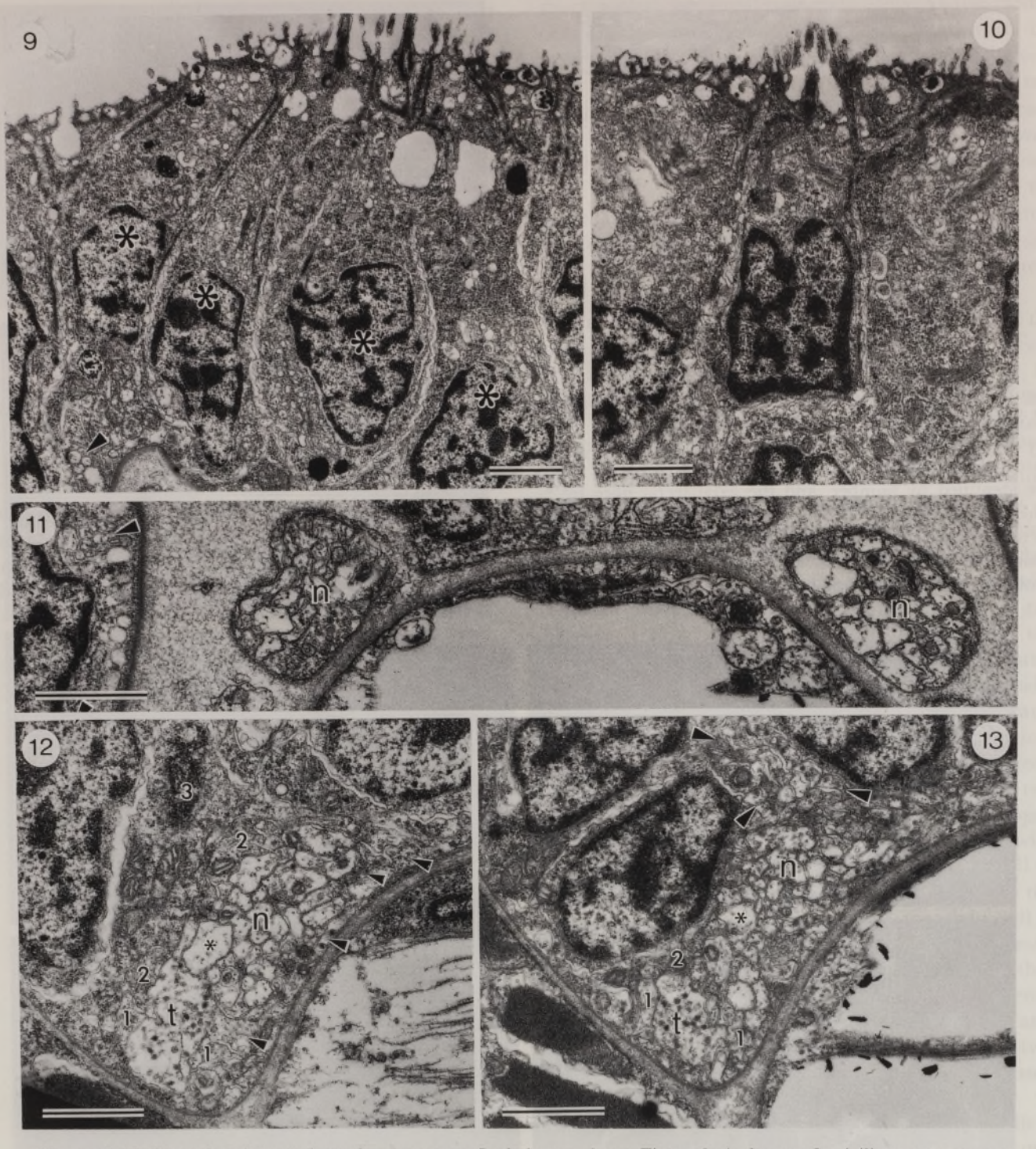
The two other fibres, one on each side, belong to the ventral-most of the tectal cells on each side (* in figures 17*a*, 20*a*). The fibres pass out the front of the cord in the rostral nerve. They appear to be dendrites: the main terminals formed by the tectal cells are clustered near the cell body, and no other neurites could be identified as potential sites of synaptic input. The other tectal cells all have similar fibres that run forward along the sides of the cord above the level of the ventrolateral tracts. These gradually approach the tracts, and finally join them near the front of the cord. Tracing has not shown how far forward the fibres travel once they have left the cord.

(*c*) *Frontal eye: receptor cells*

The frontal eye develops from cells at the very front of the neural plate. The receptor cells form two well defined transverse rows immediately behind the lip of the pigment cup (figures 15, 16). Their cilia exit the neuropore and terminate in slight swellings. The bases of the cilia are shielded by the sides of the cup. The cells themselves are simple in outline and flask-shaped. They lack the subapical processes and lateral contacts seen in nearby neurons. Each has a single basal fibre, usually with a core of microtubules. Judging from the appearance of their golgi, row 1 cells are functionally more active than row 2 cells. The row 1 fibres are also larger and have large terminals; the smaller row 2 fibres lack obvious terminals.

Six row 1 cells were identified in each of specimens 4 and 6; three on each side, symmetrically positioned with respect to the ventral midline. Only five are shown in the reconstructions because one was initially missed (it lies in the gap visible just to the right of the midline in the middle panel, figure 15). The two medial row 1 cells lie just above the rostral nerves at their point of entry into the cord. This means their fibres can enter the ventrolateral tracts directly from above. Fibres arising from the more lateral cells have to travel along the sides of the cord, approaching the tracts gradually, before entering. Large terminals erupt at intervals from the row 1 fibres. These are long and irregular in outline; they travel along the ventrolateral tracts, usually along the outside, collateral with their parent fibre. They contain scattered vesicles of diverse types (figures 12, 13), both large and small clear vesicles and dense-core granules. Contacts are formed with the axial tectal fibres and core rostral fibres at various points, but specialized junctions, e.g. synapses, were not observed. The total extent of the row 1 terminals was not determined, but they appear to be most numerous and best developed near the front of the anterior c.v.

There were ten row 2 cells in specimen 4, and about the same number in specimen 6. The basal fibres from row 2 are slender, relatively short, and terminate without forming obvious terminals. Dense-core granules occur irregularly along these fibres and in the cell body. The fibres typically terminate against the



Figures 9-13. Rostral and ventrolateral nerve tracts. Scale bars = 1 μm. Figure 9. A cluster of uniciliate sensory-type cells (*) from the tip of the rostrum; an arrow indicates the adjacent nerve. Figure 10. A second type of rostral sensory cell, with a sunken pit around the cilium. Figure 11. A section through the rostral nerves (n), taken midway between their point of origin at the rostral tip and their entry into the nerve cord. The arrow indicates a small bundle of fibres in the epithelium, a branch of one of the dorsal nerves near its anterior termination. Figures 12 and 13. Sections through the ventrolateral nerve tracts on the right side taken at two points near the middle of the anterior c.v. Figure 12 is more anterior, at about the level of row 3; figure 14 shows the same section diagramatically. Figure 13 is near the back of row 4. Both show the cluster of small rostral fibres (n), and the right axial tectal fibre (*) shown in figure 17*a*. Fibres from the receptor cells in rows 1 and 2 are indicated by number; the large terminals (t) derive from row 1. Arrows in figure 12 indicate basal neurites from row 3; arrows in figure 13 indicate the region in which neurites from other medial neurons accumulate as the tract travels through the anterior c.v.

basement membrane, but some also make repeated contacts with the row 1 terminals before doing so. In a few cases, row 2 fibres made direct specific contacts with the unpaired axial dendrite, but again, without forming obvious terminals or synapses.

The medial cells in row 2 differ somewhat from other receptor cells. They have basal fibres, but these either fail to reach the ventrolateral tract, or terminate shortly after entering it. The cells also form areas of surface contact with cells in rows 3 and 4, either by wrapping around a portion of the cell (e.g. as in figure 18*c*) or via small blunt processes. Specialized junctions were not observed.

There is reasonably good histochemical evidence

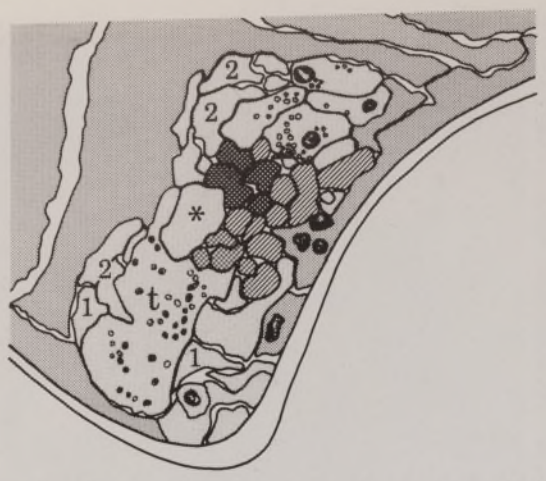


Figure 14. Diagram of the ventrolateral tract shown in figure 12. Surrounding cells and cell processes, some from row 3, are shaded a darker tone than the tract. Rostral fibres are hatched; the core fibres are cross-hatched. Row 1 and 2 fibres are identified by number, and the axial tectal fibre by *. There is one very large row 1 terminal (t) that contacts the tectal fibre in this section; at least two of the four vesicle-containing profiles just above the bundle of core fibres are probably also row 1 terminals.

that the row 2 cells are serotonergic. Holland & Holland (1993) identified a row of serotonergic anterior cells in the larval nerve cord associated with the frontal eye. A re-examination of their photos of 14d larvae shows, in side view, cells positioned exactly like the row 2 cells shown in the top panel of figure 16, with short, weakly staining fibres and no varicosities or terminals. This matches the reconstruction data quite well.

