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The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*

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Summary

We have examined neuronal differentiation and the formation of axon tracts in the embryonic forebrain and midbrain of the zebrafish, between 1 and 2 days post-fertilisation. Axons were visualised with three techniques; immunocytochemistry (using HNK-1 and anti-acetylated tubulin antibodies) and horseradish peroxidase (HRP) labelling in whole-mounted brains, and transmission electron microscopy. Differentiation was monitored by histochemical staining for acetylcholinesterase (AChE).

These independent methods demonstrated that a simple grid of tracts and commissures forms the initial axon scaffold of the brain. At 1 day, the olfactory nerve, four commissures, their associated tracts and three other non-commissural tracts are present. By 2 days, these tracts and commissures have all greatly enlarged and, in addition, the optic nerve and tract, and three new commissures and their associated tracts have been added.

Small applications of HRP at various sites revealed the origins and projections of some of these earliest axons. Retrogradely labelled cell bodies originated from regions that were also positive for AChE activity. At 1 day, HRP-labelled axons were traced: (1) from the olfactory placode through the olfactory nerve to the dorsal telencephalon; (2) from the telencephalon into the tract of the anterior commissure and also to the postoptic region of the diencephalon; (3) from the hindbrain through the ventral midbrain and diencephalon to the postoptic commissure; (4) from the dorsal diencephalon (in or near the epiphysis) to the tract of the postoptic commissure; (5) from ventral and rostral midbrain through the posterior commissure. Three new projections were demonstrated at 2 days: (1) from the retina through the tract of the postoptic commissure to the tectum; (2) from the telencephalon to the contralateral diencephalon; and (3) from the telencephalon to the ventral flexure.

These results show that at 1 day, the zebrafish brain is impressively simple, with a few small, well-separated tracts but by 2 days the brain is already considerably more complex. Most of the additional axons added onto pre-existent tracts rather than pioneered new ones supporting the notion that other axons play a crucial role in the guidance of early central nervous system (CNS) axons.

Key words: axonal guidance, pathfinding, zebrafish, neuronal differentiation.

Introduction

The embryonic formation of axon tracts is the first step in the generation of the highly complex circuitry of the CNS. This process has been investigated in considerable detail in the insect by Goodman and his associates (see Harrelson and Goodman, 1988, for a recent review), but our knowledge of these events in vertebrates is considerably more limited. This paper describes the formation of the first nerves, tracts and commissures in the presumptive forebrain and midbrain of the zebrafish, *Brachydanio rerio*.

Most previous studies of the development of early tracts in vertebrates have been done in amphibians, and the work of Herrick is particularly prominent. He used standard neurohistological methods to stain axons, and concluded that the early brain had relatively few, but well-defined, tracts and commissures (Herrick, 1937; 1938*a*,*b*,*c*). (Note that we use 'tract' to mean a bundle of axons coursing together in the CNS; we do not restrict the term to axons of common origins, destinations and functions – the definition taught in introductory neuroanatomy.) Although Herrick's techniques did not label axons at the earliest stages of axogenesis, recent studies that employed more suitable methods (Roberts *et al.* 1987; Easter and Taylor, 1989) have supported the notion that axon tracts conform to a relatively simple pattern at early embryonic stages.

In the vertebrate CNS, the retinotectal system has been a major focus for the study of early axonal outgrowth (e.g. Harris, 1986; Godement *et al.* 1987; Thanos and Bonhoeffer, 1987; Stuermer, 1988). Although the retinal axons have usually been assumed to grow across previously uncharted territory, recent

work has made this assumption questionable. Easter and Taylor (1989) have shown that in *Xenopus laevis*, the retinal axons grow alongside a pre-existing tract, and are therefore relative latecomers. This information prompted us to shift our attention to earlier stages of development, when the earliest tracts, the initial axonal scaffold, are formed. Our attention is restricted to the forebrain and midbrain; others have described the hindbrain and spinal cord (e.g., Kimmel *et al.* 1982; Nordlander and Singer, 1982; Eisen *et al.* 1986; Kuwada, 1986). We have used the zebrafish because the small size, rapid development (hatching occurs on the third day after fertilisation), and easy accessibility of its brain are convenient attributes for these studies. Axons were visualised immunocytochemically, with antibodies to a cell surface marker (HNK-1) (Kruse *et al.* 1984) and a cytoskeletal protein (acetylated tubulin) (Piperno and Fuller, 1985), and by conventional retrograde and anterograde labelling with HRP. AChE histochemistry provided an index of neuronal differentiation (Layer, 1983). Most preparations were viewed as whole-mounted brains. In addition, unlabelled brains were sectioned and viewed with both light and electron microscope.

We find that, at 1 day post-fertilisation, tracts and commissures are few in number and consistent in position, and they arise from isolated regions that are AChE positive. By 2 days, many additional axons are present, but the majority of these had added to pre-existing tracts rather than formed new ones. Our results are discussed with reference to other studies of brain development, and in terms of the mechanisms that may be responsible for guiding developing axons.

Materials and methods

General

Zebrafish embryos were obtained from our own breeding colony. Adult male and female fish were kept in the same tank, and maintained at 28.5°C on a 14 hour light/10 hour dark cycle. Fertilised eggs were collected each morning and raised in an embryo-rearing solution (ERS: 0.1% NaCl, 0.003% KCl, 0.0163% $MgSO_4$, 0.004% $CaCl_2.2H_2O$), at 28.5°C. Embryonic age was estimated by assuming that fertilisation occurred at the time of light onset, an assumption that is usually accurate to within 30 min. By convention, we considered the age of any embryo to be the age at the time the embryo left the incubator. (This introduced some uncertainty about the age of embryos that were labelled with HRP. They were generally removed from the incubator in batches of six and operated upon serially at intervals of about 10 min at room temperature, and fixed after a survival of no more than 10 additional minutes.) Embryos aged 24–27 and 48–50 h post-fertilisation are called '1 day' and '2 day' embryos, respectively.

Prior to all experimental procedures embryos were dechorionated and anaesthetised in approximately 0.03% 3-aminobenzoic acid ethyl ester (Sigma). For all procedures that used whole-mounted brains, fixed embryos were pinned down and the yolk sac, the eyes and the skin overlying the brain were removed with sharpened tungsten needles. (This procedure removed cranial nerves III–VI and the mesencephalic ganglion (if they were present) and we will not discuss these structures.) The olfactory placode was either removed with the skin at this stage, or left *in situ* apposed to the telencephalon. After the whole mounts were processed, they were dehydrated through alcohols, cleared in xylene and mounted in DPX between two coverslips, to permit them to be viewed from both sides. They were examined using a Leitz Orthoplan 2 microscope fitted with differential interference contrast optics. All preparations were sketched, and many were drawn more carefully with the aid of a *camera lucida*, and photographed.

AChE histochemistry

We used the method of Karnovsky and Roots (1964). 1-day embryos were fixed in 4% formalin buffered with either maleate (pH 6.0, 0.1 M) or Pipes (disodium salt – Sigma, pH 6.95, 0.1 M-plus 2 mM-EGTA [*N,N,N',N'*-tetraacetic acid, Sigma], and 1 mM-$MgSO_4$). Embryos prepared as whole mounts (n=18) were incubated for 2.5 h in maleate buffer (pH 6.0) with 5 mM-sodium citrate, 3 mM-copper sulphate, 0.5 mM-potassium ferricyanide and 0.5 mg ml^{-1} substrate (acetylthiocholine iodide). Reacted embryos were prepared for viewing as above. Other embryos (n=6) were prepared for sectioning as follows. After fixation they were cryoprotected in 5% then 15% maleate-buffered sucrose (pH 6.0) for 12 h and 1 h respectively, and sectioned on a cryostat at 10–20 μm. Sections were air-dried, rehydrated in maleate buffer and incubated in substrate as above for 45 min. Reacted sections were dehydrated, cleared in xylene and mounted in DPX.

HNK-1 labelling

Embryos of three ages (1 day, n=20; 34–36 h, n=10; and 2 days, n=31) were fixed in Pipes-buffered formalin for 4 h, dissected as above, washed six times in phosphate buffer (0.1 M, pH 7.2) and stored in the same buffer overnight. They were pre-incubated for 3 hours in phosphate-buffered 3% goat serum containing 0.1% Triton-X (octyl-phenoxy-polyethoxyethanol, Sigma) followed by 18 h (at room temperature, with agitation) in the monoclonal HNK-1 antibody (kindly provided by Dr Claudio Stern). This was followed by six washes in buffer and incubation overnight in goat anti-mouse IgM conjugated to HRP (1:1000 dilution in buffer with 0.1% Triton-X, Sigma). Embryos were washed six times and HRP was demonstrated using diaminobenzidine (DAB) as the chromogen. Stained embryos were prepared for viewing as above.

Control embryos (in which the primary antibody was omitted) showed no specific labelling (n=20).

Anti-acetylated tubulin labelling

2-day embryos (n=33) were stained with anti-acetylated tubulin antibody (kindly provided by Dr Gianni Piperno), using the method of Patel *et al.* (1989). Briefly, embryos were fixed in Pipes-buffered formalin, dissected, washed in phosphate-buffered bovine serum albumin (BSA, Sigma) plus 0.1% Triton-X, pre-incubated in horse or goat serum, incubated overnight with the antibody (in BSA buffer plus serum plus Triton-X) and then washed. They were then either treated as above (see HNK-1 methods) or were reacted with a Vectastain ABC kit (Vector Laboratories), according to manufacturer's instructions.

Control embryos (in which the primary antibody was omitted) showed no specific labelling (n=14).

Electron microscopy

Embryos were fixed by immersion in phosphate-buffered 3% glutaraldehyde/2% paraformaldehyde (0.075 M, pH 7.4) for 2 hours, and washed in phosphate buffer. They were post-fixed

in phosphate-buffered 1% osmium tetroxide (with 0.05 M-sucrose) for 1.5 h, washed in distilled water followed by 0.05 M-maleate buffer, and stained *en bloc* in 2% maleate-buffered uranyl acetate for 1 h, after which they were washed in maleate buffer, dehydrated through alcohols, cleared in propylene oxide and embedded in epon/Araldite.

Semithin sections were stained with toluidine blue. Ultrathin sections were mounted on formvar-supported one-hole grids, stained with Reynolds lead citrate and viewed on a Philips 300 electron microscope.

Axons were recognized by their round or oval shape, their small diameter (less than 1 μm) and the absence of ribosomes. Many profiles that satisfied these criteria also contained microtubules, smooth endoplasmic reticulum and mitochondria. Electron microscopic photomosaics of commissures in sagittal sections were made and the axons were counted. The 1-day commissures were viewed at a magnification of ×24 750, and the 2-day commissures, at ×8700. As a check on the validity of the counts made at the lower magnification, the axons in one commissure were counted independently at both magnifications. The former gave a value about 9% higher than the latter (1767 *versus* 1610). We conclude that the numbers given in the Results are probably a slight, but negligible, underestimate of the true values.

HRP labelling

Anaesthetised embryos were immobilised in 3% agar in ERS. A small hole was made with sharpenened tungsten needles in the tissues covering the brain to expose the area to which recrystallised HRP (Sigma, type VI) was to be applied. The bathing solution was drawn away, and the HRP was applied to the hole. The embryos were then removed from the agar and placed in phosphate buffer for 5–10 min, after which they were fixed in phosphate-buffered 3% glutaraldehyde for 4 h. They were dissected, the HRP reaction was carried out with DAB as the chromogen, and the embryos were mounted. In total, 210 1-day and 217 2-day embryos were labelled. Six different application sites were used, and individual *n*'s for each are given in the Results.

Results

General features

The brain is classically divided into five subdivisions (see, for example, von Kupffer, 1906). The forebrain includes two of these, the telencephalon and the diencephalon, the midbrain (mesencephalon) is the third and the hindbrain includes the metencephalon and myelencephalon. The boundaries between the subdivisions are somewhat arbitrary and alternative divisions have been suggested (Jarvik, 1980).

The embryonic zebrafish brain has several landmarks, visible at low magnification, that help to identify the subdivisions given above. Fig. 1 illustrates these landmarks and allows a comparison of the development between 1 and 2 days.

