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Stereotyped pathway selection by growth cones of early epiphysial neurons in the embryonic zebrafish

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Summary

In this report we have examined the development of one of the earliest projections in the embryonic zebrafish brain, that from the epiphysis. Epiphysial axons and growth cones were labelled anterogradely in whole-mounted brains, using either the carbocyanine dye, dil, or horseradish peroxidase (HRP). Some embryos were also either stained with anti-acetylated tubulin or HNK-1 antibodies to reveal other axons in the brain, or were secondarily sectioned for light and electron microscopy.

The epiphysial axons have a very specific projection pattern and virtually all axons grow precisely to their target regions without error. The first epiphysial growth cone extends ventrally from the epiphysis into the dorsoventral diencephalic tract at 19–20 h post-fertilisation (h PF). Several hours later, it turns rostrally to grow alongside axons in the tract of the postoptic commissure. The morphology of the leading growth cone changes in predictable ways at different locations along its pathway and these changes correlate with differences in the local environment that it encounters. In contrast to other published descriptions of other developing systems, the epiphysial growth cone is no more complex either when pioneering a pathway, or when encountering divergent axonal pathways. Indeed, it is most complex (i.e. has the greatest number of processes) when it first starts to follow the tract of the postoptic commissure. The presence and selective retention of filopodia within other axonal pathways suggests that growth cones have access to these pathways but do not select them. These observations support the notion that local guidance cues exist within the early scaffold of brain tracts.

Subsequent epiphysial axons form a tight fascicle within the dorsoventral diencephalic tract, but abruptly defasciculate from each other upon turning rostrally into the tract of the postoptic commissure. Epiphysial growth cones that enter this tract at abnormal locations still turn in the appropriate direction. Therefore, guidance cues are not restricted solely to the normal intersections but may be distributed along the length of the tracts.

The epiphysial growth cones and axons have very characteristic spatial relations to other axons in the tracts of the developing brain. They are restricted to the dorsal region of the tract of the postoptic commissure and the rostral region of the postoptic commissure. At early developmental stages, the epiphysial axons are the only axons within the dorsoventral diencephalic tract and they are located very superficially within the neuroepithelium. At later stages, they are displaced to deeper regions of the neuropil by non-epiphysial axons.

Key words: growth cone guidance, axonal pathfinding, pineal, brain development, pioneer neurons, zebrafish, epiphysis, growth cone morphology.

Introduction

During development of the nervous system, neurons make highly specific connections, frequently with very distant targets. In many systems, neuronal growth cones accomplish this navigational feat with remarkably little error (for example: Goodman *et al.* 1982; Holt and Harris, 1983; Bastiani *et al.* 1984; Tosney and Landmesser, 1985*a*; Eisen *et al.* 1986; Stuermer, 1988; Bovolenta and Dodd, 1990). Many different models have been proposed to explain how growth cones are guided and it now seems likely that many mechanisms operate to ensure accurate pathfinding (Dodd and Jessell, 1988). Even for a single growth cone, different mechanisms may operate at different points along its trajectory (for example, Tessier-Lavigne *et al.* 1988; Bovolenta and Dodd, 1990). Not only does the nature of the guidance cues vary, but the growth cone may also change its responsiveness to the cues. For instance, axons are known to regulate expression of different cell adhesion molecules at different points along their trajectory (Dodd *et al.* 1988; Landmesser *et al.* 1988; Furley *et al.* 1990), and some growth cones have an age-dependent affinity for laminin (Cohen *et al.* 1986). To unravel the complexity of guidance cues that are available to growth cones, it is important to define the

molecular and cellular environment encountered by the growth cones at different regions of their pathway (Dodd and Jessell, 1988; Harrelson and Goodman, 1988).

Growth cones are highly dynamic structures and it is believed that their morphology may depend upon the local environment through which they are advancing (for example, Harris *et al.* 1985). Changes in growth cone morphology may therefore reflect changes in the composition of the local environment. For instance, in the *Xenopus* embryo, peripheral sensory neuron growth cones become less complex when they pass from the myotome to the skin (Roberts and Taylor, 1983) and optic growth cones become more complex as they pass from the retina into the optic nerve head (Holt, 1989). Growth cone morphology may also change in response to specific guidance cues. For instance, growth cones are generally more complex in regions where they encounter divergent pathway options, such as the plexus region in the chick limb (Tosney and Landmesser, 1985*b*) and the mammalian optic chiasm (Bovolenta and Mason, 1987; Godement *et al.* 1990). Contact with specific cells that are believed to be important in pathfinding can also influence growth cone morphology (Taghert *et al.* 1982; Caudy and Bentley, 1986; Bovolenta and Dodd, 1990).

Our interest has focused on growth cone guidance and axonal tract formation in the vertebrate brain. Tract formation has been studied in greatest detail in invertebrate nervous systems in which it has been shown that a small number of early generated neurons, termed pioneers, play an important role in the establishment of axon tracts. These neurons lay down an axonal scaffold containing guidance cues that are available to later generated growth cones (Raper *et al.* 1983, 1984; review, Harrelson and Goodman, 1988). There is now evidence that similar mechanisms may operate to establish pathways in the vertebrate central nervous system (Mendelson, 1986; Kuwada, 1986; Wilson *et al.* 1990; Chitnis and Kuwada, 1990; Wilson and Easter, 1991), and indeed a population of pioneering neurons has recently been described in the mammalian brain (McConnel *et al.* 1989).

The embryonic zebrafish brain is an excellent system for the study of many aspects of vertebrate brain development (Wilson *et al.* 1990; Chitnis and Kuwada, 1990). At one day of development there is a very simple, stereotyped scaffold of axon tracts and commissures in the developing forebrain and midbrain. The scaffold comprises the axons of a small number of neurons that are present in very predictable locations. We believe that this scaffold may provide a substratum for the growth of thousands of additional axons that enter the brain during the second day of development (Wilson *et al.* 1990).

In this study, we use neuroanatomical tracing techniques combined with immunocytochemistry in a detailed examination of the development of the axonal projection from the epiphysis (pineal organ). The epiphysial projection is one of the earliest in the brain; indeed we show elsewhere that a single growth cone within this projection is responsible for pioneering one of the tracts in the early scaffold (Wilson and Easter, 1991).

We have examined the development of the epiphysial projection with the aim of understanding how epiphysial growth cones are guided, and with respect to the more general question of how axonal tracts develop in the vertebrate brain. We find that pathfinding by the epiphysial growth cones is very precise and virtually error free. We examine the morphology of the leading epiphysial growth cone at different regions of its pathway and correlate the changes that we observe with changes in directionality of growth cone extension and changes in the local environment. Furthermore, we examine epiphysial growth cone pathfinding with respect to identifying the possible distribution of guidance cues within different regions of the pathway.

Materials and methods

General

Zebrafish embryos were obtained from our own breeding colony and were raised as previously described (Wilson *et al.* 1990). Embryos at the 16 cell stage were collected and raised at 28.5°C. Animals of determined absolute age were staged prior to fixation using three descriptive features: number of somites; degree of curvature of the body axis; and amount of pigmentation. For any particular age, a range of values defined a norm, and only embryos with values appropriate to that age were used. In a few cases, embryos that developed slightly faster or slower than the general population were used; these embryos were assigned to an age group based on the physical characteristics described above. At ages beyond 24 h PF when somite addition is almost complete (Hanneman and Westerfield, 1989; Kimmel, personal communication), the rostrocaudal extent of pigmentation along the back of the animal was a good indicator of developmental stage.

Labelling of epiphysial axons and growth cones

A total of 156 embryos were used in this study, of which 63 were labelled bilaterally giving a total of 219 labelled epiphysial projections (bilaterally labelled preparations showed that axons from both sides of the epiphysis had symmetrical projection patterns). In 25 embryos, HRP was used as a tracer as described elsewhere (Wilson *et al.* 1990). In the remainder of cases, dil (1,1′-dioctadecyl-3,3,3′,3′, tetramethylindocarbocyanine perchlorate: Honig and Hume, 1986) was used as the tracer. For this technique, the embryo was fixed in Pipes-buffered 4 % formalin (pH 6.9) for 4 h and was then dissected to remove the skin and eyes, and expose the brain. DiI was recrystalised from *N*,*N*-dimethylformamide and a small fragment (5–40 μm in diameter) was manoeuvered onto the epiphysis using tungsten needles. Very small fragments enabled single, or very few cells to be labelled, and larger fragments labelled the entire epiphysis. Embryos were left overnight at 4°C to allow the dil to diffuse. The fluorescence of the labelled axons was converted to a permanent electron-dense reaction product by excitation of the dye in 0.05 % diaminobenzidene (DAB) (McConnel *et al.* 1989), after which preparations were washed, cleared in 70 % glycerol and mounted between two coverslips. All dil-labelled axons shown in the results are from photoconverted preparations.

To provide a time line for the development of the epiphysial

projection, animals were divided into 11 age groups: 19–20 (n=21); 20–21 (n=23); 21–23 (n=20); 23–24 (n=24); 24–25 (n=19); 25–26 (n=20); 27–28 (n=15); 30–31 (n=4); 32–33 (n=5); 36–38 (n=6) and 47–48 h PF (n=8). In some 36–38 and 47–48 h PF preparations (n=12), the dorsoventral diencephalic tract (DVDT) was cut (approximately 20 μm ventral to the epiphysis) prior to dil application to the epiphysis. This ensured that the only labelled axons on the lesioned side were those that had passed through the postoptic commissure (POC) from the intact side. This enabled axons from one side of the brain to be unambiguously traced to their contralateral terminations.

All labelled projections were drawn with the aid of a camera-lucida attachment fitted to a Leitz Orthoplan 2 microscope. Differential interference contrast optics were used for most observations. Throughout the results, all figures have been oriented to show a left side view of the brain; in some cases it was the left side but in others it was the right, photographically reversed.

Immunocytochemistry

Axons were labelled immunocytochemically in 52 embryos (see Wilson *et al.* 1990 for general methods). Two antibodies were used: HNK-1 (kindly provided by Dr C. Stern) and anti-acetylated tubulin (Piperno and Fuller, 1985) (kindly provided by Dr G. Piperno). Neither antibody labelled the epiphysial axons at the very earliest stages of their genesis, but both labelled the rest of the scaffold of axon tracts. This enabled us to visualise the relations between the dil-labelled epiphysial axons and the pre-existing tracts in doubly labelled preparations (n=12). Both dil- and antibody-labelled axons were revealed with DAB but in such a way as to colour them differently. The dil-labelled axons were photoconverted with DAB alone, producing brown axons. The peroxidase reaction was carried out with saturating amounts of cobalt chloride and nickel ammonium sulphate producing blue/grey antibody-labelled axons.

Electron microscopy (EM)

Embryos were prepared for EM as above except that 0.1% glutaraldehyde was added to the primary fixative in dil-labelled preparations. The embryos were photographed and drawn as whole-mounts, then fixed additionally in 3% glutaraldehyde and prepared for EM (Wilson *et al.* 1990).

Six embryos (one at 23–24 h PF, two at 36–38 h PF and three at 47–48 h PF) were serially sectioned at 1 μm with sets of serial 0.15 μm sections (approximately 25–30 sections) taken every 10 to 20 μm. In addition, HRP- or dil-labelled epiphysial growth cones either at the intersection between the DVDT and the tract of the postoptic commissure (TPOC) (n=4), or within the TPOC (n=6) were examined in five other embryos between 23 and 25 h PF. These preparations were serially sectioned either horizontally or longitudinally through the growth cone at 0.15 μm with occasional 0.5 μm sections interspersed.

DiI- or HRP-labelled profiles were easily identified by the electron-dense reaction product. We identified unlabelled axons by their small size, regular shape, presence of microtubules and absence of ribosomes. In the results, we define the boundary of an axon tract by the locations of the axons within it; so for instance the boundaries of the TPOC are defined by the most dorsal and most ventral axons within the tract. We use the term neuroepithelial cell to denote a cell with an end foot in contact with the pial surface of the brain. This definition is slightly unsatisfactory in that it may include several classes of cells, such as germinal epithelial cells and primitive 'glial' cells. However, we were unable to define any other ultrastructural features that could identify different cell types.

Analysis of growth cone morphology

In order to examine changes in growth cone morphology at different locations along the axonal pathway, 121 leading epiphysial growth cones were examined in detail. Each growth cone was drawn with the aid of a camera-lucida attachment and most were also photographed. As a measure of complexity, we counted the number of growth cone processes. A growth cone process was defined as either a lamellipodium or filopodium 2 μm or longer. In some preparations it was a subjective judgement as to where the growth cone ended and the axon began and so we limited our counts of processes to the most distal 30 μm.

We divided the epiphysial axon pathway into six regions corresponding to the different tracts and intersections encountered by the epiphysial growth cones. Fig. 1 illustrates these six zones, all of which are identifiable in wholemounts viewed with differential interference contrast optics.

