Landmarks in the anterior central nervous system of amphioxus larvae

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[Plate 1]

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SUMMARY

The anterior end of the dorsal nerve cord of amphioxus is described at the 3–4 gill slit stage based on serial transmission electron microscopy and three-dimensional reconstruction, with special attention to structures that are potential landmarks for comparing amphioxus with other chordates. The larval nerve cord is divisible, at approximately the level of the first somite, into a short anterior region, the cerebral vesicle (c.v.), and an extended posterior region that is thought to include homologues of the vertebrate hindbrain and spinal cord. The c.v., in turn, has an anterior part with a tubular neural canal and a posterior part with a keyhole-shaped neural canal similar to that found in the rest of the cord. The junction between these two parts of the c.v. is marked by a cluster of infundibular cells. The anterior c.v., whose cells have cilia that point anteriorly, includes (i) a structure we call the frontal eye, consisting of a pigment spot and transverse rows of putative receptor and nerve cells, and (ii) several small ventral commissures bridging the major nerve tracts that run ventrolaterally along either side of the nerve cord. The posterior c.v., in contrast, contains cells whose cilia point posteriorly, and includes (i) the beginnings of the floorplate, which continues posteriorly through the rest of the nerve cord, (ii) the dorsal lamellar body, made up of cells with cilia that expand into flattened lamellae, and (iii) a large ventral commissure that incorporates fibres arising from cells of the lamellar body. Where probable homologues of c.v. structures can be identified in vertebrate brain, they are found in the diencephalon, which suggests the c.v. and the vertebrate diencephalon are, to a degree, homologous.

On structural evidence, the frontal eye and the lamellar body are both ciliary photoreceptors, contrasting with the microvillar ocelli (organs of Hesse) distributed along most of the rest of the nerve cord. In young larvae the lamellar body is large and conspicuous. We suggest it functions as a high sensitivity, non-directional photoreceptor. The frontal eye is smaller, but organizationally more complex. We suggest it functions as a low sensitivity directional photoreceptor. The frontal eye is potentially of considerable interest from an evolutionary standpoint. Two issues are discussed: (i) that it is sufficiently similar in structure and organization to the paired eyes of vertebrates to indicate homology; and (ii) that its position at the extreme anterior end of the cord, together with structural and histochemical evidence, suggests it may derive from the apical organ, an evolutionarily ancient structure marking the embryonic anterior pole in diverse invertebrate phyla, whose homologue in chordates has hitherto not been identified.

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1. INTRODUCTION

A number of current schemes for vertebrate origins identify cephalochordates (amphioxus) as the sister group for vertebrates. Amphioxus may thus be the closest living relative of vertebrates and the best indication of what their immediate ancestors may have been like (Bone 1960*a*; Jollie 1973; Gans 1989). The two groups share a similar body plan that includes a dorsal nerve cord, notochord, repeating somites and a pharynx with gill slits, but amphioxus lacks most of the characteristic support structures and sense organs of the vertebrate head. The nerve cord in amphioxus nevertheless has an anterior swelling, the cerebral vesicle, that is brain-like in some respects, containing cells and groupings of cells that appear to have counterparts in the vertebrate brain. Although comparatively few in number, these have been useful in past comparative studies, which have generally supported the idea that the cerebral vesicle contains elements whose probable homologues, in vertebrates, are found in the diencephalon (Olsson & Wingstrand 1954; Olsson 1986).

The regional subdivision of the vertebrate brain and its development are currently subjects of intense interest among developmental and evolutionary biologists. The molecular methods now available permit regional homologies to be established on the basis of the expression patterns of developmentally important genes (see Holland 1992). Extending this type of analysis to amphioxus is beginning to provide important new information on ancestral patterns of chordate neural organization, specifically, concerning vertebrate rhombomeres and their counterparts in amphioxus nerve cord (Holland *et al.* 1992). To date, the central nervous system (CNS) in amphioxus is known chiefly from light microscopical studies of sections or whole mounts stained selectively for nerves by various methods (Drach 1948; Bone 1959, 1960*b*; Guthrie 1975). Electron microscopical (EM) studies have also been done, but these focus mainly on a few readily identifiable structures and cell types, notably secretory cells and photoreceptors (e.g. Olsson 1962; Nakao 1964; Eakin 1968; Welsch 1968; Meves 1973; Ruiz & Anadon 1991). Until recently, studies of the larval nervous system and the process of neural development have been hampered by the difficulty of obtaining gametes and raising the larvae.

Our strategy with amphioxus has been to examine young larval stages whose small size especially suits them to serial reconstruction at the EM level. We provide here an initial report on the 3–4 gill slit larva of *Branchiostoma floridae*. Morphology was reconstructed from serial electron micrographs examined at intermediate magnification, with emphasis on overall patterns of cell organization and distinctive features and cell types. We deal with general features of nerve cord structure (§ 4*a*), the anterior fibre tracts (§ 4*b*), the cerebral vesicle (§ 4*c*), and the frontal eye (§ 4*d*). The results clarify the nature of a number of key features recognized by past workers, and add new features of interest. These are summarized and discussed in § 5*a*. Section 5*b* deals further with the frontal eye, which we believe to be of particular evolutionary significance.

2. METHODS

The results are from *Branchiostoma floridae* larvae collected from plankton in Apalachee Bay and Tampa Bay, Florida over a period of several years, and from cultured larvae raised from gametes obtained by artificially induced spawning of ripe adults as described by Holland & Holland (1989, 1993*a*). The cultures were maintained at 25°C, first by Linda Holland through cleavage and hatching, and then by T. H. J. Gilmour, in Saskatoon, once feeding had commenced. Larvae from these cultures began metamorphosis during the fourth week.

For EM, fixation was by the semisimultaneous method (Lacalli & West 1986). Specimens were prestained with uranyl acetate and embedded in Spurr's resin. Sections were collected on slotted grids provided with a formvar support film, *ca.* eight sections per grid. Synaptek grids (J.B. EM Services, Dorval, Quebec) were used throughout because their rigidity significantly reduces damage to the support film during handling, which is crucial for serial EM work.

The results derive from four larvae, staged by body length and sectioned as shown in table 1. Somite pairs in amphioxus are staggered so that the left and right members of each pair are out of register. As a convention, the front of a given somite was marked by taking an average of the anterior position of the right and left members of the pair. Axial position along the body is given by somite number, measured from the front of each somite pair (e.g. 1.5 is halfway between the anterior ends of somites 1 and 2), with the first somite defined as in Holland *et al.* (1992).

Two main section series were obtained, comprising 1500 and 3400 sections, with a loss rate of *ca.* 5%. Micrographs were taken in a single run through each of the series at intermediate magnifications (× 3900–5200), selected in each instance to show the whole of the cord. Further work on selected regions at higher magnification is in progress, but the results reported here do not depend on high power details. The two specimens from which section series were obtained differ in fixation as follows: specimen 3 is from a planktonic collection of larvae that had suffered some prefixation trauma, causing cells to shrink, and leaving spaces between the cells and fibres. This is clearly an artefact, but it considerably simplifies the tracing process, especially for fibres that travel long distances. Specimen 4 is from culture. Cultured larvae show

Table 1. *Specimens sectioned*

specimen	length (mm)	approx. age	region cut	interval	length of series
1	1.25	4 d	*ca.* 4–15	irregular	—
2	1.3	6 d	1.0–8.3	2.5 μm	—
3	1.45	8 d	1.0–2.9	4 μm	—
			2.9–7.0	serial	3400
4	1.75	12.5 d	1.1–2.5	serial	1500

much better preservation and minimal shrinkage, but the close juxtaposition of cell processes and fibres in such specimens makes the neuropile difficult to study. The electron micrographs and reconstructions shown here, except figure 16, are of specimen 4, but results from the other specimens agree in essential features except where otherwise noted.

For reconstruction, cells and neurites were identified and traced through the micrograph series manually on 8 × 10 inch prints. Selected cell outlines were then digitized using an IBM PC and digitizing tablet, and reconstructed on a Silicon Graphics Indigo workstation using Skandha, a three-dimensional reconstruction software package developed by John Prothero and colleagues at the Department of Biological Structure, University of Washington (Prothero & Prothero 1989). To save digitizing and computing time, comparatively low resolution images were prepared that incorporate data only from selected sections. Typical imagefile size for a single cell reconstructed in this fashion is about 75 kB. The resolution limit of the digitizing tablet is 0.1 mm which, for the prints used here, translates to 0.007 μm. Digitizing profiles from every section at the resolution limit would improve the images, but generates more data than can be effectively handled. A better strategy is to adjust the resolution for each structure: fine details can be digitized at high resolution and added to lower resolution images of the cell bodies. Each Skandha imagefile then acts as a library containing all that is known about a particular set of structures or cells that can be updated and refined as the analysis proceeds.

3. ABBREVIATIONS USED IN THE TEXT AND FIGURES

an.c.	anterior commissure
ap.	apical organ
c	central canal
cm	principal commissure
d	dorsal cell
fp	floorplate
in	infundibular cells
lam	lamellar body
n	neuropile or fibre tracts
not	notochord
np	neuropore
m	mouth
p	anterior pigment spot or pigment cells
p.c.	posterior commissure
po.c.	postoptic commissure
rn	rostral nerve
R1,2,3	row 1, 2 and 3 cells
som	somite
t.b.	preoral transverse ciliary band
v.l.t.	ventral longitudinal tract
v.t.c.	ventral tegminal commissure
*	see figure legend

4. RESULTS

B. floridae larvae are functional and capable of feeding by 72 h, by which time they are about 1 mm long and have a single gill slit. The substantial increase in body size that occurs subsequently during the larval phase can be appreciated by comparing the examples shown in figures 1 and 2. Proliferative growth and cell differentiation in the nervous system is a gradual and prolonged process. The nerve cord increases in diameter, the ventricular walls become thicker, the number of fibres increases, from about 350 in a typical cord section at 12 days to roughly 20 000 in the adult (Guthrie 1975), and there is a gradual appearance of new cell types (for examples, see Holland & Holland 1993*b*). In consequence, what one can discover about neural organization from structural data depends very much on the stage chosen.

The success of our EM analysis depends on small size and limited fibre number, so only very young stages are simple enough to repay detailed study. We are concerned here with larvae having 3–4 gill slits, which in culture means larvae *ca.* 7–14 days old (figure 2*a*,*b*). During this period the larvae change only moderately in length, and there are few obvious external changes. The two anterior gill slits are large; slits 3 and 4 appear first at the ventral midline, but are slow to develop and remain comparatively small. The nerve cord does not change significantly in size, and we see

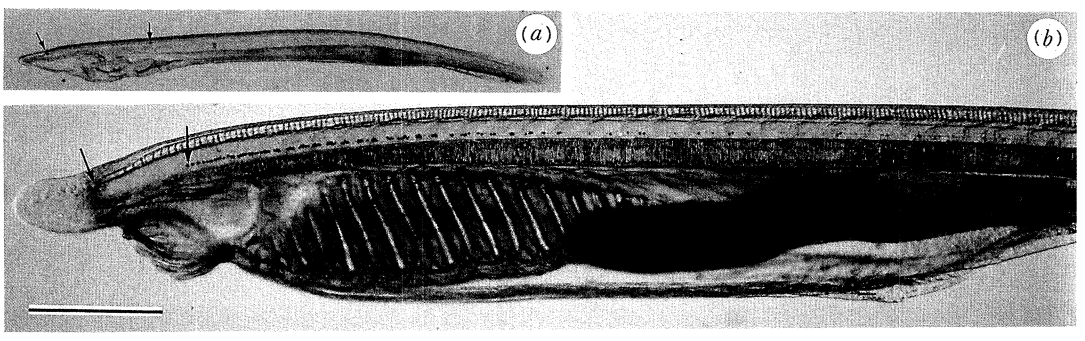


Figure 1. Larval and juvenile *Branchiostoma floridae*, both to the same scale. (*a*) 14 d larva. Arrows show the anterior pigment spot and the first of the dorsal ocelli to develop, which lies in somite 5. (*b*) Juvenile shortly after completion of metamorphosis. Arrows show the anterior pigment spot and the anterior-most of the dorsal ocelli, which now form a continuous row beginning in somite 3. None occur in the anterior cord between the arrows. Scale bar = 500 μm.

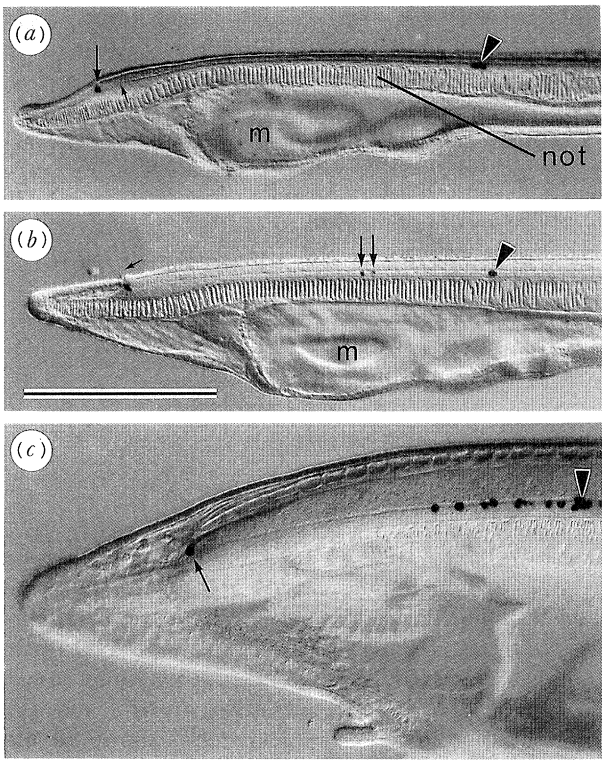


Figure 2. A stage series of *B. floridae* larvae, all to the same scale. (*a*) 6 d larva. The figure shows the anterior pigment spot (left-most arrow) and the first of the dorsal ocelli that develops (arrowhead). The small arrow indicates the point at which the central canal narrows, which is the approximate junction between anterior and posterior c.v. (*b*) 14 d larva. Shows the first dorsal ocellus as in (*a*), two more developing in somite 3 (double arrows), and cilia emerging from the neuropore (small arrow). (*c*) Late larva, an early stage of metamorphosis. There is now a continuous row of ocelli including a cluster in somite 5 (arrowhead). The arrow shows the anterior pigment spot. Scale bar = 200 μm.

few mitoses in sections. The number of fibres in the neuropile increases considerably, however, suggesting that the second week of development is a major period of neural differentiation and fibre outgrowth.

Most of the electron micrographs, and all of the reconstructions shown here, come from a serial series through a single 12.5 d larva (specimen 4, table 1), comparable in general appearance to the larva shown in figure 2*b*. Sections illustrated by micrographs are shown as seen from the front, i.e. the right side of the larva is to the left in the figures. Our series from specimen 4 extends through half of somite 2, a region covered by series at larger intervals in two other specimens. The main structures described occupy similar relative positions in all three larvae, and some of these are visible in whole larvae as well.

Anterior somite boundaries are difficult to see in whole specimens, but the larval pigment spots are easily seen and are useful landmarks. The anteriormost spot, which belongs to the frontal eye (§ 4*d*), lies at the very front of the neural tube in front of, and just below, the neuropore. A further series of pigment spots lie along the nerve cord, embedded in its ventral surface at or near the midline. These belong to the dorsal ocelli, or organs of Hesse, each of which consists of a pigment cup enclosing a receptor cell (Eakin & Westfall 1962; Nakao 1964). The first of these develops in the posterior half of somite 5, and is unusual in having two receptor cells, each enclosed separately by the single pigment cell. Additional ocelli develop subsequently, with some variation between larvae in the precise position and order of their appearance. The second one usually develops in the posterior half of somite 3, as in figure 2*b*, and none develop further forward than about the midpoint of this somite. There is thus a persistent anterior gap in the row of dorsal ocelli spanning about 2.5 somites. The absolute size of this gap increases only slightly as development proceeds, e.g. the distance between the front of the cord and first ocellus in somite 5 is *ca.* 385 μm in the specimens in figure 2*a,b*, compared with 510 μm in the post-metamorphic stage shown in figure 1*b*.

(*a*) *General features of nerve cord structure*

A section of the nerve cord showing typical features is shown in figure 3. The main fibre tracts are predominantly ventrolateral, and the cell bodies are arranged in a single ventricular layer around the central canal. All the cells of the neural tube retain their apical connection to the canal, and their cilia project into it, with the exception of one class of large axial glial cells. The latter do not, however, occur in the cerebral vesicle.

All the cells of the ventricular layer are uniciliate. Among the non-neural cells, we recognize two main types, ependymal cells and ependymoglia. Both have a similar dense, granular cytoplasm. Ependymal cells have broad areas of attachment to the basement membrane, and lack specialized processes, whereas ependymoglia have characteristic slender, fibre-filled processes that form a loose scaffolding through which tracts of axons pass. Below, in describing the cerebral vesicle, we have used the term 'support cells' for the ependyma there, because it is not clear whether these cells are all of one type or whether they differ from ependymal cells elsewhere in the cord. We also describe 'glial-like' cells in the anterior region that are clearly quite different from ependymoglia.

Neurons are scattered along the cord singly or in small clusters. They vary in appearance, but typically are paler and less uniformly granular than ependymoglial cells, and various inclusions and vesicles fill their cytoplasm. Axons are clearly distinguished from ependymoglial processes: the former contain microtubules and the latter dense fibre bundles. A number of the largest and best differentiated of the neurons lie ventrally (examples in figure 3), immediately adjacent to the floorplate. The size of these cells, together with the relative positions of their fibres in the ventrolateral tracts, suggest that they develop very early. Included among these are some very large primary motor neurons. Amphioxus lacks ventral somatic motor nerve roots entirely (Flood 1966). Instead, each muscle fibre extends a cell process to the ventrolateral surface of the nerve cord, and synapses occur across the basement membrane, as shown in figure 3.

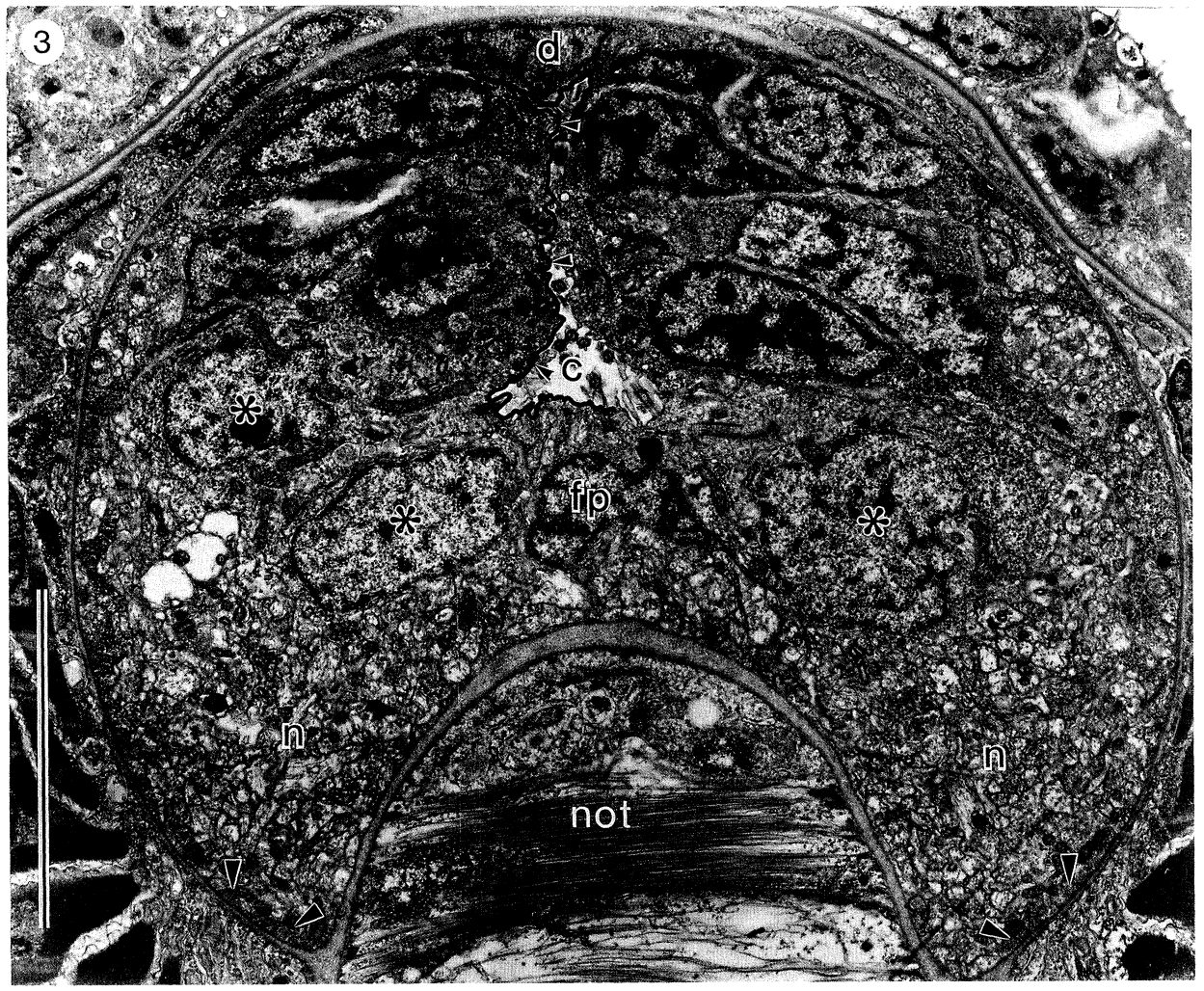


Figure 3. A section through the nerve cord near the back of somite 1. Ventrolateral nerve tracts (n) are shown, each with a zone in which neuromuscular junctions form (between arrows) with the adjacent somite. The large ventral cells (*) are neurons, and the ventral midline is occupied by a single floorplate cell (fp). The ventral part of the central canal (c) is open. It narrows dorsally, so the cells on opposite sides are directly apposed, and is capped at the top by a single dorsal cell (d). Its surface is traced on one side for emphasis (solid line, small arrows). Scale bar = 5 μm.

Floorplate cells are easily distinguished. They are small, angular and rather dense cells that form a row one or two cells wide along the base of the cord. They sit either directly on the basement membrane atop the notochord or, in commissural regions, send basal endfeet through the commissure to the basement membrane. Each floorplate cell has a single cilium projecting upward; the accessory centriole is positioned perpendicular to the cilium and posterior to it. From what is known of rootlet orientation in related larvae (Nielsen 1987), and assuming the cilia are motile, this implies a posteriorly directed beat. Cilium orientation and the implied direction of beat is more difficult to determine elsewhere in the cord, because many of the cilia are raised on projecting knobs that are twisted or deformed by adjacent cells. Cilium orientation does, however, appear to vary with cell type, and no simple generalizations are yet possible from our data.

In live larvae (figure 2), the central canal of the nerve cord is evident as a narrow channel running just above the ventral surface of the cord. In section, it is evident the canal extends almost to the dorsal surface of the cord, but dorsally the inward-facing lateral surfaces of the neural tube are pressed together leaving only a narrow slit between them. Overall, the shape is roughly that of an inverted keyhole. Where they are closely apposed, the two sides of the cord are held together by cell processes that cross the canal from either side. This occurs in the adult as well, and is illustrated in a number of published reports. Bone (1960*b*) refers to the cells involved as commissural cells, and identifies a number of distinct types. Some of the cross-connections in the larva are neurite-like (Lacalli & West 1993), which suggests a role in neural transmission, but others are not. A row of flattened dorsal cells lies along the very top of the cord (d in figure 3), and these probably assist in holding the cord together. A single such cell is found in most sections, but in two regions the cells occur in pairs: at the neuropore (figure 7), and along the lamellar body (figures 11 and 14).