A live 1-day embryo is shown in Fig. 1A. Superficial structures such as the eye, the otic capsule and the yolk sac are quite prominent, and because the animal is so transparent, several brain structures can also be discerned, such as the cerebellum and the tectal ventricle. Fig. 1B shows a whole-mounted, fixed 1-day embryo, from which the yolk sac, eyes and skin have been removed. Several structures are seen more clearly than in the live embryo. The optic recess and the ventricle leading into it separate the forebrain into telencephalon and diencephalon. The ventral flexure, bounded rostrally by the presumptive hypothalamus, and caudally by the midbrain, is prominent. The *anlage* of the pituitary is ventral to the diencephalon. Fig. 1C, a 1 μm parasagittal section, illustrates some of the same features, and gives a better view of the different subdivisions.

Fig. 1D shows a live 2-day embryo. The eye and skin have become heavily pigmented. The relative positions of the otic capsule and the eye (compare to Fig. 1A) show that the head has contracted longitudinally between 1 and 2 days. The whole-mounted, fixed brain in Fig. 1E shows more complexity than at 1 day (compare to Fig. 1B). The olfactory placode is much more prominent. The roof of the diencephalon is a thin sheet (see also Fig. 1F) with the *anlage* of the epiphysis towards its caudal end. The hypothalamus has enlarged and folded beneath the midbrain, masking the ventral flexure. Fig. 1F, a parasagittal 1 μm section of a 2-day embryo shows the ventral flexure as well as the much more elaborate ventricles.

AChE histochemistry

AChE is believed to be present transiently in neurons, including non-cholinergic ones, soon after they complete their final mitosis (Layer, 1983), and therefore has been used as a marker of differentiation.

At 1 day, cells staining for AChE were restricted to several well-defined regions as shown in the micrographs of Fig. 2 and the interpretive drawing of Fig. 10A. The reaction product appeared intracellularly as punctate deposits (Fig. 2D). In the forebrain, the label appeared in an H-shaped pattern, with one broad telencephalic band, an equally broad diencephalic band in the postoptic region, ventrocaudal to the optic stalk, and a thin band linking the two, above the optic stalk (Fig. 2A,B). The *anlage* of the pituitary was also labelled (Fig. 2C), as were several cells directly lateral to, or perhaps within, the *anlage* of the epiphysis (Fig. 2D). In the midbrain, cells along the rostral margin of the presumptive tectum (Fig. 2F) and within the ventral midbrain (Fig. 2G) were labelled. The nucleus of the medial longitudinal fasciculus (see Kimmel *et al.* 1982) is almost certainly among the stained ventral midbrain cells. In the hindbrain, clusters of labelled cells extended from the level of the cerebellum to the spinal cord (Fig. 2A,E). This pattern of staining has already been described (Hanneman *et al.* 1988; Hanneman and Westerfield, 1989); the large clusters of cells are at the centers of neuromeres, and the smaller clusters, at boundaries between adjacent neuromeres.

2-day embryos were also reacted for AChE but the reaction product was distributed so extensively that we could discern no pattern relevant to this study.

Immunolabelling

HNK-1

This antibody recognises an epitope common to a number of glycoconjugates (Tucker *et al.* 1988), includ-

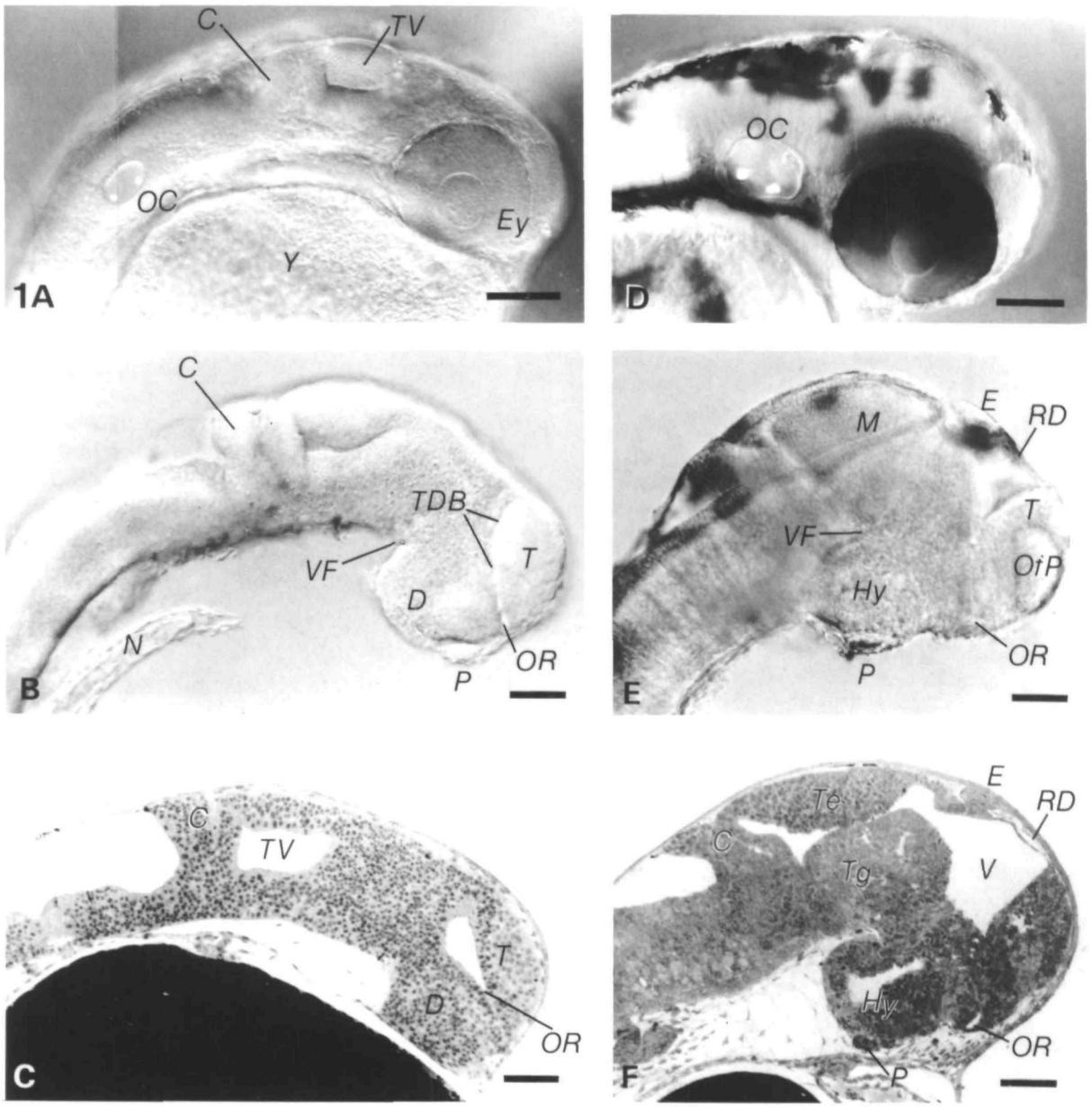

Fig. 1. General features of the zebrafish brain at 1 and 2 days. Lateral views; anterior is to the right, dorsal up. (A–C) 1 day; (D–F) 2 days. (A, D) Live embryos. (B, E) Whole-mounted fixed brains from which the eyes and skin overlying the brain have been removed. (C, F) Parasagittal 1 μm sections. Scale bars: A, D, 100 μm; B, C, E, F, 50 μm. Abbreviations: C, cerebellum; D, diencephalon; E, epiphysis; Ey, eye; Hy, hypothalamus; M, midbrain; N, notochord; OC, otic capsule; OfP, olfactory placode; OR, optic recess; P, pituitary; RD, roof of the diencephalon; T, telencephalon; TDB, telencephalic/diencephalic boundary; Te, tectum; Tg, tegmentum; TV, tectal ventricle; V, ventricle; VF, ventral flexure; Y, yolk.

ing a set of molecules involved in cell adhesion (Kruse *et al.* 1984; 1985). Drs R. Nordlander (see Nordlander, 1989) and W. Metcalfe suggested to us that this antibody labelled early axons.

The pattern of HNK-1 labelling at 1 day is shown in both the photomicrographs of Fig. 3 and the interpretive drawing of Fig. 10B. The labelled axons and cell bodies were restricted to a few discrete regions, with the rest of the brain unlabelled. Most of the tracts and commissures can be recognised from the earlier studies of Bergquist (1932) and Herrick (1938*a*,*b*,*c*). We follow their nomenclature with respect to the commissures, but we differ slightly in one convention. When we use the term, 'commissure,' we mean only the bundle of axons crossing at the midline. Once away from the midline, the bundle is referred to as the 'tract of the commissure'.

In the forebrain and midbrain, we identified four commissures and their associated tracts, and three tracts not associated with commissures. Axons originating in the dorsal and ventral telencephalon (see HRP results below), coalesced rostrodorsal to the optic stalk and formed two tracts. One of these, the tract of the anterior commissure, coursed rostroventrally and

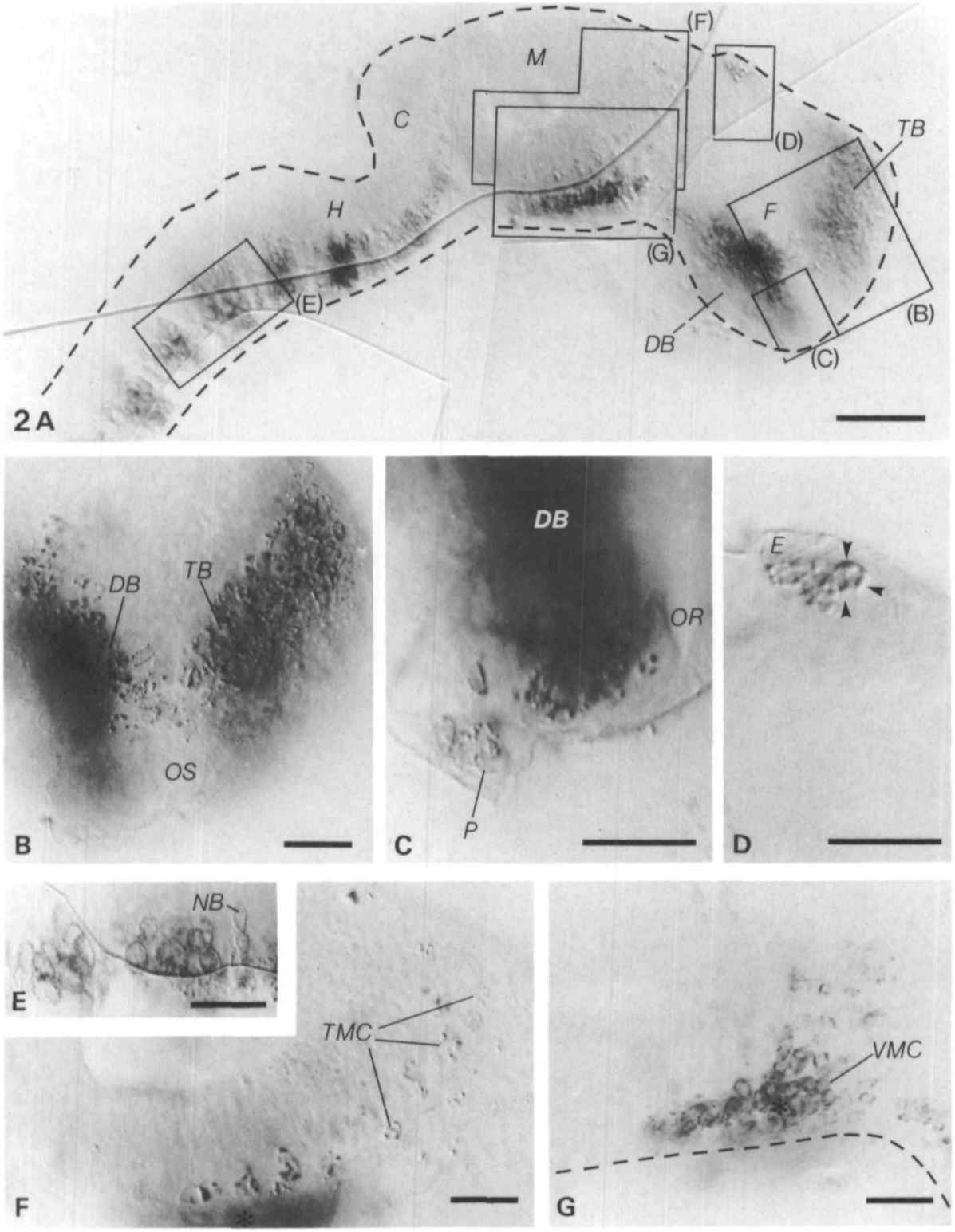

Fig. 2. AChE reaction product in a whole-mounted brain at 1 day. (A) Whole brain. Boxes indicate the fields of view in other panels, which are more highly magnified views of the same embryo at various planes of focus. The dashes outline the brain. (B) Forebrain. (C) *Anlage* of the pituitary. (D) *Anlage* of the epiphysis. The arrowheads indicate three 'packets' of AChE reaction product in a single cell. (E) The hindbrain. (F) Midbrain, superficial plane of focus. (G) Similar field of view to F but focused near the midline. Asterisks indicate equivalent positions in F and G, and the dashed line indicates the ventral surface of the midbrain. Scale bars, (A), 50 μm; (B–G), 20 μm. Abbreviations: C, cerebellum; DB, diencephalic band of reaction product; E, epiphysis; F, forebrain; H, hindbrain; M, midbrain; OR, optic recess; OS, optic stalk; NB, neuromere boundary; P, pituitary; TB, telencephalic band of reaction product; TMC, tectal marginal cells; VMC, ventral midbrain cells.