Statistical analysis was performed on the number and direction of filopodia at the different locations. T-tests were used to compare filopodial counts on growth cones at different

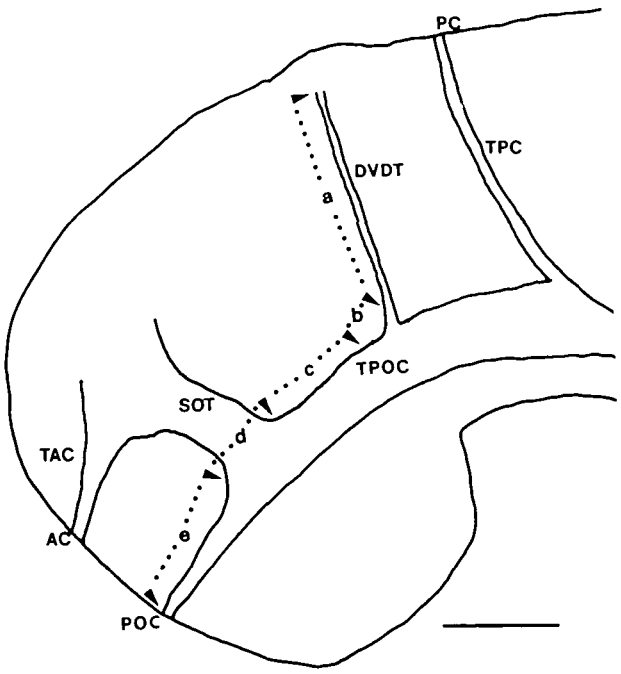


Fig. 1. Schematic of the scaffold of axons tracts in the brain. In order to examine changes in the morphology of the leading epiphysial growth cone along its pathway, we divided the epiphysial pathway into six regions. These were: within the DVDT (a); at the DVDT/TPOC intersection (b); between the DVDT/TPOC intersection and the TPOC/SOT intersection (c); at the TPOC/SOT intersection (d); between the TPOC/SOT intersection and the POC (e), and within the contralateral TPOC (the same region as (e) on the other side of the brain). Scale bar, 50 μm. Abbreviations: AC, anterior commissure; DVDT, dorsoventral diencephalic tract; PC, posterior commissure; POC, postoptic commissure; SOT, supraoptic tract; TAC, tract of the anterior commissure; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure.

locations. Chi squared tests were used to examine if filopodial extension occurred in specific directions.

Technical considerations

In the majority of preparations used for this study, axons were labelled with the lipophilic dye, diI. This dye has in recent years become a very popular tracer for labelling neurons both anterogradely and retrogradely (Godement *et al.* 1987; Honig and Hume, 1990). For our purposes, it yields results of comparable quality to HRP. We get consistent and accurate photoconversion of the fluorescent dye to a permanent reaction product which is electron dense. One cautionary note is that the density of the reaction product was always greater after the preparation had been processed for EM. This may be due to the reaction product being osmophilic (Buhl and Lubke, 1989). Therefore, to optimise the quality of the electron microscopic labelling, the preparations were only lightly photoconverted before further processing.

We found several advantages to using diI over HRP, the major one being that we were able to use the dye in fixed tissue, enabling us to accurately stage and fix groups of embryos at specific time points prior to labelling. This is not possible with HRP (see Wilson *et al.* 1990). The application of diI can also be very precise – by using extremely small crystals we are able to label single cells. DiI application also causes very little damage to the labelled cells. In fact it is possible to remove the crystal after the cells are labelled yielding good visibility of the parent cell bodies at the application site.

Results

We have previously documented many general features of brain development between one and two days PF, including a description of the simple scaffold of tracts that is present at 24 h PF (Wilson *et al.* 1990). In this study, we have examined the development of one projection, that from the epiphysis, in considerably more detail. This projection was chosen for further study for several reasons, most important of which is its suitability for axonal pathfinding studies. The epiphysial neurons that give rise to the projection are well isolated, allowing us to unambiguously label their growth cones. In addition, epiphysial axons have very stereotyped projection patterns (see below) allowing detailed examination of the pathway choices made by their growth cones.

Development of a stereotyped projection of axons from the epiphysis

The first epiphysial growth cone is present in the DVDT at 19–20 h PF (Fig. 2A). This growth cone arises from a neuron located in the caudal half of the epiphysis, and pioneers the entire DVDT (Wilson and Easter, 1991). By 20–21 h PF, the pioneering growth cone was at, or near, the junction with the TPOC (Fig. 2B) and in a few cases a second growth cone was labelled, much closer to the epiphysis. Refer to Fig. 1 for the location of the various axon tracts in the brain.

At 21–23 h PF (Fig. 2C), the leading growth cone entered the TPOC, and in every case turned in the rostral direction at the DVDT/TPOC intersection. At this stage, a second growth cone was usually labelled within the DVDT, generally trailing the first axon (Fig. 2C). Between 23 and 25 h PF (Fig. 2D,E), the leading growth cone was situated at progressively more rostral levels of the TPOC. Growth cones bypassed the supraoptic tract (SOT) and continued within the TPOC towards the commissure. The leading growth cone passed contralaterally through the POC at 25–26 h PF (Fig. 2F). By this stage of development, several other epiphysial growth cones were situated at various locations between the epiphysis and the POC (Fig. 2F).

Between 26 and 48 h PF, the epiphysial axons did not send projections to any other regions of the brain (Fig. 2G–J). The change in orientation of the epiphysial projection pattern between 24 and 48 h PF (compare Fig. 2F and J) is not due to changes in the projection patterns of the axons. Rather it is due to changes in the morphology of the brain during this period. As a result of these shape changes, the POC assumes a much more ventral location (see Ross *et al.* 1991).

In some preparations, one or more epiphysial growth cones diverged from the DVDT (Fig. 2G) resulting in the division of the DVDT into two fascicles (Fig. 2J). Also, as shown in the 36–38 h PF preparation in Fig. 3, collaterals were occasionally present on the epiphysial axons at the DVDT/TPOC and TPOC/SOT intersections. We believe that these may be the remnants of exploratory branches extended by epiphysial growth cones at these two locations (see below).

In bilaterally labelled preparations, individual bundles of epiphysial axons that passed through the POC were always in very close apposition to the ipsilateral ones from the other side. Indeed, it was not always possible to tell which axons were which near the commissure. Therefore, the contralateral projection pattern of the epiphysial axons was examined in preparations in which the DVDT had been lesioned on one side of the brain (see methods). In these preparations, all of the labelled axons remained within the bounds of the contralateral TPOC (Fig. 4A,B). The distance to which the epiphysial axons extended contralaterally varied between axons and between embryos; the furthest was near the contralateral DVDT/TPOC intersection (Fig. 4B). Varicosities were frequently present along both ipsilateral and contralateral segments of the epiphysial axons (Fig. 4A).

Growth cone morphology at different locations along the pathway

The single growth cone that establishes the DVDT usually remains at the leading edge of the epiphysial projection all the way to the contralateral side of the brain. We examined this leading growth cone at different locations along its trajectory, to look for alterations in its morphology.

The morphology of the growth cone changed substantially at different regions of its pathway. Figs 5 and 6 illustrate growth cones at six different regions of the pathway (see Fig. 1). These regions correspond to the different tracts and intersections between tracts that the epiphysial growth cones encounter along their pathway.

Table 1. *Analysis of number of growth cone processes in different regions of the pathway*

	DVDT	DVDT/TPOC intersection	DVDT/TPOC intersection to TPOC/SOT intersection	TPOC/SOT intersection	TPOC/SOT intersection to POC	Contralateral TPOC
Number of growth cones	43	21	19	11	26	5
Mean number of processes	5.3	5.5	10.1	7.3	2.2	1.4
Standard deviation	2.5	1.8	4.6	4.4	1.6	0.5
Standard error	0.4	0.4	1.1	1.3	0.3	0.6
Significantly different from previous region of the pathway?		no	yes (0.01)	no	yes (0.005)	no

See methods for detailed explanation of growth cone location. Standard deviations are given in addition to a standard error of the mean to give an indication of the variability of growth cone processes. Figures in parentheses indicate levels of significance.

As the leading epiphysial growth cone pioneered the DVDT, it was elongated in the direction of advance and frequently possessed both filopodial and lamellipodial extensions (Fig. 5A–D; Fig. 6a–k, Table 1). The axon behind the growth cone was usually smooth and possessed few processes.

Upon contacting the TPOC (Fig. 5E–G; Fig. 6l–u), the growth cone morphology changed, in that it generally became less elongated and splayed out in a rostrocaudal direction (that is, roughly at right angles to the trailing axon). The number of growth cone processes did not change significantly from the previous region of the pathway (Table 1).

The growth cone significantly increased in complexity compared to previous regions when between the DVDT/TPOC and TPOC/SOT intersections, (Fig. 5H–J; Fig. 6v–dd). It was often very elongated and the number of filopodia increased significantly (Table 1). In a few cases, the axon split into two in this region of the pathway (Fig. 6w, bb). Using differential interference contrast optics, it appeared that some filopodia wrapped around cells in the TPOC (Fig. 6v,z).

There was no significant change in growth cone morphology at the TPOC/SOT intersection (Fig. 5K; Fig. 6ee–hh). The number of growth cone processes did not change significantly (Table 1). Within both this and the previous region, there was the greatest variability in the number of growth cone processes (Table 1). Also in both regions, filopodia were evenly distributed on both dorsal and ventral sides of the growth cone (Table 2).

Growth cone complexity decreased considerably between the SOT/TPOC intersection and the POC (Fig. 5L–N; Fig. 6ii–ss, Table 1). The leading edge of the growth cone frequently displayed no processes at all (for example Fig. 6ii,oo,qq). For those axons that did possess growth cone processes, 75% were directed dorsally (Table 2). Within the contralateral TPOC, the growth cones retained their extremely simple morphology (Fig. 6tt–ww; Table 1).

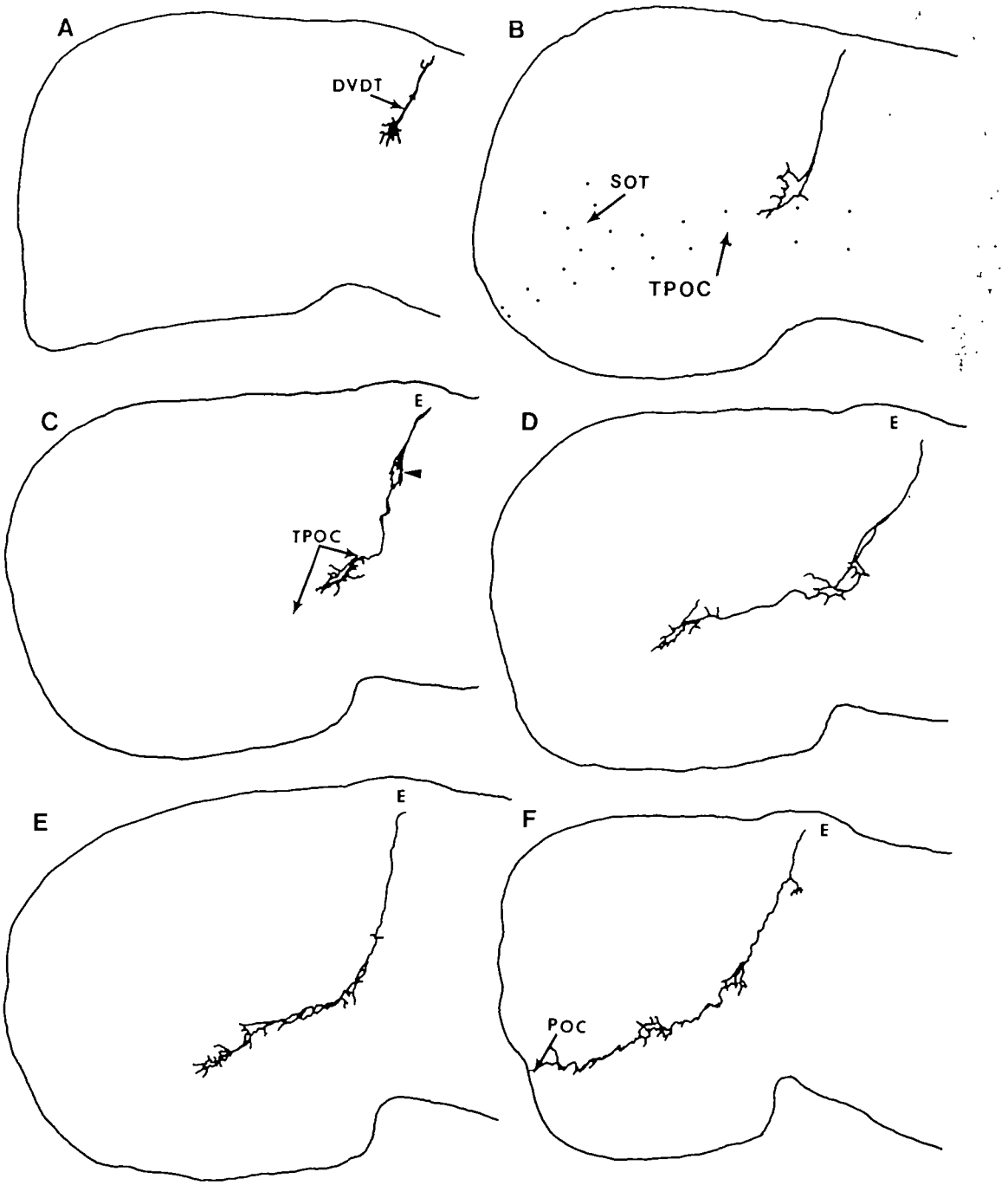
Filopodia were selectively retained at regions where the growth cone had encountered divergent axonal pathways. In 87.5% of the preparations in which the growth cone was between the TPOC/SOT intersection and the POC, filopodia or axon collaterals were retained at the TPOC/SOT intersection. Of these filopodia, 79% were directed dorsally towards the SOT (Table 2). Of epiphysial axons with growth cones within the TPOC, 56% also retained collaterals directed caudally at the DVDT/TPOC intersection.