(*d*) *Frontal eye: neurons*

Clusters of nerve-like cells lie just behind the receptor cells, separated from them by a row of ependyma. The main clusters are arranged in two transverse rows, though these are much less regular than the rows of receptor cells. There is considerable variability in the arrangement of the cells and their pattern of contacts between specimens, as shown in figure 19. Row 4 marks the front margin of a larger zone of neurons of very similar appearance. Examples are shown in figures 6–8; the cells are notable for their large rootlets and well developed golgi. Their basal axons are mostly

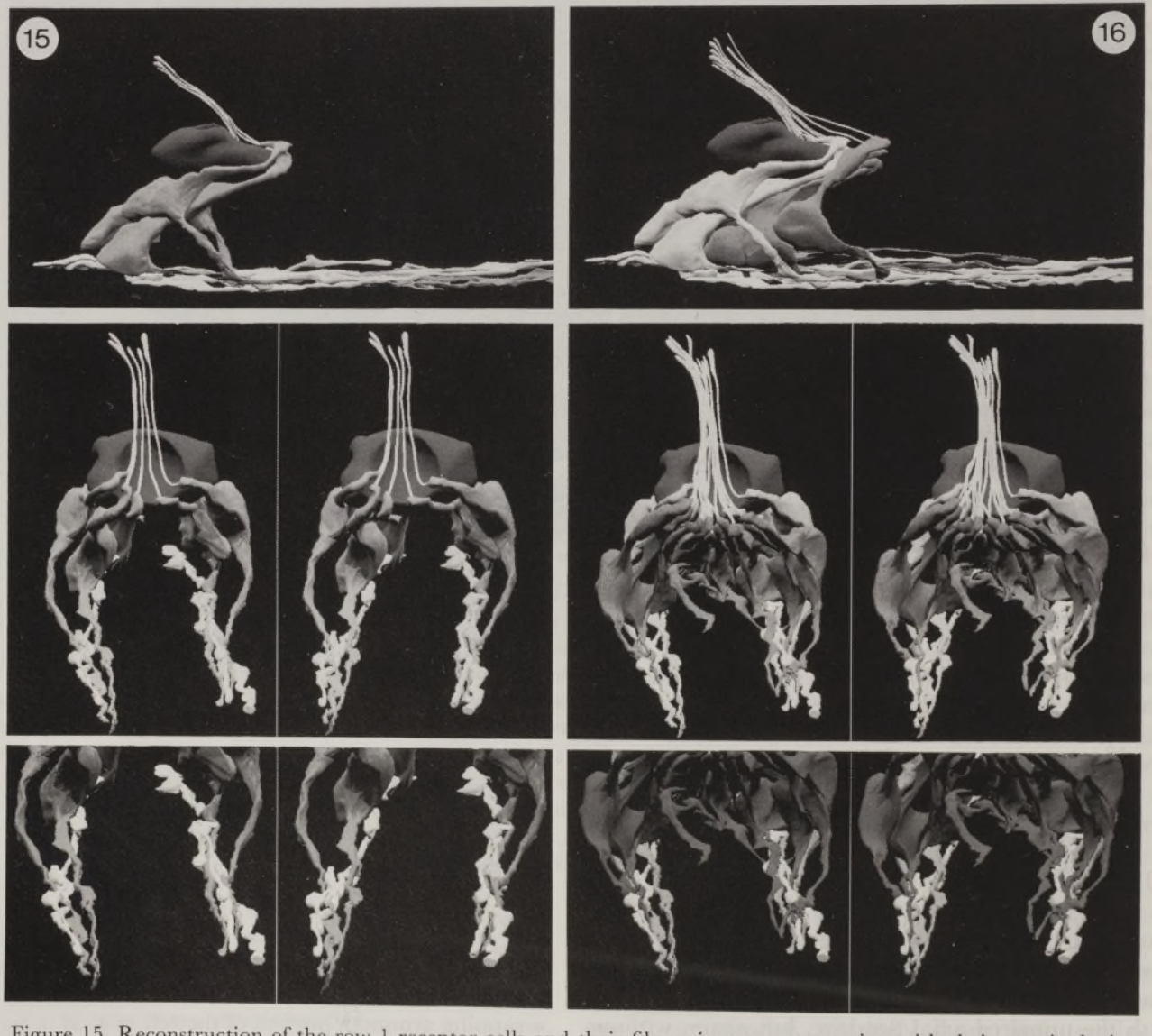
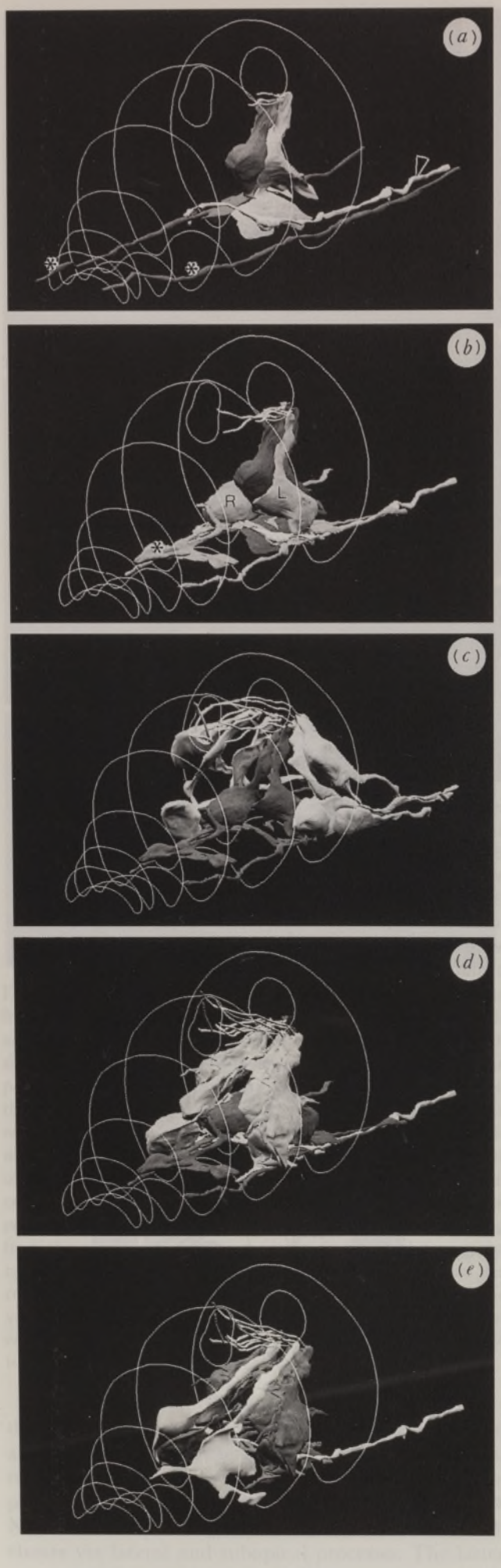


Figure 15. Reconstruction of the row 1 receptor cells and their fibres, in grey, contrasting with their terminals, in white. Top panel shows the cells and pigment cup in side view; the middle and bottom panels show stereo caudal views. Figure 16. As in figure 15, rows 1 and 2 combined; the former are lighter than the latter, and their terminals are white. The row 2 cells have tapered fibres, but lack identifiable terminals.



short, with rather small terminals. Only the two medial cells in the row show specific associations with the frontal eye. In all three specimens, the two medial cells each produces an axon that branches, sending one branch into each of the ventrolateral tracts on each side. Each branch ends in synaptic terminal filled with small clear vesicles (figure 18*e*). The left branch from both cells, in all three specimens, synapses with the large unpaired axial dendrite. The right branch terminates among fibres collected along the medial edge of the right ventrolateral tract, but no consistent target fibre could be identified.

The other constant feature between specimens is the close association between the two medial row 4 cells and a pair of medial ependymal cells (figure 17*a*, *b*). The latter are positioned precisely in the midline, and seem to be involved in some way in guiding the axons of the medial row 4 cells. This is best illustrated by the right medial row 4 cell in specimen 4, shown in figures 17*b–d* and 18*b*. This cell is unusually complex in specimen 4. It has backward-projecting axon that branches first at a point where it is sandwiched between the apposed bases of the two ependymal cells. The left branch crosses the midline through a gap between them. The branches then traverse the top surface of the ependymal cells' basal processes (visible in figure 17*a*) into the ventrolateral tracts on each side. Smaller subsidiary branches are also formed that travel forward and backward along the tracts. In addition, the cell has a large dendrite-like process that projects forward (figure 18*b*, *c*) to make basal contacts with two medial row 2 cells. The latter have poorly developed basal fibres and therefore lack effective contact with the ventrolateral tracts. The left medial row 4 cell in specimen 4 has a single branched axon, but lacks the other cell processes. In specimen 5, the right cell is again the better differentiated of the pair; in specimen 6 it is the left that is best developed, and in neither specimen is there a large anterior dendrite. The absence of the dendrite may correlate with the positioning and degree of development of the row 3 cells in these two specimens compared with specimen 4, as discussed below.

The chief distinguishing feature of the row 3 cells is their close association and reciprocal contacts with one another. There are 6 cells in specimen 4 and a similar number in specimen 6. In addition, other nerve-like cells are closely associated with row 3. Two examples are shown in figure 17*e*. The cells lie just forward of row 3; the left one is well developed with a dendrite

Figure 17. Reconstructions of rows 3 and 4, with some nearby cells. (*a*) The two medial ependymal cells, shown in constrasting grey tones. The three dendrite-like fibres encountered in this region are also shown. The axial tectal fibres (*) are paired; the unpaired dendrite (arrow) belongs to the left giant cell. (*b*) The right (R) and left (L) medial row 4 cells, with the two medial ependymal cells, shown in darker tones. (*c*) Row 4, showing the two medial cells, in dark tones, and five lateral ones. (*d*) The paired clusters of row 3 cells, in light tones, together with the medial row 4 cells, in contrasting dark tones. (*e*) The row 3 cells, in dark tones, together with two anterior nerve-like cells associated with them, in white.