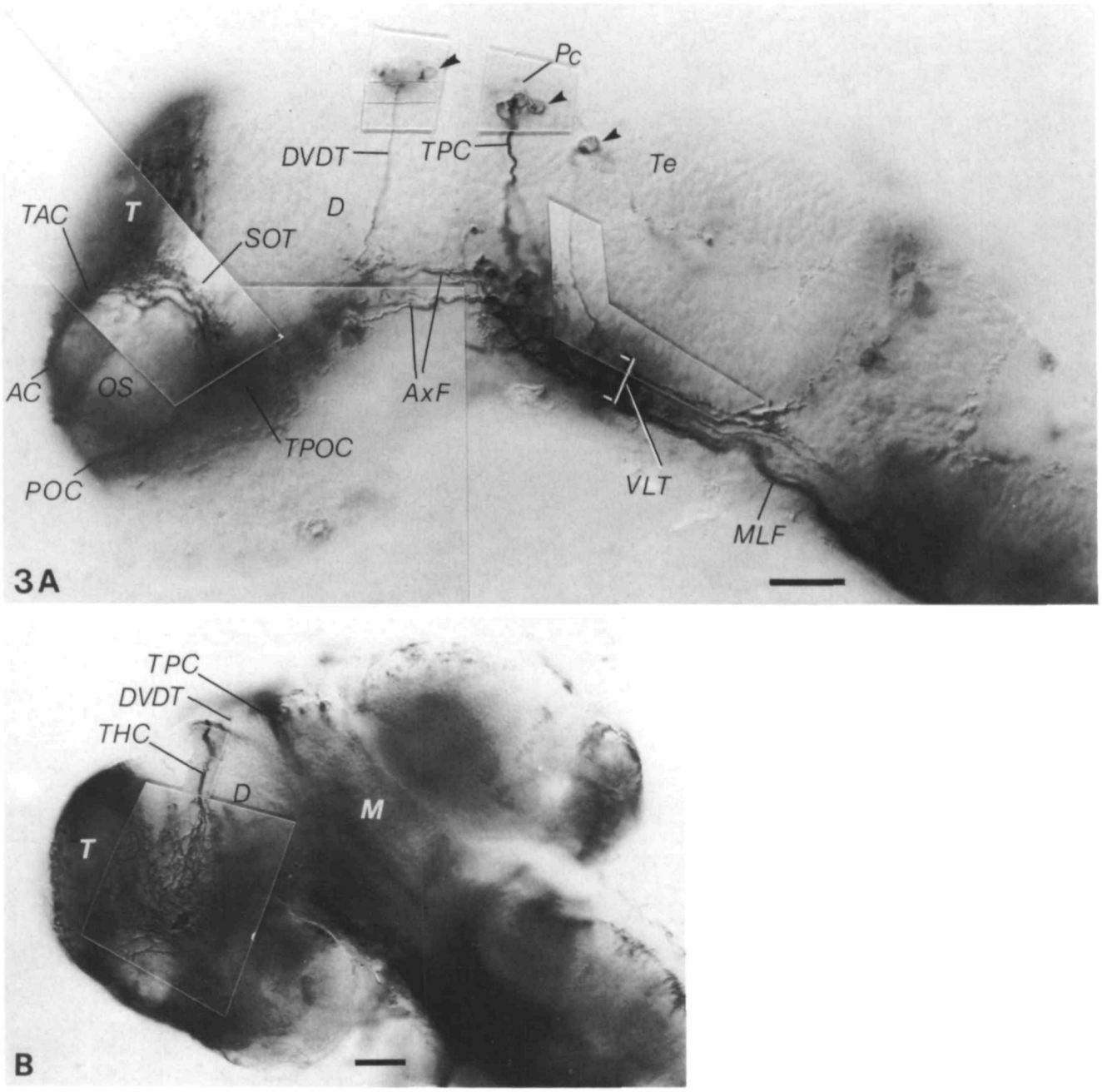

Fig. 3. HNK-1 labelling of the brain. Rostral is to the left and dorsal is up. (A) 1 day. Arrowheads indicate labelled cell bodies that are mentioned in the text. (B) Thirty-six hours. The tract of the habenular commissure has formed rostral to the dorsoventral diencephalic tract. Scale bars, 25 μm. Abbreviations: AC, anterior commissure; AxF, axon fascicles; D, diencephalon; DVDT, dorsoventral diencephalic tract; M, midbrain; MLF, medial longitudinal fasciculus; OS, optic stalk; PC, posterior commissure; POC, postoptic commissure; SOT, supraoptic tract; T, telencephalon; TAC, tract of the anterior commissure; Te, tectum; THC, tract of the habenular commissure; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure; VLT, ventral longitudinal tract.

crossed the midline as the anterior commissure. The other, the supraoptic tract, passed dorsal to the optic stalk (hence its name) and entered the diencephalon, where it met another tract, that of the postoptic commissure. This tract extended from the postoptic commissure, through a curved trajectory in the diencephalon, into the ventral midbrain. Along the way it met two tracts coursing from the dorsal side. The first (more rostral) of these was the dorsoventral diencephalic tract, whose axons were continuous with a small number of ipsilateral cell bodies either within or directly lateral to the *anlage* of the epiphysis. The second was the tract of the posterior commissure, which originates from several clusters of cell bodies in the rostral midbrain (Chitnis and Kuwada, 1990). Beyond the intersection of the tracts of the postoptic and

posterior commissures, there were several tracts, that we refer to collectively as the ventral longitudinal tract, that coursed caudally to the hindbrain. This was composed of the medial longitudinal fasciculus and the caudal extensions (see below) of the tract of the postoptic commissure (see also: Kimmel *et al.* 1982; Kuwada, 1986; Chitnis and Kuwada, 1990). Additionally, in over half the cases, one or two labelled cell bodies in the region of the presumptive tectum sent out labelled axons caudal to the tract of the posterior commissure that coursed ventrally to the ventral longitudinal tract. The fourth commissure, the ventral tegmental commissure (not illustrated in Fig. 3, but see Fig. 6D and 10B), was a small, distributed set of axons in the midbrain, with an indistinct diffuse tract directed dorsally toward the ventral longitudinal tract (see Chitnis and Kuwada, 1990).

Four related comments are important to note. First, the olfactory placode had been removed from all of the preparations labelled with HNK-1, so the olfactory nerve was not observed, although we know it to be present at this stage (see HRP results below). Second, we never saw a labelled cell body without an attached labelled axon, but we saw many more labelled axons than labelled cell bodies. Third, a labelled cell body was always close to the tract that its axon entered (Fig. 3A); that is, none of the tracts was made up of axons from dispersed, isolated cell bodies. Fourth, most of the tracts were quite compact and unitary, but the tract of the postoptic commissure, particularly in its caudal segment, was clearly divided into several distinct fascicles that merged with the ventral longitudinal tract (see Fig. 10B).

At a day and a half (34–36 h), the structures that had labelled at 1 day labelled less intensely, but a new group of axons, just rostral to the dorsoventral diencephalic tract was labelled: the habenular commissure and its associated tract (Fig. 3B).

In the 2-day embryo (not shown), the habenular commissure and tract labelled more lightly, and the other tracts and commissures were not visible at all.

In summary, the following structures were labelled by the HNK-1 antibody: the anterior, postoptic, posterior and ventral tegmental commissures and their associated tracts, and three tracts not associated with commissures, the supraoptic, the dorsoventral diencephalic and the ventral longitudinal tract (all at 1 day); and the habenular commissure and its associated tract (between 1 and 2 days).

Anti-acetylated tubulin

Acetylated tubulin is a component of microtubules, which are the first cytoskeletal elements to appear in axons (Peters and Vaughn, 1967). Piperno and Fuller (1985) raised an antibody to it, and Dr J. Kuwada discovered that it labelled early axons in zebrafish.

Chitnis and Kuwada (1990) have characterised the labelling of the zebrafish brain by this antibody at times up to 28 h post-fertilisation. The pattern that they observed is similar to that seen with HNK-1.

We have used the antibody at 2 days. The same tracts and commissures that were present at 1 day were labelled, but they were much broader, as Fig. 4A shows. In addition, several new ones were evident. Fig. 4A illustrates one of these, a caudal extension from the tract of the postoptic commissure into the hypothalamus. This is the tract of the commissure of the posterior tuberculum; it will be described further in the section on HRP labelling. Fig. 4B illustrates the others. The tract of the habenular commissure is divided into several fascicles, one directed toward the dorsal telencephalon, another into the diencephalon and several smaller ones in between. The posterior commissure was considerably enlarged, and accompanied by many new commissures more caudally in the dorsal midbrain. We assume that they are the forerunners of the distributed intertectal commissure, and we refer to them by that name. They were interconnected (Fig. 4C) and quite variable from embryo to embryo. They extended across the midline from one tectal neuropile to the other, but their cellular origins and terminations were unclear. The central tectal neuropile was heavily labelled, and individual axons and cell bodies were difficult to distinguish. From its ventral side, small fascicles (Fig. 4B) extended to enter the longitudinal tracts in the ventral midbrain. The *anlage* of the epiphysis was directly rostral to the posterior commissure and dorsal to the habenular commissure (Fig. 4D). The dorsoventral diencephalic tract originated in or near it. This tract was not associated with any commissure. Its course and relations varied between sides of the same embryo (as seen in Fig. 4D) and between embryos. In some cases, the tract angled caudally to run in association with the tract of the posterior commissure, while, in others, it associated with the tract of the habenular commissure.

In summary, the antibody to acetylated tubulin revealed one new commissure: the intertectal and several new tracts: those associated with the intertectal commissure, and the tract of the commissure of the posterior tuberculum.

Electron microscopy

Midline sagittal sections were examined with special attention to the commissures.