Growth cones that emerged from the epiphysis after the pioneer, generally did not grow independently to the TPOC, rather they followed those axons that preceded them within the DVDT. Anterograde labelling of the 'follower' growth cones within the DVDT showed that they were usually closely associated with the axon of the leading growth cone (which by these

Table 2. *Location of processes on epiphysial growth cones within the TPOC*

			TPOC/SOT intersection to POC	
% Processes	DVDT/TPOC intersection to TPOC/SOT intersection	TPOC/SOT intersection	Processes between TPOC/SOT intersection and POC	Processes retained at TPOC/SOT intersection
Dorsal	54	60	75	79
Ventral	46	40	25	21
Significantly different	No	No	Yes (0.01)	Yes (0.005)

Growth cone processes were categorised as dorsal or ventral if they emerged from the dorsal (or dorso/rostral) edge or ventral (or ventro/caudal) edge of the growth cone respectively. Processes that emerged from the leading edge of the growth cone were categorised as dorsal or ventral if they deviated from the direction of growth cone advance by more than 45°. Figures in parentheses indicate levels of significance.



stages had already turned rostrally into the TPOC) (Fig. 7). For this reason, it was often not possible to resolve their exact morphology and we did not attempt to examine systematically the morphology of these 'follower' growth cones.

Availability of an alternative axonal pathway at the SOT/TPOC intersection

Epiphysial growth cones encounter two divergent axonal pathways at the TPOC/SOT intersection; one towards the telencephalon in the SOT, and the other towards the POC in the TPOC. Do the epiphysial growth cones have access to both of these pathways or do they simply grow towards the POC because they do not have access to the SOT?

An examination of the growth cones shown in Fig. 6 (ee–ss) shows that the epiphysial growth cones project filopodia into the SOT suggesting that they had physical access to both tracts. Furthermore, in a single preparation, we observed the leading epiphysial growth cone within the wrong pathway (Fig. 8), indicating that the SOT is a permissive substratum for epiphysial growth cones. These results are strong evidence that epiphysial growth cones encounter two divergent axonal pathways at the TPOC/SOT intersection,

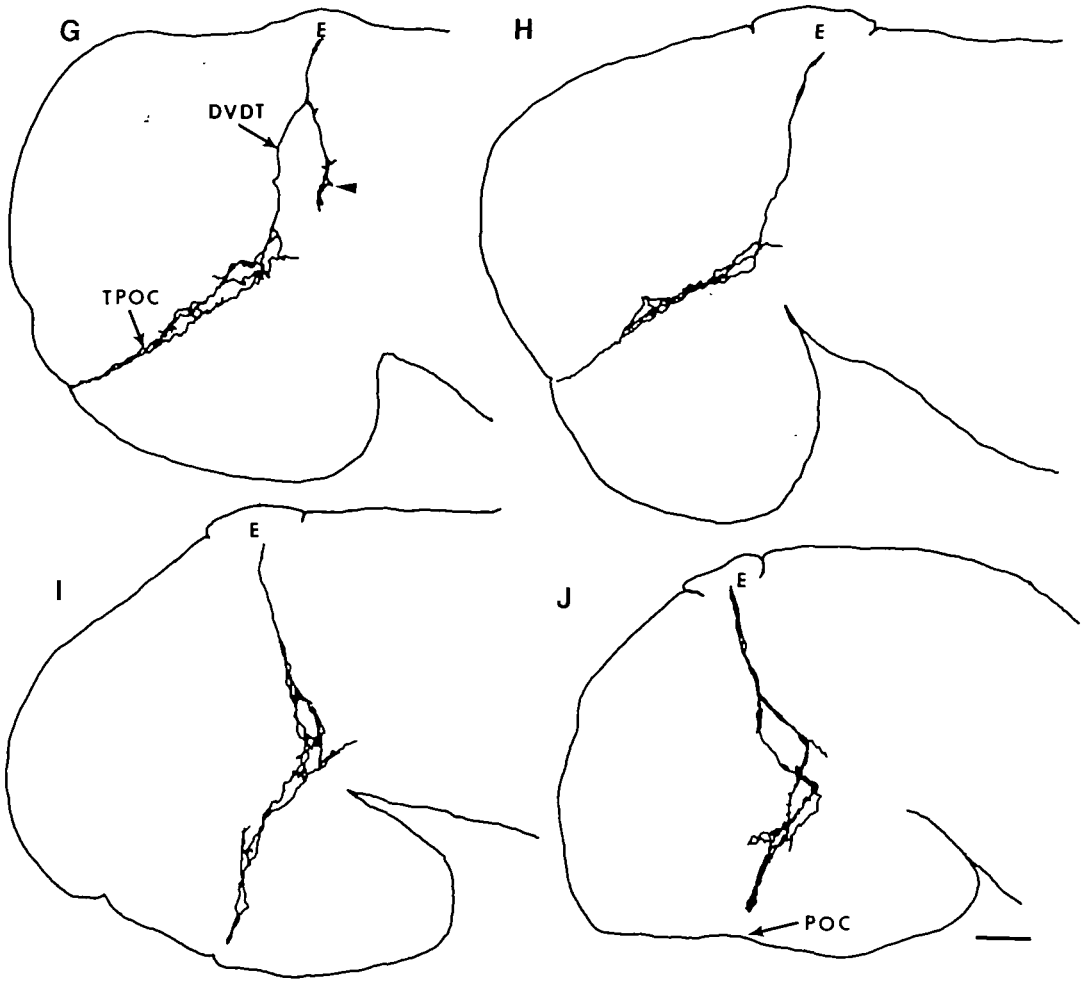


Fig. 2. Time course of development of the projection from the epiphysis. Camera-lucida tracings of whole-mounted brains in which dil had been applied to epiphysis. The fragments of dil and the outlines of the epiphysial cell bodies have been omitted from the drawings. Dorsal is up and rostral is to the left. (A) 19–20 h PF. (B) 20–21 h PF. The dots outline the approximate location of the TPOC and SOT. (C) 21–23 h PF. The arrowhead indicates a second growth cone in the DVDT. (D) 23–24 h PF. (E) 24–25 h PF. (F) 25–26 h PF. (G) 27–28 h PF. The arrowhead indicates an epiphysial axon that had split from the DVDT. (H) 30–31 h PF. (I) 36–38 h PF. (J) 47–48 h PF. Scale bar, 25 μm. Abbreviations: DVDT, dorsoventral diencephalic tract; E, epiphysis; POC; postoptic commissure; SOT, supraoptic tract; TPOC, tract of the postoptic commissure.

sample both, and almost invariably choose the TPOC over the SOT.

Relationship of epiphysial axons and growth cones to other axons in the brain

Axons are known to be an important substratum for growth cone outgrowth and indeed they may be the source of important guidance cues (Bastiani *et al.* 1984; Kuwada, 1986). We therefore examined the location of the epiphysial axons and growth cones with respect to other axons that were visualised either electron microscopically or immunocytochemically following dil application to the epiphysis.

We found that the epiphysial axons had very characteristic spatial relations to other axons in the brain. Fig. 9 illustrates these relations in combined dil- and antibody-labelled 24–25 h PF brains. In no instance did any epiphysial axons enter the nearest tract, that of the posterior commissure, about 50 μm caudal to the epiphysis (Fig. 9A). At their intersection with the TPOC, the epiphysial axons defasciculated from each other and sometimes branched. In Fig. 9B, the epiphysial axons are restricted to a single fascicle within the DVDT but abruptly defasciculate into three within the TPOC. At the DVDT/TPOC intersection, the TPOC is a broad tract containing several axonal bundles and the defasciculation at this location results in different epiphysial axons coursing along different bundles of axons in the dorsal TPOC (Fig. 9B).

Using EM, we examined the cellular environment of epiphysial growth cones (either the second or third to emerge from the epiphysis) at the location at which they turned rostrally into the TPOC. Fig. 10 shows electron micrographs from a preparation in which the second growth cone from the epiphysis had reached the DVDT/TPOC intersection. The age of the embryo

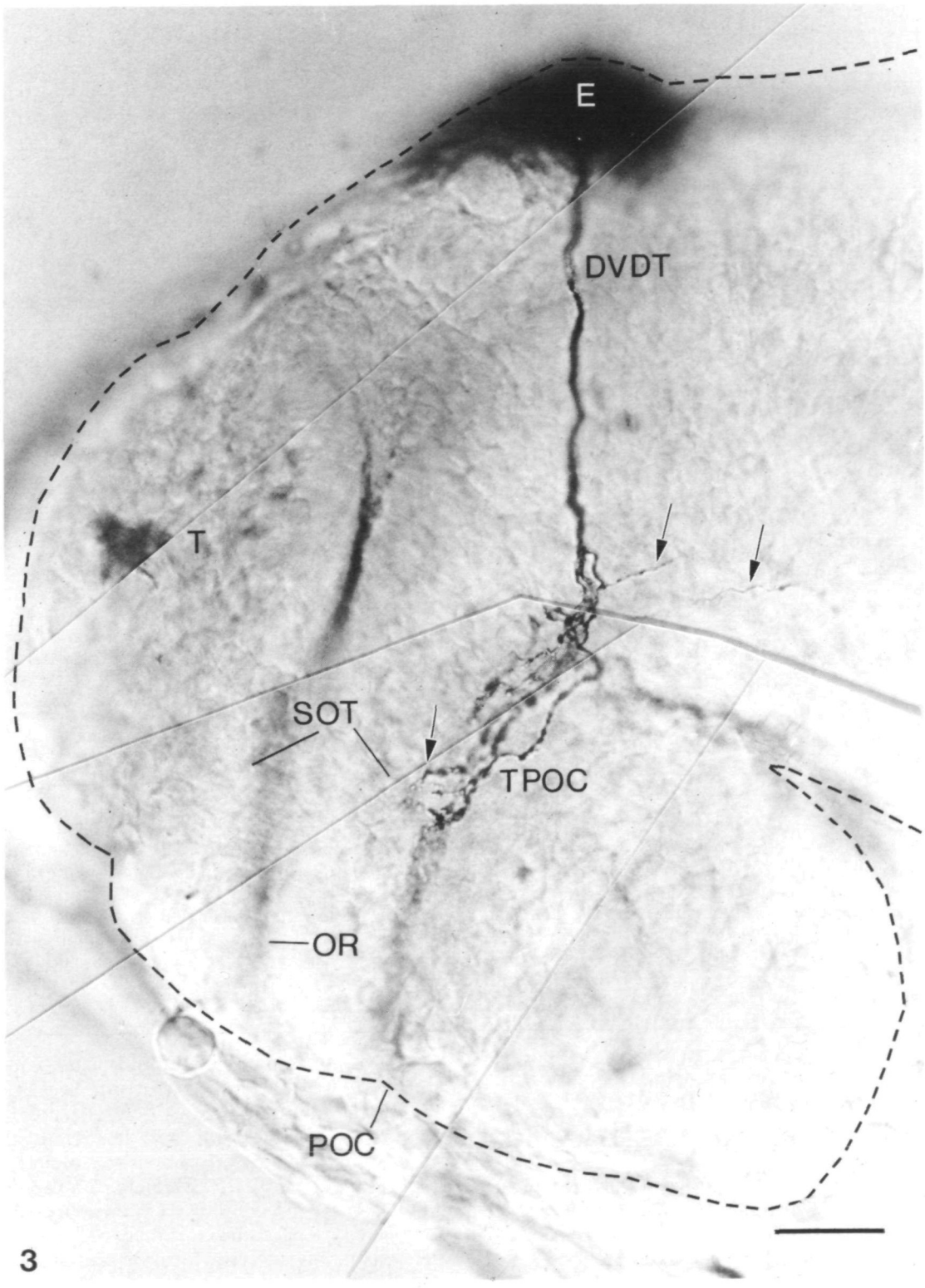
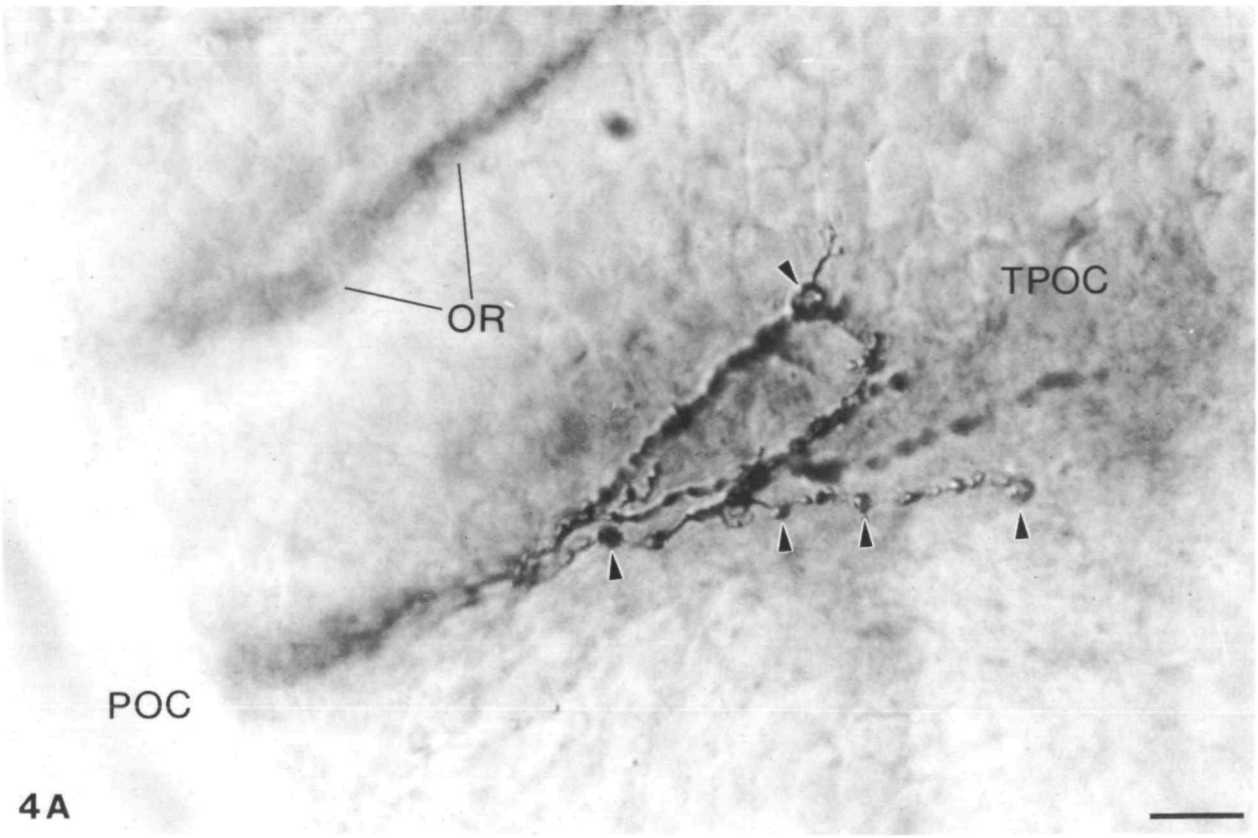


Fig. 3. Epiphysial projection at 36–38 h PF. Whole-mounted preparation in which dorsal is up and rostral is to the left. The dashes outline the brain. The epiphysis is, by this stage of development, a discrete structure in the roof of the diencephalon and is labelled darkly by the diI application. The epiphysial axons are tightly fasciculated in the DVDT, but are more widely distributed in the TPOC. Collaterals (arrows) are present on the epiphysial axons at the DVDT/TPOC and TPOC/SOT intersections. Scale bar, 25 μm. Abbreviations: DVDT, dorsoventral diencephalic tract; E, epiphysis; OR, optic recess; POC, postoptic commissure; SOT, supraoptic tract; T, telencephalon; TPOC, tract of the postoptic commissure.