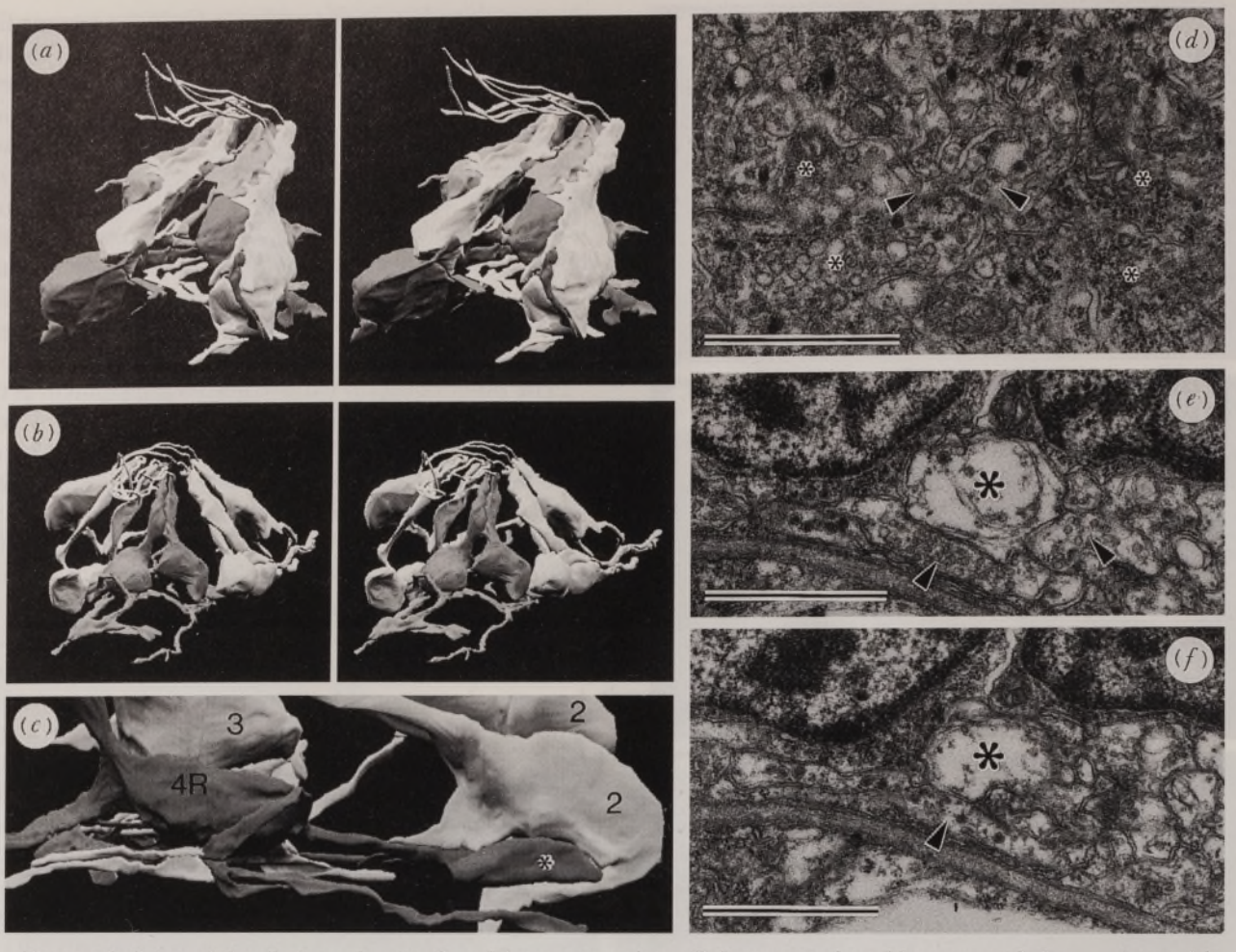


Figure 18. Selected details from the row 3 and 4 reconstructions. Cells are numbered by row, scale bars = 1 μm. (*a*) Row 3 in stereo; the cells are in contrasting tones to show their complex morphology and multiple processes. (*b*) Row 4 in stereo; shows the relative positioning of the medial and lateral cells. (*c*) Anterior dendrite (*) from the right medial row 4 cell showing how it is enveloped by the basal surface of one of the medial receptor cells in row 2. (*d*) Detail showing subapical contacts between four of the row 3 cells (*). Arrows indicate regions of contact between processes from adjacent cells, some containing vesicles. (e, f) Junctions between row 3 and 4 cell neurites and the large unpaired axial dendrite (*). The arrows in (*e*) indicate apparent synapses between medial row 4 cell terminals and the axial dendrite; the arrow in (*f*) indicates a non-synaptic contact with a row 3 basal neurite.

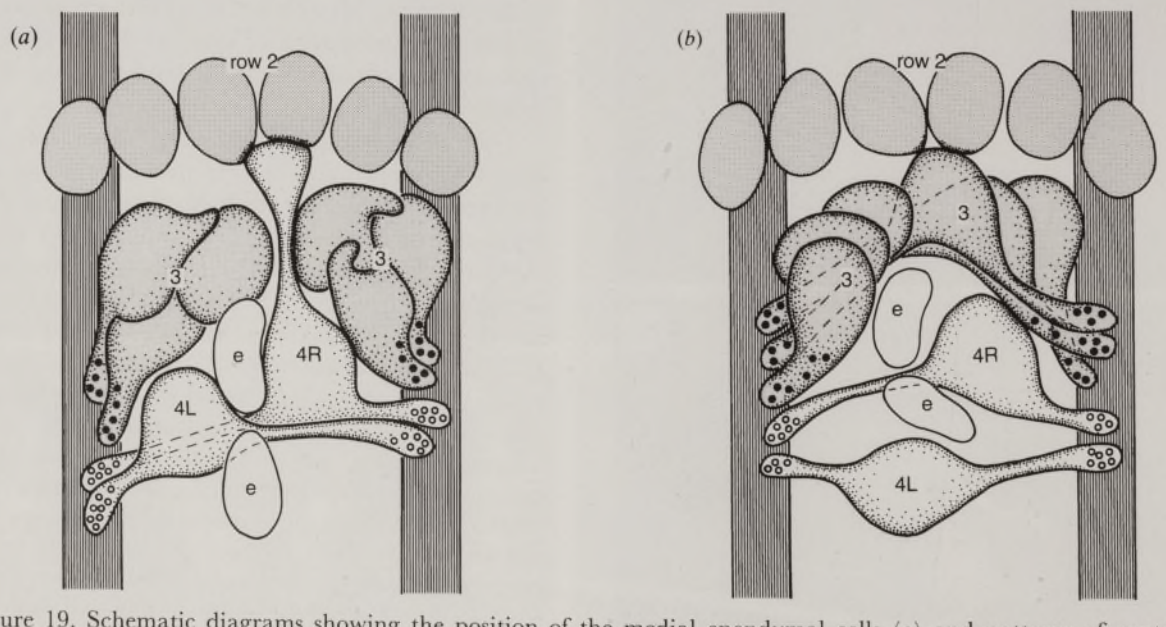


Figure 19. Schematic diagrams showing the position of the medial ependymal cells (e) and patterns of contacts between cells in rows 2, 3 and 4, indicated by number, and the paired ventrolateral nerve tracts in (*a*) specimen 4, (*b*) specimen 6.

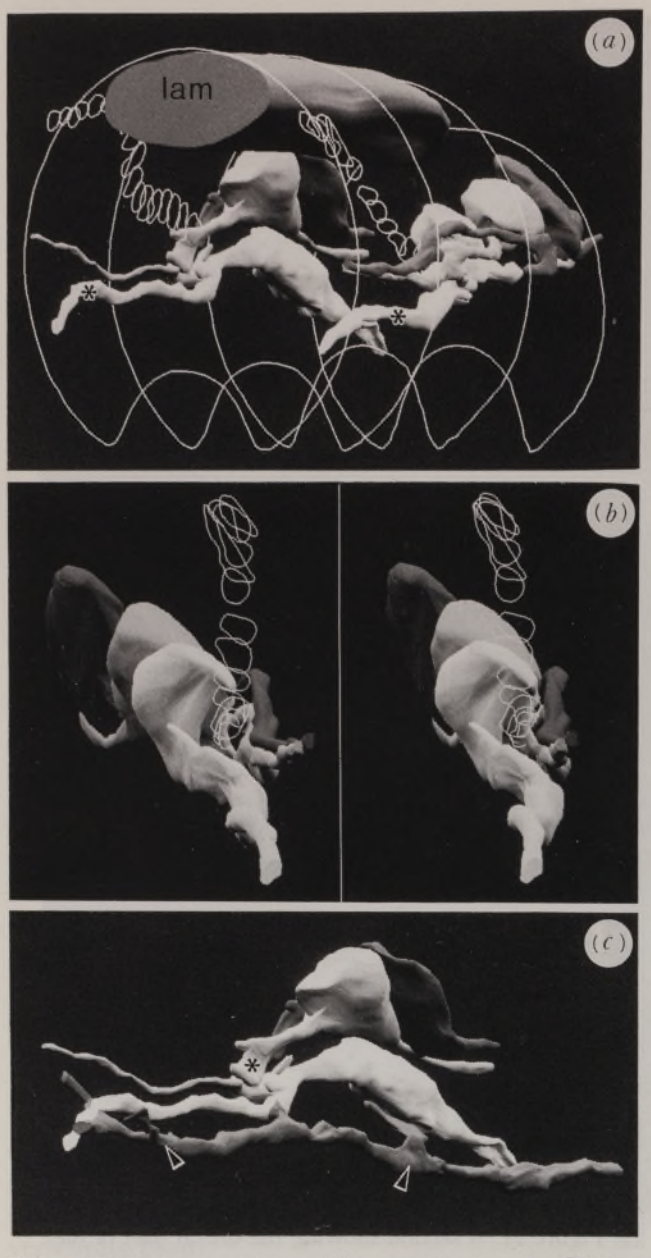


Figure 20. Reconstructions of the tectal region. (*a*) Oblique front view of the region as a whole; shows three tectal cells and their forward-projecting fibres (one not shown) on each side, in a range of grey tones. The figure includes the posterior portion of the lamellar body (lam), and, as contours, the more posterior of the two pairs of dorsal nerves. The latter enter the cord just in front of the tectal region. The ventral-most pair of fibres (*) are posterior continuations of the pair of fibres marked in figure 17*a*. (*b*) Stereo view of the tectal cells on the left side of the cord, showing how the dorsal nerve passes through a pocket formed by the terminals projecting from the proximal part of each fibre. (c) A medial view of the tectal cells on the right side of the cord. Arrows show the repeated contacts between the fibre and terminals of the ventral tectal cell and a dendrite belonging to the right giant cell. A conspicuous large terminal belonging to the middle tectal cell is marked (*).

that tracks that of the right medial row 4 cell, and an axon that forms a large terminal in the left ventrolateral tract. The row 3 cells in specimen 4 are arranged in paired clusters of three cells each (figures 17*d*, 18*a*). Numerous reciprocal contacts are formed within each cluster via lateral and subapical processes. The latter are especially well developed; the subapical region is broad and flattened, and club-shaped processes from adjacent cells enfold one another (figure 18*a*). The cells also produce irregular basal processes. These travel short distances along the ventrolateral tracts, but do not typically show the same close mutual associations as the subapical processes. Scattered dense-core granules occcur in all of the various neurites (figures 18*d*,*f*), but seldom in large concentrations. The row 3 cells are better developed in specimen 6 than in specimen 4. In the latter, only one of the three cells on the right has basal processes of significant size and extent. In specimen 6, the cells on the right have well developed processes that cross to both sides. The cells themselves push forward into the region occupied by the row 4 dendrite in specimen 4 (cf. figure 19), and form basal contacts with the medial row 2 cells.