At 1 day (n=8), axons were restricted to four commissures, the anterior, postoptic, posterior (Fig. 5A–C) and ventral tegmental (not shown); all other regions were free of axons (Fig. 5D, and see Fig. 10D). The ventral tegmental commissure was difficult to resolve, owing to its distributed nature, and we will not consider it further. The other commissures were more discrete, typically 2–5 μm wide, with rather more extracellular space than in non-commissural regions. They were occasionally divided into fascicles, and usually separated from the basal lamina by the end feet of the same processes that lined the commissure (Fig. 5A). These lining processes usually contained more vesicular profiles (Fig. 5B) than similar processes at the same depth but away from the commissure (Fig. 5D). Axons were counted in those preparations with the best preservation and orientation; the anterior commissure had 27±8 (mean±S.E.M.) (n=6); the post-

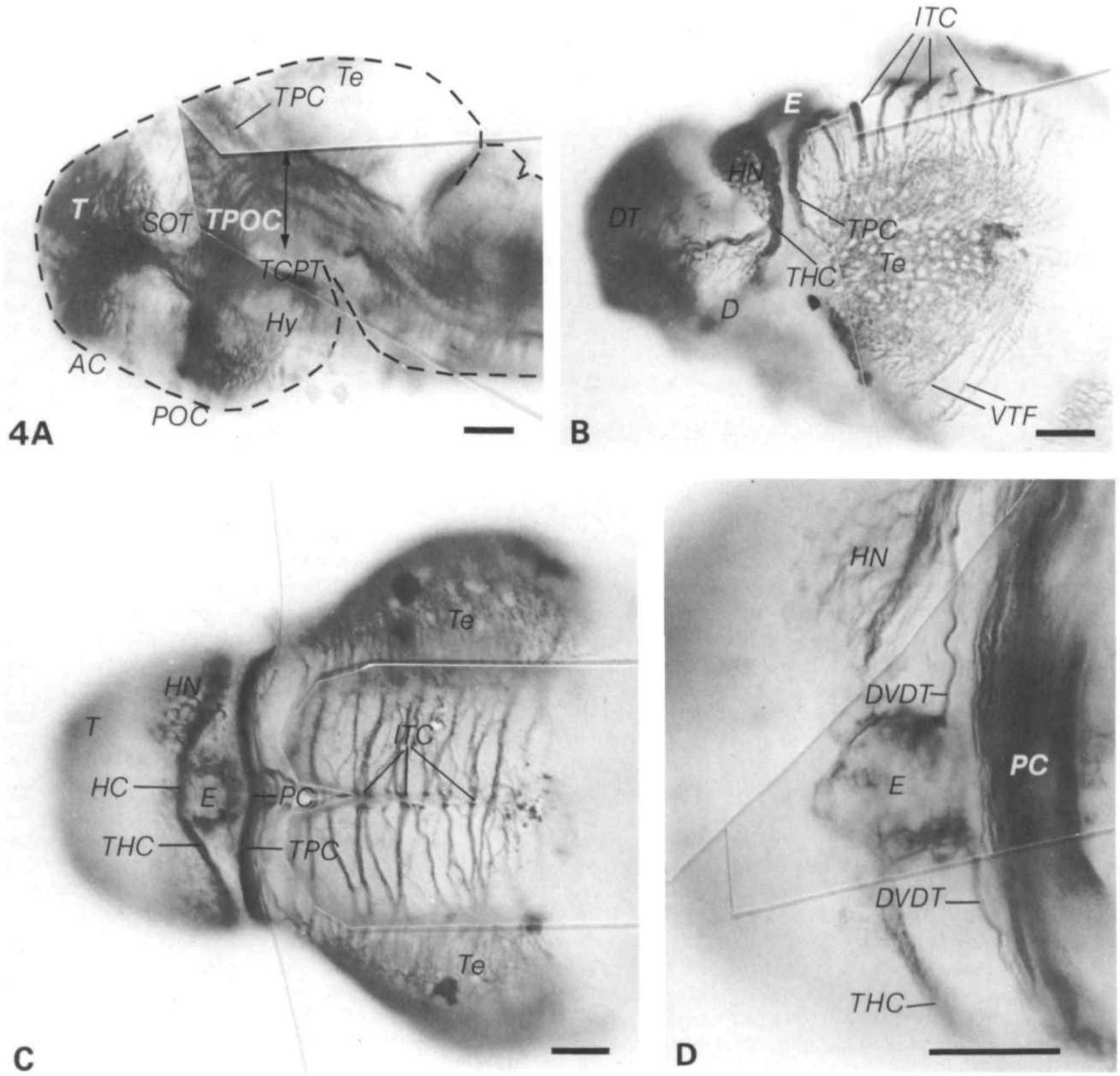

Fig. 4. Anti-acetylated tubulin labelling of the brain at 2 days. Anterior is to the left in all panels. (A) Lateral view (dorsal up) of the brain showing the major tracts described at 1 day with HNK-1 (Fig. 3). The doubleheaded arrow indicates the width of the ventral longitudinal tract. (B) Dorsolateral view (dorsal up) of the diencephalon and midbrain. (C) Dorsal view of the diencephalon and midbrain. (D) High-magnification view of the epiphysis and nearby tracts. This preparation is printed in reversed orientation to match panel C. Scale bars, 25 μm. Abbreviations: AC, anterior commissure; D, diencephalon; DT, dorsal telencephalon; DVDT, dorsoventral diencephalic tract; E, epiphysis; HC, habenular commissure; HN, habenular nucleus; Hy, hypothalamus; ITC, intertectal commissure; PC, posterior commissure; POC, postoptic commissure; SOT, supraoptic tract; T, telencephalon; TCPT, tract of the commissure of the posterior tuberculum; Te, tectum; THC, tract of the habenular commissure; TPC; tract of the posterior commissure; TPOC, tract of the postoptic commissure; VTF, ventral tectal fascicles.

optic commissure, 41±7 (n=6); and the posterior commissure, 14±3 (n=5). Fig. 8 gives individual values.

At 2 days (n=12), those commissures present at 1 day had changed appearance and grown. The cross section of the anterior commissure had elongated (Fig. 6E), and the number of axons had increased to 1454±135 (n=5). The posterior commissure was folded beneath the epiphysis, and reached from the pia to the ventricle (Fig. 6A). It contained 1703±157 axons (n=3). The postoptic commissure was divided into two portions, the caudal having much larger axons than the rostral

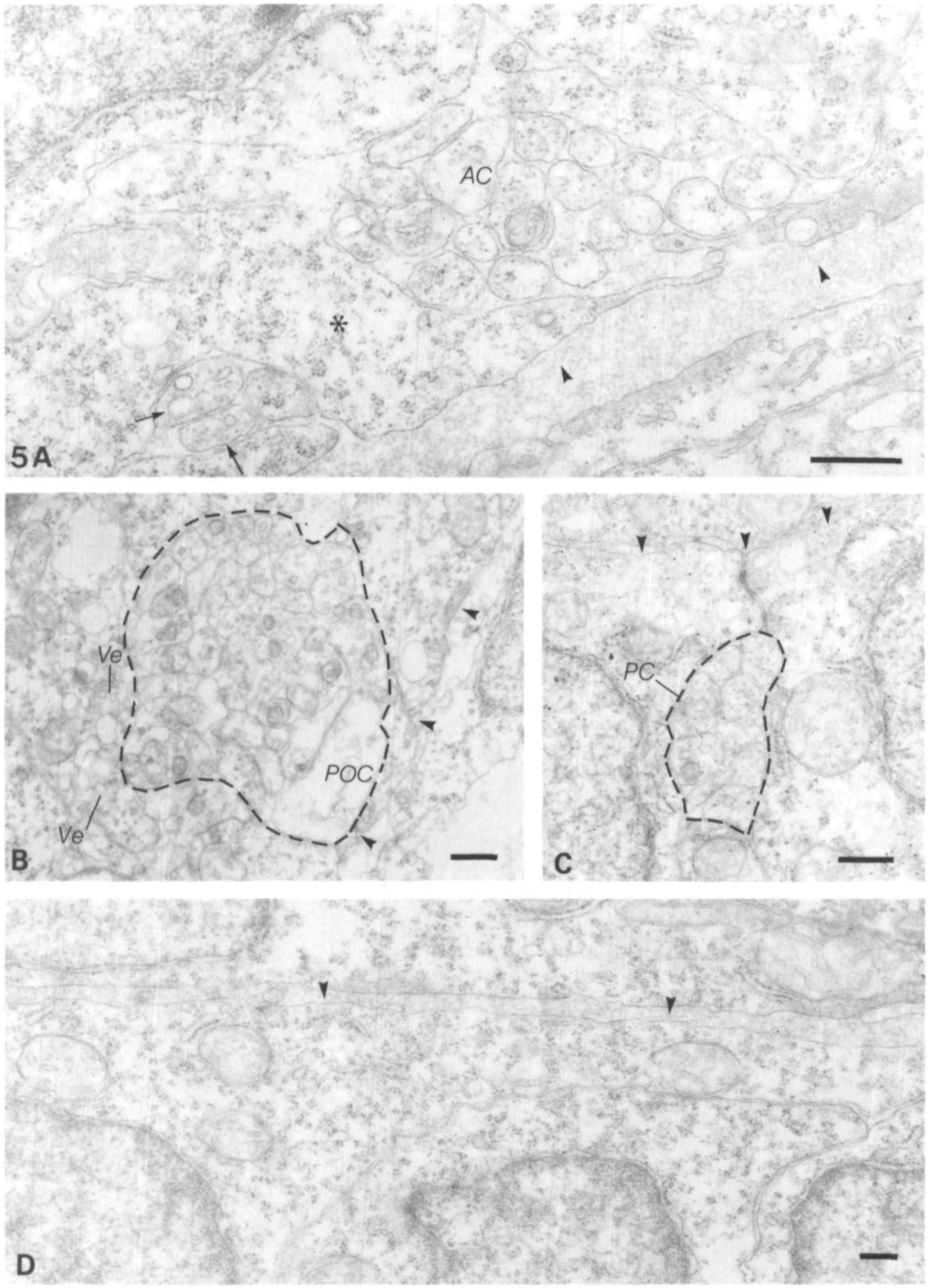

Fig. 5. Electron micrographs of commissures in the 1-day brain. Sagittal sections. Arrowheads indicate the basal lamina. The commissure is outlined in B and C. (A) Anterior commissure. Two axons (arrows) are separated from the rest of the commissure by the end foot of a cell lining the commissure (asterisk). (B) Postoptic commissure. (C) Posterior commissure. (D) A typical view of a region (in this case dorsal to the anterior commissure) that was devoid of axons. Scale bar, 0.5 μm. Abbreviations: AC, anterior commissure; PC, posterior commissure; POC, postoptic commissure; Ve, vesicles.