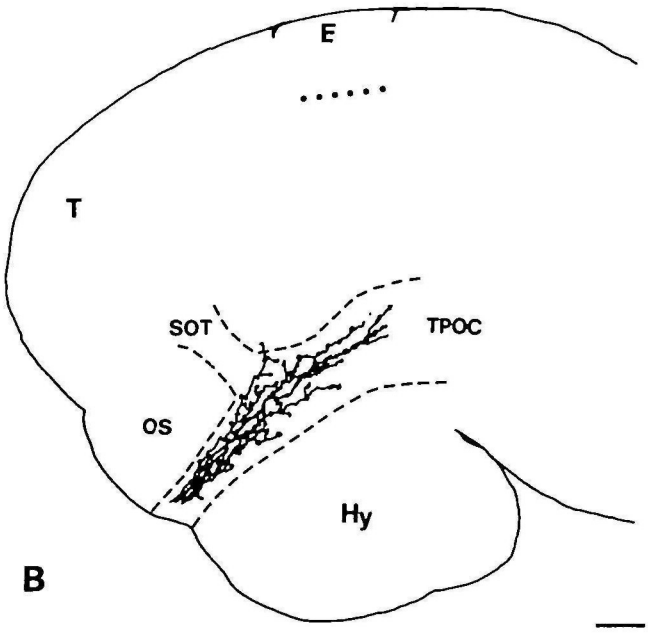


Fig. 4. Contralateral projection patttern of the epiphysial neurons. Epiphysial axons were labelled with diI. Dorsal is up and rostral is to the left. (A and B) Projection patterns of epiphysial axons on the contralateral side of the brain in 36–38 h PF embryos (the ipsilateral DVDT was cut (dots) prior to applying diI to the epiphysis in each case). (A) Photomicrograph of a whole-mounted brain. Arrowheads indicate varicosities along the axons. (B) Camera-lucida tracing summing the projections from five different preparations. The outlines of the SOT and TPOC were observed using differential interference contrast optics. The contralateral projection pattern of epiphysial axons at 48 h PF is similar to B. Scale bars, (A) 10 μm; (B) 25 μm. Abbreviations: E, epiphysis; Hy, hypothalamus; OR, optic recess; OS, optic stalk; POC, postoptic commissure; SOT, supraoptic tract; T, telencephalon; TPOC, tract of the postoptic commissure.

(23–24 h PF), and electron microscopic examination of the DVDT just proximal to the intersection (which showed two axons), allowed us to be confident that this was the second growth cone from the epiphysis. The entire growth cone was serially sectioned, allowing reconstruction of some of the cellular profiles contacted by the growth cone. Fig. 10C is a micrograph from a section through the main body of the growth cone. Much of the surface area of the growth cone is in contact with end foot processes of neuroepithelial cells. We did not observe this, or other, growth cones in contact with the basal lamina. Fig. 10D is 3.5 μm further ventral to 10C and shows the location of the leading filopodia of the growth cone. One of the filopodia is surrounded by a small fascicle of TPOC axons. Some of these axons arose from cell bodies located in the vicinity

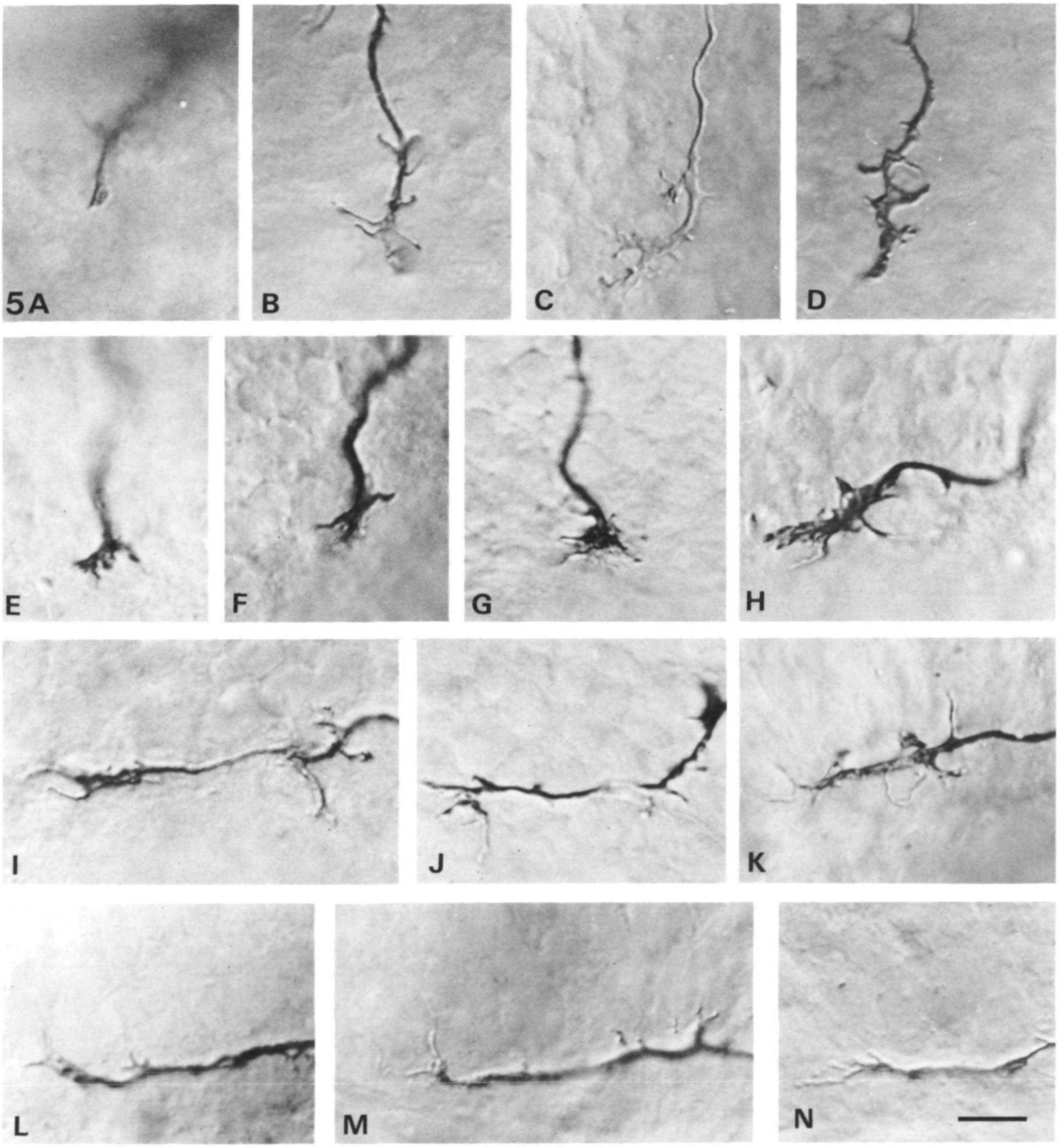


Fig. 5. (A–N) Morphology of the leading growth cone from the epiphysis at different locations along its trajectory. Whole-mounted preparations in which dorsal is up and rostral is to the left. Growth cones were labelled by dil application to their parent cell bodies. (A–D) Between the epiphysis and the TPOC. In A, the growth cone is just emerging from its parent cell body. (E–G) Midway down the diencephalon, at the DVDT/TPOC intersection. (H–N) Within the TPOC. Growth cones shown in H–J are between the DVDT/TPOC and TPOC/SOT intersections. The growth cone in K is at the TPOC/SOT intersection. (L–N) Between the TPOC/SOT intersection and the POC. Scale bar, 10 μm. Abbreviations: DVDT, dorsoventral diencephalic tract; POC, postoptic commissure; SOT, supraoptic tract; TPOC, tract of the postoptic commissure.

of the growth cone. For instance, axon (a) arose from a cell body located rostral to the main body of the growth cone (Fig. 10C).

The epiphysial axons coursed along the more dorsal of the TPOC axon bundles (Fig. 9). Several possibilities could account for the epiphysial axons being restricted to this region. Epiphysial growth cones could initially grow anywhere within the tract and then be displaced dorsally by later growing TPOC axons that are added to the ventral part of the tract. Alternatively, epiphysial growth cones could restrict their growth, from the beginning, to the dorsal TPOC. To differentiate between these possibilities, we examined epiphysial growth cones within the TPOC electron microscopically.

We found that epiphysial growth cones were located in the most dorsal regions of the TPOC. Fig. 11 shows micrographs from a 24–25 h PF preparation in which two epiphysial growth cones in the TPOC were labelled by dil application to their cell bodies in the epiphysis. The trailing growth cone had quite large filopodial and lamellipodial extensions while the leading growth cone was morphologically much more simple, with few growth cone processes (Fig. 11B). Fig. 11C is a low magnification camera-lucida tracing showing the location of the epiphysial axons within the TPOC, close to the DVDT/TPOC junction. The more dorsal epiphysial axon is located between two neuroepithelial cells and is in contact with two other small profiles which are probably axons (Fig. 11D). The ventrally located epiphysial axon is within a small fascicle of TPOC axons, which is completely surrounded by neuroepithelial cell processes (Fig. 11E). There were no other fascicles of TPOC axons dorsal to the epiphysial axons but there were others further ventrally (see Fig. 11C). Fig. 11F is a transverse section through the trailing growth cone. The body of this growth cone was sandwiched between adjacent neuroepithelial cells with its most ventral filopodia in close proximity to the dorsal fascicles of TPOC axons. At this level, the axon from the leading growth cone is still located among the dorsal TPOC axons. Fig. 11G and H show a micrograph (G) and a camera-lucida tracing (H) of a transverse section through the proximal region of the leading growth cone. The labelled profiles are among the most dorsally located axons in the TPOC, with several other axon bundles located further ventrally. These results show that the epiphysial growth cones selectively grow within the most dorsal regions of the TPOC and are not just displaced to this location by the ventral addition of later axons.

The epiphysial axons also occupied a very restricted region of the POC. Examination of their location in sectioned material showed that, as the epiphysial axons passed contralaterally, they initially occupied the rostral edge of the commissure (Fig. 12A). However, as more axons were added to the POC, they were displaced dorsally (Fig. 12B,C). Within the commissure, the epiphysial axons were considerably more tightly clustered than they had been in the TPOC (compare Fig. 12 to Figs 3 and 9). Electron microscopy of 48 h PF preparations (Fig. 12C) confirmed this and showed that the epiphysial axons were restricted to a dorsal, rostral location, deep to the optic axons (which enter the diencephalon at the rostral edge of the POC – Wilson *et al.* 1990).

Behaviour of aberrant epiphysial growth cones

Occasionally the DVDT was split into two fascicles. Analysis of growth cone behaviour in embryos which showed this aberrant projection pattern allowed us to draw several conclusions about the nature of the guidance cues available to the epiphysial growth cones.