The functional significance of the various contacts formed between receptor cells and neurons in the eye complex is not obvious from the morphology, and the pattern of contacts is quite variable. There are common features, however, with the neurons falling into two broad categories: (*i*) cells with comparatively well developed branched axons supplying both ventro-lateral tracts; and (ii) cells in clusters that form reciprocal contacts among themselves, contacts with cells in category 1, and also send neurites into the ventrolateral tracts on one or both sides. The medial receptor cells in row 2, which appear to have either limited access or no access to the ventrolateral tracts directly, may have indirect access by three routes: through contact with a row 4 cell, possibly modulated by input from row 3; through row 3 directly; or through row 4 via row 3. The variability between specimens, and between left and right sides in individual specimens, suggests these may all be redundant pathways designed simply to provide a link between medial row 2 cells and the ventrolateral tracts.

(*e*) *Tectum*

Two sets of sensory fibres enter the posterior cerebral vesicle dorsally on each side via the paired dorsal nerves. Fibres on each side combine in a single tract that travels caudally through the tectal region in transit to the primary motor centre. Some of the fibres appear to terminate in the tectal region, where a synaptic zone with a glomerular substructure is formed (figures 22-24). The tracts have not been traced thoroughly from this point on to the PMC, but some of the dendrites in the synaptic zone belong to nearby neurons whose axons form a relay pathway to the PMC, so some or all of the sensory fibres in the dorsal nerves probably terminate in the tectum.

The most distinctive cells encountered in the tectal region are the small dorsal cells (figures 20, 21) that form a single file on either side of the lamellar body. They are referred to here as tectal cells, and may be equivalent to the B cells reported by Bone (1959) in older larvae. They occupy this same position in both 6 and 8d larvae; in specimen 4 there are three on the left side and five on the right. Only three of the latter are shown in the reconstructions; the other two lie just behind these. Tectal cells are distinctive in that they lack an apical connection to the central canal. They

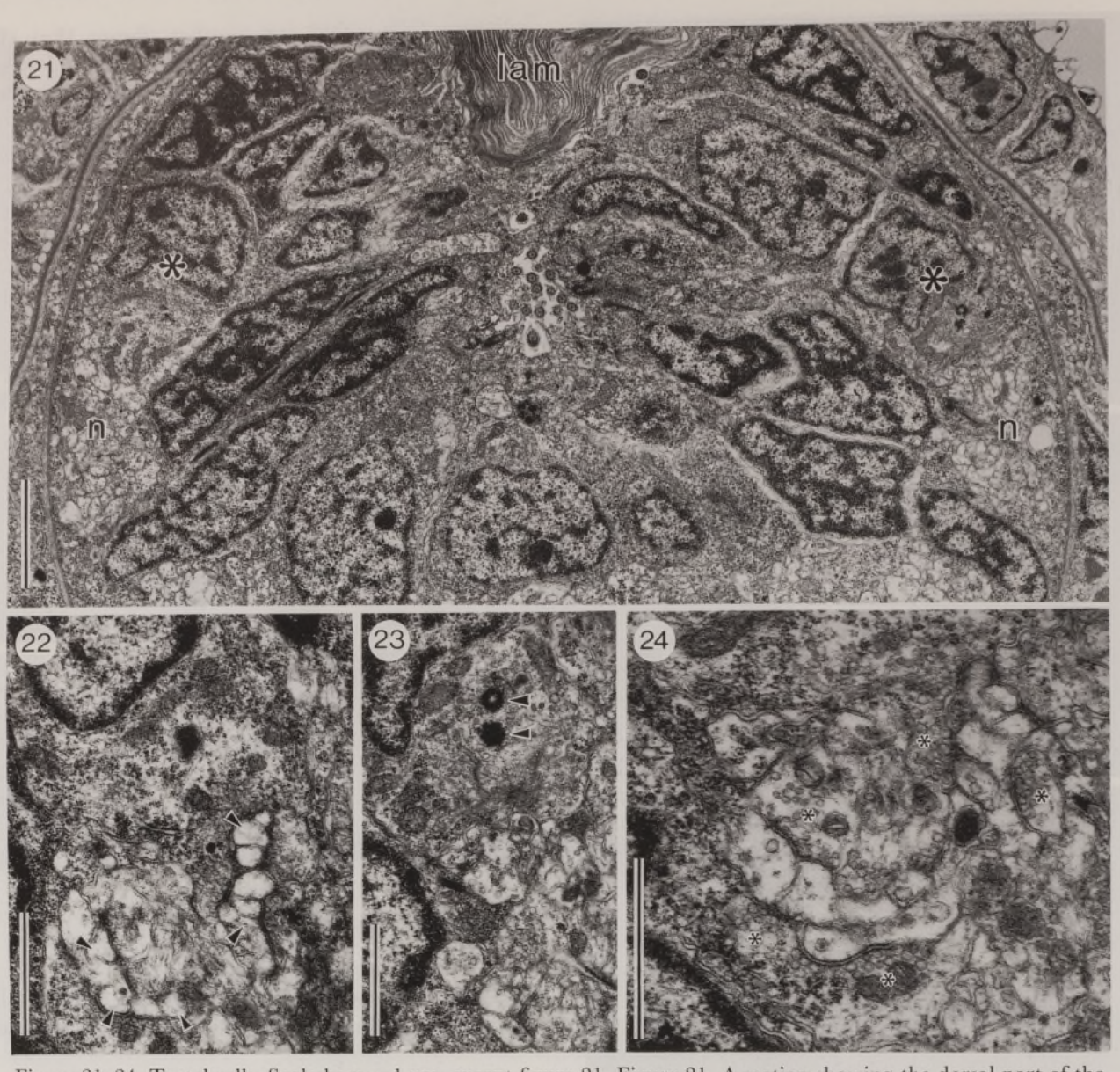


Figure 21–24. Tectal cells. Scale bars = 1 μm, except figure 21. Figure 21. A section showing the dorsal part of the cord in the tectal region, with one tectal cell (*) and the subadjacent nerve tract (n) visible on each side. Scale bar = 2 μm. Figure 22. Detail of a tectal cell and the synaptic zone immediately below it. Arrows indicate dendrites; the large terminals belong to tectal cells. Figure 23. Remnants of the ciliary basal bodies, indicated by arrows, and surrounding golgi. Both are characteristic features of the tectal cells. Figure 24. A detail of the synaptic zone. The large terminals (*) belong to tectal cells.

therefore also lack cilia, but retain the basal bodies (figure 23), which are densely stained and surrounded by a cloud of golgi cisternae and vesicles. Tectal cells are not unique in this respect. Other dorsal cells with internalized basal bodies were encountered in somite 2, in one instance with a remnant of a rootlet, but these more caudal examples develop considerably later than the tectal cells.

The golgi zone seems to serve as a focus for neurites and terminals in tectal cells. Typically, each cell has two principal neurites, a short one that extends backward, and a second long fibre that extends forward. The proximal part of the latter is elaborated into a series of club-shaped synaptic terminals, which are the main input to the synaptic zone. The terminals are packed with small clear vesicles (figure 24). The forward-projecting fibre continues, in all cases so far traced, to the level of the frontal eye. Those arising from the two ventral-most tectal cells (* in figure 20*a*) become the paired axial fibres referred to in previous sections. There are contacts also between the right ventral cell and the principal dendrite of the right giant cell (figure 20*c*); comparable contacts were not found on the left side. The forward-projecting fibres from more dorsally positioned tectal cells travel along the side of the cord in a more dorsal position. They are largely isolated from other fibres at this stage and receive no obvious synaptic input

The positioning of the tectal cell soma in relation to the synaptic zone is of special interest. The latter lies below and slightly medial to the more dorsal of the tectal cells, whereas the ventral-most tectal cell on each side is more medial still. The dorsal nerve tract is thus sandwiched between the cells and their terminals, as shown in figure 20*b*. The usual pattern throughout the rest of the nerve cord, in contrast, is for fibres to travel along the basal surfaces of neurons and ependymal cells. The tectal cells really lie outside the ependymal

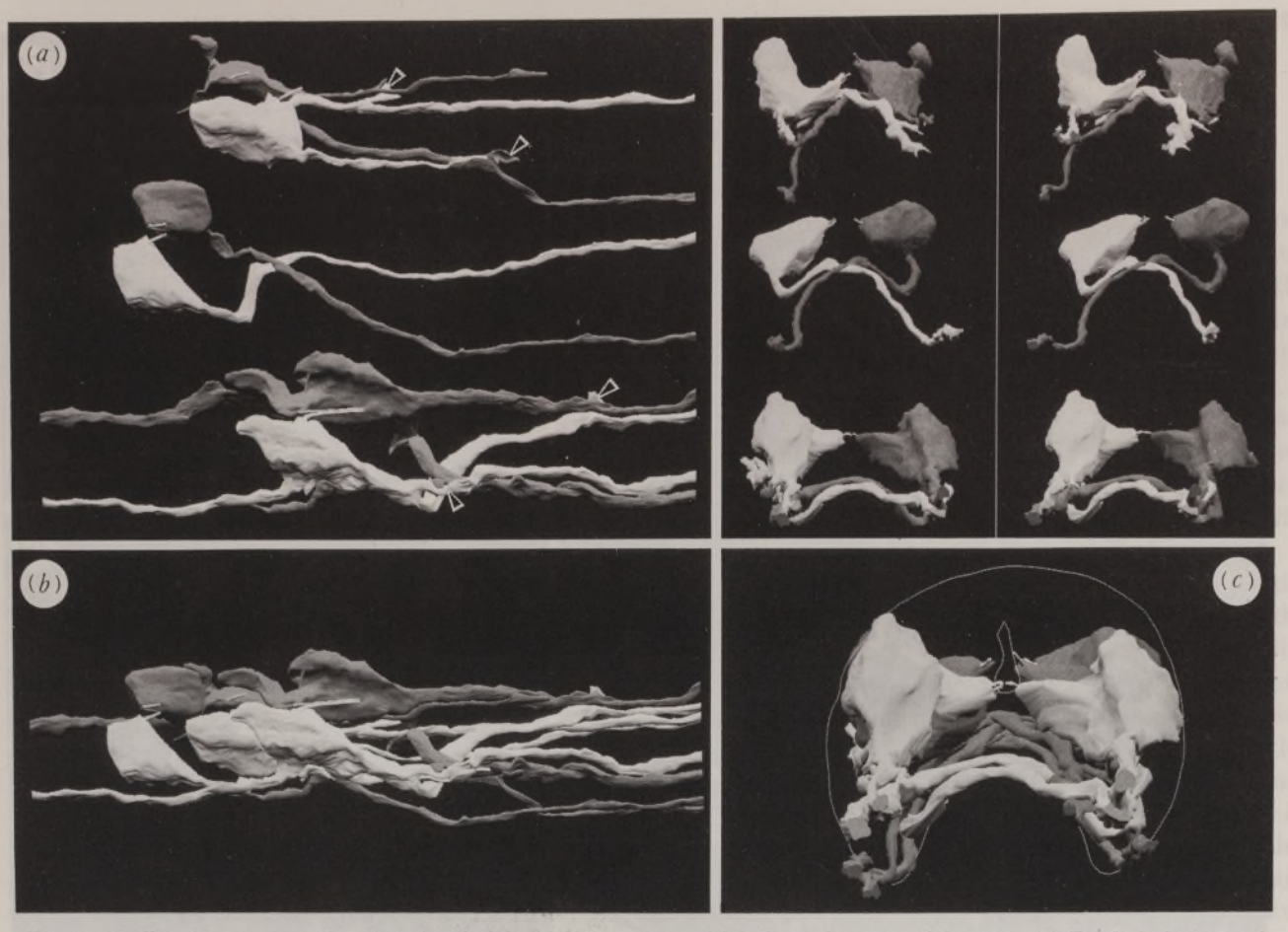


Figure 25. Reconstructions showing the six large interneurons which form the core of the PMC. (*a*) Exploded views of the PMC complex shown in side view (left panel) and from behind (right panel). From top to bottom, the panels shows the anterior and posterior pairs of large interneurons with crossed axons (arrows indicate post-synaptic terminals), and the paired giant cells (arrows indicate synapses). (*b*) Side view, and (*c*) caudal view of the entire complex.