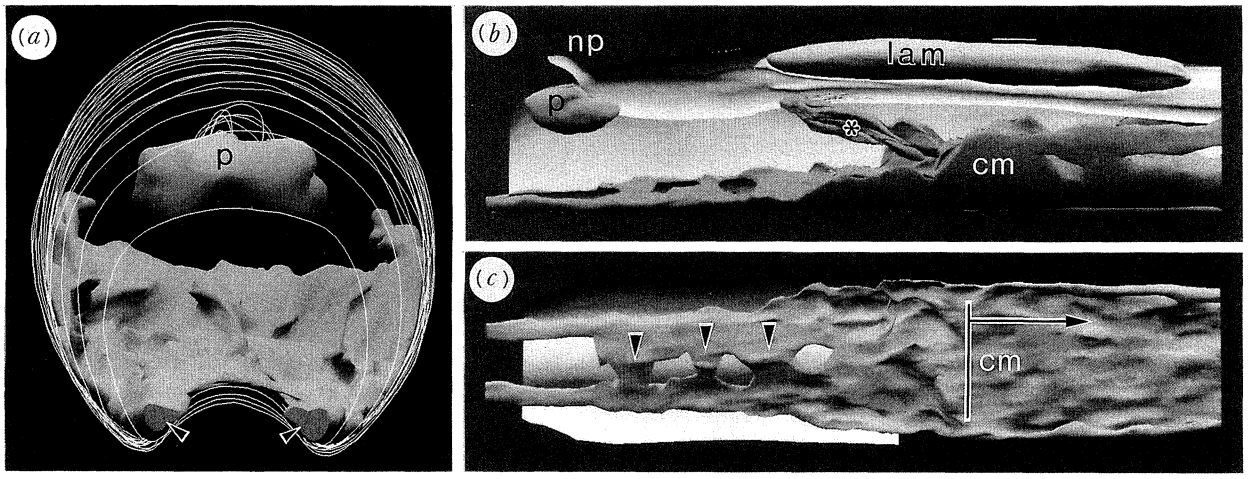


Figure 4. Reconstructions of the cerebral vesicle of specimen 4. (*a*) Front view, cord outlined as contours. Figure shows the anterior pigment spot (p) and the domain occupied by fibre tracts, including the two rostral nerves (arrows) that leave the cord at its anterior end. (*b*) Side view with the facing surface of the cord cut away. Fibre tracts, pigment spot, neuropore (np), lamellar body (lam), the cluster of infundibular cells (*), and the position of the main commissure (cm) are shown. (*c*) Top view of the fibre tracts showing positions of the small anterior commissures (arrows) and the anterior margin and approximate posterior extent of the main commissure (cm, compare with (*b*)). Magnification: (*a*) × 2830; (*b*,*c*) × 1210.

(*b*) *Anterior fibre tracts*

The main tracts in the nerve cord run along its ventrolateral margins. Fibre numbers decrease as these move forward through the cerebral vesicle, until only about a dozen remain on each side (figures 7, 9 and 11). The tracts continue forward and out of the cord at its anterior end, to form a pair of anterior, rostral nerves (figure 4). These are the largest of the peripheral nerves in the larva at this stage. The only others encountered were small, dorsal nerves, two pairs of which arise in the anterior cord as in figure 5. Other small fibre tracts can be distinguished within the anterior nerve cord, but are not dealt with here.

Numerous fibres cross from one side of the nerve cord to the other at various points along its length, passing beneath the floorplate singly or in small groups. There is no clear order to the way these crossings are positioned, e.g. no obvious segmental pattern, and only one large commissure overall. This lies in the anterior region near the back of somite 1 (position shown in figures 4–6). Its fibres occupy almost the whole lower half of the cord (figure 14), disrupting the connections between ependymal and floorplate cells and the basal lamina. This makes it difficult to determine whether there is, in fact, a proper floorplate in this region. Our interpretation is that it is probably present but discontinuous. Individual floorplate-type cells occur, but cells of other types are found in the ventral midline interspersed between them.

In front of the large commissure lie several much smaller, commissure-like cross-connections. We can identify three such commissures in specimen 4 (figure 4*c*), but do not know how constant this number is between larvae. The anterior-most is associated with the cells of the frontal eye (§ 4*d*).

(*c*) *The cerebral vesicle*

The term cerebral vesicle (c.v.) has been used to refer to the expanded anterior region of the nerve cord evident in adult amphioxus. This region contains a number of distinctive structures not found elsewhere in the cord. Its anterior part, in the adult, is cylindrical with a central canal that expands dorsally (the 'dorsal dilation') and ventrally (the infundibulum), before narrowing again at the beginning of the nerve cord proper. The dorsal roof of the c.v. contains cells whose cilia produce lateral stacks of membranous lamellae. These are thought to act as photoreceptors and, as a group, to form an organ that may be a homologue of the vertebrate pineal (Eakin 1968; Ruiz & Anadon 1991). Clusters of peculiar giant cells with microvilli, the Joseph cells, lie just behind the lamellar cells (Welsch 1968; Watanabe & Yoshida 1986). These are probably also photoreceptors, but have no obvious counterparts in other chordates. The floor of the c.v. has a distinctive cluster of secretory infundibular cells that produce Reissner's fibre, a non-cellular strand that is presumed to be carried down the central canal by ciliary beat (Olsson 1962, Obermüller-Wilén 1976). Nothing clearly marks the posterior end of the c.v., and its size and extent are variously interpreted in different accounts.

A diagrammatic dorsal view of the larval c.v. and anterior cord from our specimens, showing somite registry and the main landmarks, is shown in figure 5. A midsagittal view is illustrated in figure 6. Figures 7–15 show selected sections, at levels indicated in figure 5, and various details. In live larvae (figure 2*a*,*b*), the c.v. is visible as a slightly expanded region of the anterior neural tube with a thickened ventral wall. It extends some 75–80 μm before tapering to more typical cord dimensions by somite 2. The central canal

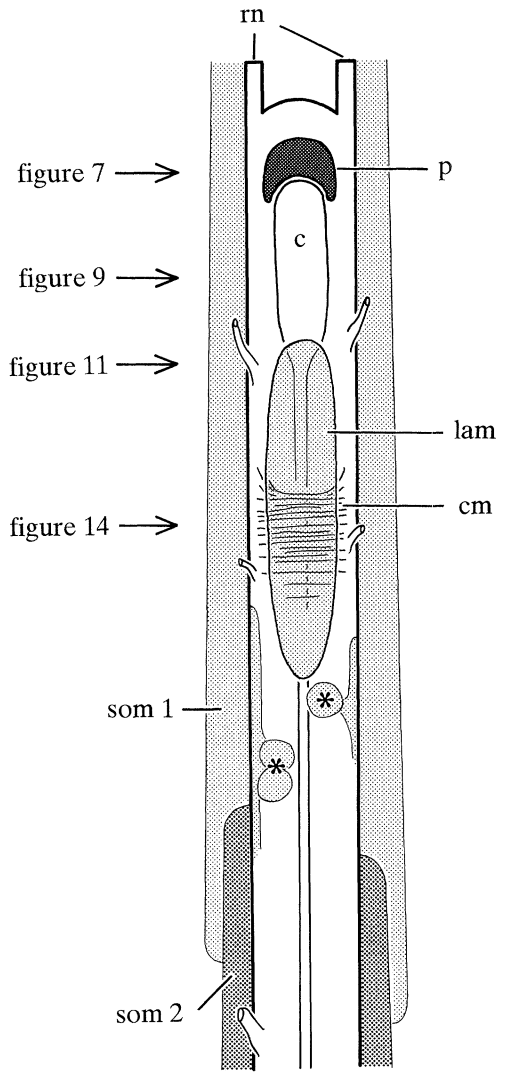


Figure 5. Top view of the anterior end of the nerve cord showing how its main structures are positioned: rostral nerves (rn), pigment spot (p), central canal (c), dorsal lamellar body (lam), commissure (cm), cell bodies (*) of the anterior-most motor neurons with the anterior extent of their synaptic fields indicated. The small dorsal roots in this region are shown but not labelled.

is comparatively spaceous for the first 25–30 μm, and then narrows (small arrow in figure 2*a*), so that the open part of the canal forms a narrow channel.

There is a clear internal subdivision of the cerebral vesicle into anterior and posterior parts. The anterior c.v. has a central canal that is uniformly circular in section, displaced dorsally, and packed with cilia (figure 9). Tall, flask-shaped cells occupy its ventral and ventrolateral walls, while more dorsal cells are flatter and less numerous. Together the cells form an uninterrupted epithelial tube without a floorplate or any similarly distinctive midsagittal structures. The open portion of the central canal appears to narrow towards the back of the anterior c.v. In fact, it expands dorsally in this region, but as elsewhere in the cord, the two sides of this dorsal expansion are closely apposed, the gap between them is bridged by apical processes from cells on both sides (e.g. figure 13), and the lumen is essentially obliterated. Beginning near the front of the posterior c.v., the dorsal-most part of the canal dilates to accommodate the lamellar body, a sausage-shaped mass of membranous lamellae that extends the whole length of the posterior c.v., a distance of about 50 μm in 12 d larvae (figures 4*b*, 11 and 14). The lamellae arise in parallel stacks (figure 15) from the sides of cilia belonging to cells ranged in a row along each side of the lamellar body. The individual lamellar cells have been described by other authors, but a large structure formed by ordered rows of lamellar cells has not. The structure probably fragments later in development, judging from the scattered, irregular arrangement of lamellar cells in the adult (Meves 1973).

We as yet find no distinctive feature to mark the posterior limit of the cerebral vesicle, but the posterior end of the lamellar body is a convenient provisional landmark. The lamellar body terminates just in front of the cell bodies of several large primary motor neurons that innervate the first somites (figure 5), but there is otherwise no sudden transition in this region in terms of cord morphology or organization.

In contrast, the junction between the anterior and posterior c.v. is quite clear, the precise landmark

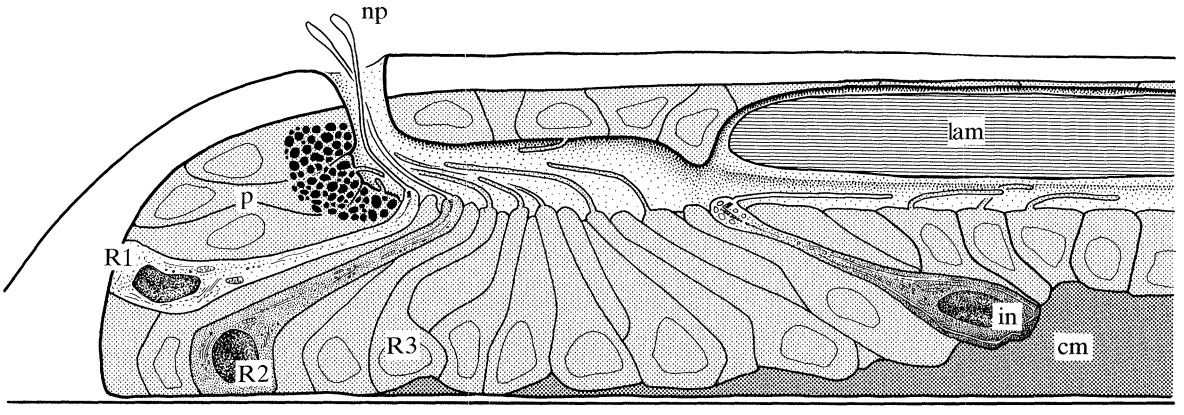
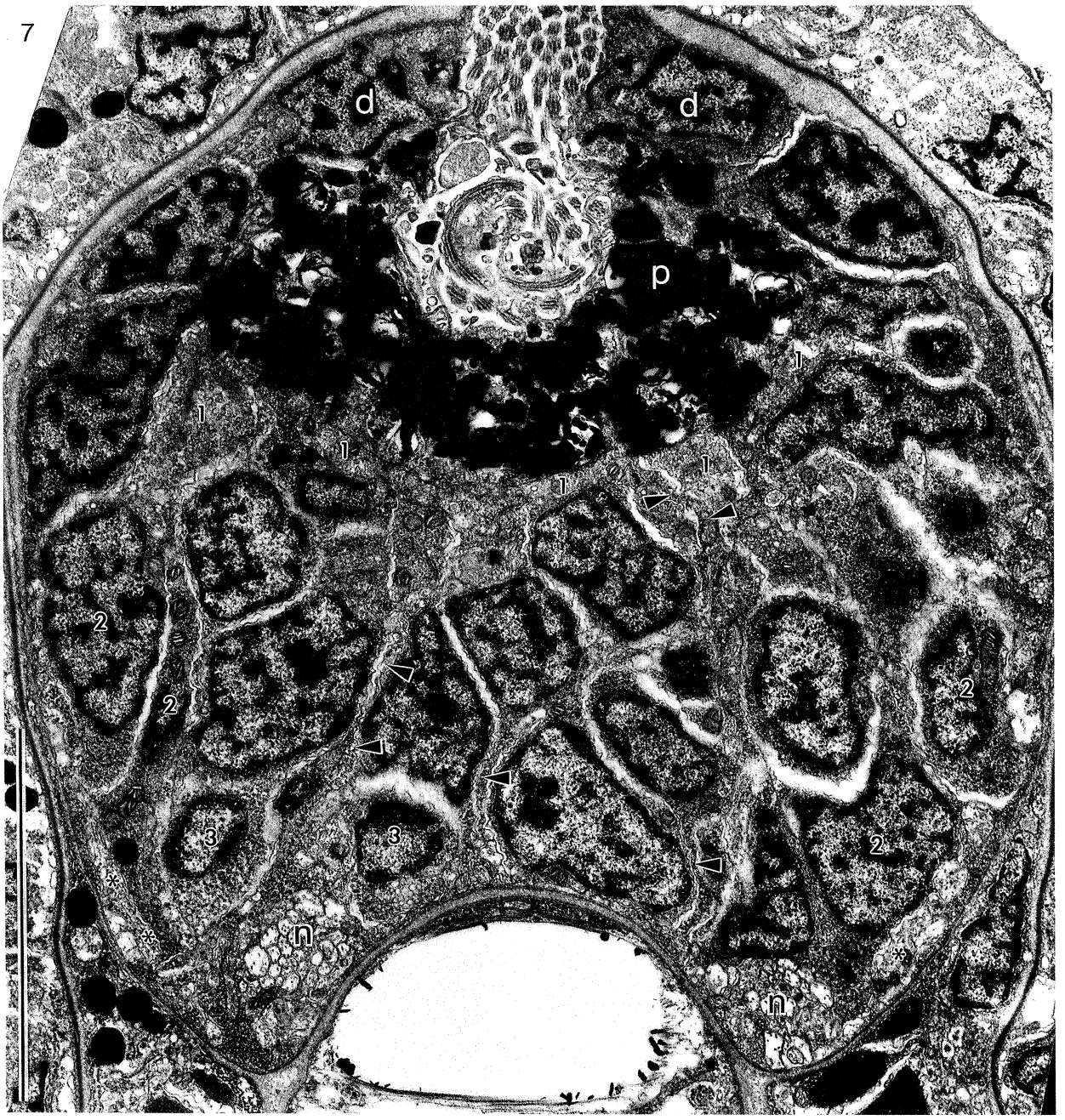


Figure 6. Cut-away side view of the anterior two-thirds of the cerebral vesicle at 12.5 d. Shows pigment cell cluster (p), neuropore (np), lamellar body (lam), an infundibular cell (in), commissural fibres (dark shading, main commissure at cm), and anterior cells belonging to rows 1–3 (R1–R3).



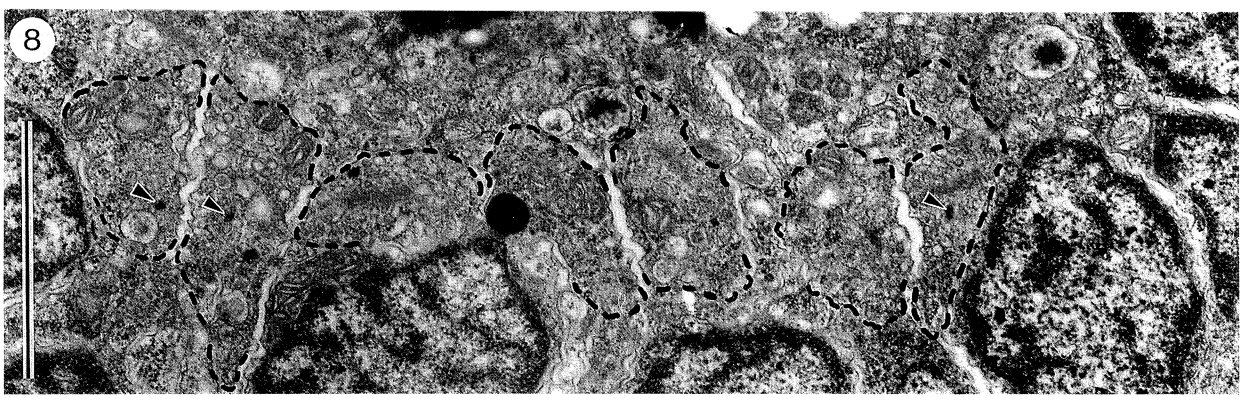
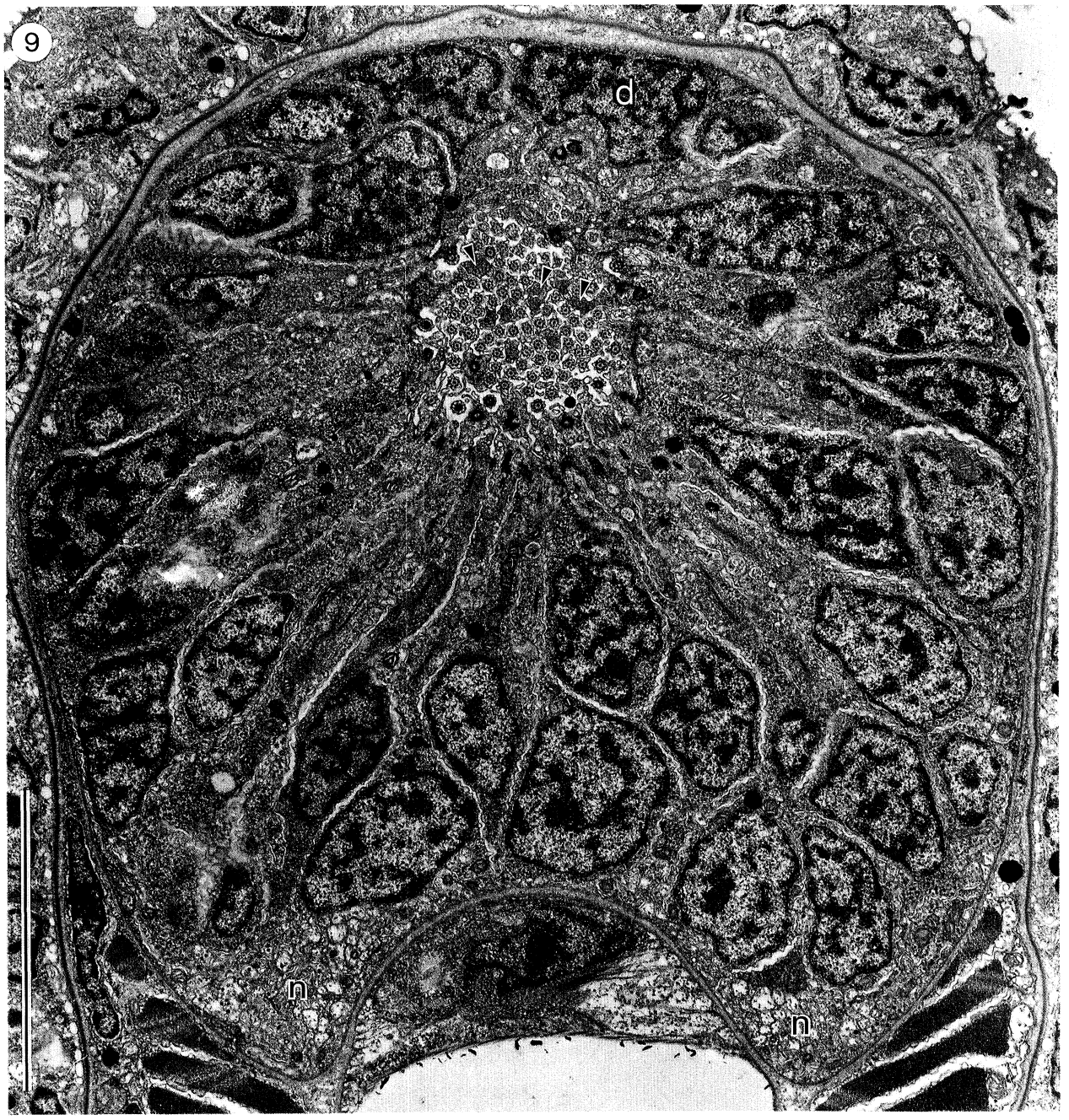


Figure 7. A section through the cord at the level of the pigment spot (p). Shows the nuclei of several row 2 and 3 cells (labelled 2 and 3) and tapering apices of row 1 cells (labelled 1). Most of the cells are, however, supporting or glial-like cells. Arrows indicate some of the curtain-like processes of the latter as described in the text. Shows also the ventrolateral tracts (n) and descending basal processes from two row 1 cells (small *s). Scale bar = 5 μm.

Figure 8. Detail just below the pigment spot, near its posterior margin, at the level of the tapering apices of the row 2 cells. Shows a transverse row of seven of these (cell boundaries partially outlined), some with rootlets (arrows). Scale bar = 2 μm.



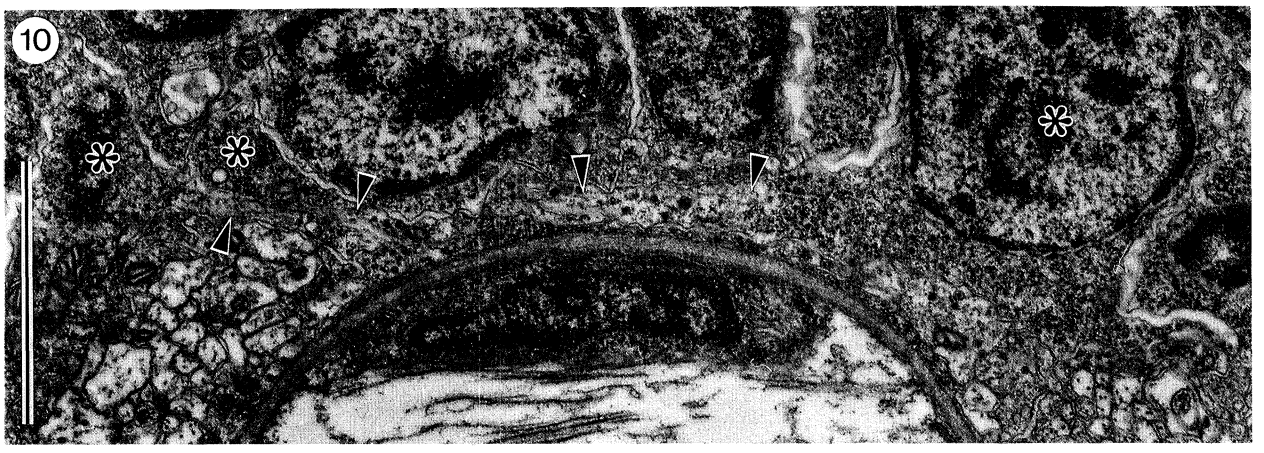


Figure 9. A section 10 μm behind the posterior lip of the pigment spot showing typical arrangement of cells in the anterior c.v. The central canal is circular and packed with cilia; the ventrolateral tracts are still small. Arrows indicate several examples of cilia where the axoneme is replaced by a uniformly dense matrix. None of those in this section are much larger than an average cilium in diameter, but much larger ones do occur. Scale bar = 5 μm.