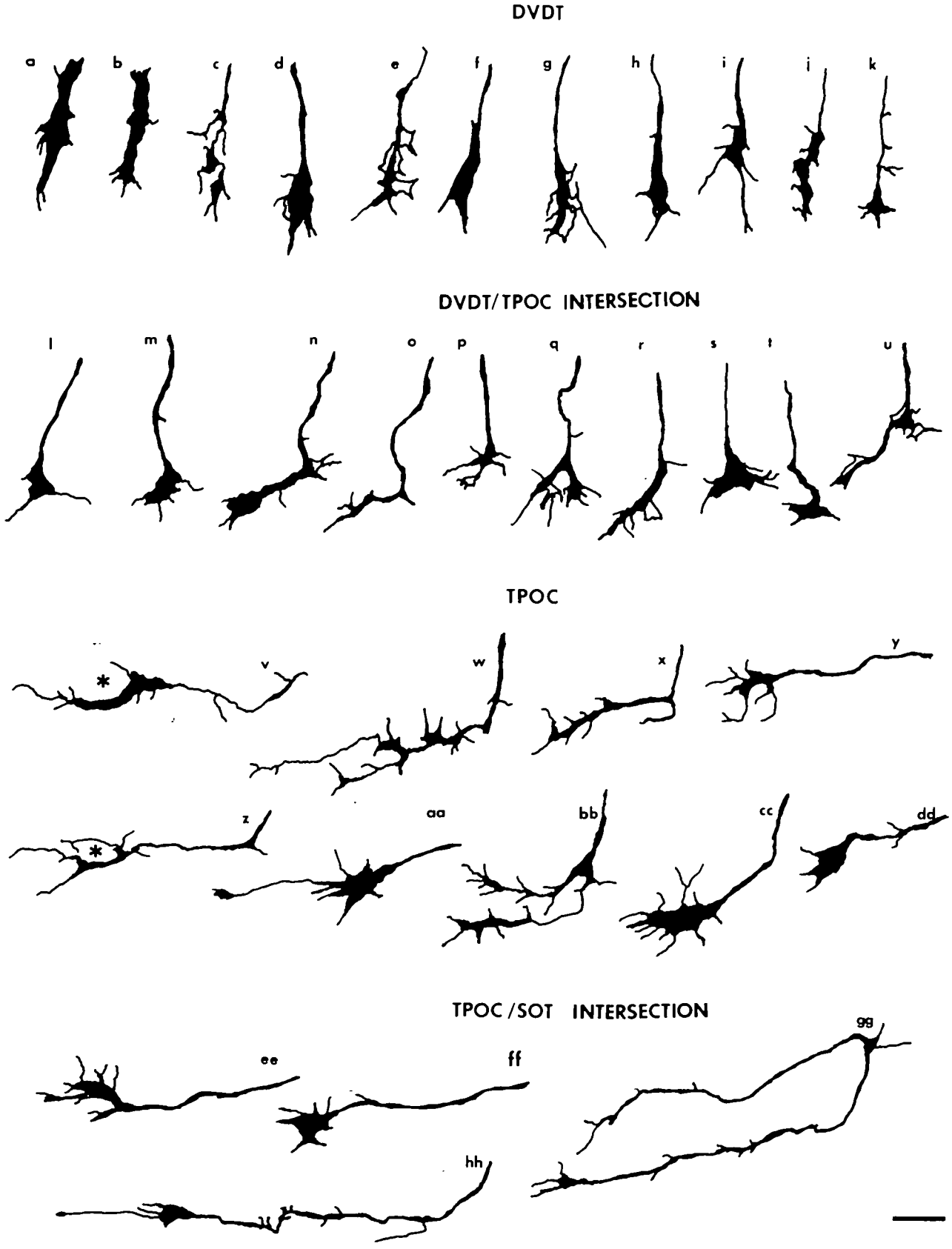
The growth cone that pioneers the DVDT is not guided by any other axons (Wilson and Easter, 1991). We believe that later epiphysial growth cones can also navigate independently of other axons to the TPOC because they occasionally took routes towards the TPOC independent of other axons ($n=24$; in two other cases there were two entirely distinct fascicles of axons running all the way from the epiphysis to the TPOC.). Fig. 13A shows a divided DVDT in a 24 h PF preparation. Once it has split from the DVDT the aberrant growth cone maintains a fairly straight trajectory and continues independently towards the TPOC. In no instance did we observe an epiphysial growth cone growing away from the TPOC.

The specific turning behaviour exhibited by epiphysial growth cones at the DVDT/TPOC intersection suggests that local guidance cues exist at this location. We assessed whether these guidance cues were restricted to the normal DVDT/TPOC intersection by examining the behaviour of epiphysial axons that entered the TPOC at inappropriate locations following divergence from other epiphysial axons in the DVDT (Fig. 13A). We examined 15 of these preparations at stages after these aberrant growth cones had reached the TPOC. If the cues that enable the epiphysial axons to turn rostrally are restricted to the normal DVDT/TPOC intersection then we might expect that the aberrant growth cones would choose inappropriate pathways when they contact the TPOC in the wrong location. Alternatively, if cues are not restricted, then we would expect the growth cones to turn in the appropriate rostral direction at the abnormal intersection.

We found that all of the more caudally directed epiphysial axons turned in the appropriate rostral direction, no matter where they contacted the TPOC. Fig. 13B shows a 36 h PF preparation in which an epiphysial axon contacts the TPOC approximately 40 μm caudal to the normal DVDT/TPOC intersection. The aberrant axon turns rostrally at this location and rejoins the other epiphysial axons in the more rostral part of the TPOC. These results demonstrate that the guidance cues that enable the epiphysial growth cones to turn rostrally are not restricted to the normal DVDT/TPOC intersection.

Addition of non-epiphysial axons to the DVDT

In many systems the axons that establish pathways, termed pioneers, are suggested to have a role in



providing a substratum for the growth of other populations of later developing axons (for example, Klose and Bentley, 1989; Kuwada, 1986; Easter and Taylor, 1989; Wilson *et al.* 1990). If the epiphysial axons have such a role then we would expect to see an increase in the size of the DVDT over time as more

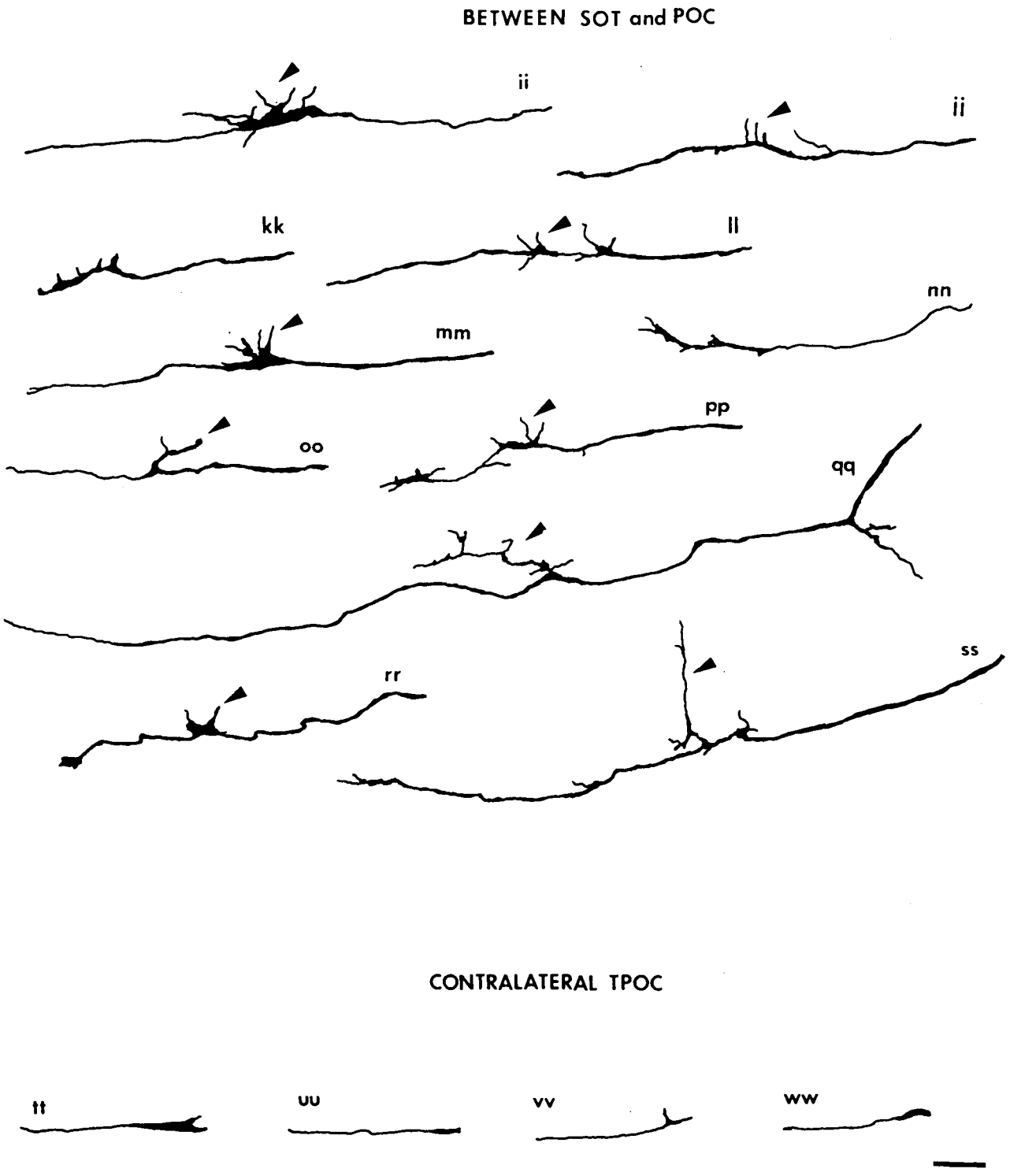
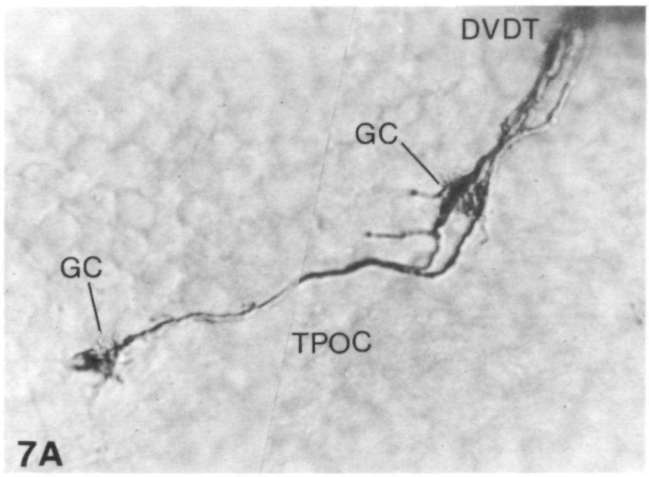
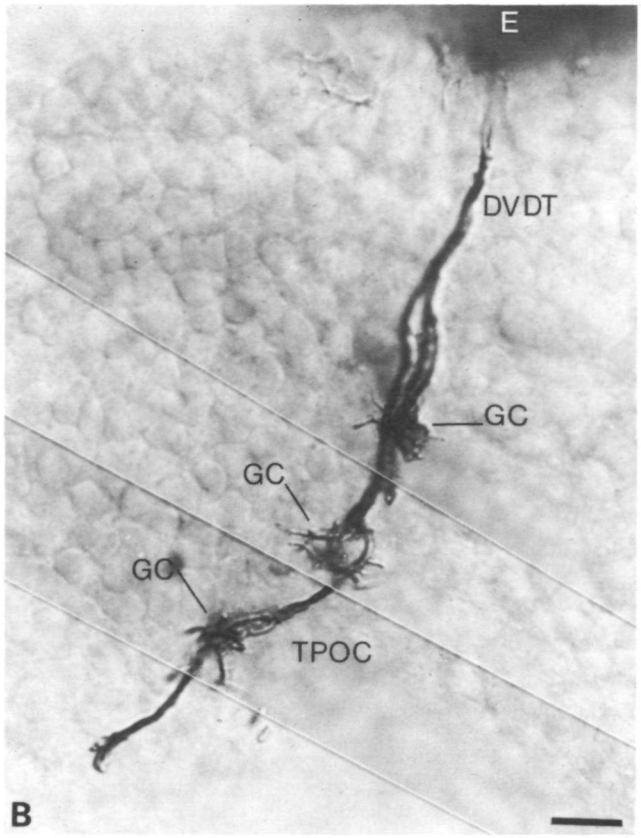


Fig. 6. Camera-lucida tracings of leading epiphysial growth cones at different locations along their pathway. Dorsal is up and rostral is to the left. (a–k) Within the DVDT. In a and b, the growth cones are just emerging from their cell bodies. (l–u) At the DVDT/TPOC intersection. (v–dd) Between the DVDT/TPOC and TPOC/SOT intersections. Asterisks indicate cell bodies around which filopodia were wrapped. (ee–hh) At the TPOC/SOT intersection. (ii–ss) Between the TPOC/SOT intersection and the POC. Arrowheads indicate side branches at the TPOC/SOT intersection. (tt–ww) In the contralateral TPOC (the leading edge of the growth cone is now to the right). Scale bar, 10 μm. Abbreviations: DVDT, dorsoventral diencephalic tract; POC, postoptic commissure; SOT, supraoptic tract; TPOC, tract of the postoptic commissure.

Fig. 7. 'Follower' epiphysial growth cones within the DVDT. Whole-mounted preparations in which dorsal is up and rostral is to the left. Growth cones were labelled anterogradely by dil application to parent cell bodies in the epiphysis. (A) 22–23 h PF. Two growth cones are labelled, the leading one has turned rostrally into the TPOC, the trailing growth cone is almost at the DVDT/TPOC junction. (B) 23–24 h PF. Three growth cones are labelled. The leading growth cone is within the TPOC and is more elongate than the two trailing growth cones (one of which is at the DVDT/TPOC junction, the other is still in the DVDT). Scale bar, 10 μm. Abbreviations: DVDT, dorsoventral diencephalic tract; E, epiphysis; GC, growth cone; TPOC, tract of the postoptic commissure.





axons enter this tract. Therefore, we examined the DVDT electron microscopically at 23–24, 36–38 and 47–48 h PF to see if the early epiphysial axons had been joined by any axons of non-epiphysial origin.

Electron microscopy at 23–24 h PF (Fig. 14A) showed that only two or three axons were present in the DVDT, confirming our light microscopic impression. The axons were located within 1–2 μm of the pia and were completely enveloped by neuroepithelial end foot processes (Fig. 14A). At 36–38 h PF, ultrathin sections taken just ventral to the emergence of the axons from the epiphysis showed a small tightly fasciculated bundle of axons surrounded by neuroepithelial processes (Fig. 14B). However, at more ventral levels, many other axons of non-epiphysial origin coursed alongside the epiphysial axons within the DVDT (Fig. 14C). By 48 h PF (Fig. 14D), the DVDT had enlarged considerably, and the labelled epiphysial axons, which had been superficial, were now separated from the pial surface by many unlabelled axons. Therefore, at early developmental stages, the DVDT consists solely of a small number of epiphysial axons but as the embryo ages, many axons of non-epiphysial origin are added to the tract, displacing the epiphysial axons to deeper regions of the neuropil.