layer, separated from it by the gap through which the dorsal nerves pass. An inner tract of fibres and synapses is thus formed that is subependymal, but not basal, resembling in this respect the cortical tracts seen in vertebrate brain.

(*f*) *Primary motor centre*

The region extending from the back of the lamellar body through the front part of somite 2 is occupied by a number of well differentiated ventral motor neurons and interneurons arranged in pairs. The three pairs of large interneurons shown in figure 25, which lie in the overlap zone between somites 1 and 2, are the most conspicuous. They comprise a pair of giant cells, and a quartet of smaller cells whose axons all meet as they cross the midline (figure 25*c*). Similar large cells were found in the same region is 6 and 8d larvae, though only the two giant cells and one other pair of large interneurons could be identified with any confidence. The region as a whole is referred to here as the primary motor centre (PMC) because of its evident importance as a target for sensory input, and because it contains the largest concentration of motor neurons discovered so far in the anterior cord. The motor neurons and their patterns of innervation are not yet fully analysed, but it is clear from specimen 3 that the main cells responsible for innervating the anterior somites are located in this region, and that both motor neurons and interneurons extend forward to just under the back of the lamellar body. Undulatory swimming develops in amphioxus larvae from less complex locomotory patterns, involving at first a side-to-side bending motion in which the myotomes along one side all contract together. The startle response (§4b) also develops early. The PMC is the best candidate so far for controlling these early locomotory activities.

The giant cells are displaced from the ependymal layer (figure 26), but they retain an apical connection to the central canal, as well as a cilium. The rootlet is associated with well developed golgi (figure 28). Each of the cells has a large axon that branches; one branch descends on the ipsilateral side while the other crosses and descends on the contralateral one. At their point of first contact large synapses are formed; each contralateral branch synapses with the ipsilateral branch arising from the opposite cell (figure 25*a*). Collateral branches and additional series of large synapses (figure 27) are formed at intervals by both sets of axons as they descend along the ventrolateral tracts. Both cells also have large forward-projecting dendrites and some subsidiary dendritic branches. Those arising from the right cell go no further forward than the back of posterior c.v. The left giant cell, in contrast, has a single large dendrite that travels forward to the frontal eye, as described above. Dendrites from both cells receive synaptic input all along their length from a variety of sources including a series of synapses from the core rostral fibres.

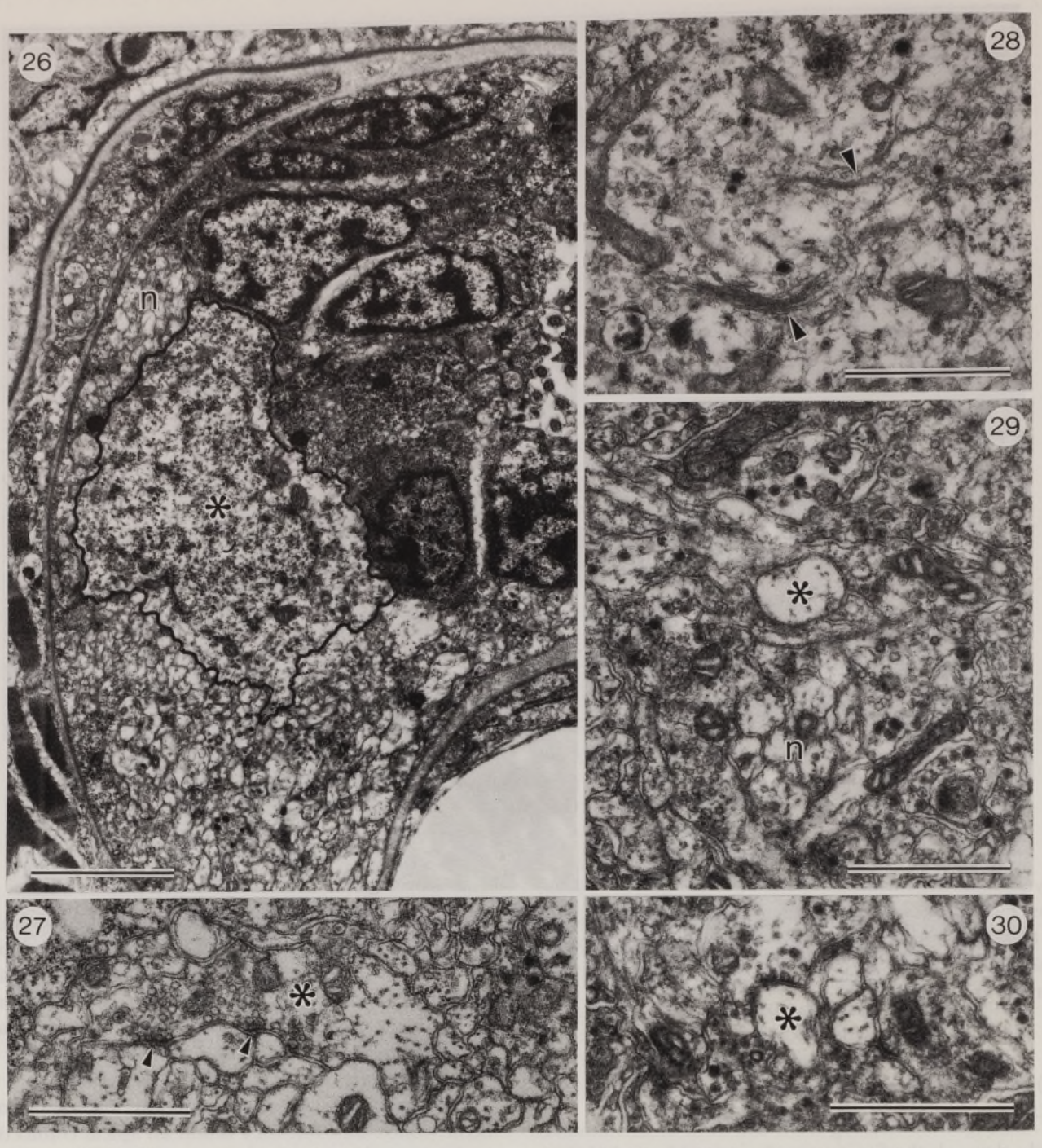


Figure 26–30. The PMC giant cells. Scale bars = 1 μm, except figure 26. Figure 26. A section through the soma of the right giant cell, outlined for emphasis, showing the posterior continuation of the dorsal nerve tract (*n*). Scale bar = 2 μm. Figure 27. A typical giant cell synapse (*); arrows indicate zones of membrane specialization. Figure 28. Golgi complex near the base of the apical rootlet; arrows indicate stacks of cisternae. Figure 29. The main forward-projecting dendrite (*) of the left giant cell in transit through the ventral commissure. A bundle of rostral core fibres (n) lies just below. Figure 30. As in figure 29, a nearby section showing a synapse to the giant cell dendrite.

Axons from the other two pairs of large interneurons all cross, at a point near the front of the giant cells, and descend on the contralateral side. All four axons meet at a single point in the midline (figure 25*c*), a very distinctive feature. Their axons penetrate more deeply into the ventrolateral tracts than those of the giant cells, and form large synaptic varicosities at intervals. The synapses resemble those of the giant cells, but very probably have different postsynaptic targets. There are differences also in the synaptic input the two pairs of cells receive. The anterior pair lacks obvious dendrites but each soma is covered with synaptic terminals, some of which come from the dorsal nerve tracts. Each of the cells of the posterior pair produces dendrites from both its cell body and, on the contralateral side, its axon. These are targets for giant cell synapses, which suggests that at least two of the four cells may be controlled by giant cell input.

Based on their morphology, the PMC interneurons appear to be involved in coordinating the response to sensory input by generating one or more descending signals via several possible pathways. Figure 31 summarizes the input paths and circuitry so far discovered. These include: (*i*) direct contact from core rostral fibres to the giant cells on both sides; (ii) direct contacts from a variety of sources, including medial

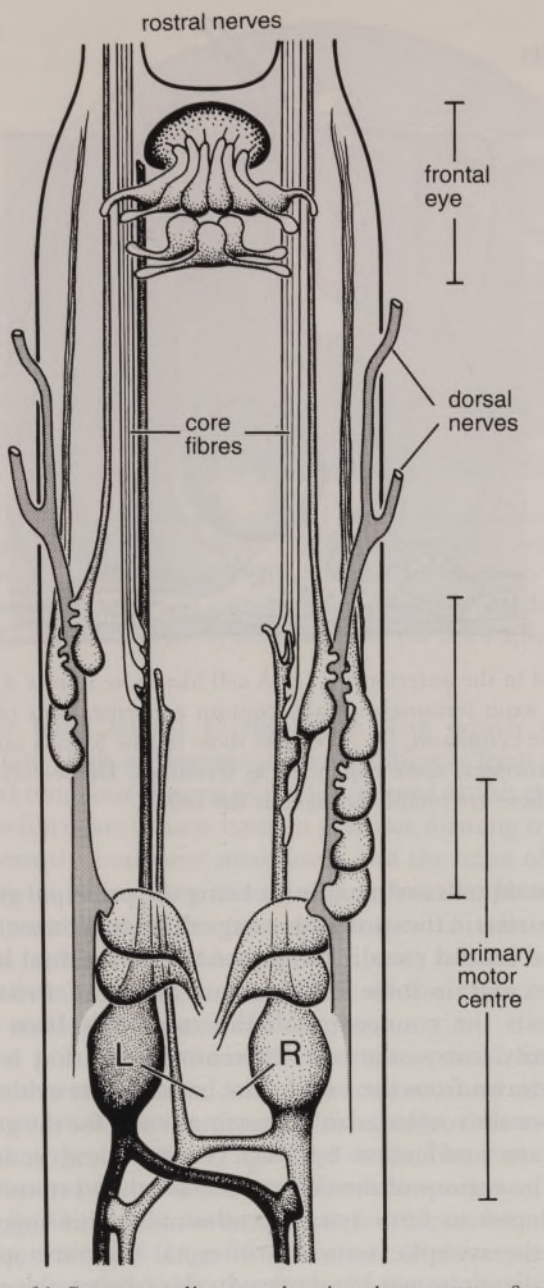


Figure 31. Summary diagram showing the patterns of contact between the frontal eye and rostral nerves, and cells in the tectum and PMC.