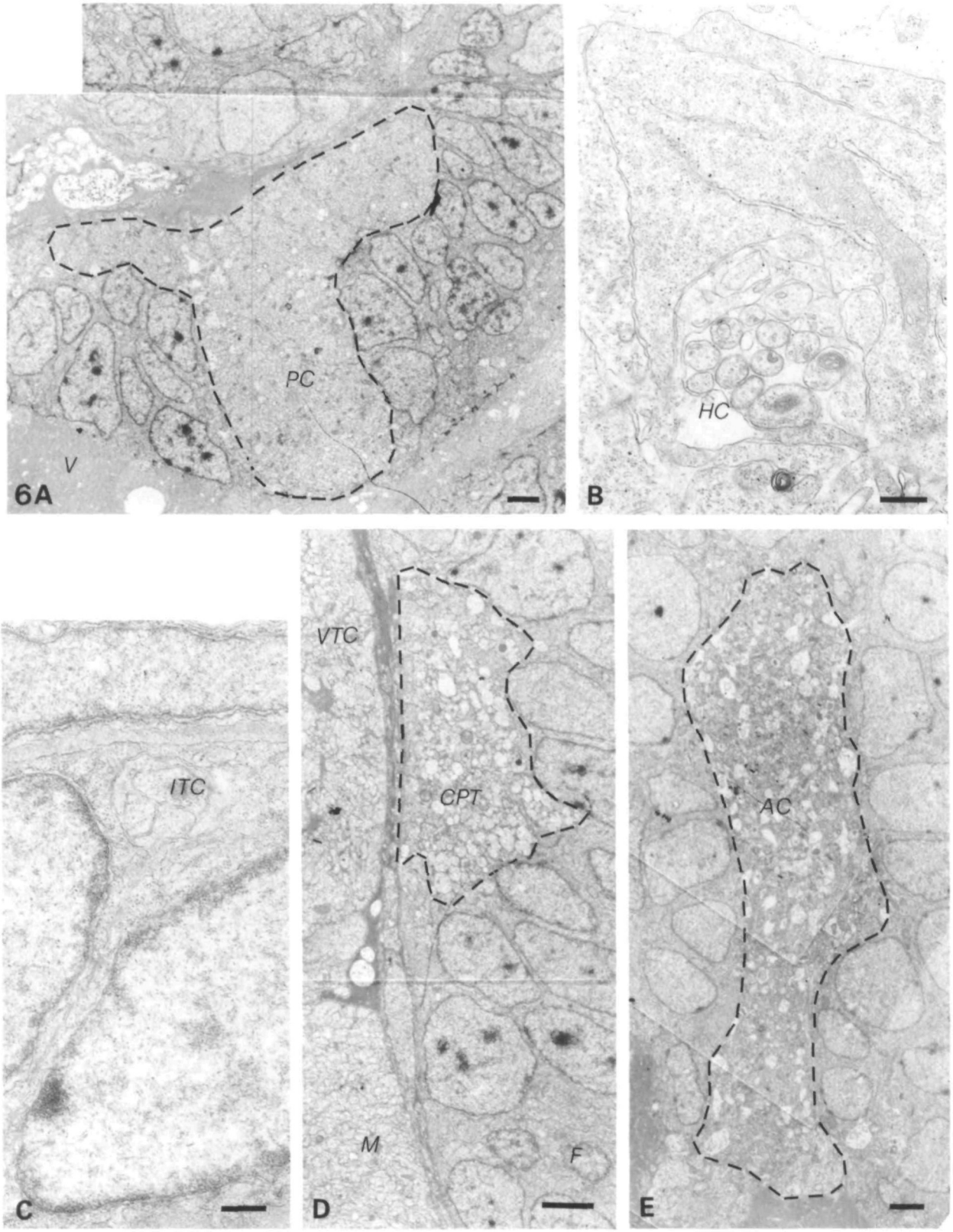

Fig. 6. Electron micrographs of commissures in the 2-day brain. Sagittal sections. Arrowheads indicate basal lamina. (A) Posterior commissure. Compare to Fig. 5C. (B) Habenular commissure. (C) Small intertectal commissure (see Fig. 4C). (D) Commissure of the posterior tuberculum. The caudal forebrain is folded back against the ventral midbrain at this position (see Fig. 1E, 13). (E) Anterior commissure. Compare to Fig. 5A. Scale bar (A, D, E), 2 μm; (B, C), 0.5 μm. Abbreviations: AC, anterior commissure; CPT commissure of the posterior tuberculum; E, epiphysis; F, forebrain; HC, habenular commissure; ITC, intertectal commissure; M, midbrain; PC, posterior commissure; V, ventricle; VTC, ventral tegmental commissure.

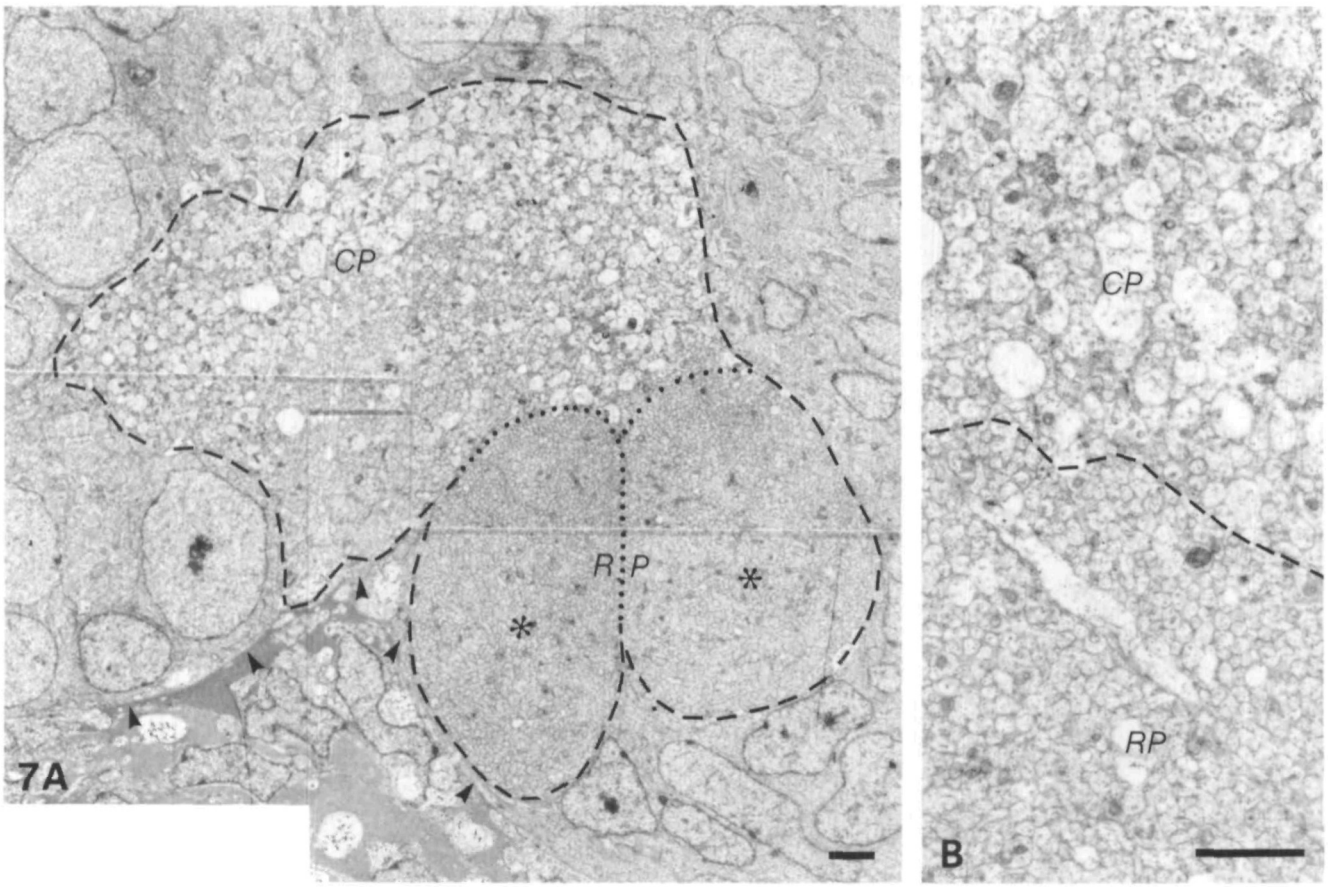

Fig. 7. Electron micrographs of the postoptic commissure at 2 days. Sagittal sections. Arrowheads indicate basal lamina. Rostral is to the right. (A) Photomosaic of the entire commissure (outlined). Asterisks indicate the two fascicles of optic axons that form the chiasm. (B) Higher magnification of the junction (dashes) between rostral and caudal portions of the commissure. Scale bar, 2 μm. Abbreviations: CP, caudal portion of the postoptic commissure; RP, rostral portion of the postoptic commissure.

(Fig. 7). Although the two portions were clearly distinct, no continuous barrier, such as a glial partition, separated them (Fig. 7B). The caudal portion contained 1877±198 axons (n=4), the rostral, 2263±363 (n=4). The rostral portion was divided into two fascicles (Fig. 7A). They were traced in serial 1 μm sections taken from one side of the midline. One was traced to the optic stalk, and was presumed to be the optic nerve from the ipsilateral retina. The other was traced up the wall of the diencephalon, in association with the tract of the postoptic commissure, and was presumed to be the optic tract from the contralateral retina. The rostral pair of fascicles in association with the postoptic commissure was therefore the optic chiasm. Fig. 8 gives the individual axon counts.

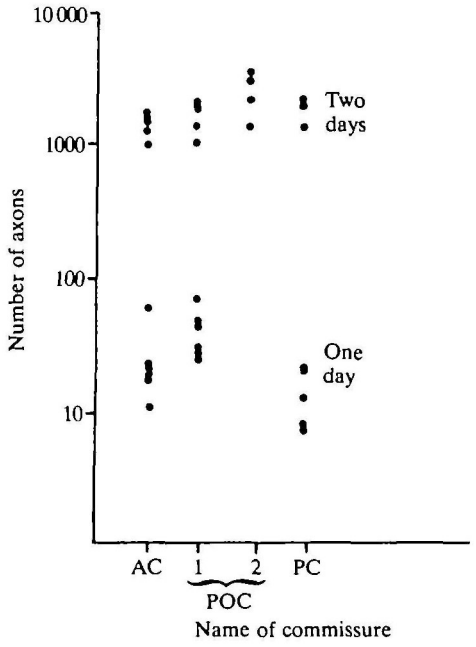

Fig. 8. Growth of the anterior, postoptic and posterior commissures between 1 and 2 days. Each dot represents the number of axons counted in the same commissure of different preparations. At 2 days the axons in the postoptic commissure are counted as two groups (1 – 'non-optic' axons and 2 – optic axons); see text for further explanation. Note that the number of axons is plotted on a logarithmic axis. AC, anterior commissure; POC, postoptic commissure; PC, posterior commissure.

Three new commissures were also present at 2 days. In the dorsal diencephalon, there was usually a discrete group of axons rostral to the posterior commissure, probably the habenular commissure (Fig. 6B, and see Figs 3, 4). In some preparations, it could not be discerned; either it had not formed or it was continuous with the posterior commissure. Caudal to the posterior commissure were a series of small dorsal midbrain commissures (Fig. 6C), which constituted the distributed intertectal commissure already described with antibody labelling (Fig. 4). A new ventral commissure, the commissure of the posterior tuberculum, was present just rostral to the ventral flexure and separate from the diffuse ventral tegmental commissures which lay across from it (Fig. 6D). It will be described in more detail below (see Figs 10, 13).

HRP analysis of the developing tracts

General features

HRP was applied to regions of the brain at many different locations within the forebrain and dorsal midbrain in both 1- and 2-day embryos. Initially the application sites were random but, as our knowledge of the brain improved, we aimed at particular tracts, commissures or nuclei. The application site was located in the reacted whole mount by the lump of reaction product debris; its size varied from several cell diameters to nearly an entire subdivision of the brain. Just under half of the preparations contained labelled processes that were long enough to be identified as axons; others contained labelled cells, usually neuroepithelial-like, that lacked axons. Some preparations contained no labelled cells.

The morphology of the individual axons and their relation to the tract in which they coursed differed at the two time points. At 1 day, most anterogradely labelled axons were tightly confined to the tract and terminated in growth cones with extensive filopodia and lamellipodia (Fig. 9). They were quite broad, up to about 10 μm across. At 2 days (not shown), many of the axons were not so tightly confined; these 'strays' tended to terminate diffusely or in fine branches, rather than in growth cones, suggesting that they were no longer *en route*, but were at or near their target sites (see, for example, Harris *et al.* 1987).

The results of HRP labelling are first summarised and then presented in six sections corresponding to the six application sites that gave labelled axons. They are: the olfactory placode, the telencephalon, the postoptic commissure, the eye, the dorsal diencephalon and the posterior commissure. For each section '*n*' refers to the number of preparations with labelled axons, not the total number of applications performed.

In 1-day embryos, the results obtained with HRP fit very well with the results obtained by other methods. Labelled axons were always confined to one or more of the tracts and commissures that had been identified with immunolabelling and electron microscopy (Fig. 10). The differential interference contrast optics on the microscope often enabled us to recognize the tracts and commissures in the HRP material even when they lacked labelled axons. Retrogradely labelled cell bodies were always in AChE-positive regions (Fig. 10A), but a greater number of cells reacted positively for AChE than were labelled by either HRP or HNK-1. This is consistent with previous results that suggested that AChE is present in cells several hours before they elaborate axons (Hanneman *et al.* 1988).

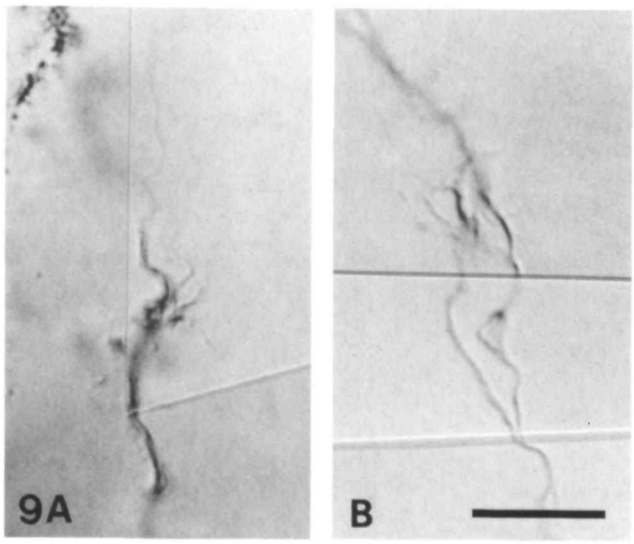

Fig. 9. HRP-labelled growth cones in the brain at 1 day. In each panel, the application site was below the region shown in the photograph but orientation varies between panels. (A) Growth cone in the tract of the posterior commissure labelled by HRP application to the posterior commissure (see Fig. 17). (B) Growth cone in the tract of the postoptic commissure labelled by HRP application to the dorsal diencephalon (see Fig. 16). Scale bar, 10 μm.