Discussion

This report shows that epiphysial growth cones navigate very precisely during embryonic development of the zebrafish brain. In the discussion, we will focus much of our attention on how the morphology and behaviour of the epiphysial growth cones sheds light on the distribution and nature of guidance cues available to growth cones in the embryonic brain.

Axonal pathfinding by the epiphysial neurons

The epiphysial axons exhibited very precise pathfinding and showed no evidence of exuberant or inappropriate projections. Navigational accuracy is a feature common to many systems and is usually achieved by growth cones making specific pathway choices rather than by random growth followed by pruning of inappropriate axons (for example, Holt and Harris, 1983; Tosney and Landmesser, 1985*a*; Eisen *et al.* 1986; Wilson *et al.* 1988). Even in systems where pruning is important in establishing final connections, growth cones are still remarkably selective in their pathway and target choices. For example, although rat optic axons make many targeting errors within the lateral geniculate, the majority nevertheless accurately pathfind from the eye to the geniculate (Simon and O'Leary, 1990).

One of the most striking features of the epiphysial projection is the invariant rostrally directed growth at the TPOC. This provides strong evidence for the presence of directional cues at this location. However, it is unlikely that guidance cues are restricted to the normal DVDT/TPOC intersection. The absolute rostrocaudal level at which the DVDT intersects with the TPOC is slightly variable yet in all cases epiphysial axons turn rostrally. Indeed, in cases where an

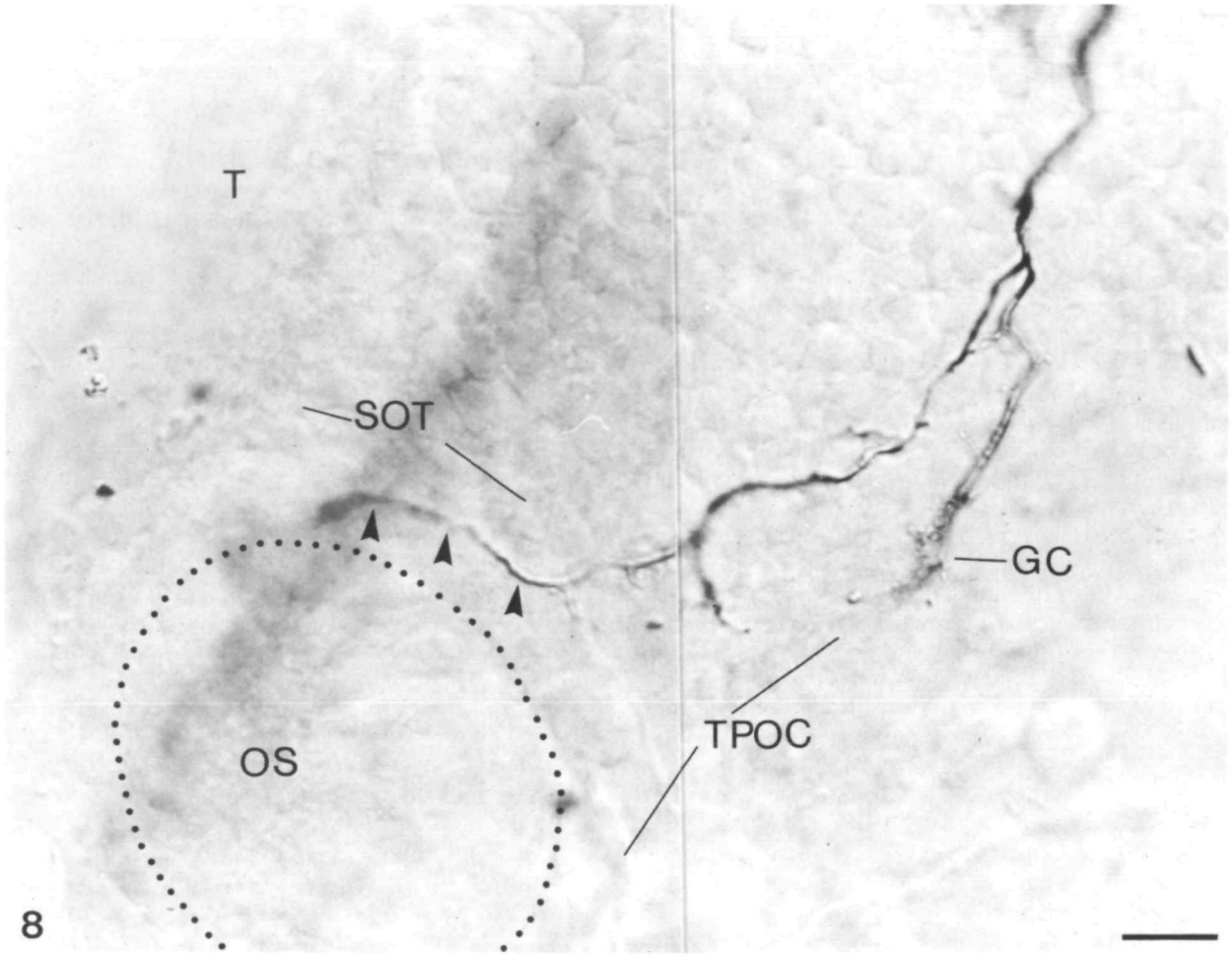


Fig. 8. Aberrant epiphysial projection into the SOT. Whole-mounted preparation in which dorsal is up and rostral is to the left. The leading epiphysial growth cone (arrowheads) has entered the SOT, rather than continuing within the TPOC. A second epiphysial growth cone has a normal trajectory within the TPOC. The dots show the approximate outline of the optic stalk. Scale bar, 10 μm. Abbreviations: GC, growth cone; OS, optic stalk; SOT, supraoptic tract; T, telencephalon; TPOC, tract of the postoptic commissure.

epiphysial axon intersects the TPOC considerably further caudally to normal (Fig. 13B), it nevertheless turns in the appropriate direction. This suggests that the guidance information necessary to allow the epiphysial growth cones to turn rostrally is distributed along the length of the TPOC and is not restricted to the normal DVDT/TPOC intersection.

The epiphysial axons and growth cones are associated with bundles of TPOC axons when they enter the TPOC and so these axon fascicles are possible candidates for the location of the guidance cues. Evidence for specific guidance cues being located on axon fascicles comes from studies of the developing insect CNS in which advancing growth cones selectively associate with different axon fascicles (Ghysen, 1978; Raper *et al.* 1983; Bastiani *et al.* 1984). An explanation of this specific pathfinding is provided by the labelled pathways hypothesis which states that the early axonal fascicles are differentially labelled and that later growth cones can read these labels and navigate accordingly (Goodman *et al.* 1982). This hypothesis now has strong evidence to support it from observations showing selective distribution of different cell adhesion molecules on different axonal pathways (Harrelson *et al.* 1988).

The epiphysial axons and growth cones were restricted to the dorsal regions of the TPOC and the rostral region of the POC. Several possibilities could account for this. For instance, the epiphysial growth cones may have a selective affinity for axons (and perhaps also cell bodies) in the dorsal part of the TPOC, or alternatively they may be located in the dorsal TPOC because this region is the first to be encountered. Given that epiphysial growth cones are quite large in relation to the TPOC we think that it would be unlikely that the growth cones remain associated with dorsal axons unless these axons offer a preferable substratum to those located further ventrally. To resolve this issue fully, it may be necessary to manipulate the epiphysial cells experimentally such that their growth cones initially encounter ventral TPOC axons and then see if they still retain an affinity for the dorsal TPOC. The

epiphysial axons are not the only ones that show location-specific growth within the TPOC. Optic axons form a distinct fascicle on the rostral edge of this tract (Wilson *et al.* 1990; see Easter and Taylor, 1989, for similar results in *Xenopus*).

Almost invariably, later epiphysial growth cones extend along axons that preceded them to the TPOC. This fasciculation results in the DVDT being a tight bundle of epiphysial axons and is support for the role of axons in providing a substratum for later growth cones to extend from the epiphysis to the TPOC. This fasciculative mode of growth of the epiphysial axons is in contrast to the ventrally directed extension of commissural growth cones in the spinal cord of rat (for example; Bovolenta and Dodd, 1990), chick (Holley and Silver, 1987), fish (Kuwada *et al.* 1990), and frog (Roberts *et al.* 1988) in which, at early stages, the growth cones have independent trajectories towards the ventral midline.

The first epiphysial growth cone does not use other axons as a substratum and at least some later epiphysial growth cones can also navigate without an axonal substratum to the TPOC, as evidenced by the fact that later growth cones occasionally diverge from the DVDT yet still grow towards the TPOC. Other classes of neurons in the fish central nervous system are also able to navigate independently of the axons that normally precede them. Primary motor neurons are able to grow independently into the periphery (Eisen *et al.* 1989; Pike and Eisen, 1990), and individual Rohon Beard cell growth cones can traverse regions of spinal cord devoid of other Rohon Beard axons (Kuwada, 1986). It should be noted however, that the growth cones of a later developing class of neurons cannot navigate normally in the absence of these same axons (Kuwada, 1986).

As yet we do not know what role the epiphysial axons play in the guidance of the non-epiphysial axons that enter the DVDT at later developmental stages. We have previously proposed that the early scaffold of tracts in the zebrafish brain may provide a substratum for the growth cones of many additional axons that enter the brain at later stages of development (Wilson *et al.* 1990). We should be able to resolve the role played by epiphysial axons in the guidance of later axons by ablating the epiphysis and examining pathfinding by the later axons in the absence of the epiphysial axons.

Growth cone morphology

The morphology of the leading epiphysial growth cone changes in predictable ways at different locations along its pathway. What might be responsible for these shape changes? One possibility is that changes in the local environment might influence the shape of the growth cone (Roberts and Taylor, 1983; Bovolenta and Mason, 1987; Holt, 1989; Bovolenta and Dodd, 1990). Furthermore, growth cone morphology may be dependent upon specific extrinsic guidance cues (Caudy and Bentley, 1986) and so we might also expect growth cone morphology to become more complex in regions where divergent pathways are encountered (see: Tosney and Landmesser, 1985*b*; Godement *et al.* 1990). Do the changes in growth cone morphology that we observe correlate with changes in the microenvironment of the growth cone, or with the presence of divergent pathways?

As it pioneers the DVDT, the leading epiphysial growth cone is exclusively in contact with neuroepithelial cells and extracellular matrix in the sub-pial region of the brain (Wilson and Easter, 1991). It has been shown that, in some systems, the leading growth cones within a pathway are more complex than later growth cones which may follow other axons (Lopresti *et al.* 1974; Nordlander, 1987). We did not examine the morphologies of later epiphysial growth cones and so we do not know if they are any more or less complex than the leading growth cone. However, the leading growth cone is unusual in that it only pioneers the DVDT and then joins other axons within the TPOC. This change from being a pioneer to a 'follower' is accompanied by an increase rather than a decrease in the number of growth cone processes. Therefore, our results do not support the notion that pioneering growth cones are necessarily any more complex than follower growth cones.

The leading epiphysial growth cone must encounter specific guidance cues at at least two pathway intersections along its trajectory. At the DVDT/TPOC intersection, the growth cone is faced with several pathway options; in theory, it could turn rostrally or caudally along the TPOC, or it could continue ventrally. Similarly, upon reaching the SOT, the growth cone faces a pathway option between the TPOC and the SOT. We see no significant changes in growth cone complexity (as measured by the numbers of growth cone processes) at either of these locations. This is in contrast to other systems in which growth cone complexity increases at regions where divergent pathways are encountered (Tosney and Landmesser, 1985*b*; Bovolenta and Mason, 1987; Caudy and Bentley, 1986).

Growth cones in the TPOC between the DVDT/TPOC and TPOC/SOT intersections had variable morphologies and possessed significantly more filopodia than in previous regions. The TPOC is a relatively broad tract and once within it, epiphysial growth cones extend amongst cell bodies and fascicles of axons in the dorsal part of the tract. If the dorsally located TPOC fascicles of axons offer favourable substrata for the growth cone then the increased complexity may reflect increased sampling of these fascicles by growth cone filopodia.