Figure 10. A section 1.5 μm behind the posterior lip of the pigment spot at the level of the first small commissure. Basal processes (arrows) from three row 3 cells (*) appear in the section. Scale bar = 2 μm.

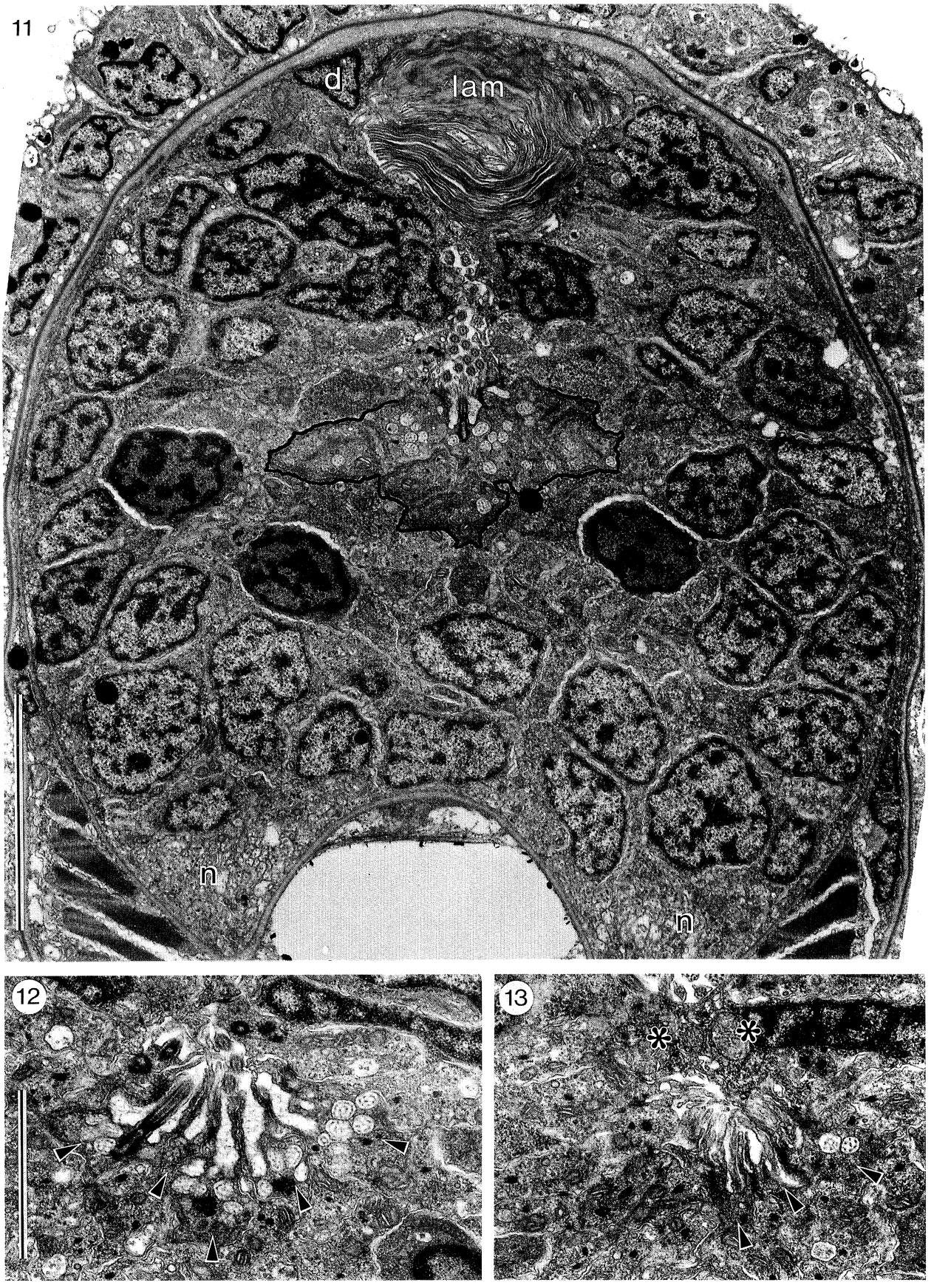


Figure 11. A section at the level of the infundibular cells, shown in outline. The section cuts the anterior part of the lamellar body (lam). The single vertical cilium belongs to the posterior-most of the infundibular cells; the ciliary profiles above it come from others. Scale bar = 5 μm.

Figures 12 and 13. Sections just anterior to figure 11, both to the same scale. Figure 12 shows the apical surfaces, cilia, and associated secretory vesicles of three infundibular cell apices (arrows). Figure 13 is *ca.* 1 μm further forward and shows the anterior-most part of three infundubular cells (arrows). In front of their cilia, they have microvilli that fill the open portion of the central canal, which is otherwise devoid of cilia. Apical cell processes (*) bridge the canal above this point. Scale bar = 2 μm.

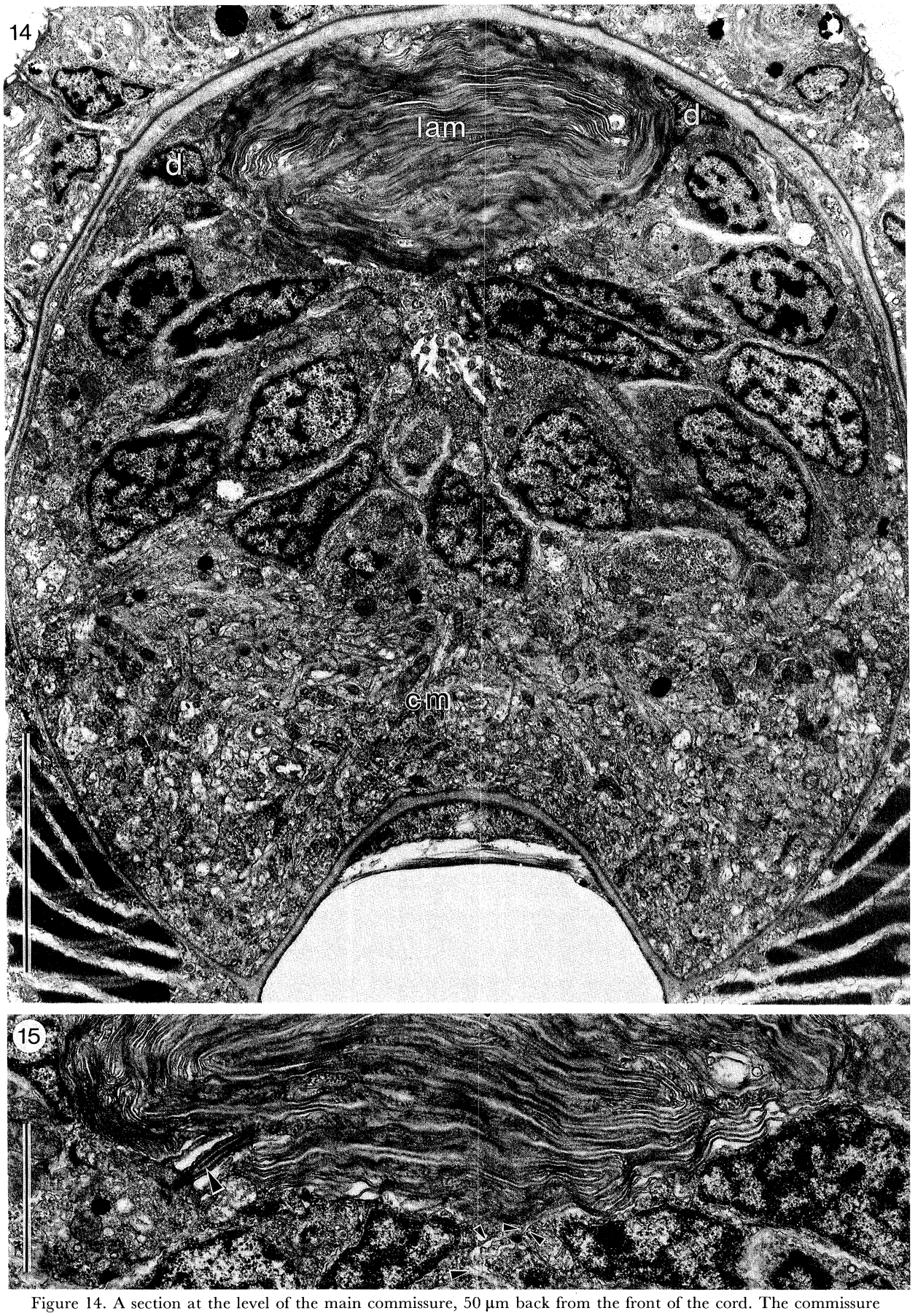


Figure 14. A section at the level of the main commissure, 50 μm back from the front of the cord. The commissure occupies the lower third of the cord, while the lamellar body (lam) occupies most of the upper third. Scale bar = 5 μm.

Figure 15. Detail of the lamellar body showing a cilium of one of the cells and lamellae arising from it. Small arrows show the ventral continuation of the very narrow central canal. Scale bar = 2 μm.

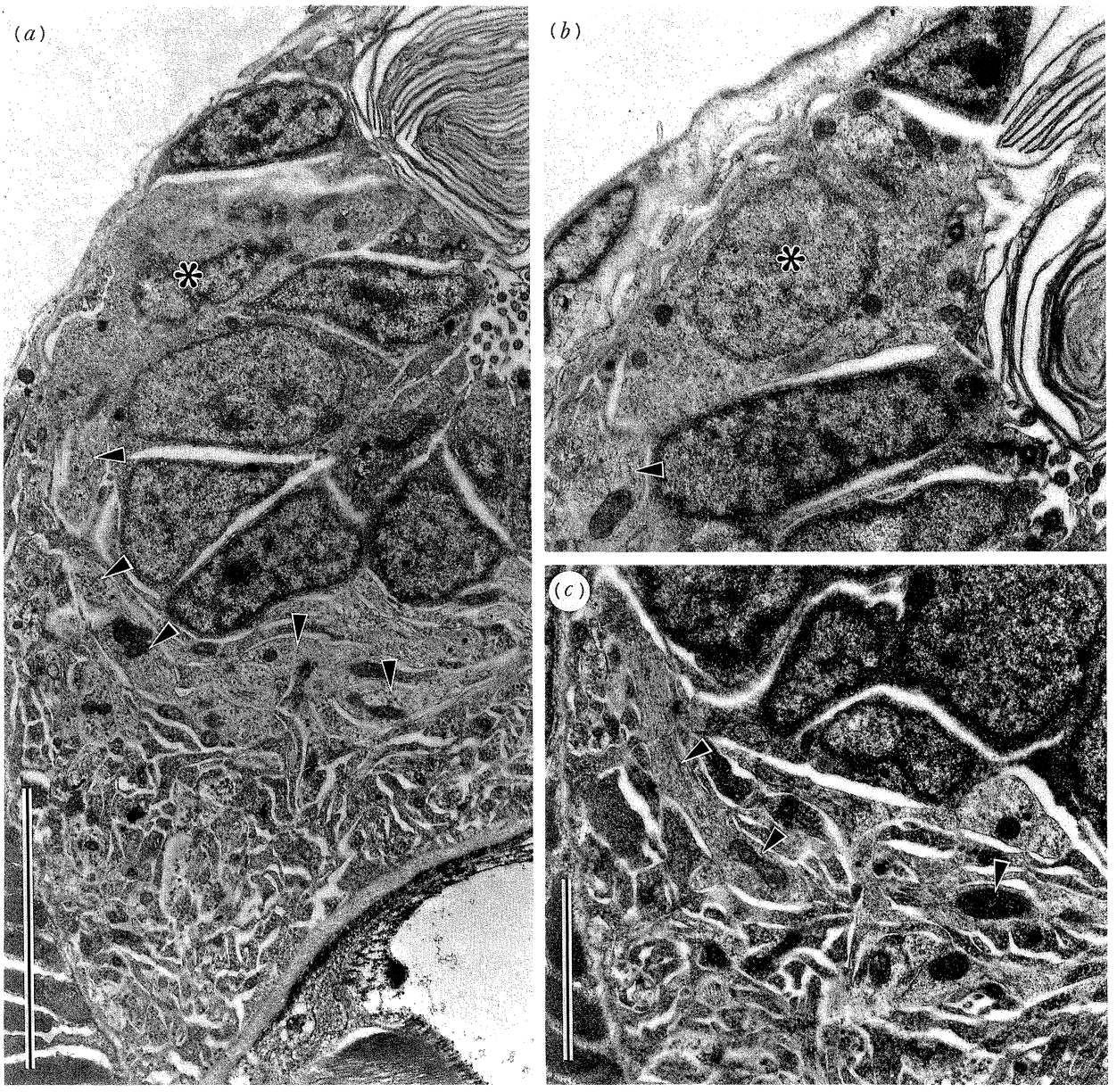


Figure 16. Sections of specimen 2 at the level of the anterior part of the main commissure. Shows descending fibres (arrows) from the soma (*) of cells responsible for the lamellar body. (*a*) Scale bar = 5 μm. (*b*,*c*) Scale bar = 2 μm.

being the infundibular cells. In specimen 4 there are nine such cells forming a single cluster. These have a very characteristic ultrastructure (figures 11–13) with distinctive apical granules, a sunken pit around each cilium through which the granules are evidently expelled, and extensive flat cisternae of rough ER. The cells are more numerous, but otherwise identical in older larvae, as described by Olsson (1962). Their apices project into the open part of the central canal at its narrowest point. Just anterior to this point, for a short distance, the central canal is devoid of cilia (figure 13). This is because cells positioned further forward have anteriorly projecting cilia, while the infundibular cells, together with two other cells lying just above on either side, are the anterior-most cells in the c.v. to have backwardly projecting cilia. Assuming these cilia beat, the inferred direction of beat based on rootlet structure is also backward, i.e. posteriorly: their accessory centrioles are precisely positioned just behind the ciliary basal body perpendicular to the long axis of the canal. We have made a preliminary examination of rootlet orientation in the anterior chamber of the cerebral vesicle, and cells in its ventral and ventrolateral walls have cilia with the opposite orientation: their accessory centrioles lie in front of the cilium and rootlet, although not always with the same precise alignment with respect to the body axis. The implication is that the infundibular region represents a point of transition between that part of the c.v. having cilia that project forward, and the rest of the neural tube, in which the cilia of at least those cells forming the floor of the cord project and beat posteriorly. Our interpretation is that the infundibular cells should be considered the anterior-most cells of the ventral part of the posterior c.v. Dorsally the point of transition is less obvious.

In side view, the infundibular cells, along with the cells around them, are steeply inclined, such that their apices lie considerably forward of their bases (figures 4*b* and 6). Assuming these cells define the boundary between anterior and posterior c.v., the boundary itself is then also inclined. This appears to be the way the comparatively numerous cells of the sides and floor the anterior c.v. are accommodated within it without producing a ventral bulge: the cells' bases are much wider than their apices, and are displaced forward at the front of the anterior c.v., and backwards at the back (figure 5). The effect is most pronounced in anterior- and posterior-most cells of the anterior c.v., i.e. the row 1 and 2 cells (§ 4*d*) and the infundibular cells. Only cells near the middle (figure 9) are oriented more-or-less vertically.

The bases of the infundibular cells lie buried within the commissure, which suggests that it, too, lies somewhere near the boundary between the anterior and posterior c.v. The commissure extends further back from this point than it does forward, and so is probably best thought of as belonging to the posterior c.v. We have not yet traced its fibres in detail in specimen 4. In specimens 2 and 3, however, some of the largest of these derive from the cells lying along the sides of the lamellar body that supply its lamellae. These cells, along with the infundibular cells, are the most distinctive and best differentiated cells in the cerebral vesicle in these two specimens. Their fibres are large and easily traced (figure 17). Similar large fibres occur in specimen 4. We conclude that the cells of the dorsal lamellar body are major contributors to the commissure. Most cells further forward in the anterior c.v., e.g. those associated with the frontal eye, although they produce some neurites, are probably no more than minor contributors to the commissure.

(*d*) *The frontal eye*

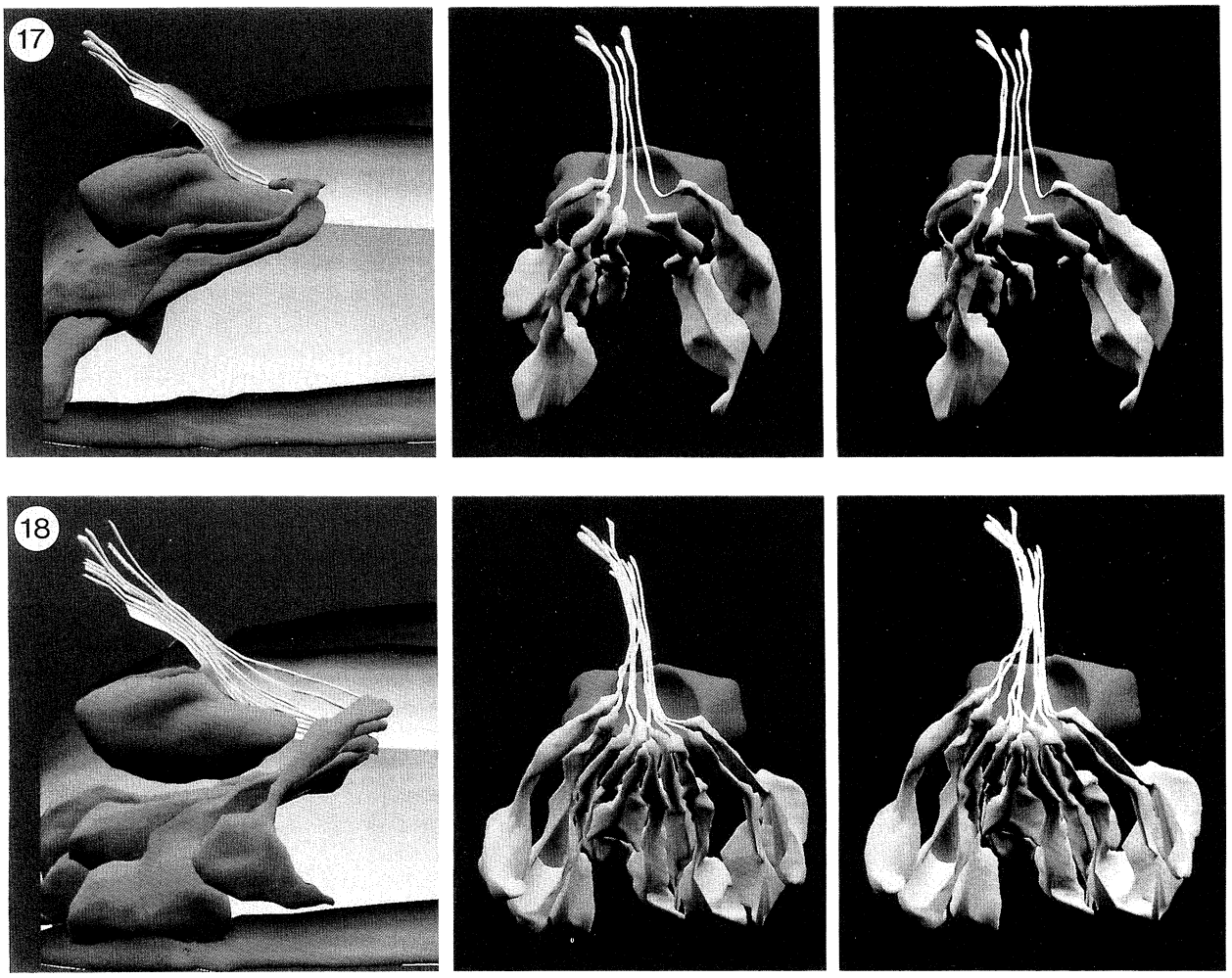
We refer here to the medial anterior pigment spot and the cells associated with it by the term 'frontal eye', although past authorities have disagreed on the nature and significance of this structure (see § 5). We are especially interested in the frontal eye because it contains a distinctive cluster of serotonin-containing cells, and serotonin is a key marker for the apical organ in invertebrate larvae.

The pigment cup is the most anterior part of the eye and its most obvious feature. The pigment granules fill the apical cytoplasm of a cluster of cells whose apical surfaces form the upwardly curving front surface of the neuropore (figures 6 and 7). During neurulation, the neuropore points forward, which means the precursors of the pigment cells lie at the extreme front of the neural tube in a ventromedial position. In the larva, the neuropore is shifted so that it opens dorsally. The walls of the pore itself are formed by two dorsal cells (figure 7), one located on each side of the neuropore, but only a thin extension from these cells continues around the front of the pore. In specimen 4, the pigment cup arises from nine cells arranged in three rows of three cells each. Two cells that are similar, except for having no pigment granules, lie close by, on either side of the third row. They appear to be part of the same cluster. The pigment cup is concave, open dorsally and posteriorly, and is remarkably constant in size, shape and orientation between larvae. The pigment cells have short cilia that lie in a coil at the very front of the central canal (figure 7). These cilia do not leave the neuropore.

Moving progressively back along the ventral wall of the cerebral vesicle, we find cells of several types arranged in a series of transverse rows. We have so far examined only the first three rows in detail (R1–R3, figure 6), and only at intermediate power, but this is sufficient to define their basic characteristics and relative positions unambiguously. The rows are each shown separately in figures 17–19, and a selection of the glial-like cells associated with them is shown in figure 20. The cells are shown together, in various combinations, in figure 21.

Rows 1 and 2 (figures 17, 18 and 21*a*,*b*) consist of flask-shaped cells of rather simple outline, five in row 1 and ten in row 2. They can be distinguished from surrounding support cells because the latter make direct contact with the basal lamina along their basal and lateral surfaces (together they form a contiguous surface). In contrast, the row 1 and 2 cells are encapsulated by these support cells and do not contact the basal lamina. The nuclei of the row 1 and 2 cells lie in front of, or near the front of the pigment spot, so the elongate apical part of each cell must pass beneath it (those of the row 1 cells pass directly adjacent to the pigment cells). Row 1 cells are paler in appearance, but are otherwise ultrastructurally similar to row 2 cells. The cilia of all fifteen cells are of similar length and pass out the neuropore. The shaft of each is well defined with a smooth, circular outline except near the end, where each cilium expands to form a bulbous swelling. The latter may be fixation artefacts, but are nevertheless characteristic. From preliminary evidence, each of the row 1 and 2 cells appears to have a single neurite-like basal process that extends into the region of the first small commissure. We have not traced these in detail, but two examples, both from row 1, are shown in figure 7. The apex of each row 1 cell projects into the central canal separately, i.e. the apices of the row 1 cells are separated from each another by those of surrounding support cells. A further row of support cells then separates the apices of the row 1 cells from those of row 2, but the latter lie side-by-side in direct contact (figure 8). Row 2 cells each have a prominent ciliary rootlet, and both row 1 and 2 cells have a well developed apical Golgi producing numerous vesicles. Neither is true of the surrounding support cells or glial-like cells.

Behind rows 1 and 2, and separated from them by more intervening support cells, is a row of simple nerve-like cells (row 3). We have identified four such cells by their basal processes, but there could be more in the anterior region that we have missed. The row 3 cells so far identified sit directly atop the first small commissure just below the posterior margin of the pigment spot. They have rounded basal nuclei and a very thin layer of perinuclear cytoplasm, and so are small and round in external view (figure 19). Each



Figures 17–20. Reconstructions of cell rows associated with the anterior pigment spot. Left panel in each figure shows a side view of the anterior end of the cord (compare with figure 4*b*); right panels are stereopairs seen from directly behind the pigment spot.