In 2-day embryos, the huge increase in the number of axons precluded investigation of all projections, so we concentrated on a subset.

HRP application to the olfactory placode

Olfactory receptor cells and their axons were labelled by applying HRP directly into the external cavity of the placode. Labelled embryos were prepared for viewing with the placode either removed or in place. When the placode was left in place, the morphology of the placodal cells was often preserved and, in some cases, apical projections to the external environment were seen (Fig. 11A; compare Klein and Graziadei, 1983). Removal of the placode made the olfactory projection more visible (Fig. 11B).

Labelled axons arose from the basal ends of the placodal cells and coalesced to form the olfactory nerve. At 1 day, axons were traced onto the mid- to dorsal telencephalon (n=7). This is probably the presumptive olfactory bulb, but it has not evaginated at this stage. The axons had multiple terminal branches, some of them tipped with growth cones bearing lamellipodia and filopodia.

Many more axons were labelled at 2 days (n=12). Groups of them formed glomerulus-like terminations

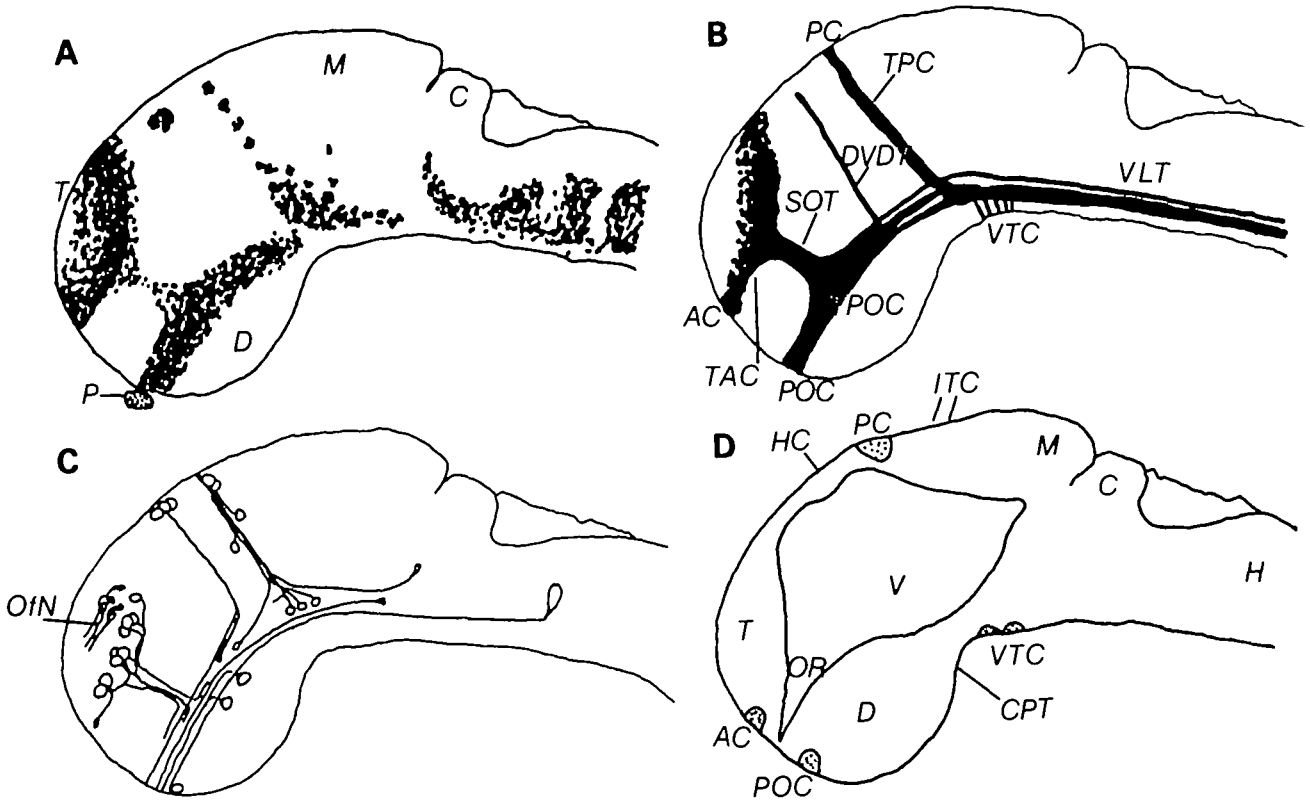

Fig. 10. Semi-diagramatic summary drawings of whole-mounted brains labelled for AChE (A), HNK-1 (B), and HRP (C), and a sagittal section from electron microscopy (D) at 1 day. Anterior is to the left, dorsal up. (A) AChE-positive cell bodies. (B) HNK-1 labelled tracts. (C) HRP-labelled cells and axons. The locations of cell bodies are approximate and are not intended to represent every neuron that projects an axon at this stage. Nomenclature of tracts is the same as in B. (D) Commissures in the forebrain and midbrain (stippled). The presumptive locations of commissures that develop between 1 and 2 days are indicated with arrows. Abbreviations: AC, anterior commissure; C, cerebellum; CPT, commissure of the posterior tuberculum; D, diencephalon; DVDT, dorsoventral diencephalic tract; H, hindbrain; HC, habenular commissure; ITC, intertectal commissure; M, midbrain; OfN, olfactory nerve; OR, optic recess; P, pituitary; PC, posterior commissure; POC, postoptic commissure; SOT, supraoptic tract; T, telencephalon; TAC, tract of the anterior commissure; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure; V, ventricle; VLT, ventral longitudinal tract; VTC, ventral tegmental commissure.

on the dorsal telencephalon (Fig. 11B), but individual axonal terminations could not be resolved.

In no preparation did any labelled axons project beyond the telencephalon, as would be expected if the terminal nerve (von Bartheld, 1987) were present at this stage.

In no preparations were any cell bodies in the brain retrogradely labelled.

HRP application to the telencephalon

We have no information on the fate map of the telencephalon, so we make no distinction in this section between subregions of that structure. We could not distinguish any intrinsic telencephalic tracts; HRP applications labelled axons that descended to the diencephalon (Figs 12, 13).

At 1 day (n=12) anterogradely labelled axons coalesced in an area rostral and dorsal to the optic stalk. As Fig. 12 illustrates, most of them projected into the supraoptic tract, and a few extended into the tract of the anterior commissure, but failed to reach the midline. We could not discern whether or not the individual axons bifurcated. At the intersection of the supraoptic tract and the tract of the postoptic commissure, axons projected in both directions, ventrally toward the postoptic commissure (not illustrated) and caudally toward the midbrain. In some cases, a thin veil between two branches at a bifurcation site suggested that a single axon had branched upon entering this tract. In other cases, bifurcations may have been due to the separation of two previously tightly fasciculated axons. In two cases, a few axons extended across the tract of the postoptic commissure and entered the territory of the developing hypothalamus. These relatively rare axons were probably forming the tract of the commissure of the posterior tuberculum.

In none of the 1-day preparations were any neurons retrogradely labelled outside the telencephalon.

At 2 days (n=11) labelled axons entered the same tracts as at 1 day, but they were much more numerous, projected much farther, and formed one new tract and commissure (Figs 12C, 13). The greater projection distances are revealed in an examination of individual

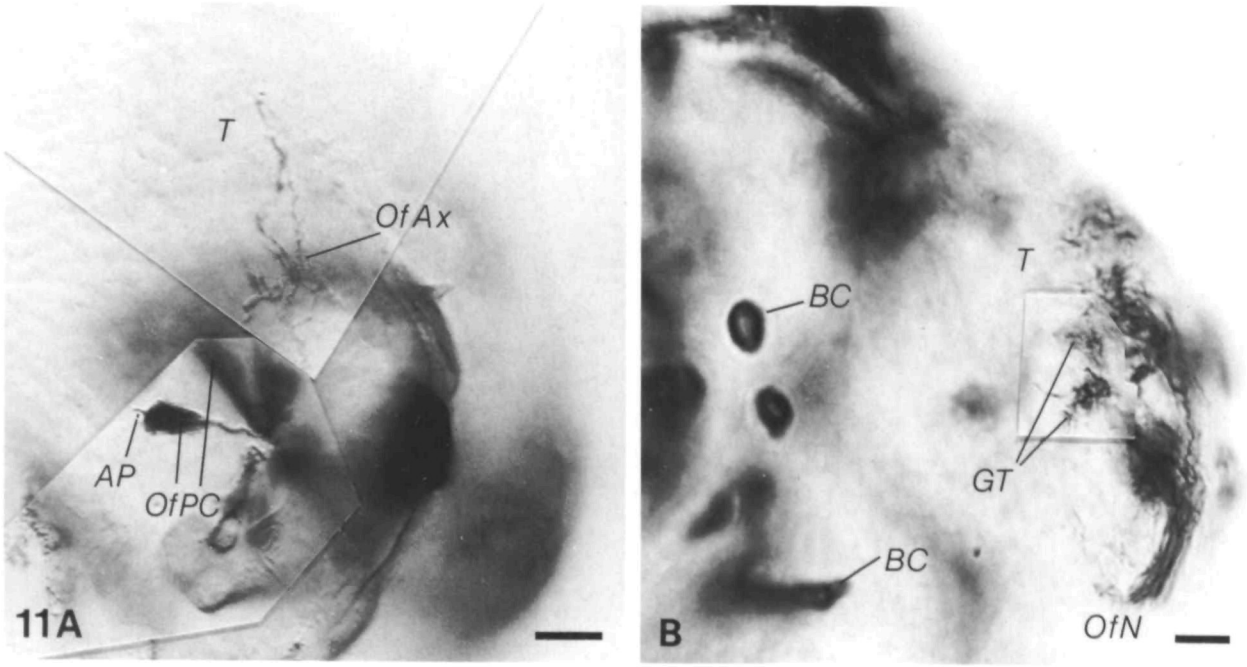

Fig. 11. Labelled axons and cell bodies following HRP application to the olfactory placode. Whole-mounted brains. Rostral is to the right and dorsal is up. (A) HRP-filled placodal cells and their axons in a 1-day embryo. The olfactory axons innervate the dorsal telencephalon. (B) Olfactory nerve and terminals in a 2-day embryo. The placode was removed during dissection, severing the olfactory nerve. Axons form glomerulus-like terminations. The dark staining in the rest of the brain is associated with endogenous peroxidase activity in blood vessels and cells. Scale bars, 10 μm. Abbreviations: AP, apical process; BC, blood cells; GT, glomerulus-like terminations; GC, growth cone; OfAx, olfactory axons; OfN, olfactory nerve; OfPC, olfactory placode cell; T, telencephalon.

tracts. Axons in the tract of the anterior commissure projected across the midline and entered the contralateral supraoptic tract, as Fig. 13 shows. Those that entered the ipsilateral supraoptic tract, and then the caudal limb of the tract of the postoptic commissure, projected farther. The axons in the supraoptic tract that projected beyond the intersection with the tract of the postoptic commissure, rare at 1 day, were far more numerous at 2 days. They extended across the hypothalamus, and entered the commissure of the posterior tuberculum (Fig. 12C). These axons had been noted previously with anti-acetylated tubulin labeling (Fig. 4A).

In two cases, one or several retrogradely labelled neurons were present in the postoptic region of the diencephalon following HRP application to the telencephalon at 2 days. However, the majority of preparations did not label any cells retrogradely.

HRP application to the postoptic commissure

This procedure (n=18) labelled axons both anterogradely and retrogradely at 1 day. As Fig. 14 illustrates, all were restricted to the commissure, its tract and the ventral longitudinal tract in the midbrain and hindbrain.