Perhaps the most dramatic change in growth cone morphology occurred beyond the TPOC/SOT intersection. In this region the growth cone had very few filopodia, and in some cases was virtually indistinguishable from the trailing axon. What features might account for this change? One factor that may be important is that the dimensions of the TPOC change significantly from being a multifascicled tract in the mid-diencephalon, to a tighter single fascicle of axons approaching the commissure (Wilson *et al.* 1990; Chitnis and Kuwada, 1990). The physical size of the

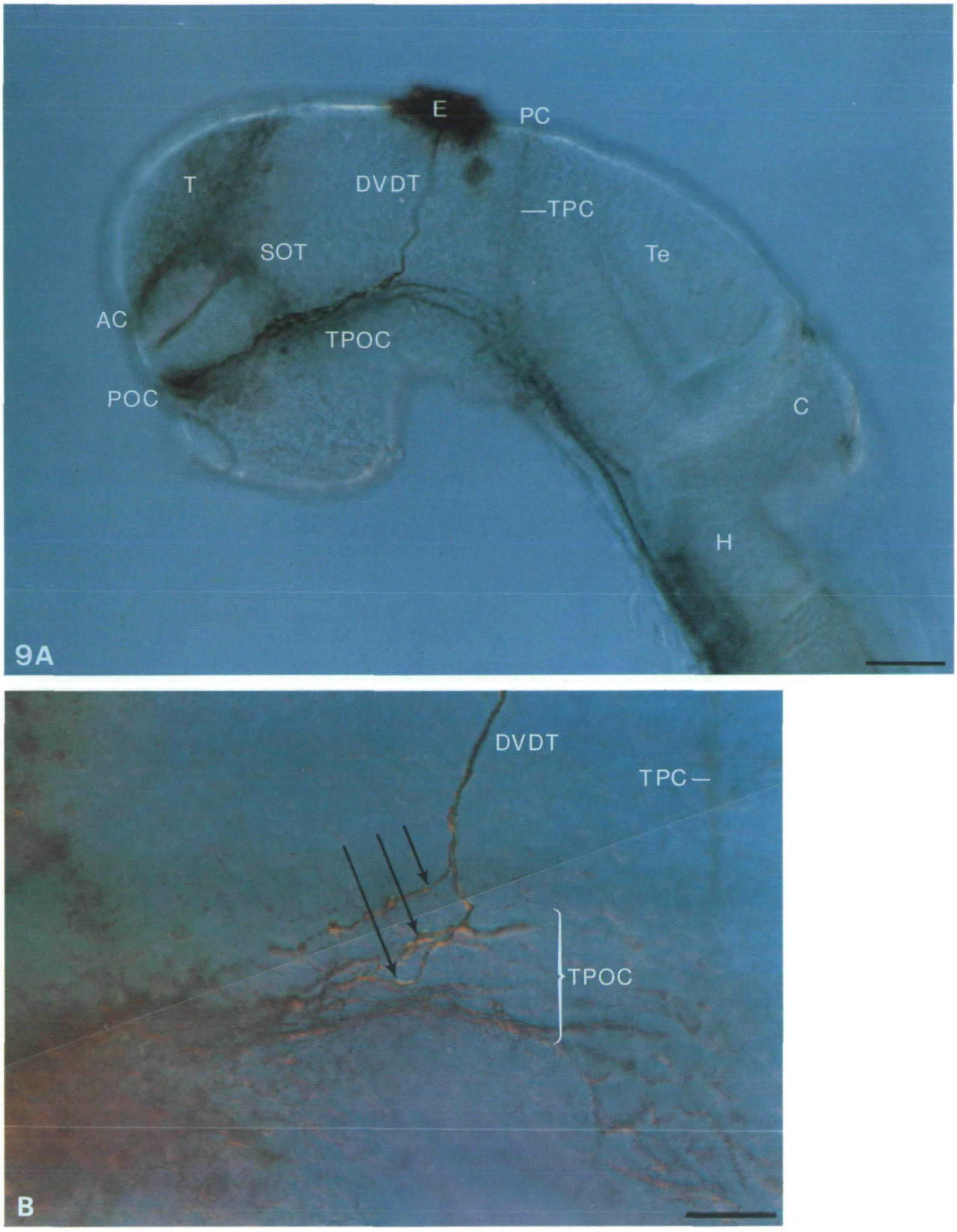


Fig. 9. Epiphysial axons in relation to other axons at 24–25 h PF. Whole-mounted preparations in which dorsal is up and rostral is to the left. Epiphysial axons (brown) were labelled by dil application to their cell bodies in the epiphysis and the other axons in the brain (grey) were labelled with an antibody to acetylated tubulin. In A, all of the tracts present at this stage (see Wilson *et al.* 1990) are labelled, although the TPC is poorly visible at this focal plane. (A) Epiphysial axons course ventrally in the DVDT to the TPOC, where they turn rostrally, towards the POC. (B) Epiphysial axons form a tight bundle in the DVDT, but defasciculate upon entering the TPOC. Epiphysial axons (arrows) are located dorsally within the TPOC, but different epiphysial axons run alongside different bundles of TPOC axons. Scale bar, (A) 50 μm; (B) 20 μm. Abbreviations: AC, anterior commissure; C, cerebellum; DVDT, dorsoventral diencephalic tract; E, epiphysis; H, hindbrain; PC, posterior commissure; POC, postoptic commissure; SOT, supraoptic tract; T, telencephalon; Te, tectum; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure.

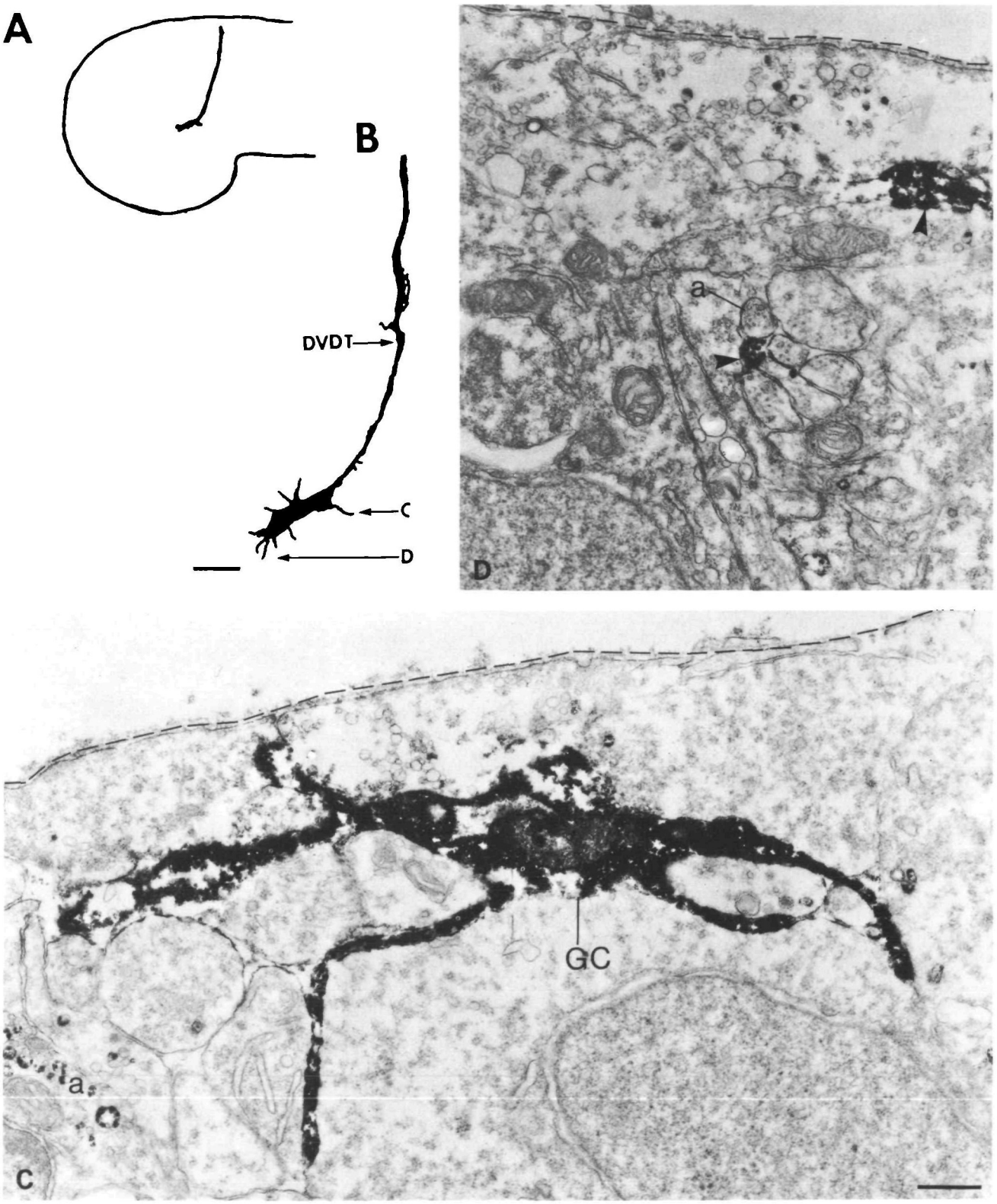


Fig. 10. Epiphysial growth cone at the DVDT/TPOC junction. The growth cone was labelled by HRP application to its parent cell body in the epiphysis. (A) Outline drawing of the brain and labelled axon from a whole-mounted preparation prior to electron microscopic processing. Dorsal is up and rostral is to the left in A and B. (B) Camera-lucida tracing of the labelled axon and growth cone. The arrows indicate the approximate levels of the sections shown in C and D. (C and D) Electron micrographs of transverse sections through the growth cone. The surface of the brain is up and rostral is to the left. The section shown in D is approximately 3.5 μm ventral to that in C. The arrowheads indicate leading filopodia of the growth cone in D. The label (a) refers to profiles that are discussed in the text. Scale bar, (B) 5 μm, (C, D) 0.5 μm. Abbreviations: DVDT, dorsoventral diencephalic tract; GC, growth cone; TPOC, tract of the postoptic commissure.