Figure 17. Row 1.

Figure 18. Row 2.

sends a flattened process into the adjacent commissure (figure 10). These resemble other neurites in the commissure, but are short, unbranched, and rather blunt. The cells' cilia extend only as far forward as the base of the neuropore. They are irregular in outline, with a ruffled membrane, and the terminal portions of several are enlarged and flattened, the axoneme being replaced by a considerable volume of dense matrix. A few small profiles of this type are visible in figure 9. Such profiles occur commonly in the anterior c.v., and some are quite large. They are clearly not just fixation artefacts, but specialized ciliary tips of some kind.

We find other nerve-like cells behind row 3, some with neurites that enter the other small commissures. We have not examined these in detail except to note the occurrence of additional transverse rows and clusters of cells of similar type that warrant further investigation.

Supporting cells in the anterior c.v. appear to be of several types, but only one of these was particularly distinctive. We refer to them here as anterior glial-like cells. Four examples are shown in figure 20. They have basal processes that expand to form flattened regions of contact with the basal lamina, especially along the ventrolateral surface of the cord, and they enclose parts of the ventrolateral nerve tracts. Neither of these features is, in itself, unusual. However, the cells also have thin curtain-like extensions that pass between the cells surrounding them (figure 7), and this is distinctive. Their cytoplasm is dense and granular basally, becoming somewhat disorganized and extracted-looking apically, often with irregular membranous bodies of the kind associated with lysosomal activity. The flattened extensions have a clear, somewhat vesiculate appearance that differs from the rest of the cell, and from other surrounding support cells. Although connections to the lateral and ventrolateral surfaces of the nerve cord are seen throughout its length, curtain-like extensions between cells near the ventral midline of the cord are not common except in the anterior c.v.

When traced, the cilia of most of the supporting and glial-like cells proved to be short stubs that tapered to small threads after about 1 μm. Some of the cells had longer cilia, but these appeared degenerate, and typically adhered to the sides of the neuropore. In

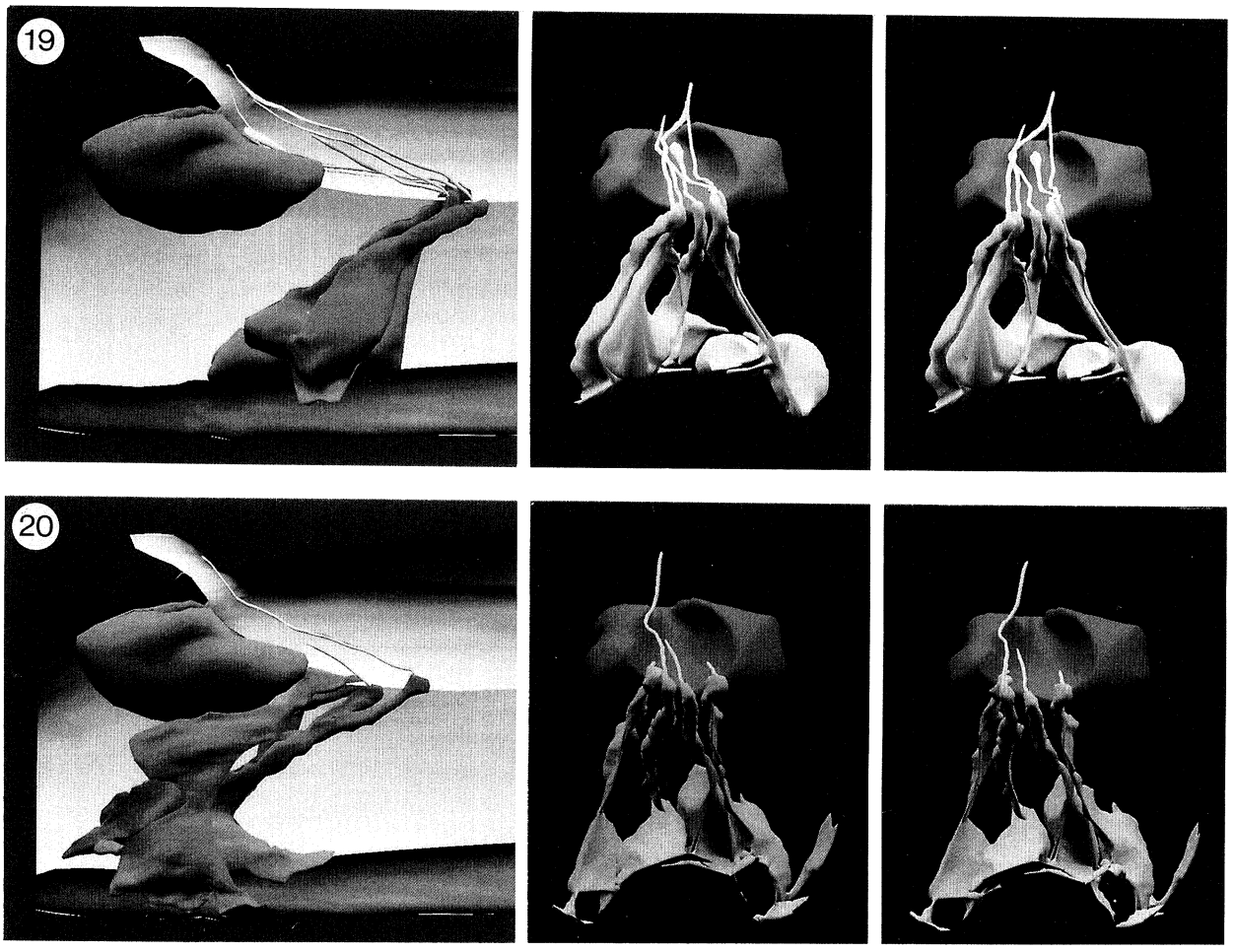


Figure 19. As in figure 17, row 3.

Figure 20. Anterior glial-like cells.

contrast, most of the other cilia in the canal lie free in its center. None of the support cell cilia leave the neuropore. Of the cilia that do (*ca.* 25 cilia in specimen 4), 15 belonged to the row 1 and 2 cells described above, six to cells lying along the dorsal and dorsolateral surfaces of the canal near the neuropore, and the remainder to a row of cells behind row 3 that we have yet to examine in detail.

5. DISCUSSION

(*a*) *Summary and general remarks*

Previous work on the amphioxus CNS has recognized a distinct anterior region, the cerebral vesicle, that is brain-like in some respects. Whether it is homologous with any specific part of the vertebrate brain has been a matter of considerable debate, but it does contain structures that appear to have counterparts in the brain. Chief among these are the secretory cells of the infundibular region, the dorsal photoreceptors, and the frontal eye. The infundibular cells are responsible for producing Reissner's fibre in amphioxus, which in vertebrates is formed by the dorsal subcommissural organ. In embryonic fish, however, the substance of the fibre comes partly from the infundibular region, which places the homology between infundibular cells in amphioxus and the vertebrate infundibulum on a comparatively sound footing (Sterba *et al.* 1983; Olsson 1993). The dorsal roof of the amphioxus c.v. contains two types of putative photoreceptors: lamellar cells and microvillar Joseph cells. The former are identical ultrastructurally to photoreceptor cells in the pineal of lower vertebrates (cf. Eakin 1968; Pu & Dowling 1981; Ruiz & Anadon 1991), which suggests the lamellar body and pineal may be homologues. Finally, the frontal eye, by which we mean the anterior pigment spot and the cells associated with it, has been interpreted as a homologue of the paired lateral eyes of vertebrates, although past authorities have disagreed on this question (reviewed by Lönnberg 1924). There is as yet no evidence to prove the frontal eye is a functional photoreceptor, but there are significant structural and organizational similarities between the frontal eye and vertebrate eyes (§ 5*b*), and positional similarities as well. Vertebrates form a single medial eye in exceptional circumstances, e.g. due to mutation or teratogens. Such eyes invariably lie at the anterior, ventral tip of the brain in essentially the same position as the frontal eye in amphioxus (Adelmann 1936). This is also the initial location, in vertebrates, of the normal embryonic eye rudiment. The vertebrate infundibulum, the pineal, which derives from the epiphysis, and the eye rudiments, are all

located in the diencephalon of the brain, i.e. in the posterior part of the forebrain (figure 22*a*). The anatomical evidence thus supports, in general terms at least, the idea that the cerebral vesicle contains structures whose probable homologues in vertebrates lie within the diencephalon.

A related question concerns whether homologues of vertebrate midbrain and hindbrain are to be found in amphioxus. Recent work by Holland *et al.* (1992) identifies an amphioxus Hox gene, with an anterior limit of expression at the somite 4–5 boundary, whose vertebrate counterpart marks the boundary between rhombomere 4 and 5. This strongly supports the idea that the hindbrain is basically derived from the part of the nerve cord that, in amphioxus, innervates the anterior somites. There thus appears to be a region of the amphioxus nerve cord extending back from the cerebral vesicle, through a number of somites, that cannot properly be considered homologous with vertebrate spinal cord, and a region further back that probably is. Whether there is any counterpart to the midbrain in amphioxus, or to the midbrain-hindbrain junction, which is an important molecular landmark in vertebrates, remains to be determined.

The EM results reported here on the larval c.v. are consistent with previous work on the adult, and clarify some unresolved issues as well. For example, the nature of the dorsal chamber, or 'dilation' of the central canal, has been a puzzle. It has been interpreted in some accounts as a chamber entirely separate from the central canal. It is evident from our results that it arises simply as a secondary expansion of the dorsal part of the main canal, which is otherwise very narrow. Further, while the c.v. is defined as a distinct region of the CNS primarily on the basis of the structures it contains, a more fundamental distinction, in terms of cellular organization, seems to be that between the anterior part of the c.v. and all the rest of the CNS. The former is tubular, its cells are arranged in predominantly transverse rows and their cilia project forward. The posterior c.v., like the rest of the cord, is folded laterally and held together by apical cell processes that traverse the central canal. Its two sides are separated by a floorplate, discontinuous in the posterior c.v., but continuous further back, whose cilia project backwards. Cells of a given type (e.g. the lamellar cells), are arranged in axial rather than transverse rows. The infundibular cells mark the junction between these two very different regions.

Our observations on the larval c.v. provide some further support for the supposition that the c.v. and the vertebrate diencephalon are homologues. Figure 22*b* shows the main structural landmarks we recognize in the larval c.v. For comparison, figure 22*a* shows a representative embryonic brain from a lower vertebrate including the early axonal tracts. The latter are now comparatively well known, mainly from work on zebrafish (Wilson *et al.* 1990; Ross *et al.* 1992). As in the adult, the main landmarks in the larval c.v. in amphioxus are the infundibular cells, the dorsal lamellar cells, which form a large lamellar body, and the frontal eye. The lamellar cells establish early axonal connections to the main commissure. Early connections are also formed in vertebrate embryos between structures in the roof of the diencephalon, specifically the posterior commissure and the epiphysis, and the developing ventrolateral tracts and commissures. There is a massive growth of such fibres in mouse, originating both from the diencephalon and midbrain (Easter *et al.* 1993). The main fibre tracts in amphioxus continue forward as a pair of rostral nerves. The latter could be counterparts of the terminal and/or olfactory nerves in vertebrates, which together form the anterior-most elements of the cranial nerve series. The only large commissure in amphioxus lies just behind the infundibular cells, near the junction between the anterior and posterior c.v. The only early commissure obvious in the post-infundibular region in vertebrates is the rather diffuse ventral tegmental commissure. Based on the amphioxus results, this commissure may be more significant than its comparatively small size would suggest.

Defining the posterior extent of the cerebral vesicle in our specimens is problematic. For the early stage examined here, we place the boundary near the first of the large ventral motor neurons, which clearly mark the beginning of somite innervation. In vertebrates, the anterior-most somatic motor nerves originate in midbrain nuclei and supply the eye muscles. This reinforces the association of the c.v. as defined here, with structures found forward of the midbrain in vertebrates. If there is also a midbrain homologue in amphioxus, it could conceivably lie just behind the c.v. and incorporate the above-mentioned large motor neurons.

A considerable proportion of the cerebral vesicle in young larvae is evidently concerned with light reception, as putative photoreceptors are among its most conspicuous features. The lamellar body is the largest in relative terms, occupying roughly two-thirds of the length of the c.v., and is a major source of nerve fibres. The expectation is that the larvae should have a well developed light response, but there is only limited behavioural evidence to show this. Adult amphioxus react to light, but the response is usually attributed to the dorsal ocelli, and the anterior end of the body is relatively insensitive to light (Guthrie 1975). In contrast, the larva becomes sensitive to light and to changes in light intensity at about the time the anterior pigment spot appears (Bone 1958). By this stage the first dorsal ocellus would also be developing, but the lamellar body is larger and better differentiated than either the ocellus or the frontal eye. Of the three types of putative photoreceptors in the young larva, the lamellar body therefore appears the one most likely to be responsible for the initial photoresponse. The role of this response in the natural environment is probably to keep the larvae away from surface waters during the day. Amphioxus larvae clearly can regulate their position in the water column on a diurnal basis (Wickstead & Bone 1959; Webb 1969), which means an organ for measuring light intensity over a considerable depth range is needed. The lamellar body, with its huge area of membrane surface, would appear to be ideally suited to this task

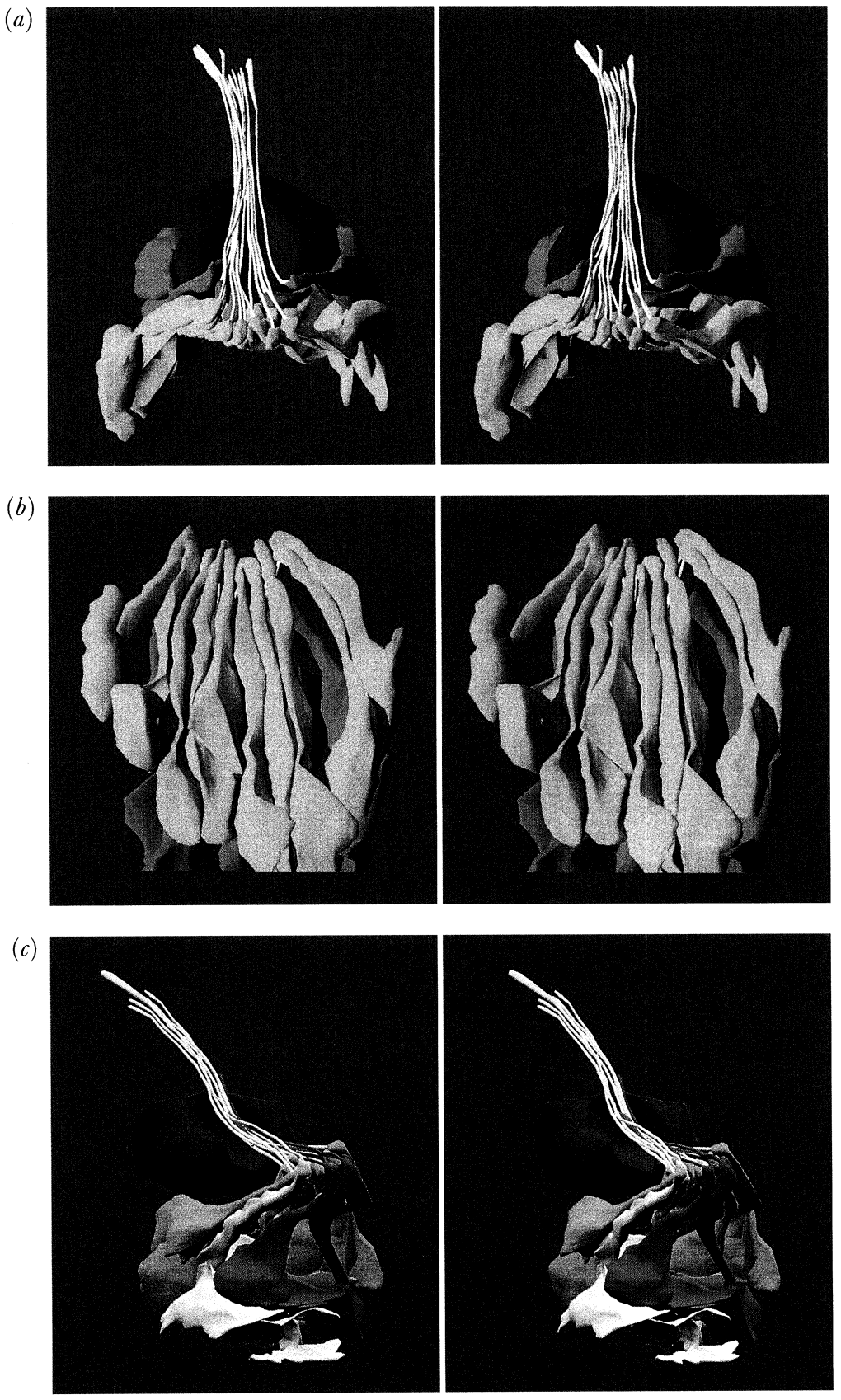


Figure 21. Stereopairs. (*a*) Cells of rows 1 (dark blue) and 2 (light blue), seen from behind the pigment spot (red) and slightly above the longitudinal axis. (*b*) Cells of rows 1 and 2, from below, anterior end facing up. (*c*) Selected cells from row 1 (blue), row 3 (purple), and the glial-like accessory cells (yellow), relative cell positions.

if, as its structure suggests, it functions as a high sensitivity non-directional light receptor. Both the frontal eye and the dorsal ocelli are structured more as directional sensors. The former, with its simple, unmodified cilia, is probably the least sensitive of the three systems to light, suggesting it may be designed to modulate daytime behaviour at the surface or in shallow water.

(*b*) *The frontal eye: comparative and evolutionary aspects*

The singular position of this structure at the anterior end of the nerve cord makes it especially interesting from an evolutionary standpoint. Among authors who have accepted it as a proper eye, some (e.g. Ayers 1890) considered it a starting point for the

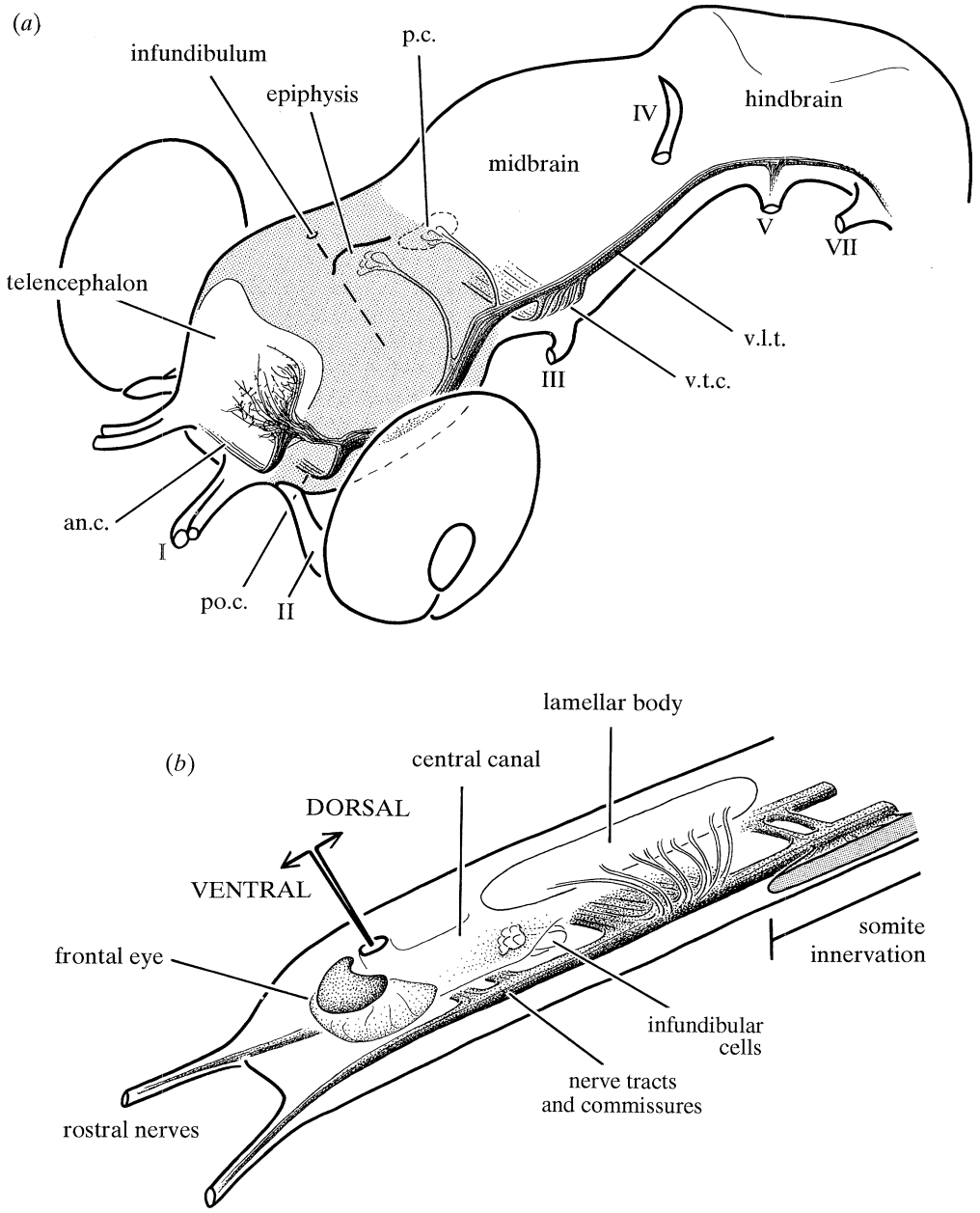


Figure 22. Comparison of vertebrate brain and amphioxus cerebral vesicle. (*a*) Anterior part of developing brain of a representative lower vertebrate, based loosely on zebrafish and showing the fibre tracts at 24 h according to Wilson *et al.* (1992). The diencephalon is shaded; the infundibulum occupies its posterior floor as indicated by the dashed straight line. Cranial nerves are identified by roman numerals; the terminal and olfactory components of nerve I are shown as two separate adjacent nerves. The approximate position of the subcommissural organ, below the posterior commissure (p.c.), is shown by a dashed oval. (*b*) The principal landmarks in the larval cerebral vesicle of amphioxus, based on this paper. The neuropore, though dorsal, marks the original anterior margin of the neural plate, so it separates the dorsal and ventral surfaces of the neural tube (roof and floor) as shown. The Joseph cells (not shown) develop later in the roof of the cord, behind the lamellar body.