cells in the frontal eye, to the dendrite of the left giant cell; (iii) direct contacts from row 1 receptor cells to the tectal cells via the forward-projecting fibres produced by the latter; (iv) direct contacts from tectal cells on the right side to all three PMC cells on that side; and (*v*) an indirect pathway from the dorsal nerves to the PMC, to which the tectal cells have an input. The frontal eye and PMC are thus linked by three pathways, from the medial receptor cells and neurons directly to one giant cell, to both giant cells indirectly via contacts between receptor cells and rostral core fibres, and to the PMC on both sides via connections through the tectum. It is an interesting point that if additional receptor cells were to develop as the larva grew, pathway (iii) would likely be the one that would enlarge to accommodate this. Fibres from the more lateral of the receptor cells would necessarily make first contact, as they developed, with the more lateral of the tectal fibres that project forward along the sides of the cord, rather than with ventral ones. A pair of comparatively substantial lateral connections to the tectum, a kind of receptor-tectal pathway, could be established in this way.

4. DISCUSSION

(*a*) *Summary and general remarks*

This paper reports the results obtained to date from a serial EM study of the larval nerve cord in amphioxus. A comparatively detailed understanding of the organization of the nervous system is slowly emerging from this project. The overall layout of the anterior end of the nerve cord, showing its principal landmarks, is shown in figure 2. In general terms, three separate regions can be recognized, each with its own organizational characteristics and distinctive cell types: the anterior c.v., the posterior c.v. and, just behind the latter, the primary motor centre (PMC).

The anterior c.v. is the anterior-most part of the nerve cord, extending from the frontal eye to the infundibular cells. It is a simple cylindrical epithelial tube, thickened ventrally, where the cells are both more numerous and better differentiated. The anterior c.v. lacks the medial floorplate that elsewhere divides the cord into symmetrical halves. The cells are mainly flask-shaped uniciliate cells arranged predominantly in transverse rows, or more nerve-like variants of this simple cell type. Some of the cells undoubtedly retain a basic sensory function, but others appear to have been modified to perform local integrative and relay functions as part of larger units, which then function as simple sense organs, e.g. as in the frontal eye and balance organ. The main nerves in the anterior c.v. are the paired ventrolateral tracts, to which the cells in this region contribute various types of neurites.

Nerve cells in the anterior c.v. fall broadly into two categories depending on the types of cell processes they form. Particularly common are cells that look like primary sensory cells (figure 32*a*), resembling those found in invertebrates generally and in a few specialized situations in vertebrates, e.g. in olfactory epithelium. Each has a cilium and an axon that forms terminals and, in some cases, synapses. Otherwise the cells have few other cell processes except, in some instances, in the subapical zone. The more lateral cells in row 4 of the frontal eye are good examples of this type. Less common are cells without axons, but with far more extensively developed basal, lateral and subapical neurite-like processes (figure 32*b*); the row 3 cells in the frontal eye are examples. Neurites in such cells are generally short and form extensive local plexes. Vesicles may be present, usually as dense-core granules, but there are no obvious junctional specializations. In very general terms, specialized synaptic junctions and clear vesicles are most often associated with acetylcholine or amino acid transmitters and fast transmission, whereas active golgi, granular vesicles and unspecialized terminals are more typically associated with slow transmission involving neuropeptides (Zimmerman 1993; Golding 1994). The paucity of any of the usual correlates of fast transmission in the anterior c.v., coupled with the development of numerous local plexes, suggests that the anterior c.v. may be a region in which slow, integrative

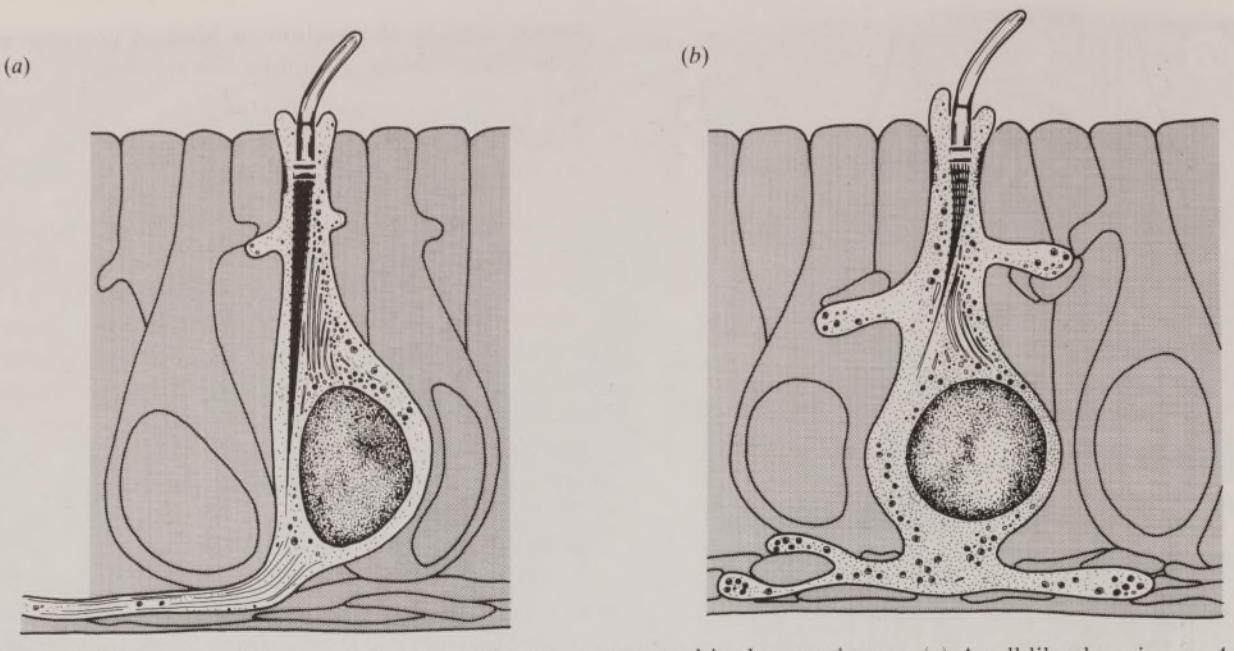


Figure 32. Examples of the two main nerve cell types encountered in the anterior c.v. (*a*) A cell like those in row 4, with a basal axon and comparatvely few lateral processes. The axon terminals usually contain concentrations of vesicles, usually clear vesicles, which are not found elsewhere in the cytoplasm. (*b*) A cell like those in row 3, with no obvious axon, but with extensive basal, lateral and subapical processes, some expanded as terminals. Dense-core granules are the most commonly encountered vesicle type, and these are found throughout the cell.

activities involving extensive reciprocal interactions between cells predominate over more rapid relay functions via specific pathways.

The posterior c.v. begins behind the infundibular cells and extends to the end of the lamellar body, where the cord begins to narrow. The posterior c.v. resembles the rest of the cord in having a central canal shaped like an inverted keyhole, open in the centre and narrow at the top, where the two sides are held together by an interlacing network of apical cell processes. The floorplate also begins in the posterior c.v., and continues caudally through the rest of the cord. The dorsal part of the posterior c.v. is occupied by the lamellar body, two pairs of dorsal nerves and the tectum. Its ventral part, which is rather short, is largely taken up by the ventral commissure, which contains fibres from the lamellar body. The ventral commissure is the largest commissural structure by far in the larva. Fibres crossing the midline elsewhere do so individually or in small groups. The cells of the posterior c.v. consist of a few well defined types associated with particular structures, e.g. the lamellar cells, tectal cells, and ventral commissural neurons. They do not share the uniform primitive appearance of cells in the anterior c.v., and are typically arranged in axial, rather than transverse, rows. Synapses are present at many points, often with large concentrations of clear vesicles, as in the case of the tectal cells, which suggests more specific and faster modes of transmission are employed. From the circuitry, the two main functions of the posterior c.v. would appear to be concerned with photoreception and response to touch. The former involves the lamellar body and the ventral commissure, but the ultimate targets or fibres from the lamellar body have yet to be determined. The latter involves a relay of signals from the dorsal nerves to the PMC via the tectum, incorporating input from the tectal cells.

Tectal cells are notable for being the principal group of neurons in the cord at this stage that lack connections to the central canal. The presence of a residual basal bodies and, in some instances, a remnant of a rootlet, suggests the connection to the canal has been secondarily lost as the cell differentiates i.e. that it has withdrawn from the canal. The basal bodies evidently replace the rootlet as an organizing centre for the golgi, and are surrounded by golgi cisternae and vesicles. Nearby regions of the cell surface are then extensively developed to form synaptic terminals. This suggests that the synaptic terminals of tectal cells are apical specializations, not basal ones. In this respect, the cells' polarity is evidently reversed over that seen in most other cells in the nerve cord, which have basal axons.