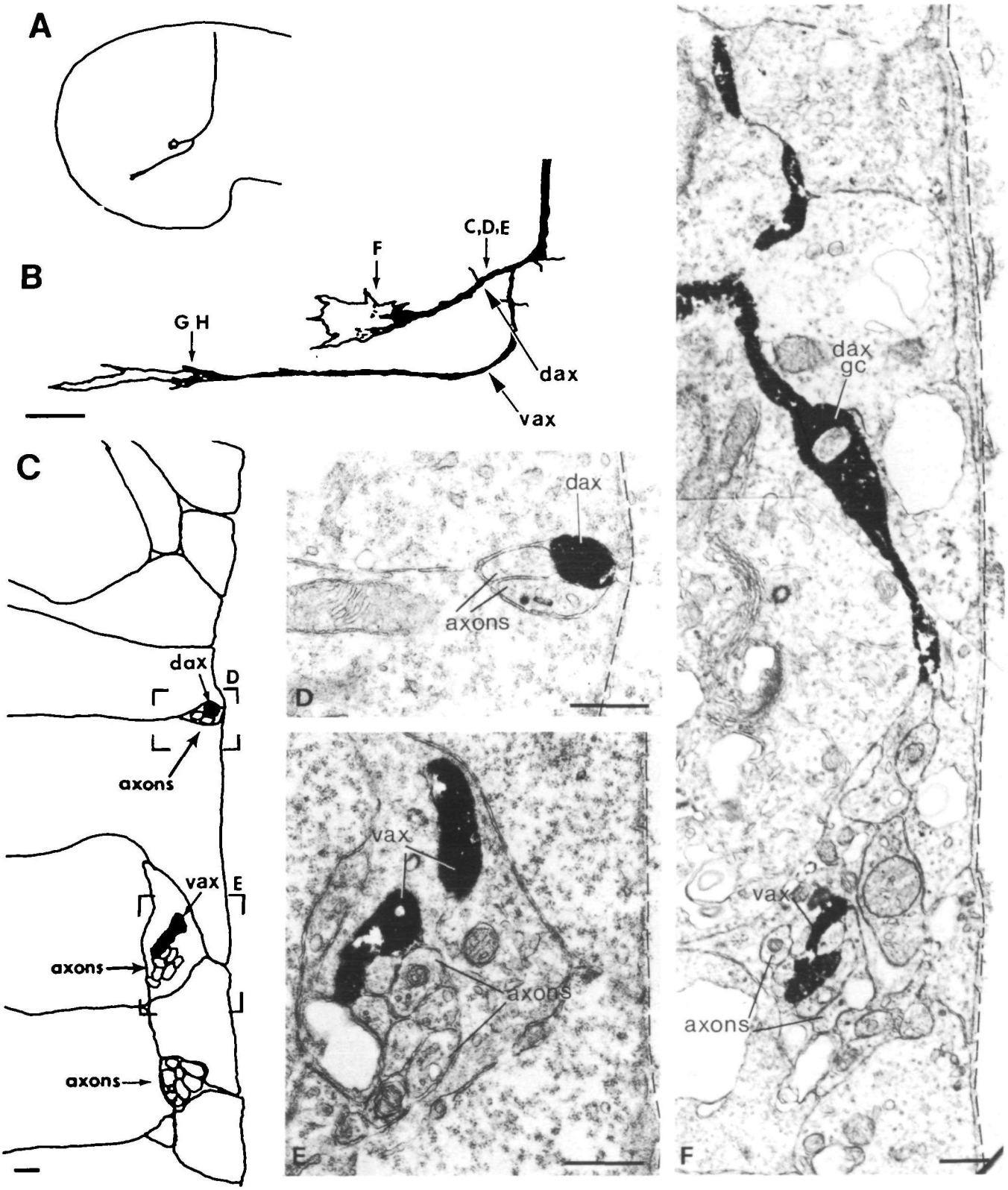
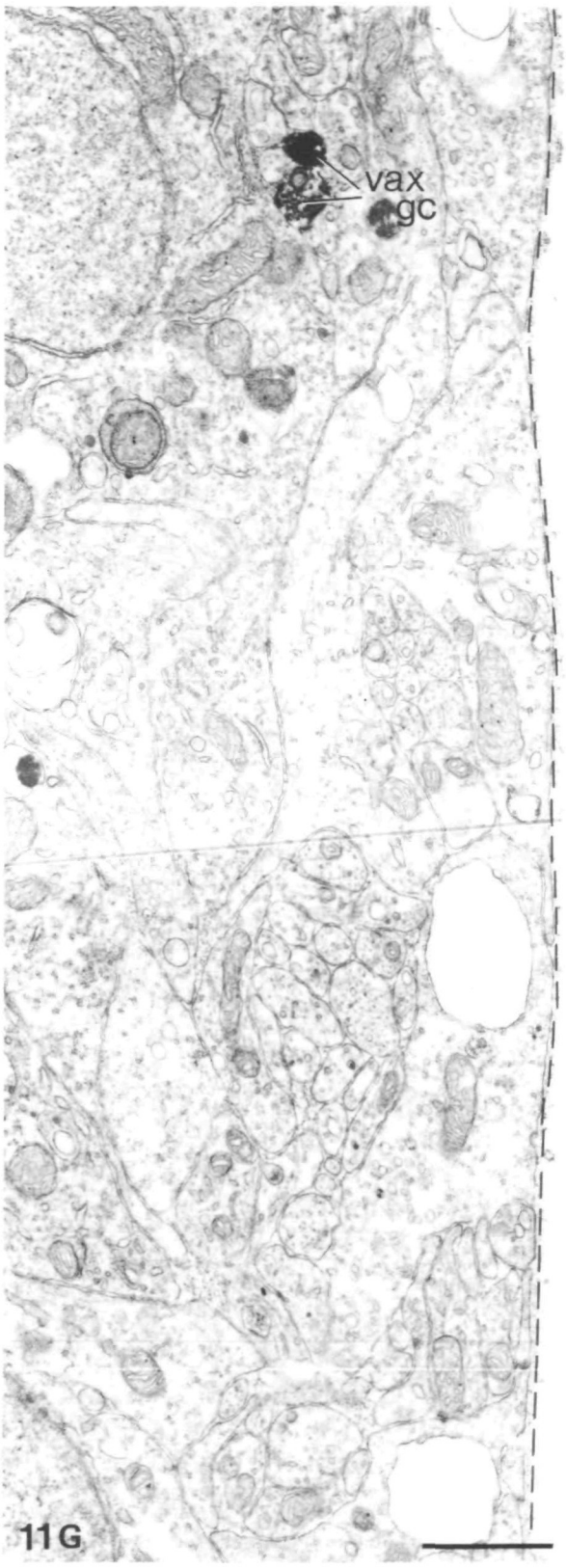
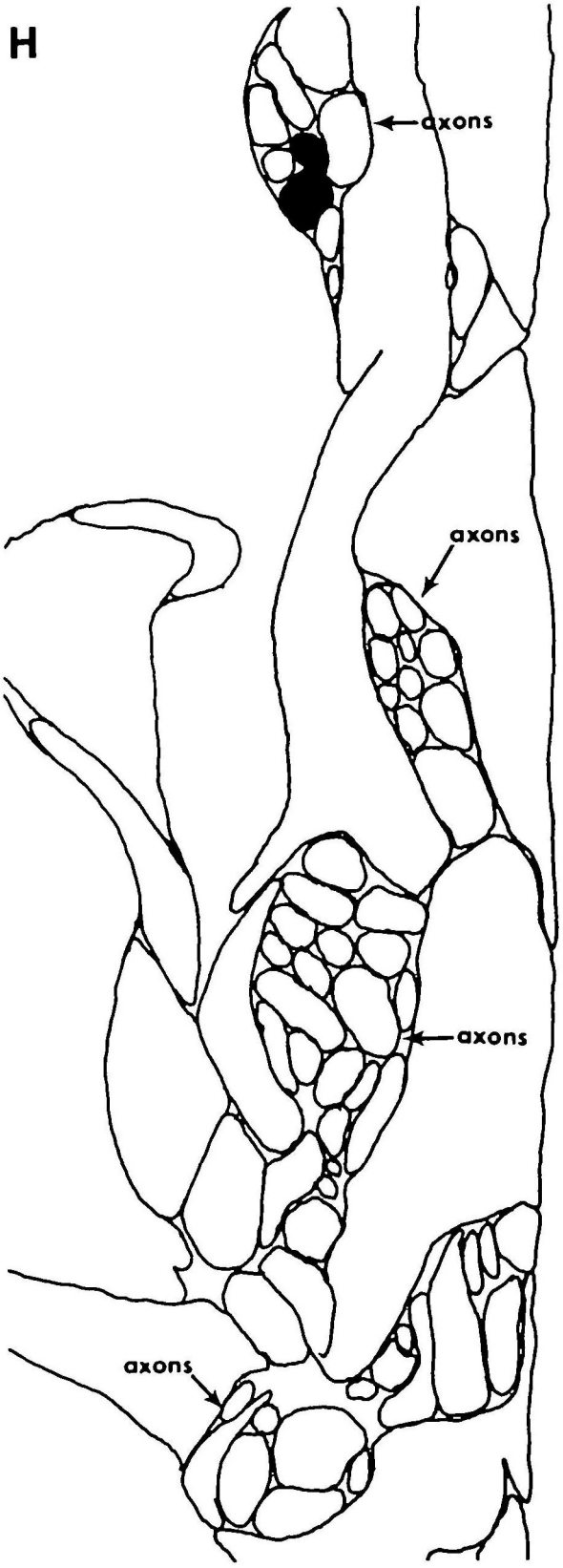
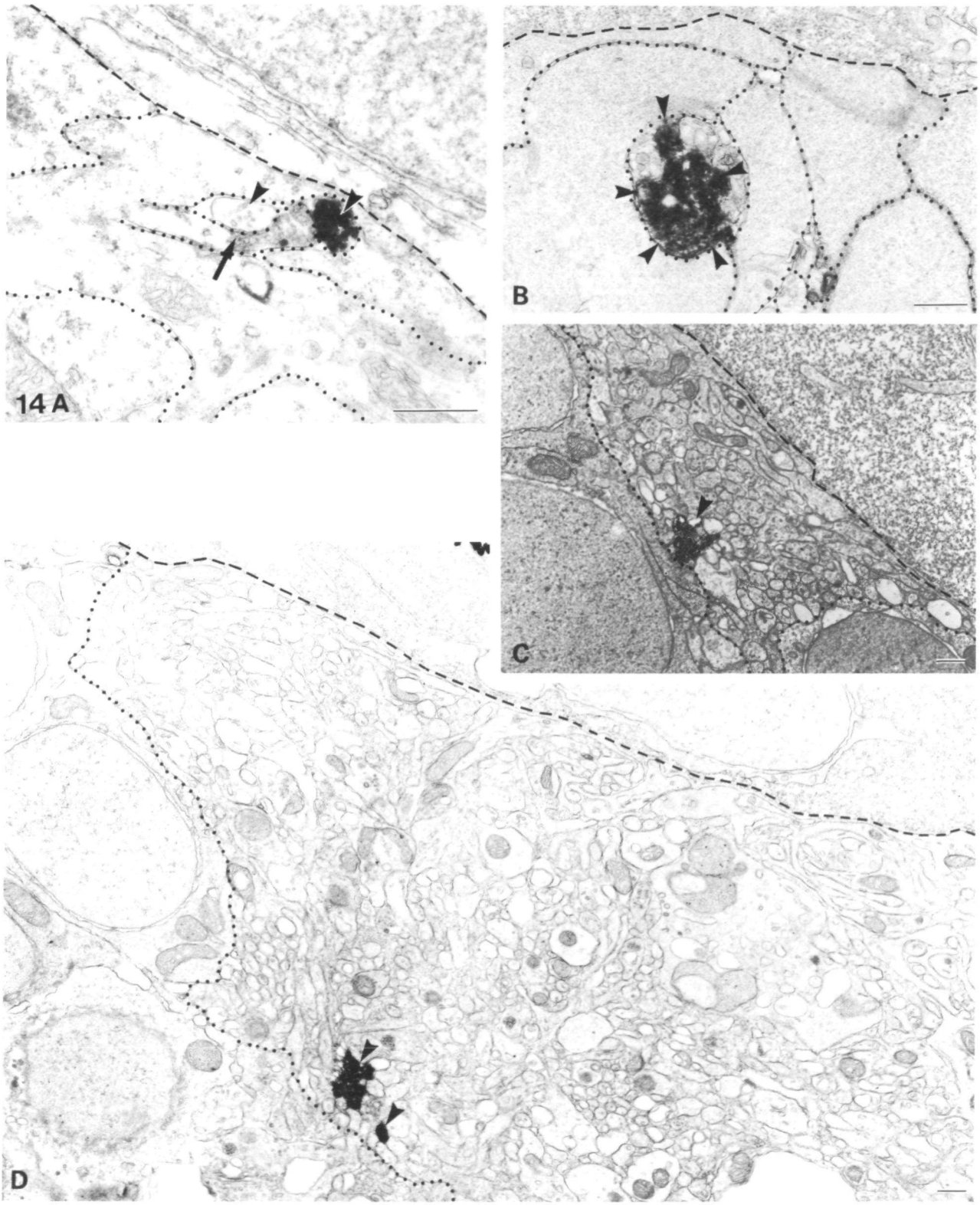


Fig. 11. Epiphysial growth cones within the TPOC. The growth cones were labelled by HRP application to their parent cell bodies in the epiphysis. (A) Outline drawing of the brain and labelled axons from a whole-mounted preparation prior to electron microscopic processing. Dorsal is up and rostral is to the left in A and B. (B) Camera-lucida tracing of the labelled axons and growth cones. The arrows indicate the approximate levels of the sections shown in C–H. (C and G), Camera-lucida tracings and (D, E, F, and H), electron micrographs of transverse sections through the axons and growth cones. The surface of the brain is towards the right of the panels and dorsal is up. (C) Low-magnification camera-lucida tracing of an electron micrograph showing the location of dax and vax within the TPOC close to the DVDT/TPOC junction. The boxes outline the regions shown in D and E. (D and E) High magnification micrographs from the ultrathin





section subsequent to C. In D, dax is in contact with two axons. In E, vax is in contact with a larger fascicle of dorsal TPOC axons. (F) Micrograph showing the dorsal TPOC at the level of the trailing growth cone. (G) Micrograph and (H), camera-lucida tracing of the TPOC at the level of the proximal part of the leading growth cone. The labelled profiles are located among the most dorsal of the fascicles of axons in the TPOC. Scale bars, (B) 10 μm; (C–H) 1 μm. Abbreviations: dax, dorsal epiphysial axon; dax gc, growth cone of the dorsal epiphysial axon; DVDT, dorsoventral diencephalic tract; TPOC, tract of the postoptic commissure; vax, ventral epiphysial axon; vax gc, growth cone of the ventral epiphysial axon.



tract may therefore influence the morphology of the growth cone. It would be interesting to know if there are also changes in the cell surface properties of cells lining the tract near the commissure. Our results suggest that these cells may be less permissive for growth cones near the commissure in that we do not see any evidence of growth cones elaborating processes between or around these cells as was observed in the DVDT and to a lesser extent, the caudal TPOC.

One of the most interesting features of the growth cone morphology was the distribution and selective retention of filopodia. For instance, on growth cones

Fig. 14. Development of the DVDT. Electron micrographs of preparations in which dil was applied to the epiphysis. Sections were cut transverse to the DVDT (horizontally through the brain). The surface of the brain is outlined with dashes. The dots outline cellular profiles in A and B, and they outline the DVDT in C and D. (A) 23–24 h PF. Only two axons (arrowheads) are present in the DVDT. Serial reconstruction of consecutive sections showed that the arrowed process belonged to a neuroepithelial cell. (B) 36–38 h PF, section cut just ventral to the emergence of axons from the epiphysis. The epiphysial axons (arrowheads, approximately 13 are labelled) are a tightly fasciculated bundle surrounded by neuroepithelial cell processes. (C) 36–38 h PF, section cut further ventrally within the DVDT than in B. The labelled epiphysial axons (arrowhead) remain tightly fasciculated, but at this level they are joined by other axons of non-epiphysial origin. (D) 47–48 h PF. By this stage of development the epiphysial axons (arrowheads, approximately 11 were labelled) are surrounded by many more axons than at 36–38 h PF. Scale bars, 0.5 μm.

located at the junction with the SOT, there is no significant difference between the number of filopodia directed towards and away from the SOT. However, after the growth cone has passed, many of the processes that were directed towards the SOT are retained. Also, at the DVDT/TPOC intersection many axons retained small caudally directed collaterals. At both the DVDT/TPOC and TPOC/SOT intersections, the abortive branches are directed along alternative axon pathways. This suggests that there may be a hierarchy of preferred axonal pathways for the growth cones and the eventual axon morphology and trajectory is a result of a stepwise selection of different pathways encountered *en route* to the target.

In summary, our results show that the morphology of the leading epiphysial growth cone changes in a very predictable manner at different regions of the pathway. These changes are correlated with changes in the local environment encountered by the growth cone, but are not consistent with the growth cone being any more complex in regions where it is either pioneering a pathway or facing divergent pathway choices.

Displacement of older axons to deeper locations

A further striking observation was the progressive displacement of the epiphysial axons to deeper regions of the neuropil at all regions of their pathway. Within the DVDT, the epiphysial axons are displaced from being separated from the basal lamina by a single end-foot process at 23–24 h PF to being 5–6 μm deep to the pia by 47–48 h PF. Similarly, within the POC, the epiphysial axons initially occupy a superficial rostral position. However, later axons (predominantly optic) displace the epiphysial axons to the deepest regions of the commissure. These results provide strong support for our belief that many axonal projections that appear deep to the surface of the brain in the adult may initially start off superficial and subsequently be displaced deep by the addition of later axons and perhaps also by cell migrations (Easter *et al.* 1984; Easter and Taylor, 1989; Wilson *et al.* 1990; see also, Kroger and Schwartz, 1990).

Possible functional roles of the epiphysial projection

In *Xenopus* larvae, the pineal organ (epiphysis) mediates the first light-evoked behavioural responses (Roberts, 1978; Foster and Roberts, 1982) but the circuitry behind this response has not been elucidated. There is also early differentiation of photoreceptive capability in the epiphysis of some species of fish (Ekstrom *et al.* 1983; Ostholm *et al.* 1987, 1988). These results have led us to think that the epiphysial projection that we have described may be involved in circuitry that generates early light-evoked behaviour in the zebrafish embryo. We are currently examining the epiphysis at early developmental stages to see if it becomes photoreceptive before the retinas.

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