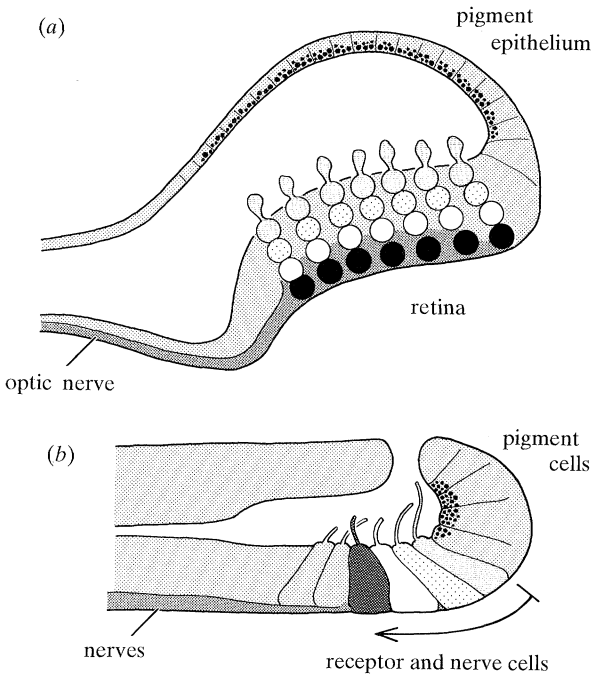


Figure 23. Comparison of vertebrate eye with the frontal eye of an amphioxus larva, schematic. (*a*) A section taken medially along the optic stalk through the developing eye of a typical vertebrate. The diagram emphasizes the topological relationship between the pigment epithelium, retina and optic nerve, which form a dorsal-to-ventral series in the medial plane of the optic vesicle. The layering of cells in the retina is shown by differential shading. (*b*) The comparable situation in amphioxus cerebral vesicle. The sequence from pigment cells through putative receptor and nerve cells to fibres is again dorsal to ventral. The arrangement of cells is retina-like, but each cell type is represented only by a single transverse row, and all cells retain their connection to the central canal.

evolution of vertebrate eyes, and took the single eye to be ancestral to the paired condition. Others (e.g. Todaro 1888) considered the amphioxus eye a relict of a more complex eye or eyes in earlier chordates, which implies the unpaired condition may have arisen secondarily by fusion. Amphioxus is clearly specialized in various ways for burrowing, and this has probably involved a narrowing of the head and loss of some cephalic sensory structures. Making two eyes into one, or reducing the size of a single large photoreceptor, could have accompanied this process.

Our morphological results do not help with respect to the question of which condition, one eye or two, is more primitive. They do, however, provide some further support for the supposition that amphioxus and vertebrates eyes are homologous, based on structural and organizational similarities. The eye in amphioxus (figure 23*b*) has a pigment cup produced by cells derived from the ependyma. Behind these lie precisely positioned rows of flask-shaped cells with long cilia and basal neurites, and further back lie additional rows and clusters of cells that are more typically nerve-like. The cells are separated and supported by glial-like cells, and have basal neurites and processes that form simple plexus-like commissures. Assuming these elements together constitute a functional photoreceptor, signal processing would be expected to follow an anterior-to-posterior sequence, with the first two rows being the receptor cells, and cells behind these forming the beginning of an interneuronal relay system. The neural retina in vertebrates (figure 23*a*) is an extension of the brain, and receptors are modified ventricular (ependymal) cells whose receptive processes (rods and cones) derive from cilia. The pigment epithelium lies dorsal to the retina, and the optic nerve emerges ventrally and runs along the lower surface of the optic stalk. The cells in the retina form a series of separate layers held together by specialized glia (Müller cells), and signals are processed through these in sequence, from receptors through sets of locally connected interneurons to the ganglion cells whose fibres form the optic nerve. The sequence parallels that seen in amphioxus, similar types of cells appear to be involved, and their positions with respect to the structure as a whole are also very similar.

A major difference between amphioxus and vertebrates lies in the nature of the image that the eye could potentially form. Vertebrate eyes have two-dimensional receptor fields that form a proper image. This is possible because the processing units in the retina are removed from the ventricular surface and packed into layers. In contrast, comparable cells in amphioxus retain their connections to the central canal. This means receptor arrays can never form more than single rows, so the receptor field is one-dimensional. This is a major limitation of the amphioxus design, but the latter may represent an intermediate step in the evolution of image-forming eyes from simple ocelli.

A second evolutionary issue concerns the origin of the frontal eye. The auricularia hypothesis of Garstang (1894) derives the nerve cord in chordates from dorsally converging ciliary bands in an ancestral larva something like the modern auricularia or tornaria (figure 24*a*,*b*). This hypothesis is unproven, but is widely accepted as a reasonable possibility, and there is some recent morphological support for it (Lacalli *et al.* 1990; Crowther & Whittaker 1992; Lacalli & West 1993). Our interest here is specifically in what happens at the anterior end of the neural tube at neurulation. Folding the lateral parts of the ciliary band system together in Garstang's scheme produces a neural groove or tube whose sides are separated ventrally by an intervening strip of epithelium, derived from the original dorsal surface of the larva, which then forms the floorplate. Further, because cells in ciliary bands are differentially distributed across the width of the bands, cells of a given type should lie in axial rows along it, except where the cord incorporates transverse band elements that cross the dorsal midline. There we expect to find cells arranged in transverse rows, and no floorplate, which is precisely the situation in the anterior c.v. in amphioxus.

Auricularia-type larvae have two anterior transverse band elements, either of which could be responsible for the organization we see in the anterior c.v.: the preoral transverse band, which forms the anterior

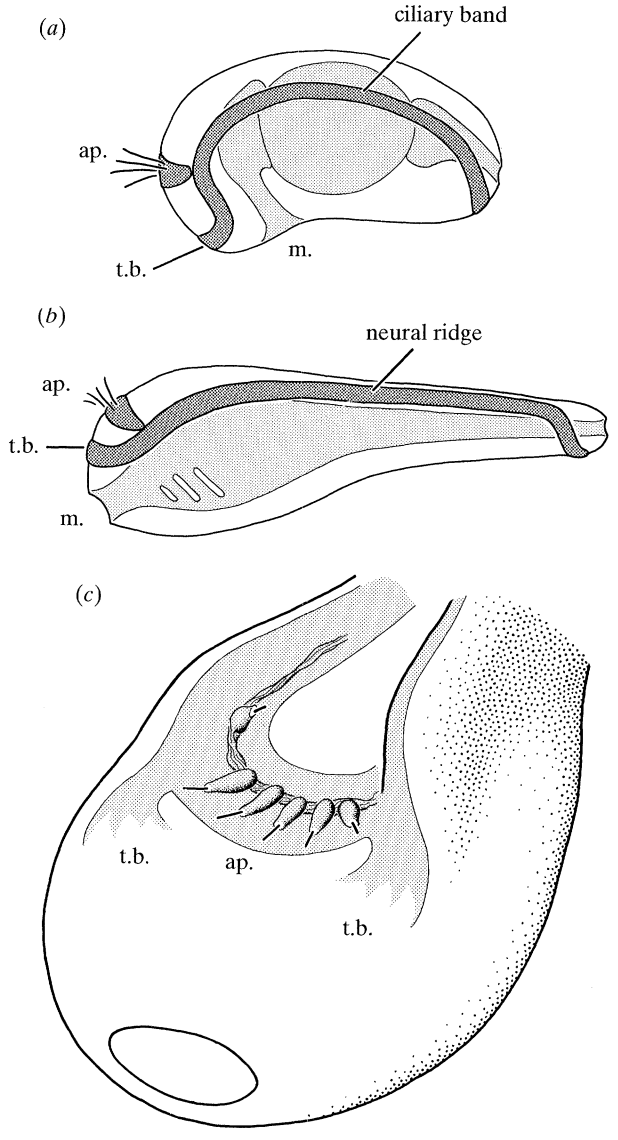


Figure 24. A diagrammatic summary of the main features of Garstang's auricularia hypothesis, after Garstang (1894). (a) A dipleurula or auricularia-type larva, showing ciliary bands and apical organ (ap.). The preoral transverse band (t.b.) lies forward of the apical organ and defines a ventral preoral field. (*b*) Ancestral chordate in which the ciliary bands are converted to neural ridges. The question that concerns us here is the fate of the two anterior transverse elements, the apical plate and the preoral transverse band, and whether both or only the former are incorporated into the CNS at neurulation. (*c*) Neurulation according to our interpretation of the amphioxus evidence, which suggests that the anterior-most cells of the neural tube are derived from apical plate. They form transverse rows as shown. Ciliary band elements, i.e. the preoral transverse band, lying forward of (ventral to) the apical plate would, in this scheme, remain outside the CNS.

loop connecting the lateral bands, and the apical organ, which bridges between them. Garstang recognized this, and it is clear from his later writings (e.g. Garstang & Garstang 1926) that he favoured placing sensory elements from the apical plate at the front margin of the nerve cord, and ventral preoral structures like the preoral transverse band outside it (figure 24*c*). Our evidence supports this interpretation, and we suggest further that cells derived from the ancestral apical organ may have been incorporated into the frontal eye as receptor cells and neurons. We consider this a possibility worth serious consideration for three reasons.

1. If the preoral transverse band were to form the anterior margin of the cord, a second zone of transversely organized cells derived from the apical organ would be expected further back, which to date we have not found.

2. Crinoid larvae, the closest larva to the auricularia type having a persistent apical tuft, have apical organs in which ciliary orientation is reversed in relation to the surrounding epithelium: the apical cilia point forward, while surrounding cilia beat backward (Lacalli & West 1986). Phoronid larvae are similar in this respect (Lacalli 1990), but the situation in most other larval types is not known. This suggests, nevertheless, that the apical organ may represent a distinct epithelial domain with its own ciliary organization. The existence of an anterior region in the cord with reversed cilium orientation, as in the anterior c.v., whose cilia point forward, is easily understood if this area arises from the apical organ.

3. The neurotransmitter serotonin is found widely in invertebrate apical organs, including those of all echinoderm larvae so far examined, and, according to a recent immunohistochemical study by Holland & Holland (1993*b*), in the frontal eye of amphioxus larvae. It is not known whether serotonin occurs in the apical plate of tornaria larvae, which, however, do have apical photoreceptors. The serotonin-containing cells in echinoderm larvae typically form a single transverse row, and have basal processes that form a small local plexus (Bisgrove & Burke 1986; Burke *et al.* 1986; Nakajima 1988). In amphioxus, a cluster of serotonergic 'anterior cells' develops just behind the anterior pigment spot at 2 d that persists through the whole larval period. Their arrangement in young larvae, as an approximately transverse row of 10–15 cells, is shown in figure 2H of Holland & Holland (1993*b*). In older larvae they form a somewhat larger cluster of cells with a basal plexus-like arrangement of neurites that extends back along the ventral surface of the cord for a short distance. Because slightly different stages were used in this study and that of Holland & Holland, we cannot identify their serotonergic cells in our EM specimens with certainty. The serotonin is clearly not localized in ependymal and glial-like cells, however, which leaves little else in the relevant region except our putative receptor and nerve cells, i.e. cells in rows 1–3.

We suggest, on this basis, that the frontal eye in amphioxus may be derived from the primitive apical organ, which would then occupy the ventral anterior margin of the nerve cord in this organism. The original preoral band elements, lying ventral to the apical pole in ancestral larvae, would not then have been incorporated into the cord. This is a reminder that a significant amount of neurogenic tissue may have been left outside the cord at neurulation in primitive chordates. Garstang considered this tissue a

possible source for the vertebrate hypophysis. It is a possible source also for the olfactory placodes.

Overall, the foregoing links the anterior pole and apical organ of primitive metazoan larvae with the eye in amphioxus, and ultimately with retinal cells in vertebrates. The apical organ could conceivably be the original source for both photoreceptors and some classes of neurons in the retina.

We are especially indebted to Linda Holland and T.H.J. Gilmour for rearing larvae and providing specimens for this study. We also thank the Director and staff at the Florida State University Marine Laboratory at Turkey Point and John Lawrence (University of South Florida) for providing laboratory facilities; John Prothero and the staff of the Computer Graphics Unit, Department of Biological Structure, University of Washington for assistance using Skandha; Ragnar Olsson, Stephen Easter, Peter Holland and two anonymous reviewers for comments and criticisms; and Lyna Eng, Jocelyn Fowke and Sanjay Bakshi for technical help. This work was supported by NSERC Canada.

REFERENCES

Adelmann, H.B. 1936 The problem of cyclopia I., II. *Q. Rev. Biol.* **11**, 161–182, 284–304.

Ayers, H. 1890 Concerning vertebrate cephalogenesis. *J. Morph.* **4**, 221–245.

Bisgrove, B.W. & Burke, R.D. 1986 Development of serotonergic neurons in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Devel. Growth Diff.* **28**, 569–574.

Bone, Q. 1958 Observations on the living larva of amphioxus. *Pubbl. Staz. Zool. Napoli* **30**, 458–471.

Bone, Q. 1959 The central nervous system in larval acraniates. *Q. Jl micros. Sci.* **100**, 509–527.

Bone, Q. 1960*a* The origin of the chordates. *Zool. J. Linn. Soc.* **44**, 252–269.

Bone, Q. 1960*b* The central nervous system in amphioxus. *J. comp. Neurol.* **115**, 27–64.

Burke, R.D., Brand, D.G. & Bisgrove, B.W. 1986 Structure of the nervous system of the auricularia larva of *Parastichopus californicus*. *Biol. Bull.* **179**, 450–460.

Crowther, R.J. & Whittaker, J.R. 1992 Structure of the caudal neural tube in an ascidian larva: vestiges of its possible evolutionary origin from a ciliated band. *J. Neurobiol.* **23**, 280–292.

Drach, P. 1948 Embranchement des Céphalocordés. In *Traité de zoologie*, vol. 11 (ed. P. P. Grassé), pp. 931–1037. Paris: Masson & Cie.

Eakin, R.M. 1968 Evolution of photoreceptors. In *Evolutionary biology*, vol. 2 (ed. T. Dobzhansky, M. K. Hecht & W. C. Steere), pp. 194–242. Amsterdam: North Holland.

Eakin, R.M. & Westfall, J.A. 1962 Fine structure of photoreceptors in amphioxus. *J. Ultrastruct. Res.* **6**, 531–539.

Easter, S.S., Ross, L.S. & Frankfurter, A. 1993 Initial tract formation in the mouse brain. *J. Neurosci.* **13**, 285–299.

Flood, P.R. 1966 A peculiar mode of muscular innervation in amphioxus. *J. comp. Neurol.* **126**, 181–218.

Gans, C. 1989 Stages in the origin of vertebrates: analysis by means of scenarios. *Biol. Rev.* **64**, 221–268.

Garstang, S.L. and Garstang, W. 1926 On the development of *Botrylloides* and the ancestry of vertebrates. *Proc. Leeds Philos. Lit. Soc., Sci. Sect.* **1**, 81–86.

Garstang, W. 1894 Preliminary note on a new theory of the phylogeny of the Chordata. *Zool. Anz.* **27**, 122–125.

Guthrie, D.M. 1975 The physiology and structure of the nervous system of amphioxus, *Branchiostoma lanceolatum*. In *Symp. Zool. Soc. Lond.* no. 36 (ed. E. J. W. Barrington & R. P. S. Jefferies), pp. 43–80. London: Academic Press.

Holland, N.D. & Holland, L.Z. 1989 Fine structural study of the cortical reaction and formation of the egg coats in a lancelet, *Branchiostoma floridae*. *Biol. Bull.* **176**, 111–122.

Holland, N.D. & Holland, L.Z. 1993*a* Embryos and larvae of invertebrate deuterostomes. In *Essential developmental biology, a practical approach* (ed. C. Stern & P. W. H. Holland), pp. 21–32. Oxford: IRL Press.

Holland, N.D. & Holland, L.Z. 1993*b* Serotonin-containing cells in the nervous system and other tissues during ontogeny of a lancelet, *Branchiostoma floridae*. *Acta Zool.* **74**, 195–204.

Holland, P.W.H. 1992 Homeobox genes in vertebrate evolution. *BioEssays* **14**, 267–273.

Holland, P.W.H., Holland, L.Z., Williams, N.A. & Holland, N.D. 1992 An amphioxus homeobox gene: sequence conservation, spatial expression during development and insights into vertebrate evolution. *Development* **116**, 653–661.

Jollie, M. 1973 The origin of chordates. *Acta Zool.* **54**, 81–100.

Lacalli, T.C. 1990 Structure and organization of the nervous system in the actinotroch larva of *Phoronis vancouverensis*. *Phil. Trans. R. Soc. Lond.* B **327**, 655–685.

Lacalli, T.C., Gilmour, T.H.J. & West, J.E. 1990 Ciliary band innervation in the bipinnaria larva of *Pisaster ochraceus*. *Phil. Trans. R. Soc. Lond.* B **330**, 371–390.

Lacalli, T.C. & West, J.E. 1986 Ciliary band formation in the doliolaria larva of *Florometra* I. The development of normal epithelial pattern. *J. Embryol. exp. Morph.* **96**, 303–323.

Lacalli, T.C. & West, J.E. 1993 A distinctive nerve cell type common to diverse deuterostome larvae: comparative data from echinoderms, hemichordates and amphioxus. *Acta Zool.* **74**, 1–8.

Lönnberg, E. 1924 Das Nervensystem. In *Bronn's Klassen und Ordnungen des Tier-reichs, Pisces* (Pt. 1), Bk. 1 (*Einleitendes, Leptocardii und Cyclostomi*) (ed. E. Lönnberg, G. Favaro, B. Mazejka & M. Rauther), pp. 171–200. Leipzig: Akademische Verlagsgesellschaft.

Meves, A. 1973 Elektronenmikroskopische Untersuchungen über die Zytoarchitektur des Gehirns von *Branchiostoma lanceolatum*. *Z. Zellforsch. mikros. Anat.* **139**, 511–532.

Nakajima, Y. 1988 Serotonergic nerve cells of starfish larvae. In *Echinoderm biology* (ed. R. D. Burke, P. V. Mladenov, P. Lambert & R. L. Parsley), pp. 235–239. Rotterdam: A.A. Balkema.

Nakao, T. 1964 On the fine structure of the amphioxus photoreceptor. *Tohoku J. exp. Med.* **82**, 349–369.

Nielsen, C. 1987 Structure and function of metazoan ciliary bands and their phylogenetic significance. *Acta Zool.* **68**, 205–262.

Obermüller-Wilén, H. 1976 The infundibular organ of *Branchiostoma lanceolatum*. *Acta Zool.* **57**, 211–216.

Olsson, R. 1962 The infundibular cells of amphioxus and the question of fibre-forming secretions. *Arkiv Zool.* **15**, 347–355.

Olsson, R. 1986 Basic design of the chordate brain. In *Indo-Pacific fish biology* (ed. T. Uyeno, R. Arai, T. Taniuchi & K. Matsuura), pp. 86–93. Tokyo: Ichthyological Soc. Japan.

Olsson, R. 1993 Reissner's fiber mechanisms: some common denominators. In *The subcommissural organ* (ed. A. Oksche, E. M. Rodriguez & P. Fernandez-Liebrez), pp. 33–39. New York: Springer.

Olsson, R. & Wingstrand, K.G. 1954 Reissner's fibre and

the infundibular organ in amphioxus. *Univ. Bergen Arb. (Naturvitensk rekke)* **14**, 1–14.

Prothero, J.S. & Prothero, J.W. 1989 A software package in C for interactive three-dimensional reconstruction and display of anatomical objects from serial section data. *Nat. Computer Graphics Assn. Conf. Proceedings* **1**, 187–192.

Pu, G.A. & Dowling, J.E. 1981 Anatomical and physiological characteristics of pineal photoreceptor cells in the larval lamprey, *Petromyzon marinus*. *J. Neurophysiol.* **46**, 1018–1038.

Ross, L.S., Parrett, T. & Easter, S.S. 1992 Axogenesis and morphogenesis in the embryonic zebrafish brain. *J. Neurosci.* **12**, 467–482.

Ruiz, S. & Anadon, R. 1991 The fine structure of lamellate cells in the brain of amphioxus. *Cell Tiss. Res.* **263**, 597–600.

Sterba, G., Fredricksson, G. & Olsson, R. 1983 Immunocytochemical investigations of the infundibular organ in amphioxus. *Acta Zool.* **64**, 149–153.

Todaro, F. 1888 Sur l'origine phylogénétique des yeux des vertébrés et sur la signification des épiphyses et des hypophyses de leur cerveau. *Arch. ital. Biol.* **9**, 55–57.

Webb. J.E. 1969 On the feeding and behavior of the larva of *Branchiostoma lanceolatum*. *Mar. Biol.* **3**, 58–72.

Welsch, U. 1968 Die Feinstruktur der Josephschen Zellen im Gehirn von Amphioxus. *Z. Zellforsch. mikros. Anat.* **86**, 252–261.

Wickstead, J.H. & Bone, Q. 1959 Ecology of acraniate larvae. *Nature, Lond.* **184**, 1849–1851.

Wilson, S.W., Ross, L.S., Parrett, T. and Easter, S.S. 1990 The development of a sample scafford of axon tracts in the brain of the embryonic zebrafish, *Brachydanio revio*. *Development* **108**, 121–145.

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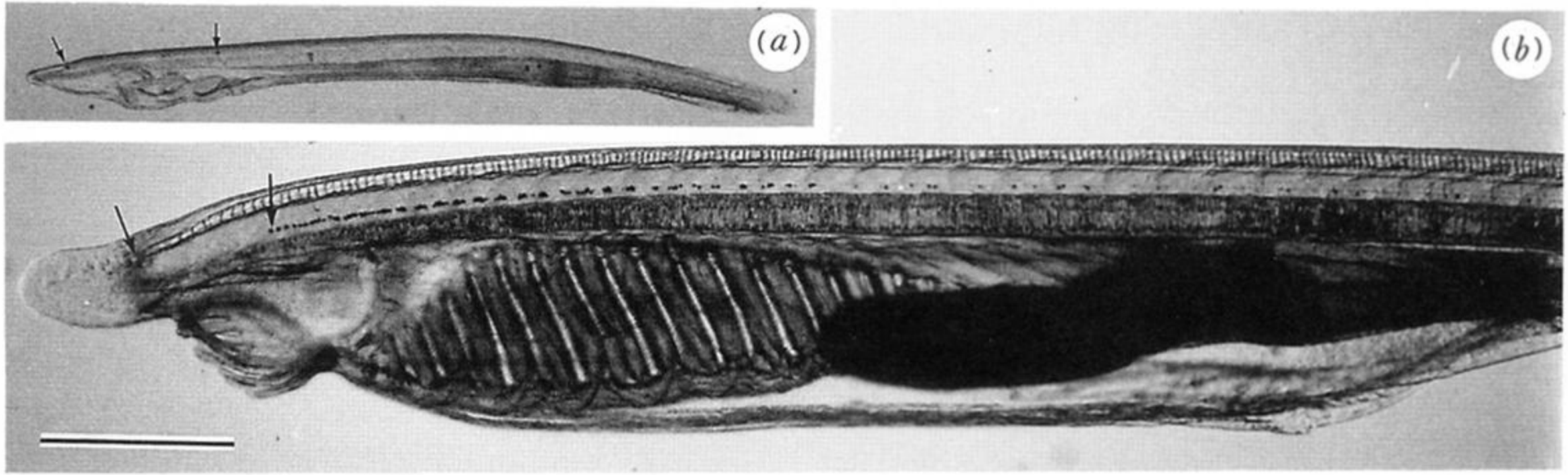


Figure 1. Larval and juvenile *Branchiostoma floridae*, both to the same scale. (*a*) 14 d larva. Arrows show the anterior pigment spot and the first of the dorsal ocelli to develop, which lies in somite 5. (*b*) Juvenile shortly after completion of metamorphosis. Arrows show the anterior pigment spot and the anterior-most of the dorsal ocelli, which now form a continuous row beginning in somite 3. None occur in the anterior cord between the arrows. Scale bar = 500 μm.