The tectal cell is probably best understood as a more extreme version of a neuronal cell type that occurs more commonly in the dorsal part of the amphioxus nerve cord. Throughout the cord, dorsal cells are encountered with apical specializations in the form of long processes that cross the central canal (figure 33). There are various types (Bone 1959), some of which are probably responsible for holding the two sides of the cord together in the dorsal region. The giant Rohde cells are the most distinctive and best developed of the neurons. Each has a cross-luminal apical process with a mass of small neurites at its tip, whereas the soma has both dendrites and a large axon. It is not clear whether the apical processes in such cells are efferent, or receptive, or both. Tectal cells seem have carried the process of apical specialization a step further, by transforming the apical processes into well defined terminals. If at the same time, the cells withdraw from the central canal and shift to the side of the cord, their terminals can form arrays that face inward to already-established nerve tracts. In this way a rudimentary cortical arrangement can be formed, with the cell

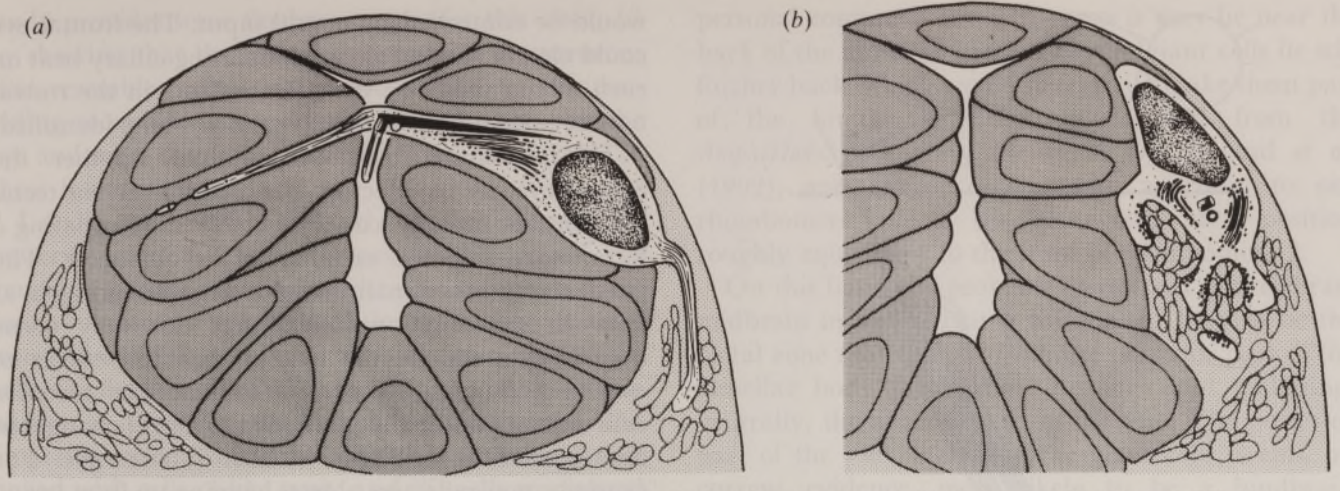


Figure 33. Cells from the dorsal part of the amphioxus cord, showing two types of apical specializations. (*a*) A dorsal cell typical of those found throughout the cord, with slender apical processes that cross the central canal (adapted from Lacalli & West 1993). (*b*) A tectal cell, from the cerebral vesicle. The cilium and apical surface are retracted from the canal, and synaptic terminals arise from the cell surface in close association with the basal bodies, suggesting the terminals arise by elaboration of the cell's apical surface.

bodies lying lateral to the tracts with which they associate. This arrangement is very different from that seen throughout the rest of the larval cord at this stage, where fibres are always basal in position, forming tracts between the basement membrane and the bases of the cells from which they arise. Judging from amphioxus, it is possible that the first appearance of cells with apical terminals could have been directly related to the evolutionary origin, in chordates, of the capacity to form cortical structures of this type.

The primary motor centre contains paired sets of large motor neurons and interneurons, and is the anterior-most centre from which large descending axons project to the rest of the cord. The PMC thus appears to be a control centre of some importance, and is by far the largest such centre so far discovered in the larvae examined. Input from variety of sources seems to be channeled to the PMC from the rostral nerves, the frontal eye, the dorsal nerves, via the tectum, and the lamellar body. The primary integrative role of the PMC may be to generate an appropriate response dependent on the balance of inputs as discussed in §4b.

In summary, the anterior part of the cord has three distinctive regions, each evidently associated with particular functions. The cerebral vesicle is primarily sensory, with markedly different organization and cell types in its anterior and posterior halves; the PMC is evidently an integrative command centre. There is no indication that there is any underlying, more fundamental plan in the anterior cord to suggest, for example, that it is organized into repeating elements or neuromeres. The most nearly comparable example of a regionally subdivided 'brain' among among primitive chordates is probably that seen in the tunicate tadpole larva. The tadpole larva has an anterior sensory vesicle, with an eyespot and otolith, that can be interpreted as an asymmetrical and much reduced counterpart of the cerebral vesicle. Just behind this is a slight swelling, the visceral 'ganglion', containing cells with descending axons that innervate the tail muscles (Nicol & Meinertzhagen 1991). This ganglion may be the equivalent, in the tadpole, of the amphioxus PMC. Amphioxus, however, appears to be a much more informative representative of the primitive chordate condition than the tadpole. Its nervous system is far less reduced and modified, and in young larvae its organization is still comparatively simple, which makes it far easier to make meaningful comparisons with other chordates, including vertebrates.

(*b*) *Functional aspects*

The principal and most obvious reflex pathway identified here, for which the morphlogical evidence is comparatively unambiguous, is the link between core rostral fibres and the giant cells. The circuitry of the PMC has not yet been fully traced but from preliminary data it seems likely that its probable function of the giant cells is to trigger the startle reponse, which can be evoked by touching the tip of the rostrum. The first coordinated muscle contractions of which the larva is capable are weak side-to-side flexings. By the stage examined here, the larvae can generate two quite different locomotory responses: bouts of swimming and the startle response. Young larvae swim by means of phased side-to-side contractions of the whole body, at about one tenth of a second per cycle. This progressively changes to a true propagated wave of contraction in older larvae. The startle response is typically a single rapid twitch resulting from a nearly simultaneous contraction of somites on both sides of the body, but with one side sufficiently delayed that the body arcs first to one side, then straightens out. The result is to move the larva suddenly to one side, after which it may remain motionless or initiate a bout of swimming. The interval between the initial contraction of one side, and the restoring contraction of the opposite side, is on the order of 0.01 s (M. D. Stokes, personal communication), much faster than the normal phasing of the swimming contractions.

Of the various descending neurons in the PMC, the giant cells are the most obvious candidates to trigger the startle response. These cells innervate both sides of the cord massively, their axons and terminals being the

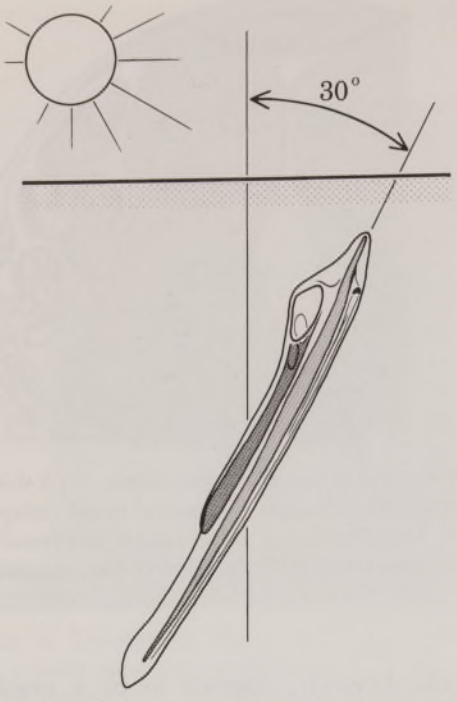


Figure 34. Hovering posture in amphioxus larvae, maintained by ciliary beat, from Stokes & Holland (1995*b*). The larvae orient slowly to light so as to maximally shade the frontal eye.

largest in the cord. Each also synapses with the ipsilateral fibre of the other, but not its contralateral fibre. This suggests a means by which a consistently asymmetrical descending signal could be generated that would ensure that one side always contracted marginally sooner than the other. Assuming the synapse between giant cells is excitatory, activating either would initiate a double signal on one side and a single signal on the other. This could strengthen and/or speed the response on one side. There are other asymmetries at the level of the motor innervation, however, that could perform the same function.

The frontal eye straddles the rostral nerves at their point of entry into the cord, and fibres from the frontal eye are major inputs to these nerves. This suggests a role in modulating the function of the rostral fibres. The frontal eye is structured like a directional photoreceptor but its function has puzzled past authorities, some of whom doubted it functioned as a photoreceptor at all. It is probably of little use during swimming because the larvae rotate as they swim. There is recent evidence on larval feeding behaviour, however, that indicates something of the conditions under which the frontal eye may operate. According to Stokes & Holland (1995*b*), larvae will feed while suspended in the water column if given suitable conditions and opportunity. They adopt a characteristic posture, shown in figure 34, and can remain more-or-less motionless, maintaining their position using only cilia. They also orient to light while hovering so that the frontal eye is maximally shaded, but the posture of the body with respect to the water surface ensures that the eye is comparatively well shaded regardless of angular orientation. Ciliary metachronal waves are initiated in the rostral area, so this is probably also the point where the strength of the beat would be controlled by neural input. The frontal eye could clearly play a role in modulating ciliary beat on each side of the body via efferent fibres in the rostral nerves, but suitable fibres have not yet been identified.

The remaining question concerns whether the frontal eye, by its effect on the PMC, either via tectal pathways or directly, could be involved in initiating a locomotory response or altering its character. The particular posture maintained during hovering is at least circumstantial evidence that this may occur because it positions the eye so that the effects of overhead illumination are minimized. The receptor cells are then optimally positioned to detect changes in illumination from below without interference. For example, non-reflective objects looming up from below against a reflective background, e.g. in shallow water, would produce an intensity decrease; reflective objects looming up from below aganst a dark background would produce an increase. In either situation, there should be a sudden change in the relative illumination of well shaded medial receptors compared with less effectively shaded lateral ones. The morphological evidence for separate output paths from medial and lateral receptor cells is consistent with at least the possibility that eye is structured to enable the output of the lateral and medial receptor cells to be compared.

Even if the eye can detect illumination changes due to looming objects, and assuming the object is potentially hazardous (e.g. a predator) it is not clear how the larva should be expected to respond. Young larvae are transparent and essentially invisible so long as they remain motionless. Swimming provides an escape, but generates vibrations and makes the larva easily visible to predators with eyes. The startle response i.e. a sudden twitch, may be a compromise solution, because it rapidly moves the larva without requiring a conspicuous bout of swimming. With this in mind, one possibile function for the frontal eye could be to increase the sensitivity of the startle response, while simultaneously suppressing swimming. Even from the preliminary data reported here on pathways to and from the PMC, it is clear that sufficient complexity is built in to the system that modulatory effects of this type would be possible.