The anterogradely labelled axons never extended beyond the midbrain. Most terminated in growth cones, and some had small side branches along the axonal shaft (Fig. 14B). The locations of the cell bodies of these axons were unknown; they may have been either contralateral or near the midline, close to the application site.

Two groups of somata were retrogradely labelled on both sides of the midline (Fig. 14A). The first was in the diencephalon, always alongside the tract of the postoptic commissure. The size of this group varied widely, typically from 3 to 5 but up to 20. Cells in this region reacted positively for AChE (see Fig. 2A). The second group was in the hindbrain; it was smaller than the first and was seen in only two cases, two somata in one case, one in the other. Two cells were at the level of the otocyst, and the other was slightly rostral to it. These cells were not labelled artifactually, as they were traced through a labelled axon to the postoptic commissure.

HRP was not applied to the postoptic commissure at 2 days.

HRP injection into the eye

At 1 day, retinal ganglion cells have not elaborated axons (Stuermer, 1988; Burrill *et al.* 1989), so HRP was not injected into the eye at this age. Injections at 2 days (n=16) labelled optic axons that were observed in both whole mounts and horizontal plastic sections (Fig. 15). They exited the optic stalk and entered the diencephalon at the postoptic commissure as shown in Fig. 15A and B. They maintained a tight fascicle just beneath the surface on the rostral edge of the commissure and tract (Fig. 15B and C), including its intersection with the

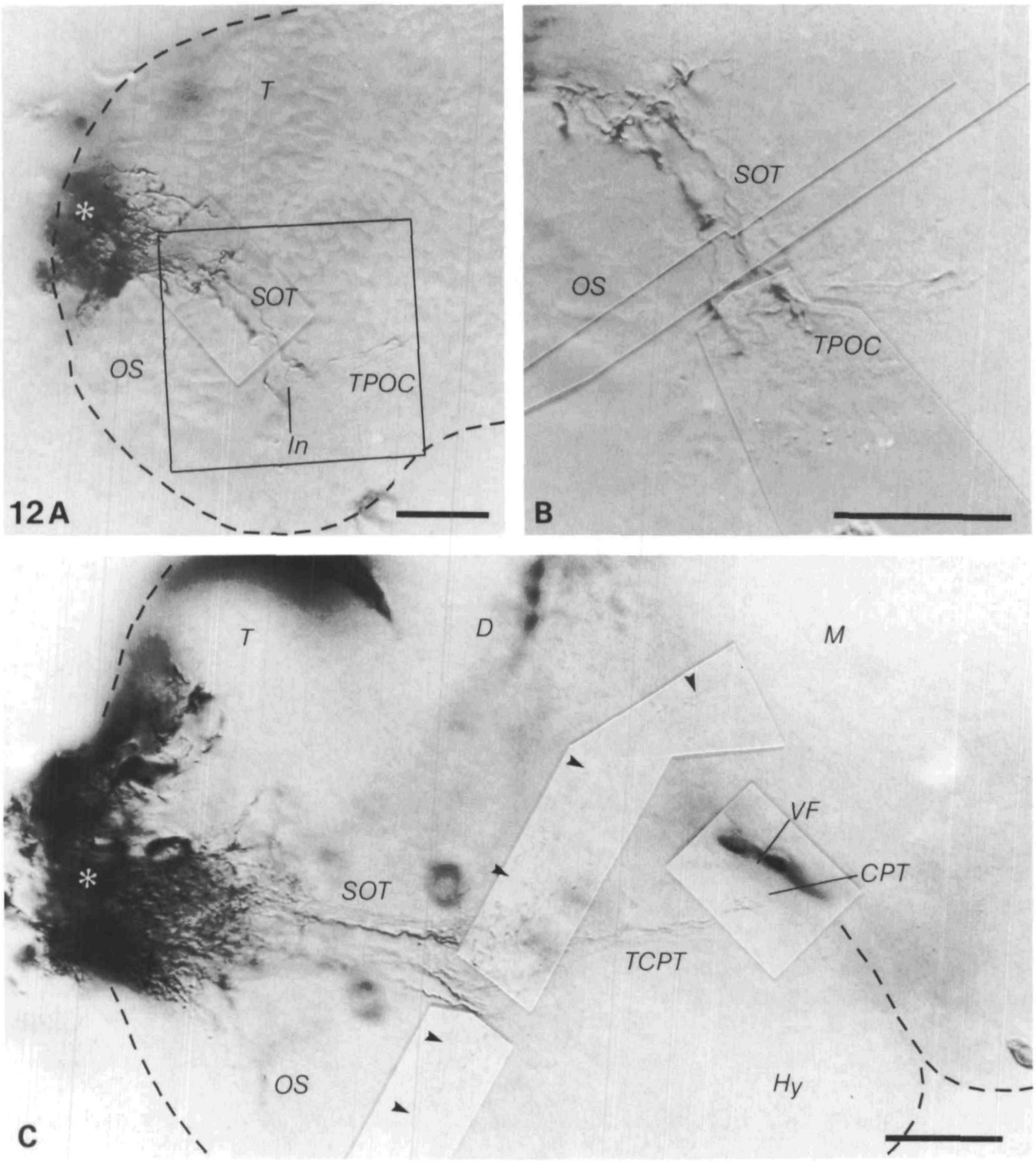

Fig. 12. Labelled axons following HRP application to the telencephalon at 1 day (A, B) and 2 days (C). Asterisks indicate application sites. Whole-mounted brains. Rostral is to the left, dorsal is up. Dashes outline the brain. (A) Axons entering the supraoptic tract, and turning into the tract of the postoptic commissure. Boxed region is shown in B. (B) Higher magnification detail of A. (C) At 2 days axons are projecting into the tract of the commissure of the posterior tuberculum in addition to the tracts mentioned above. Arrowheads indicate the trajectories of axons within the tract of the postoptic commissure. Scale bars, 25 μm. Abbreviations: CPT, commissure of the posterior tuberculum; D, diencephalon; Hy, hypothalamus; In, intersection between tracts; M, midbrain; OS, optic stalk; SOT, supraoptic tract; T, telencephalon; TCPT, tract of the commissure of the posterior tuberculum; TPOC, tract of the postoptic commissure; VF, ventral flexure.

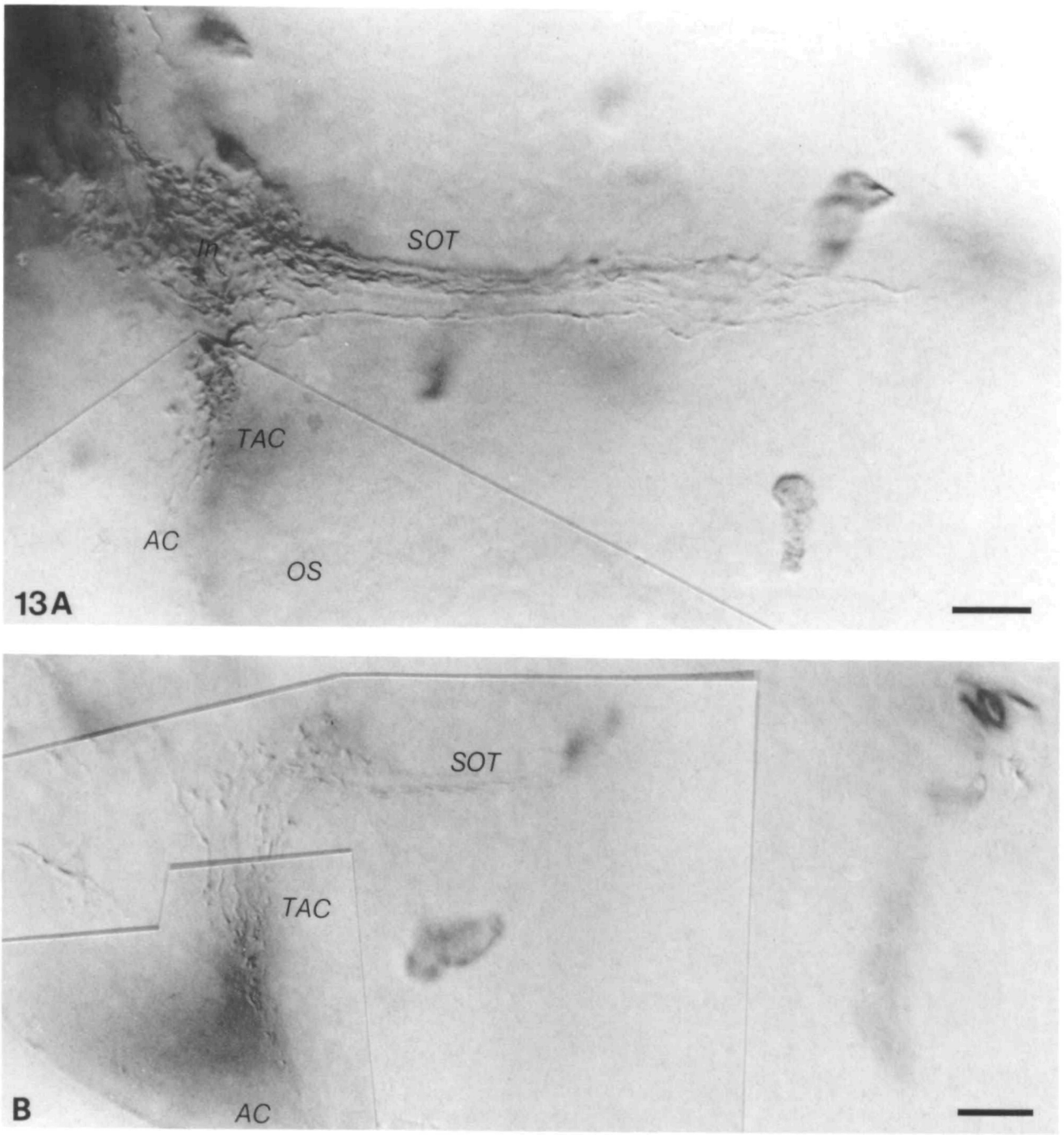

Fig. 13. Labelled axons following HRP application to the dorsal telencephalon (upper left in A) at 2 days. Whole-mounted brain. Rostral is to the left and dorsal is up. (A) Ipsilateral to the application site, showing axons in the intersection, the supraoptic tract and the tract of the anterior commissure. (B) Same preparation focused through the brain to the contralateral side. Axons emerge from the anterior commissure and enter the contralateral supraoptic tract. Scale bars, 10 μm. Abbreviations: AC, anterior commissure; In, intersection between tracts; OS, optic stalk; SOT, supraoptic tract; TAC, tract of the anterior commissure.

supraoptic tract (Fig. 15D). The axons began to fan out dorsally at approximately the forebrain–midbrain boundary, with the leading axons just beginning to invade the tectum (Fig. 15A).

No cell bodies in the brain were labelled by intraocular HRP.