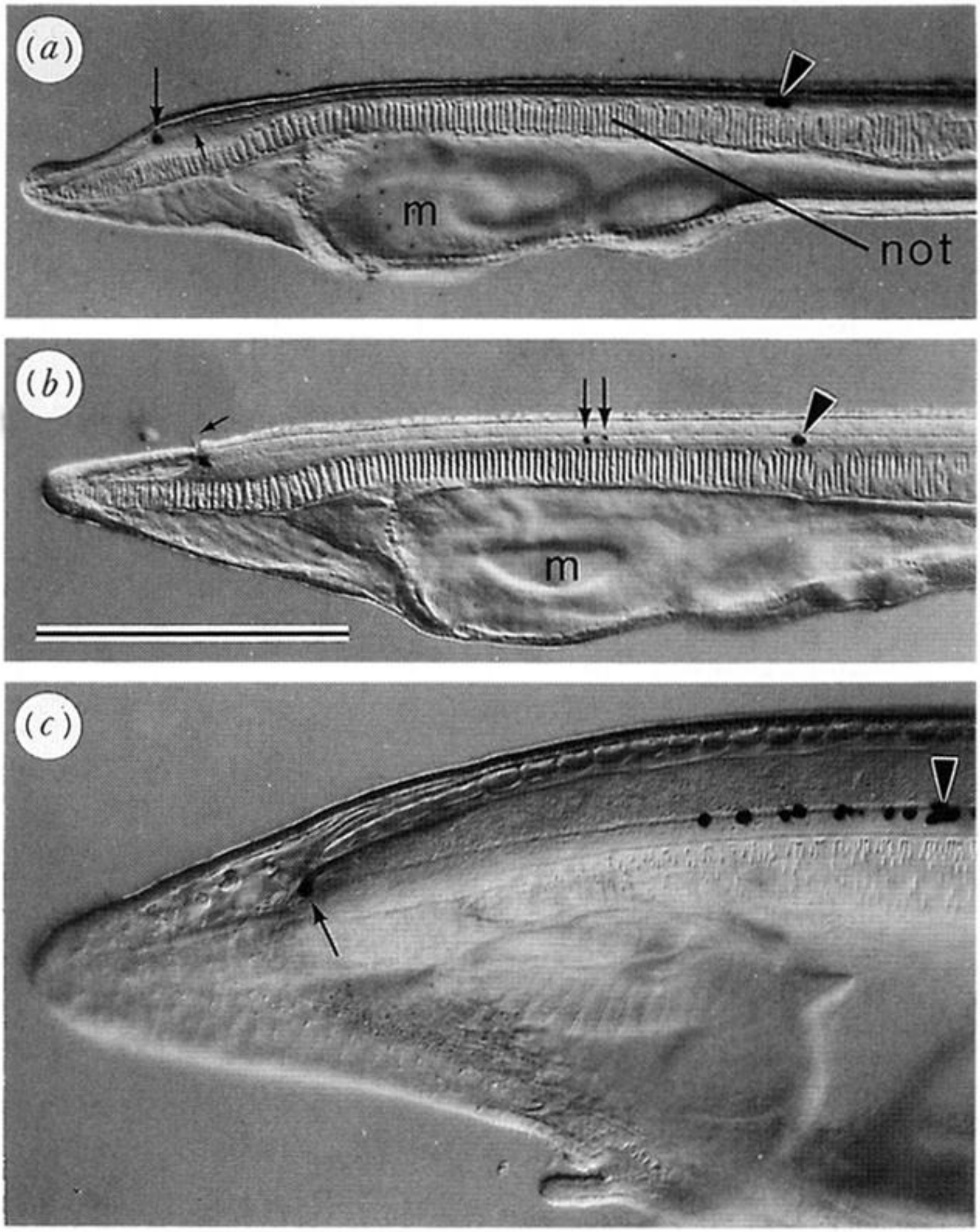


Figure 2. A stage series of *B. floridae* larvae, all to the same scale. (*a*) 6 d larva. The figure shows the anterior pigment spot (left-most arrow) and the first of the dorsal ocelli that develops (arrowhead). The small arrow indicates the point at which the central canal narrows, which is the approximate junction between anterior and posterior c.v. (*b*) 14 d larva. Shows the first dorsal ocellus as in (*a*), two more developing in somite 3 (double arrows), and cilia emerging from the neuropore (small arrow). (*c*) Late larva, an early stage of metamorphosis. There is now a continuous row of ocelli including a cluster in somite 5 (arrowhead). The arrow shows the anterior pigment spot. Scale bar = 200 μm.

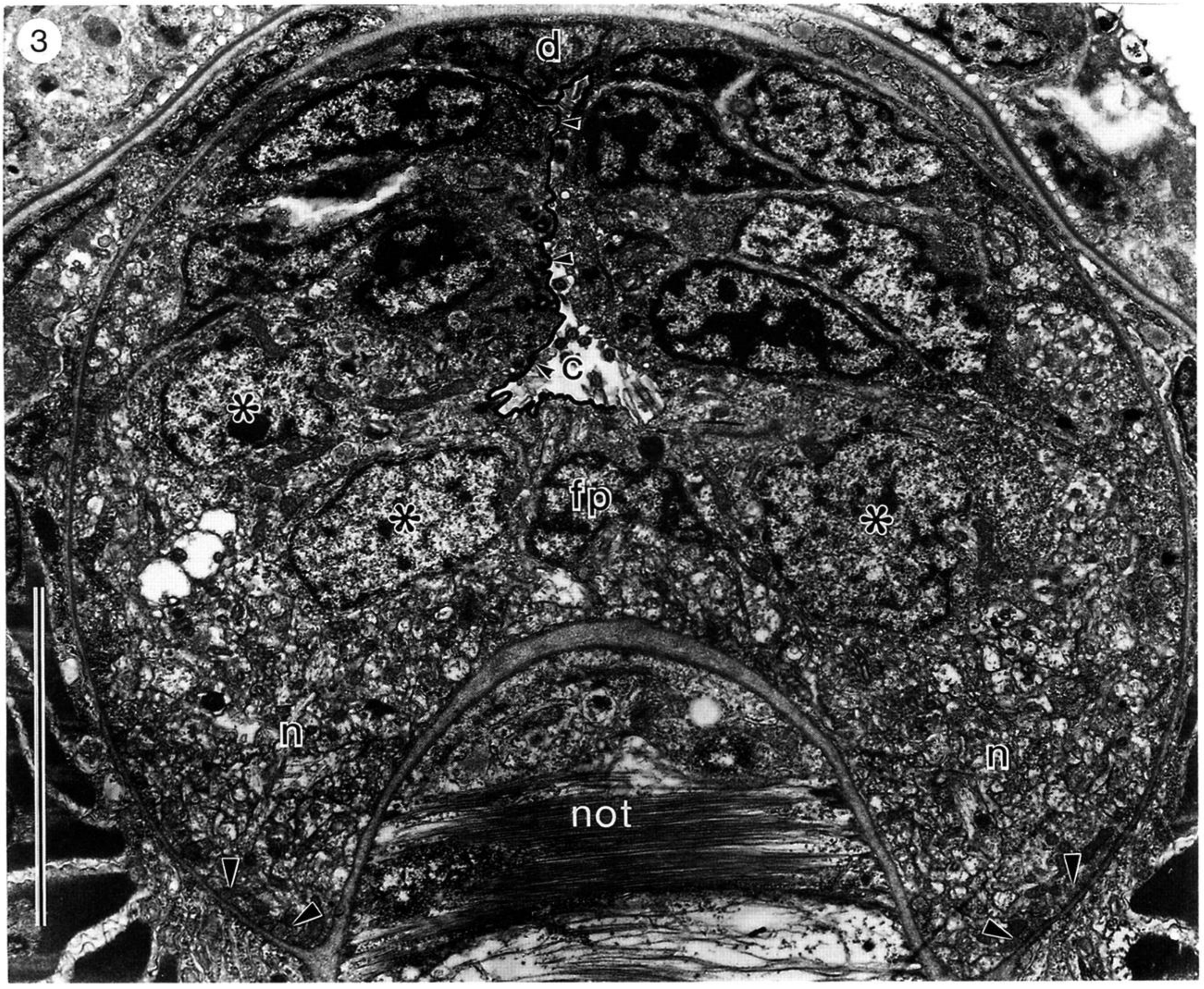


Figure 3. A section through the nerve cord near the back of somite 1. Ventrolateral nerve tracts (n) are shown, each with a zone in which neuromuscular junctions form (between arrows) with the adjacent somite. The large ventral cells (*) are neurons, and the ventral midline is occupied by a single floorplate cell (fp). The ventral part of the central canal (c) is open. It narrows dorsally, so the cells on opposite sides are directly apposed, and is capped at the top by a single dorsal cell (d). Its surface is traced on one side for emphasis (solid line, small arrows). Scale bar = 5 μm.

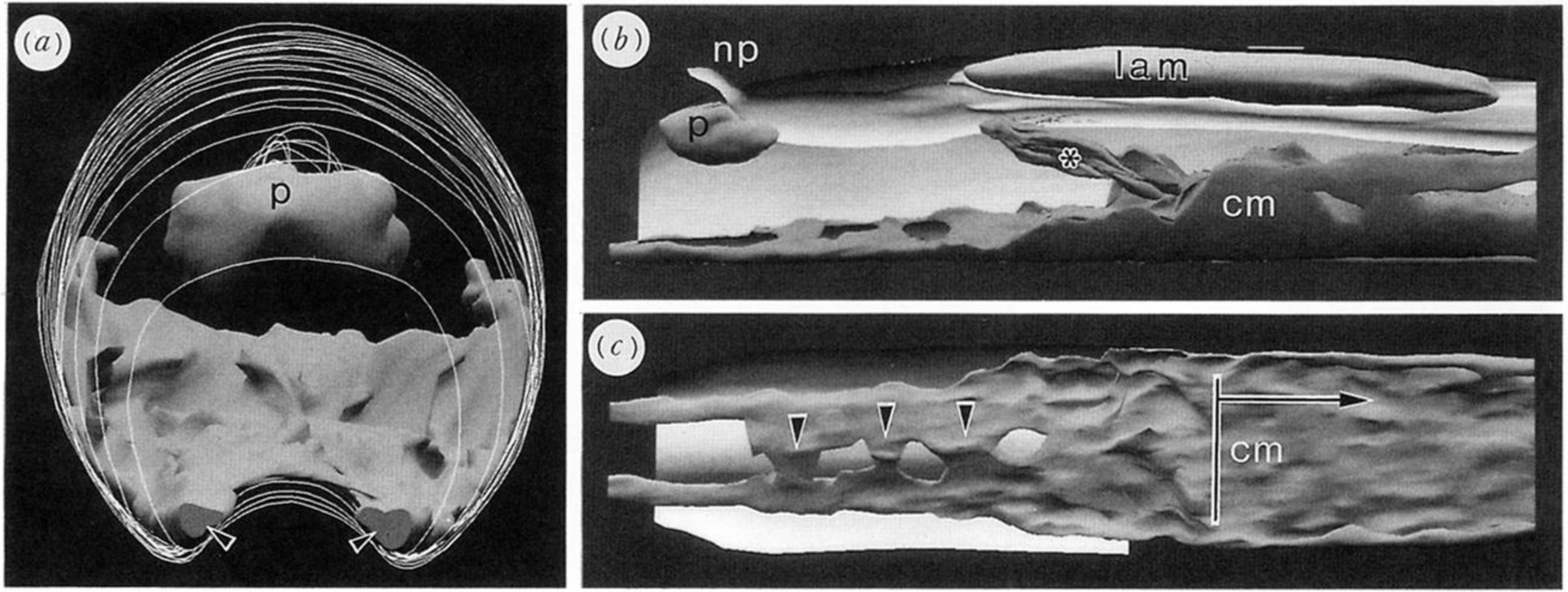
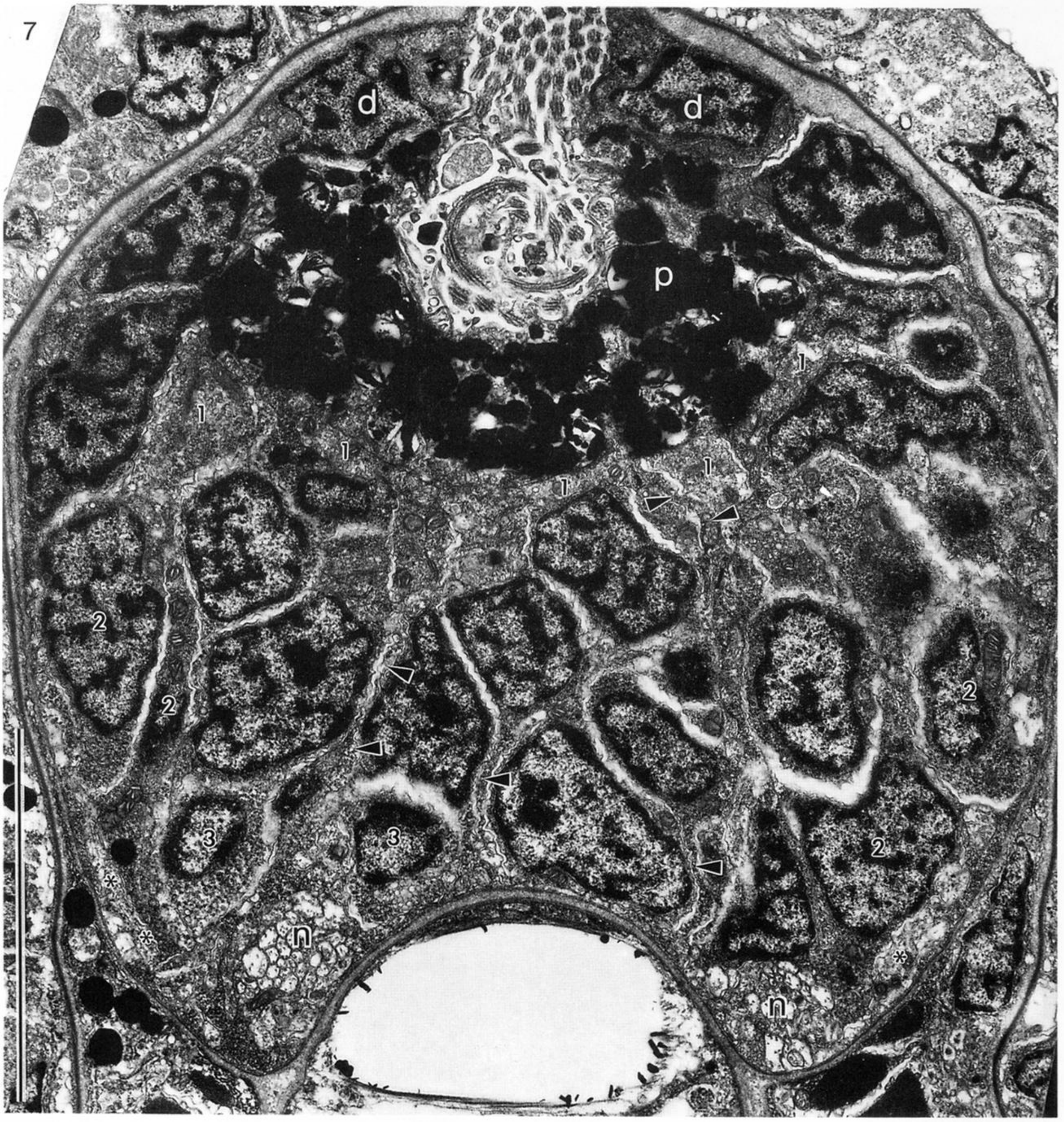


Figure 4. Reconstructions of the cerebral vesicle of specimen 4. (*a*) Front view, cord outlined as contours. Figure shows the anterior pigment spot (p) and the domain occupied by fibre tracts, including the two rostral nerves (arrows) that leave the cord at its anterior end. (*b*) Side view with the facing surface of the cord cut away. Fibre tracts, pigment spot, neuropore (np), lamellar body (lam), the cluster of infundibular cells (*), and the position of the main commissure (cm) are shown. (*c*) Top view of the fibre tracts showing positions of the small anterior commissures (arrows) and the anterior margin and approximate posterior extent of the main commissure (cm, compare with (*b*)). Magnification: (*a*) × 2830; (*b*,*c*) × 1210.



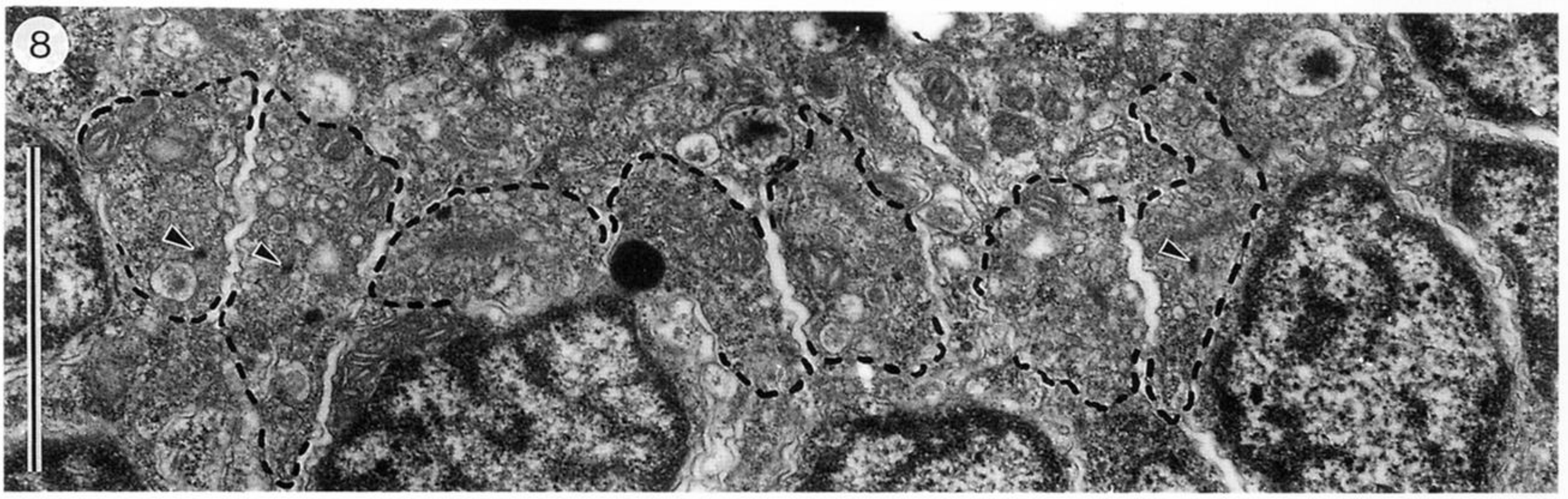
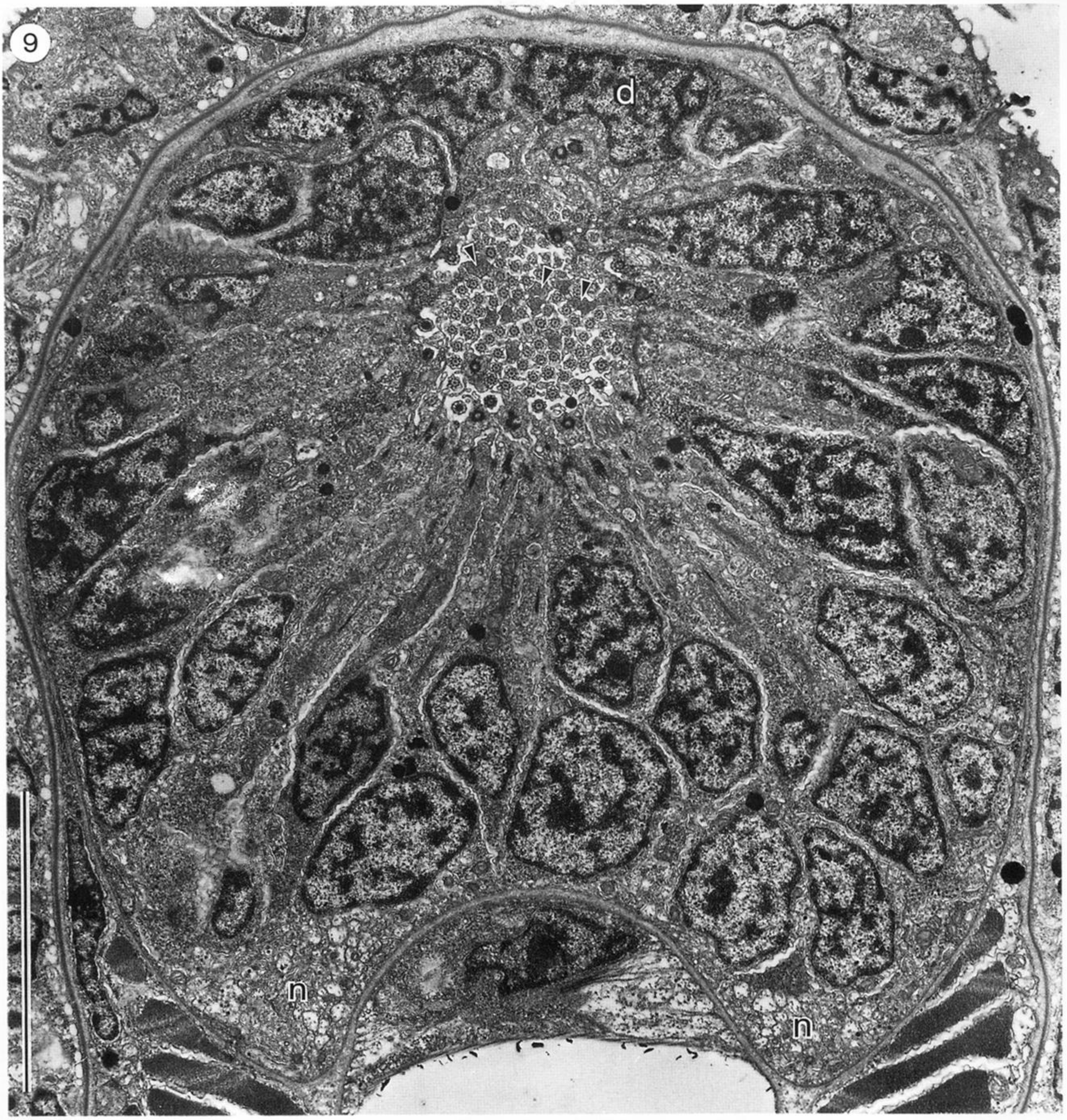


Figure 7. A section through the cord at the level of the pigment spot (p). Shows the nuclei of several row 2 and 3 cells (labelled 2 and 3) and tapering apices of row 1 cells (labelled 1). Most of the cells are, however, supporting or glial-like cells. Arrows indicate some of the curtain-like processes of the latter as described in the text. Shows also the ventrolateral tracts (n) and descending basal processes from two row 1 cells (small *s). Scale bar = 5 μm.

Figure 8. Detail just below the pigment spot, near its posterior margin, at the level of the tapering apices of the row 2 cells. Shows a transverse row of seven of these (cell boundaries partially outlined), some with rootlets (arrows). Scale bar = 2 μm.