(*c*) *Comparison with vertebrates*

The precise relationship between the anterior end of the amphioxus nerve cord and vertebrate brain i.e. whether the two are homologous, has been a subject of long controversy. Recent molecular studies on Hox gene expression have provided convincing evidence that the segmental region of the cord extending from behind the cerebral vesicle maps to vertebrate hindbrain (Holland *et al.* 1992; Holland & Graham 1994). This leaves the forebrain and midbrain to be accounted for. Lacalli *et al.* (1994) have shown that the cerebral vesicle contains structures whose most probable counterparts in vertebrates lie in the diencephalon of the forebrain. One of these is the frontal eye, whose vertebrate counterpart would be paired lateral eyes or, more precisely, the eyes plus the ventral structures that link them, the optic stalks and chiasmatic ridge. This

study provides some further support for this view; (*i*) by showing that the frontal eye contains interneurons that resemble retinal interneurons; and (ii) by providing morphological evidence for links between the eye and more caudal parts of the brain comparable to those found in vertebrates.

Lacalli *et al.* (1994) pointed out that the antero-posterior sequence of cell types in the frontal eye generally parallels that across the retina, from its distal (outer) surface to its proximal (inner) surface. Retinal interneurons fall very generally into two classes: (*i*) cells chiefly concerned with lateral integration within particular layers in the retina, e.g. horizontal and amacrine cells, which are profusely branched and linked with cells of like type; and (ii) cells specialized for relay between layers and output, e.g. bipolar and ganglion cells. Neurons in the eye complex in amphioxus are also of two general types. The row 3 cells are like horizontal and amacrine cells in forming extensive lateral contacts with cells of like type, and they lack axons. Row 4 cells, in contrast, are more like bipolar cells and ganglion cells in being specialized for axial relay and output functions. There is, however, no clear one-to-one correspondence between cells types in the frontal eye and those in retina: row 3 cells appear to have a bipolar-like relay function in some instances, and row 4 cells combine some features of bipolar cells (short axons) with others more typical of ganglion cells (crossing the ventral midline). It is possible that cells in and around row 3 represent a primitive version of the distal retina as a whole, but with the various cell types as yet incompletely diversified. Row 4 would then represent a rudimentary ganglon cell/chiasma complex. In most vertebrates, the ganglion cells are separated from distal retinal cells by a plexus, but most of the ganglion cells in the lamprey retina are integrated into the inner nuclear layer (Fritzsch & Collin 1990). Their association with other retinal cells is therefore much closer, and more like the compact arrangement seen in amphioxus.

The second feature of interest is the link between the frontal eye and more caudal regions of the nerve cord. The optic nerve in lower vetebrates projects to the tectum, which forms the roof of the vertebrate midbrain. The midbrain is also the source of somatic motor nerves that innervate the derivatives of the first two somites, which form extrinsic eye muscles, and includes a ventral plexus of interneurons (the reticular formation) that extends into the hindbrain. The reticular formation in lower vetebrates contains several types of giant reticulospinal neurons: Müller cells in lamprey; and Mauthner cells, which are best known in fish and amphibian larvae. In lamprey, such cells occur both in the midbrain and hindbrain (Nieuwenhuys 1977), and it is not immediately clear which of these is the more likely counterpart of the amphioxus PMC. The midbrain-hindbrain boundary is marked in vertebrates by the rhombencephalic isthmus, which is an important organizing centre involved in the control of midbrain development (Marin & Puelles 1994). The precise location of the isthmus homolog in amphioxus is not yet known, but preliminary data on *engrailed* expression (L. Z. Holland, personal communication) suggests it may lie near the back of the lamellar body. The PMC giant cells lie still further back, which would necessarily make them part of the hindbrain. Counting forward from the *AmphiHox*-3 boundary identified by Holland *et al.* (1992), and taking each somite as equal to one rhombomere, places the giant cells at a position roughly equivalent to the front of rhombomere 2.

On this basis, the probable conterpart of vertebrate midbrain in amphioxus is interpreted here to be the tectal zone that extends from the posterior part of the lamellar body for a short distance and including, ventrally, the anterior part of the PMC. The posterior part of the PMC, including the giant cells, seems, on current evidence, more likely to be a hindbrain structure. If the giant cells do indeed lie at the level of rhombomere 2, very little space would be left between there and the tectum for the either rhombomere 1 or the isthmus. With respect to the latter, this is understandable if the isthmus became important only with the evolution of an expanded midbrain in vertebrates in conjunction the first appearance of image-forming eyes. In amphioxus, with its minute frontal eye and correspondingly small tectum, a smaller and less conspicuous isthmus may suffice.

Although the zones identified in the amphioxus 'brain' are organizationally similar to certain parts of the vertebrate brain, they are tiny in terms of scale and cell numbers. It is nevertheless possible, on the data available, to construct a provisional map showing how the main zones identified in the early larva might map onto vertebrate brain. The result, shown in figure 35, marks out a set of interconnected domains that encompass the eyes and optic chiasma, the dorsal epiphysis, the floor of the midbrain, and longitudinal and lateral tracts connecting these regions. The domains correspond roughly with expression patterns of a number of developmentally important genes, notably *sonic hedgehog* (Ericson *et al.* 1995), *axial* (Macdonald *et al.* 1994), *nk*2.2 (Barth & Wilson 1995) and *Wnt8b* (Kelly *et al.* 1995). This suggests the cells and tissue domains most critical from a developmental standpoint may also be comparatively ancient ones. Patterns of gene expression have been interpreted as suggesting an underlying segmental structure in forebrain and midbrain comparable to that of hindbrain (reviewed by Puelles & Rubenstein 1993). The amphioxus data provides no support for this idea, however. The expansion of the forebrain during evolution could have involved the stepwise addition of blocks of tissue that would resemble segments, without being part of an actual segmental series. This accords more with the interpretation of Macdonald *et al.* (1994), who question whether the subdomains identified in forebrain represent true segments.

A final point concerns the formation, in the tectal region in amphioxus, of what appears to be a rudimentary tectal cortex. The issue is closely related to current ideas concerning the intrinsic polarity of nerve cells and the difference between axons and dendrites. Primitive sensory cells of the type seen in many invertebrates and invertebrate larvae have basal axons and apical cilia. Yet current evidence from

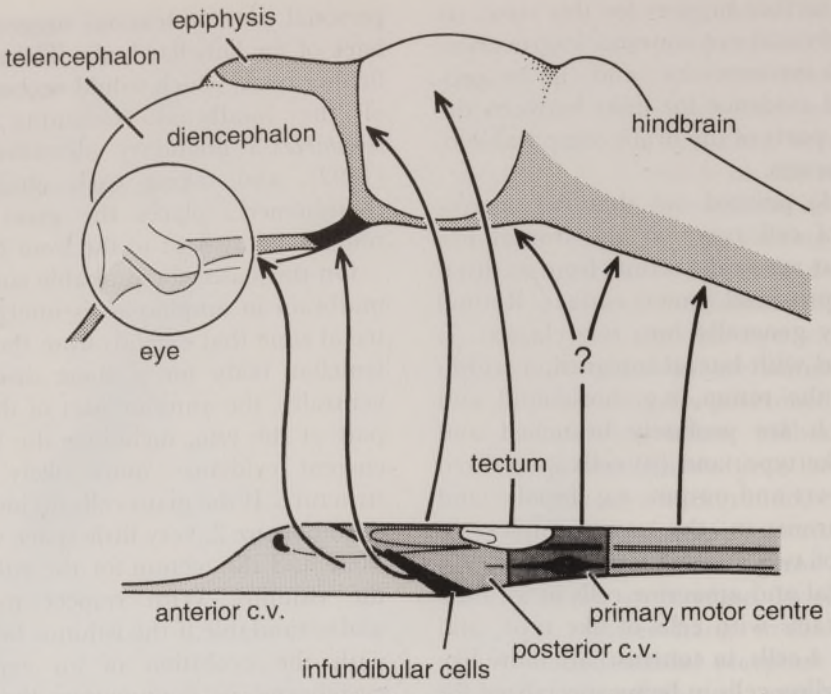


Figure 35. A proposal for how the amphioxus cerebral vesicle and PMC might map to the vertebrate brain. The anterior c.v., with its axial ventrolateral tracts and frontal eye, probably maps in large part to structures in or close to the floor of the diencephalon, including the longitudinal tracts extending from the postoptic commissure, and structures associated with the eyes, e.g. retina, optic stalk and chiasmatic ridge. The early connections established between these structures are comparatively well known for zebrafish (e.g. Ross *et al.* 1992). The infundibular cells are generally thought to be homologues of the infundibulum, and the lamellar body of the epiphysis (i.e. the pineal), which communicates with the longitudinal nerves and ventral commissures in both amphioxus and vertebrates via lateral fibre tracts. The organizing centre for the commissure in amphioxus is positioned considerably behind the infundibular region, so comparable tracts in vertebrates would presumably be those joining the longitudinal nerves behind the infundibulum, as shown in the figure. The amphioxus PMC includes cells whose vertebrate counterparts would probably be found in the reticular formation, which extends along the floor of both the midbrain and hindbrain. Precisely how the PMC maps to this region is uncertain (indicated by ?). Current evidence is at least consistent with the idea that the tectum and the anterior part of the PMC just below it map to the midbrain, and the posterior PMC, including the giant cells, to the hindbrain. The precise position of the isthmus homologue in amphioxus, if one exists, is not clear, but a likely position would be between the tectum and the giant cells, which means it could be quite small.

hippocampal cells, cultured from vertebrate forebrain, suggests that axons there are derived from the apical secretory compartment i.e. from the apex of the cell (Dotti & Simon 1990; Mundigl & Camilli 1994). In amphioxus there is a clear distinction between neurons in the ventral compartment of the cord, which have basal axons and apical cilia, and more dorsal cells, many of which have well developed apical neurites, as shown in figure 33. The tectal cells provide an example of how, in the dorsal compartment, the cell apex may be withdrawn from the central canal and secondarily elaborated to form sets of synaptic terminals. Whether a true axon is formed in this instance is not clear, but the inversion process appears to be associated with the formation of cortical structures. This may be an indication of how the development of dorsal structures in forebrain and midbrain and specifically cortical structures, has depended upon the evolution of new neuronal cell types with inverted polarity, in which neurites developed from the cell's apical surface became secondarily specialized to function as axons. It also provides a rationale for the comparatively widespread occurrence of dendrodendritic synapses in certain parts of the brain, e.g. between cells in the olfactory bulb (Farbman 1992) and in the optic tectum (Szekely & Lazar 1976). If both the axons and dendrites of such cells are apical specializations, their failure to conform to rules governing axons and dendrites in more conventionally organized neuronal circuits would be less surprising. It could, in fact, account for some of the unexpected complexities of dendritic function in vertebrates CNS (e.g. Barinaga 1995), providing a basis for the evolution of novel modes of neural integration.

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