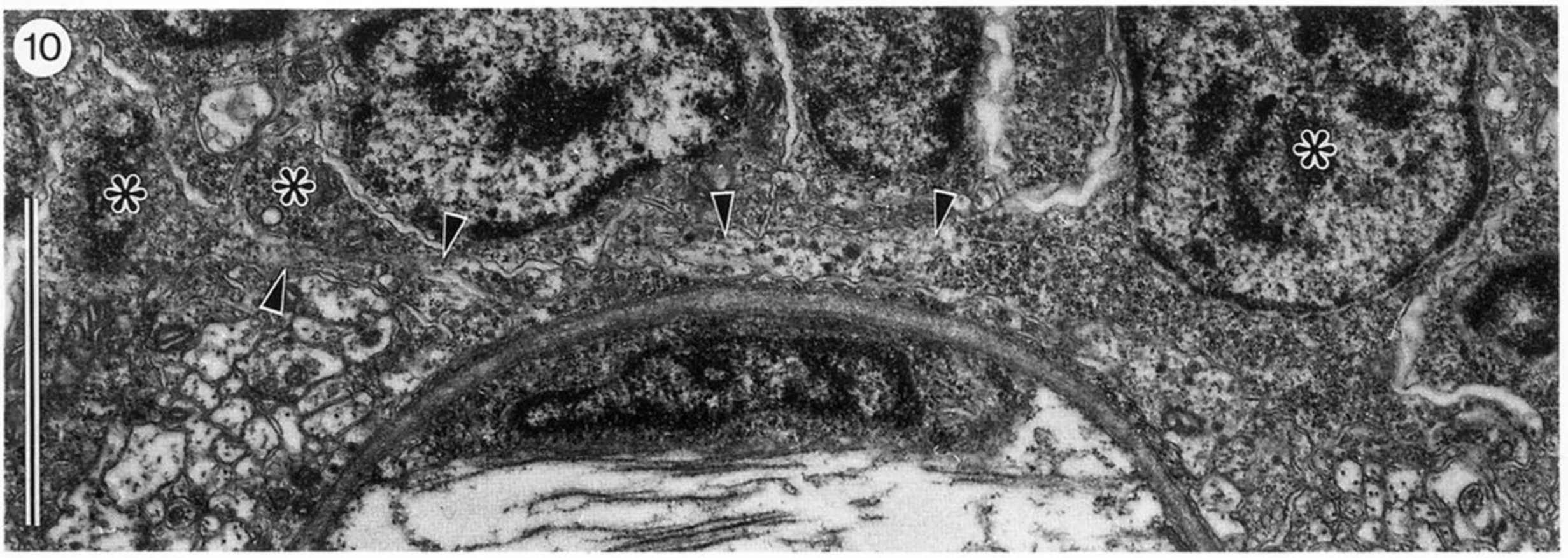
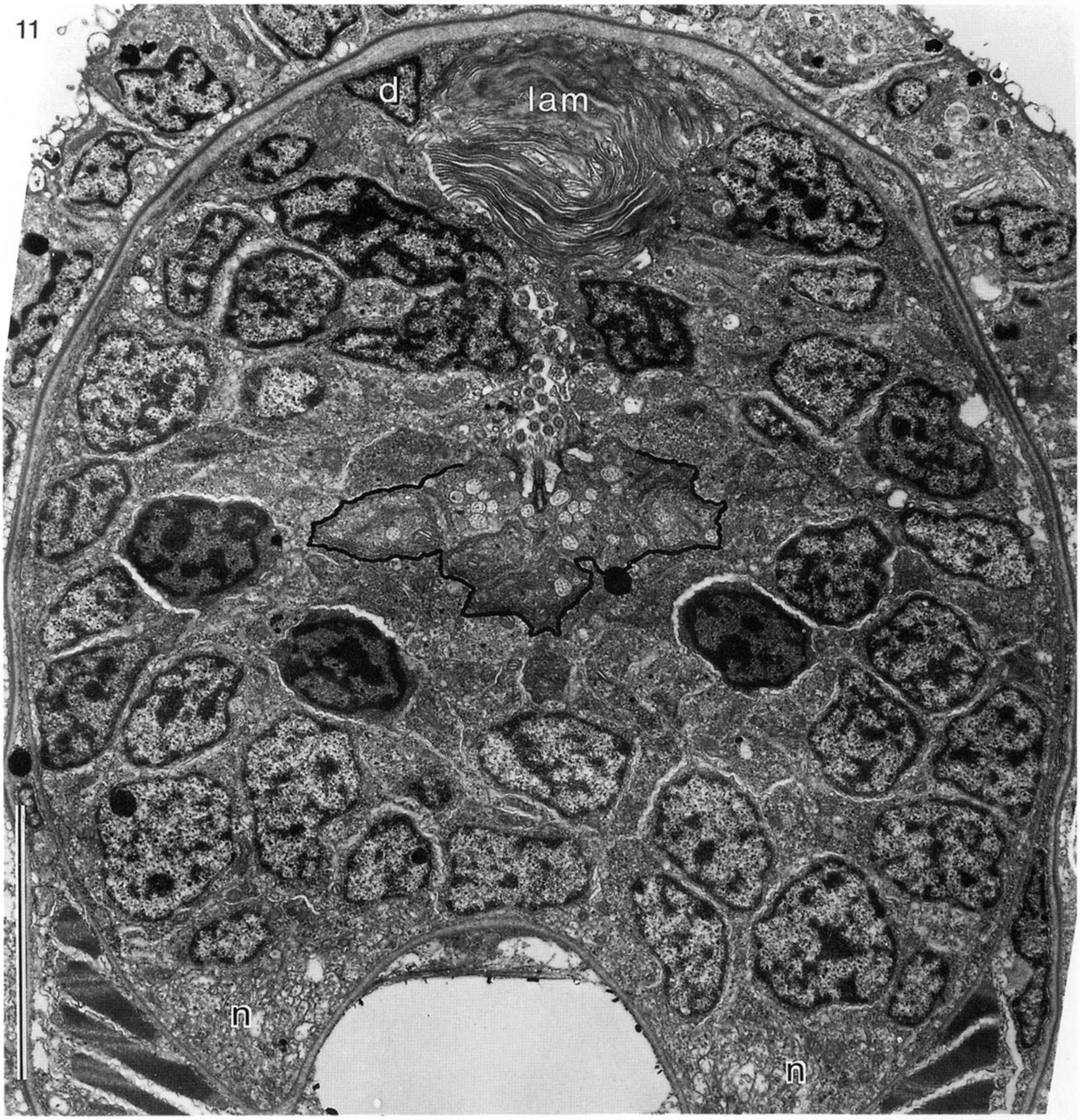
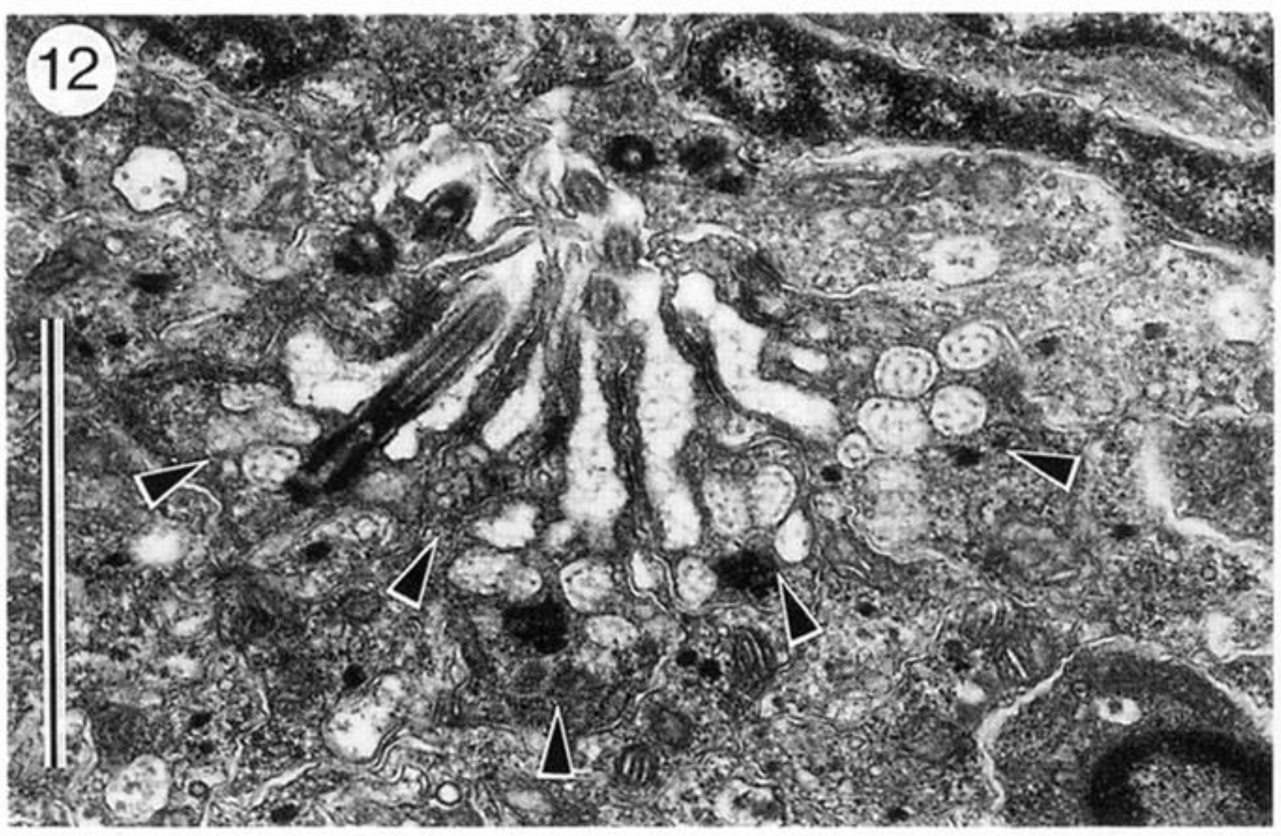


Figure 9. A section 10 μm behind the posterior lip of the pigment spot showing typical arrangement of cells in the anterior c.v. The central canal is circular and packed with cilia; the ventrolateral tracts are still small. Arrows indicate several examples of cilia where the axoneme is replaced by a uniformly dense matrix. None of those in this section are much larger than an average cilium in diameter, but much larger ones do occur. Scale bar = 5 μm.

Figure 10. A section 1.5 μm behind the posterior lip of the pigment spot at the level of the first small commissure. Basal processes (arrows) from three row 3 cells (*) appear in the section. Scale bar = 2 μm.





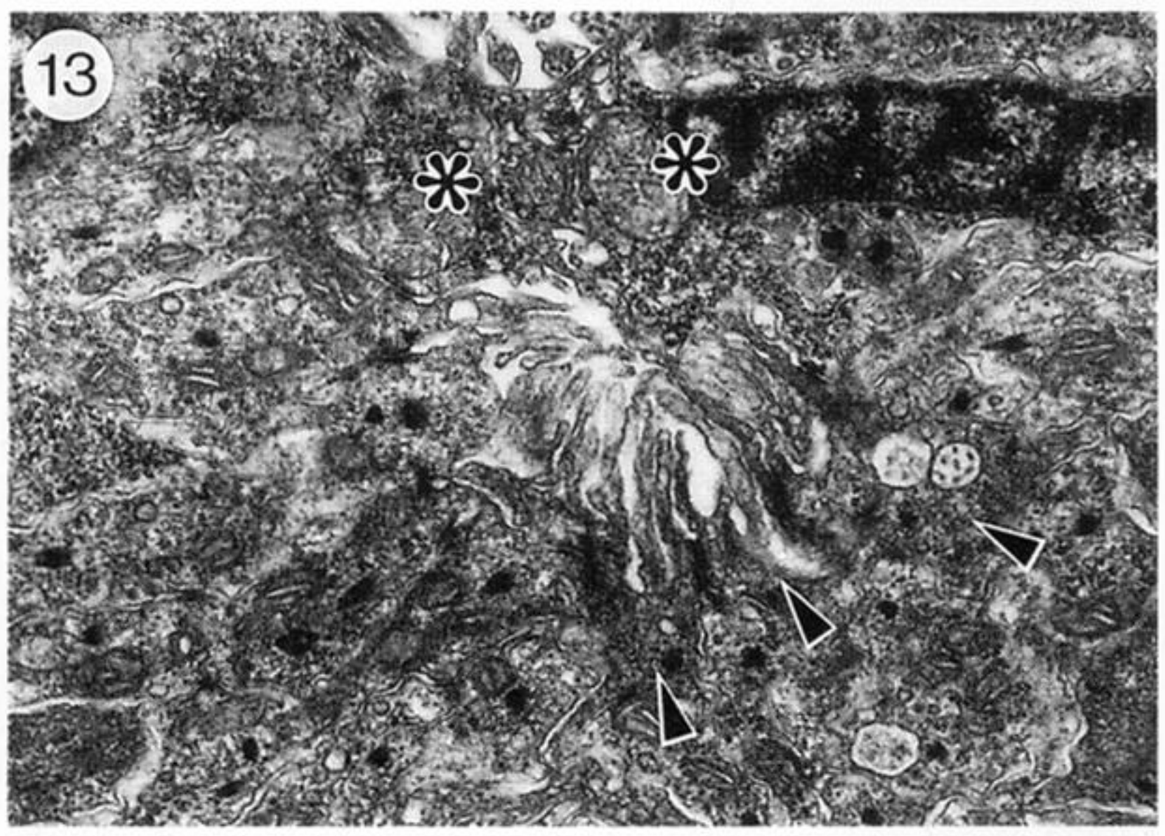


Figure 11. A section at the level of the infundibular cells, shown in outline. The section cuts the anterior part of the lamellar body (lam). The single vertical cilium belongs to the posterior-most of the infundibular cells; the ciliary profiles above it come from others. Scale bar = 5 μm.

Figures 12 and 13. Sections just anterior to figure 11, both to the same scale. Figure 12 shows the apical surfaces, cilia, and associated secretory vesicles of three infundibular cell apices (arrows). Figure 13 is *ca.* 1 μm further forward and shows the anterior-most part of three infundubular cells (arrows). In front of their cilia, they have microvilli that fill the open portion of the central canal, which is otherwise devoid of cilia. Apical cell processes (*) bridge the canal above this point. Scale bar = 2 μm.

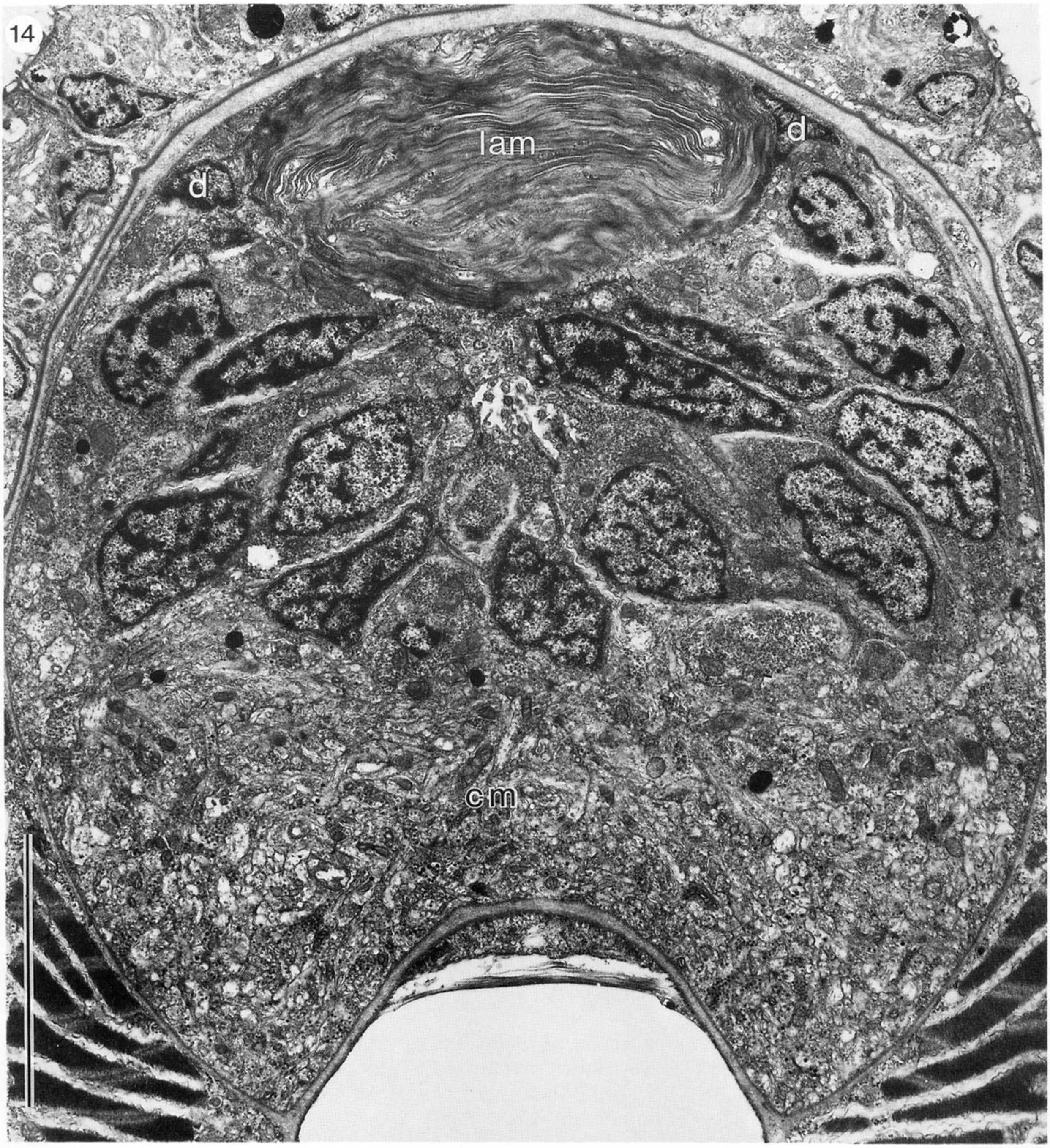


Figure 14. A section at the level of the main commissure, 50 μm back from the front of the cord. The commissure occupies the lower third of the cord, while the lamellar body (lam) occupies most of the upper third. Scale bar = 5 μm.

Figure 15. Detail of the lamellar body showing a cilium of one of the cells and lamellae arising from it. Small arrows show the ventral continuation of the very narrow central canal. Scale bar = 2 μm.

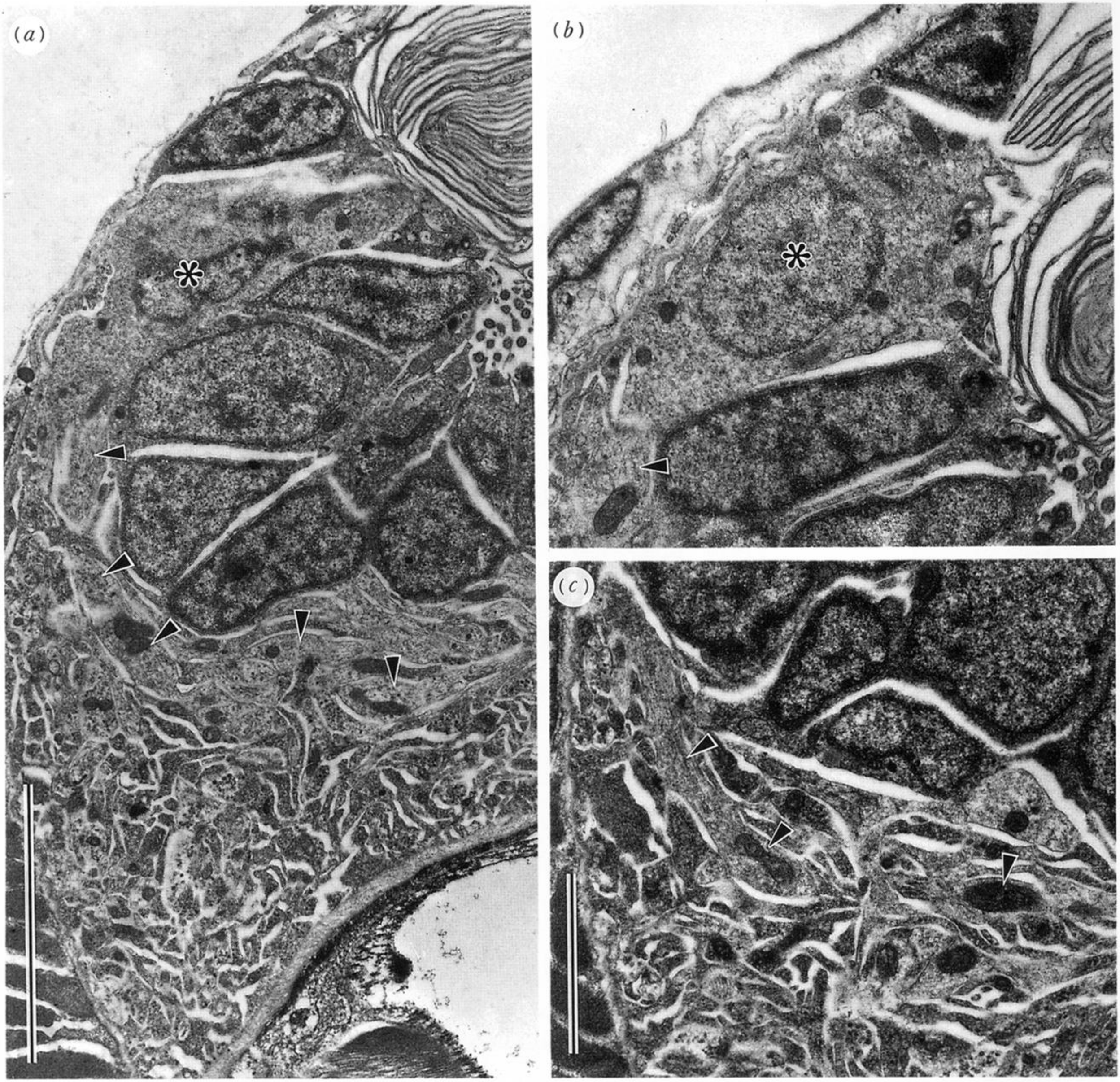
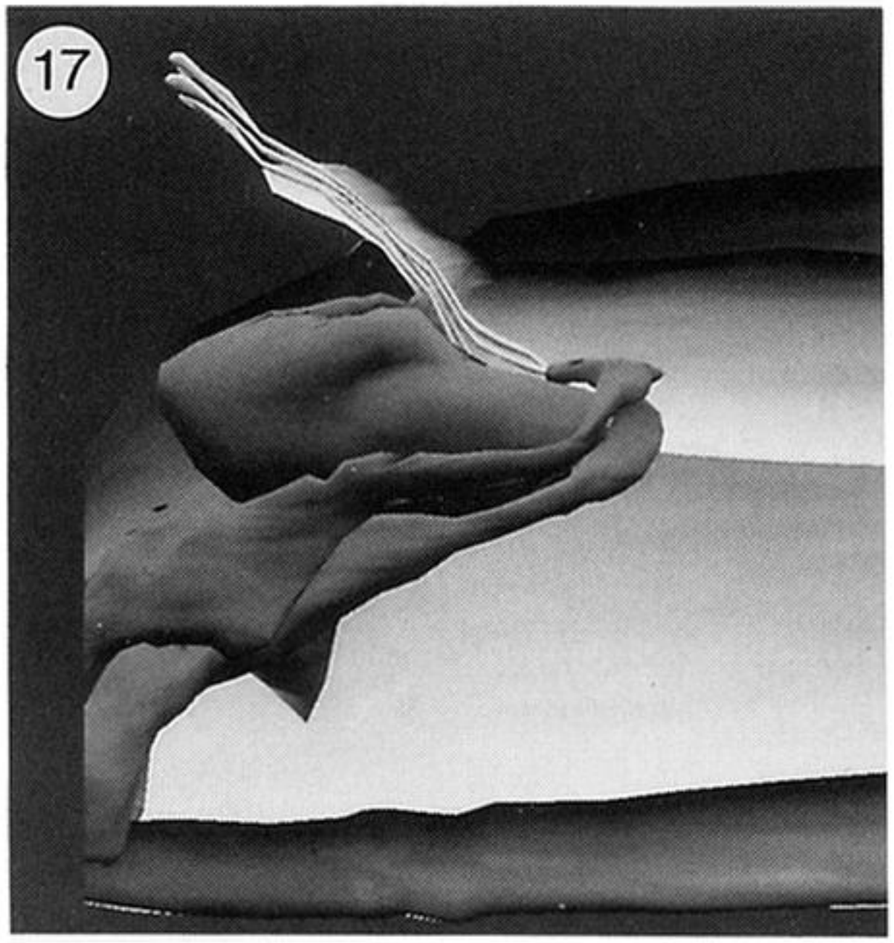
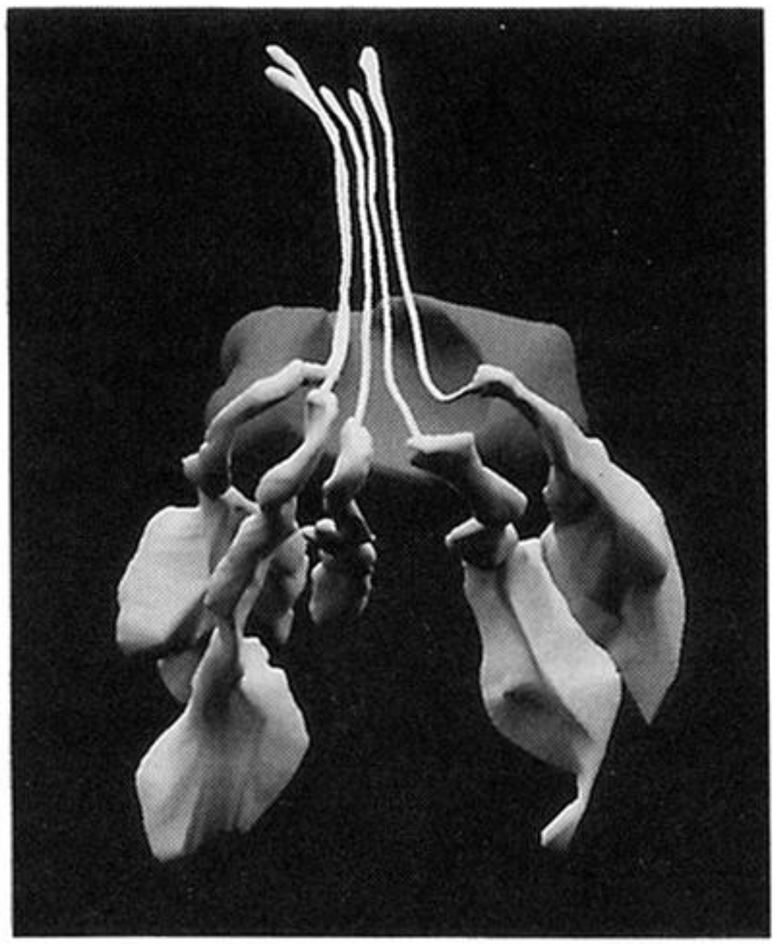
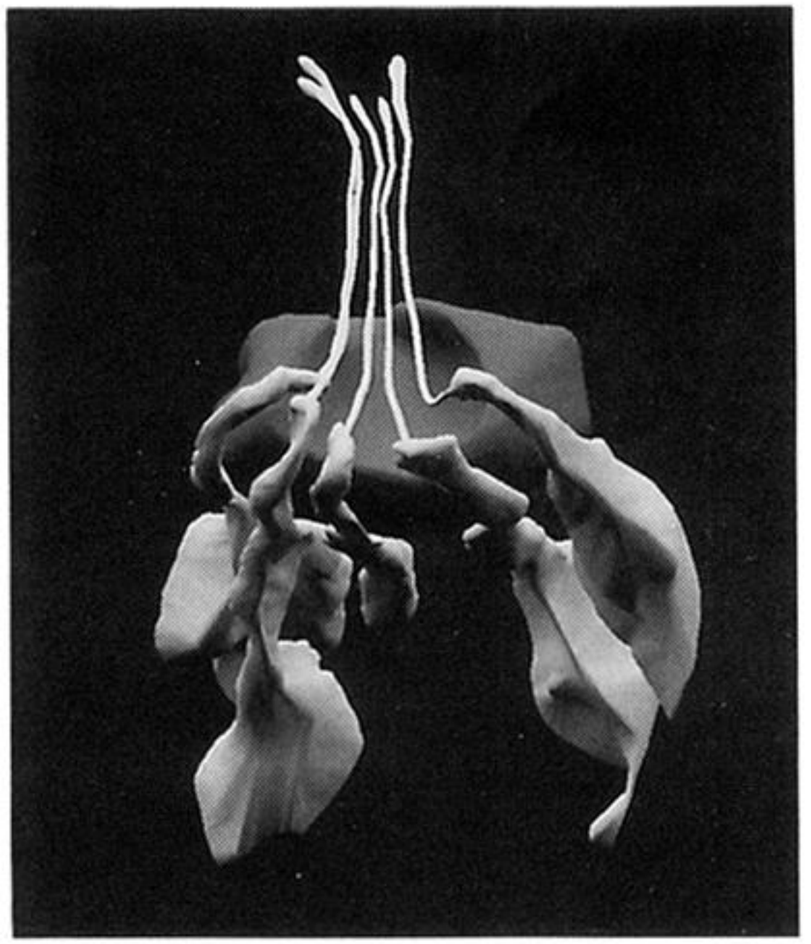
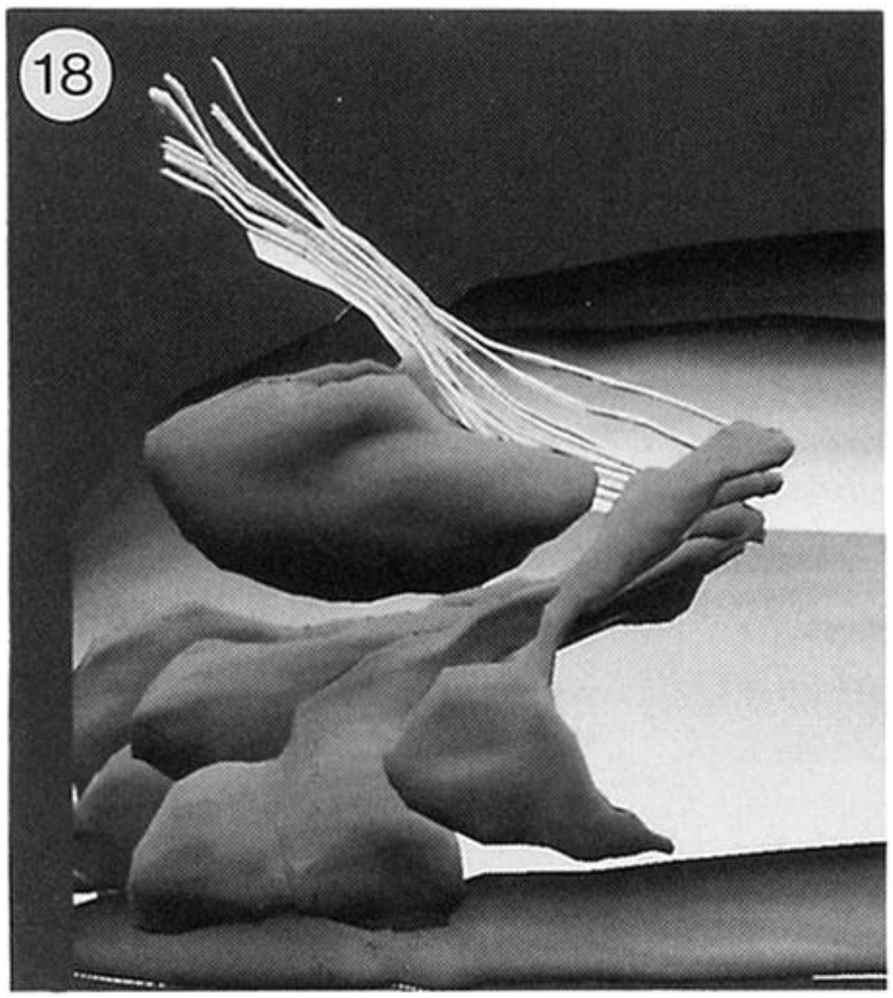


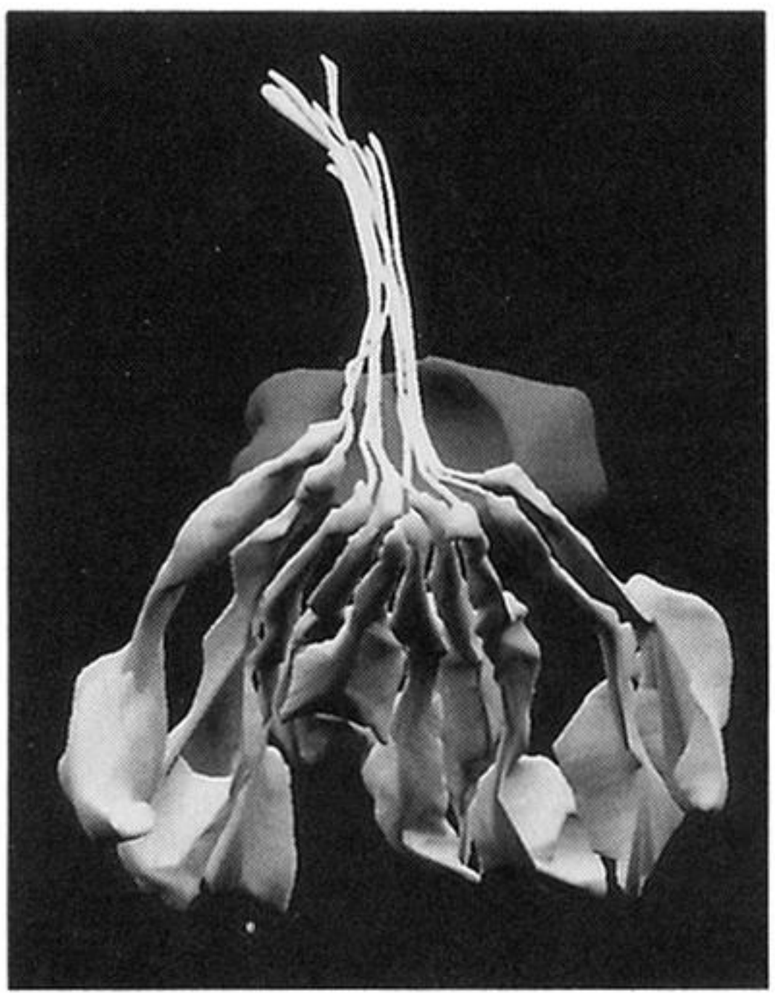
Figure 16. Sections of specimen 2 at the level of the anterior part of the main commissure. Shows descending fibres (arrows) from the soma (*) of cells responsible for the lamellar body. (*a*) Scale bar = 5 μm. (*b*,*c*) Scale bar = 2 μm.

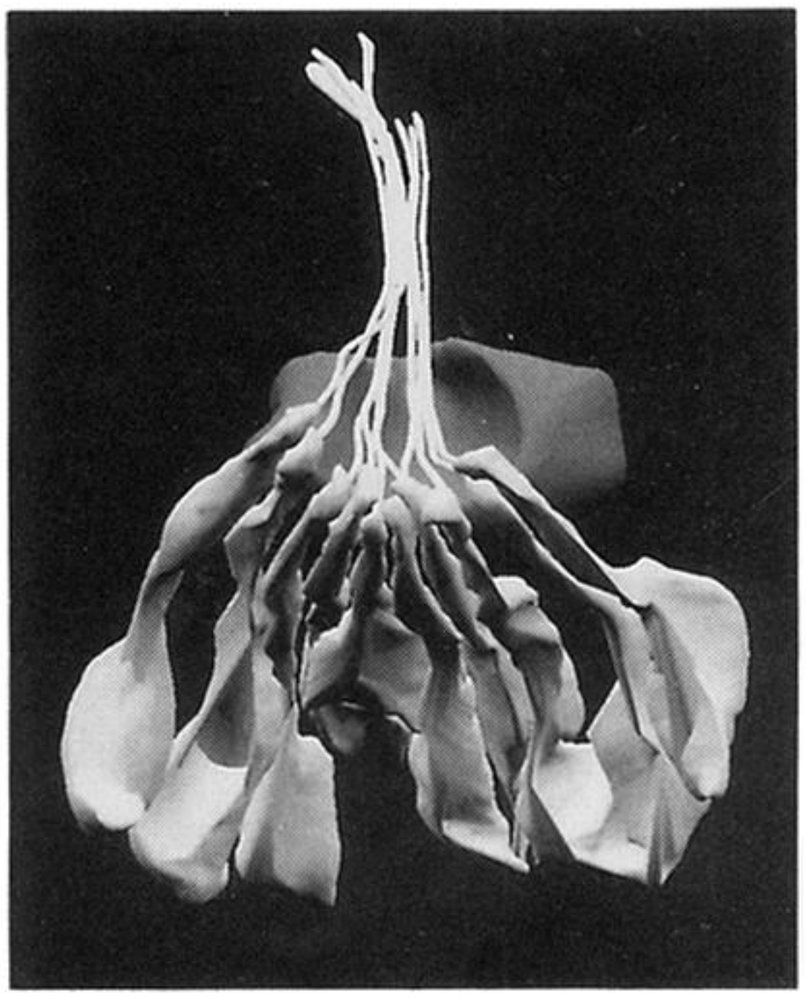








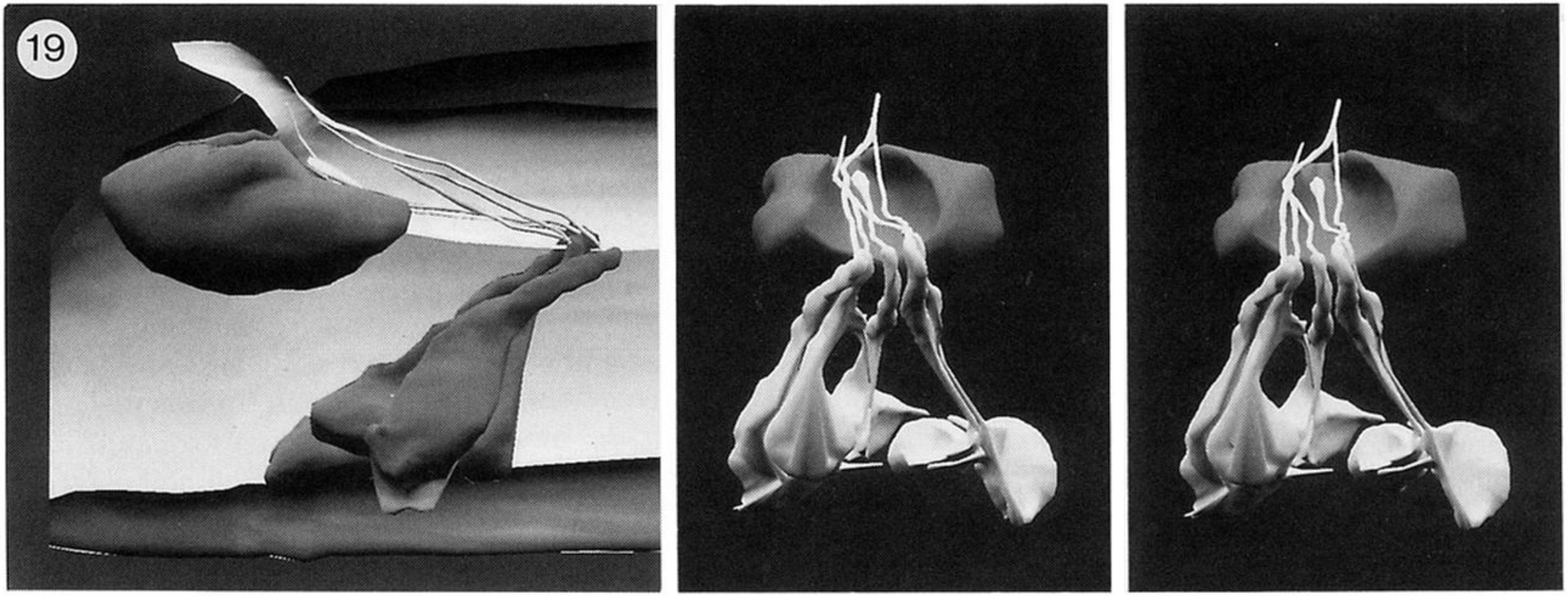




Figures 17–20. Reconstructions of cell rows associated with the anterior pigment spot. Left panel in each figure shows a side view of the anterior end of the cord (compare with figure 4*b*); right panels are stereopairs seen from directly behind the pigment spot.

Figure 17. Row 1.

Figure 18. Row 2.



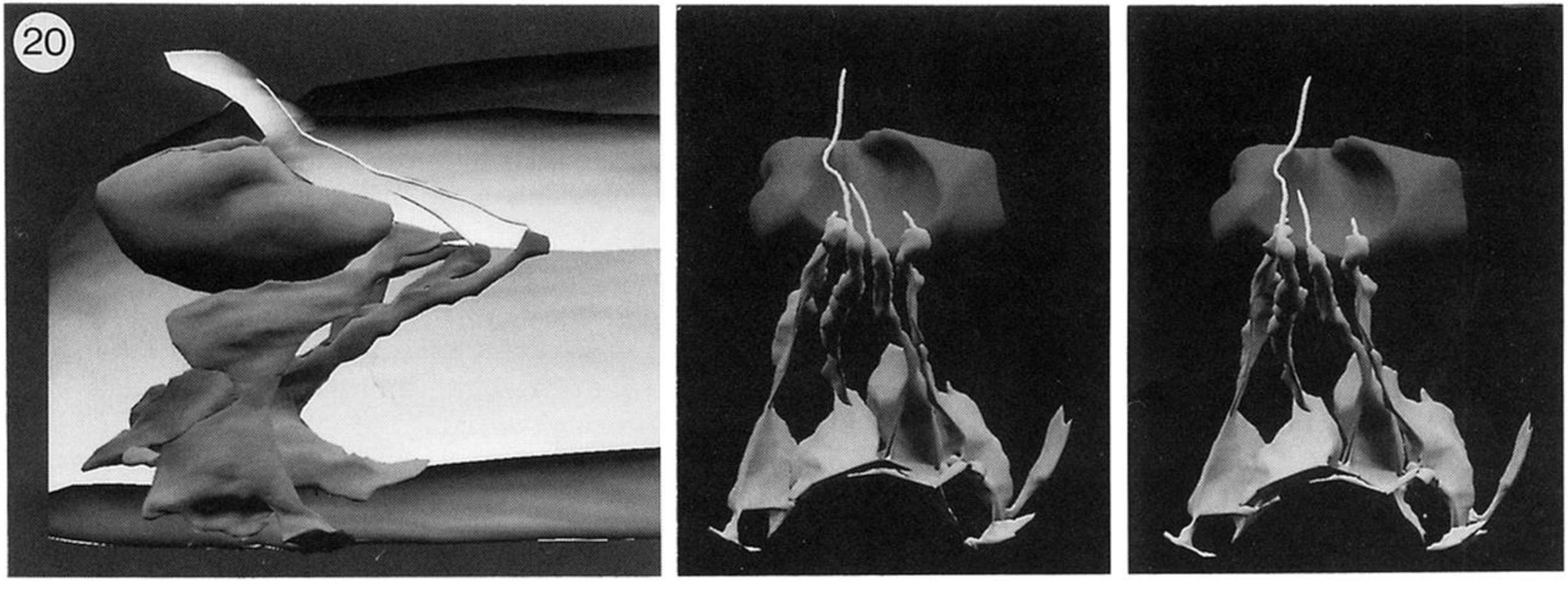
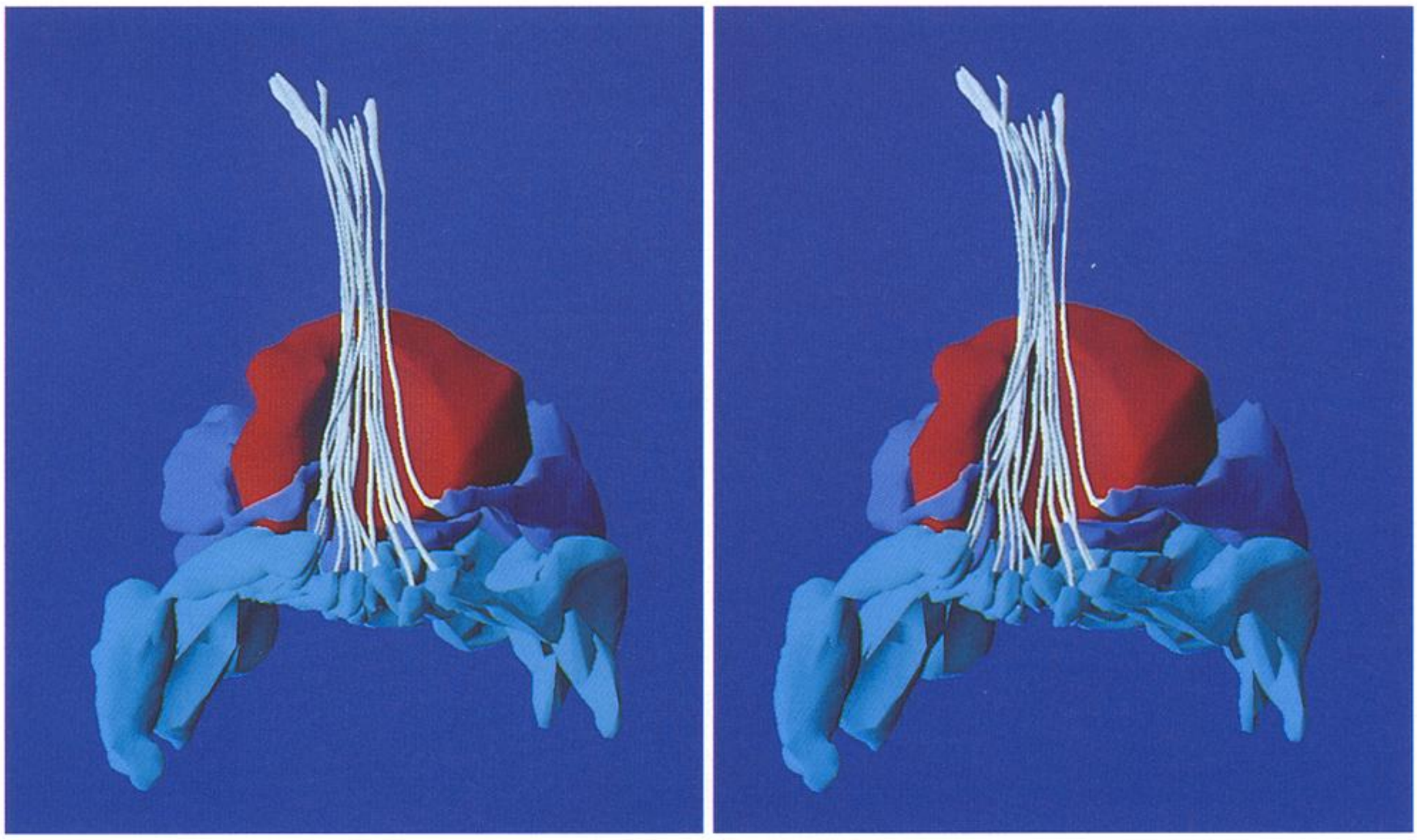


Figure 19. As in figure 17, row 3.

Figure 20. Anterior glial-like cells.

(*a*)



(*b*)

(*c*)

Figure 21. Stereopairs. (*a*) Cells of rows 1 (dark blue) and 2 (light blue), seen from behind the pigment spot (red) and slightly above the longitudinal axis. (*b*) Cells of rows 1 and 2, from below, anterior end facing up. (*c*) Selected cells from row 1 (blue), row 3 (purple), and the glial-like accessory cells (yellow), relative cell positions.