HRP application to the dorsal diencephalon

Application of HRP to the region of the *anlage* of the epiphysis at 1 day (n=16) always labelled a very small number of axons. In some cases, they were bilateral, and in others, unilateral. They extended ventrally in the dorsoventral diencephalic tract as a tightly fasciculated

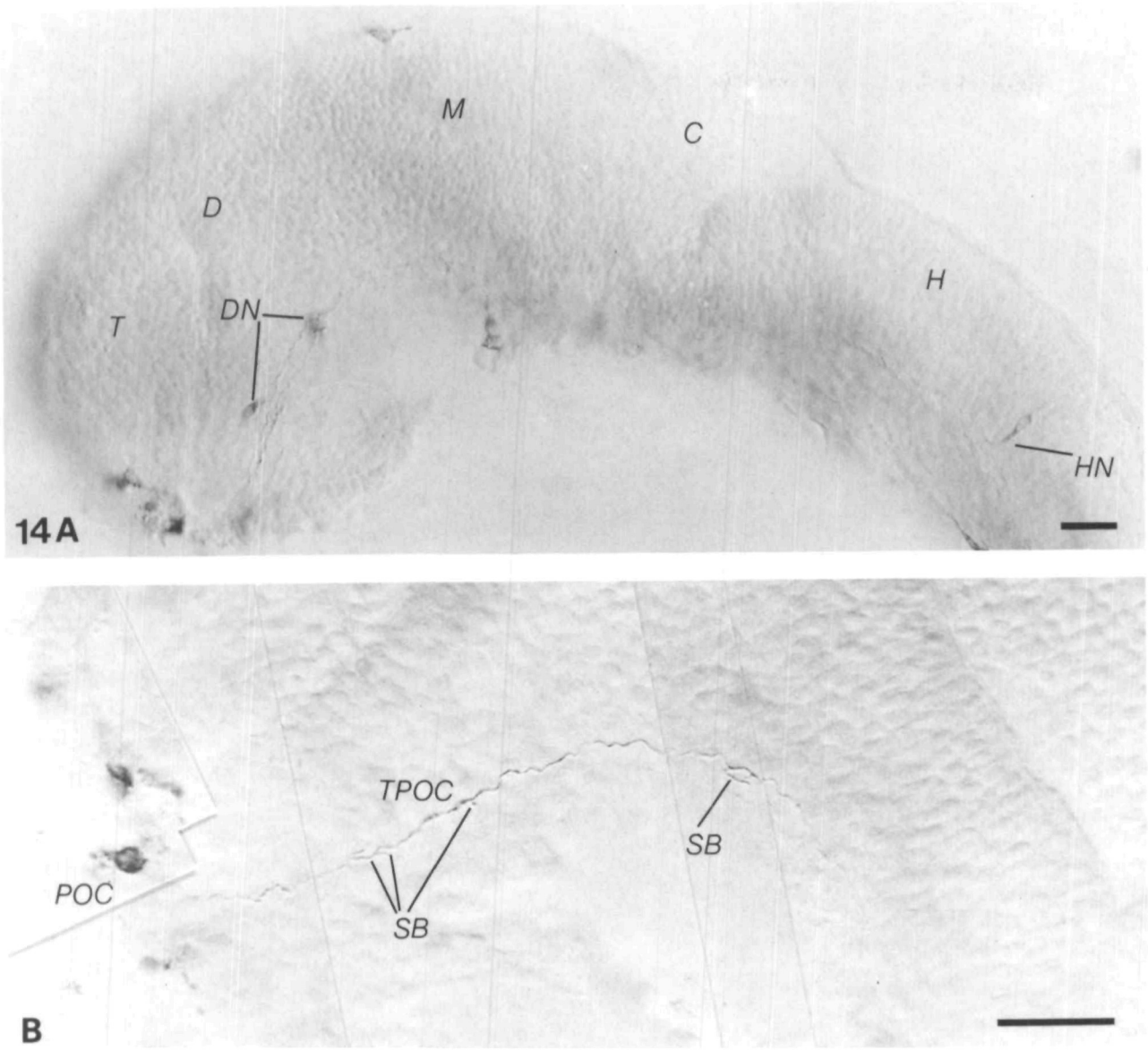

Fig. 14. Labelled cell bodies and axons following application of HRP to the postoptic commissure at 1 day. Whole-mounted brains. Rostral is to the left and dorsal is up. (A) A few axons in the tract of the postoptic commissure are labelled both anterogradely and retrogradely. Several neurons in the diencephalon are labelled as is a single neuron in the hindbrain. (B) Photomosaic of a single anterogradely labelled axon (different preparation) in the tract of the postoptic commissure. Scale bars, 25 μm. Abbreviations: C, cerebellum; D, diencephalon; DN, diencephalic labelled neurons; H, hindbrain; HN, hindbrain labelled neuron; M, midbrain; POC, postoptic commissure; SB, side branches; T, telencephalon; TPOC, tract of the postoptic commissure.

bundle. At the intersection with the tract of the postoptic commissure they defasciculated and, in many cases they appeared to branch, often quite profusely (Fig. 16). In the great majority of cases, all turned rostroventrally toward the postoptic commissure and, in a small minority of cases (n=2), one turned caudally (Fig. 16A). The leading growth cones were at slightly different positions in different preparations. Some were found ipsilaterally as in Fig. 16, whereas others extended into the contralateral tract of the postoptic commissure and, in one case, all the way to its intersection with the contralateral dorsoventral diencephalic tract.

The cell bodies of these labelled axons could not be discerned in the HRP preparations. They must have been near the midline in the application site, because all of the axons that were traced away from the application site terminated in growth cones, and there were no labelled cell bodies distant from the application site. They may have been those relatively few cells in or near the epiphysis that were labelled by HNK-1 and/or positive for AChE.

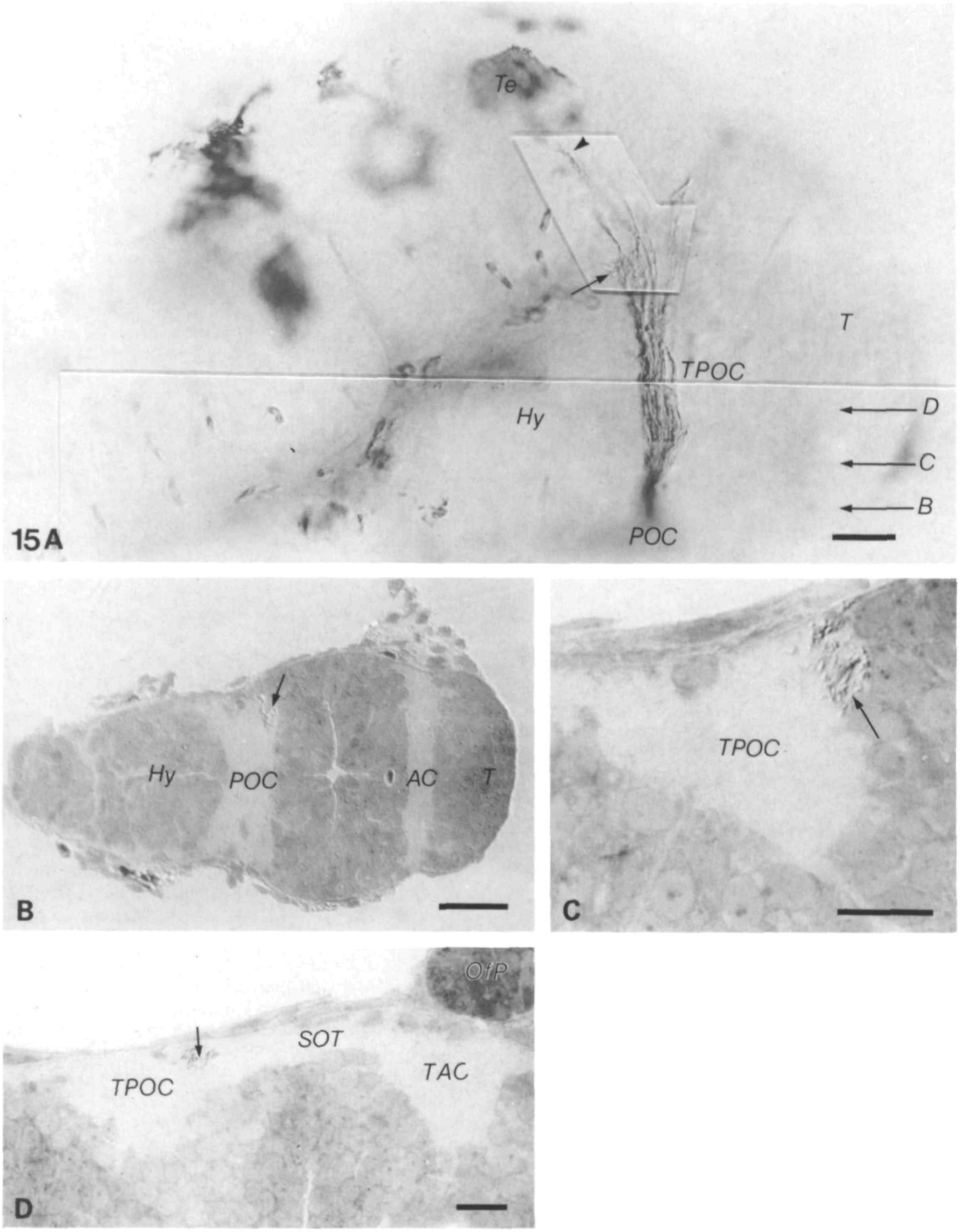

Fig. 15. Labelled retinal ganglion cell axons following HRP injection into the left eye at 2 days. (A) Whole-mounted brain. Lateral view, rostral is to the right and dorsal up. Axons fan out dorsally towards the tectum at approximately the forebrain/midbrain boundary (short arrow); the leading axons (arrowhead) are just beginning to invade the tectum. Long arrows indicate the approximate dorsoventral levels of sections in B–D. (B–D). Horizontal 1 μm sections of a preparation similar to that in A. Rostral is to the right. Arrows indicate labelled axons. (B) In the postoptic commissure. (C) In the tract of the postoptic commissure. (D) In the tract of the postoptic commissure at the level at which this tract meets the supraoptic tract (see Fig. 12A). Scale bars: (A, B), 25 μm; (C, D), 10 μm. Abbreviations: AC, anterior commissure; Hy, hypothalamus; OfP, olfactory placode; POC, postoptic commissure; SOT, supraoptic tract; T, telencephalon; TAC, tract of the anterior commissure; Te, tectum; TPOC, tract of the postoptic commissure.

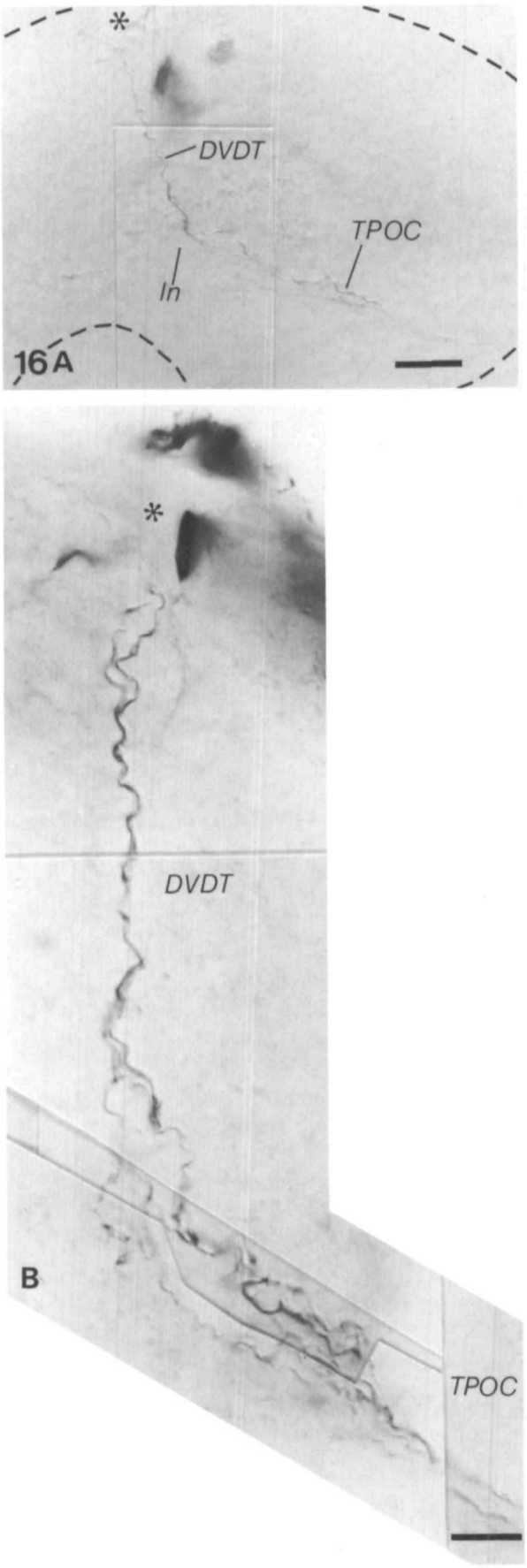

Fig. 16. Labelled axons following HRP application to the dorsal diencephalon at 1 day. Whole-mounted brains. Rostral is to the right and dorsal is up. Asterisks mark the application sites. (A) Most labelled axons turn rostrally from the dorsoventral diencephalic tract into the tract of the postoptic commissure. Dashes outline the brain. (B) High magnification view (different preparation) of axons defasciculating as they enter the tract of the postoptic commissure from the dorsoventral diencephalic tract. Scale bars: (A) 25 μm; (B) 10 μm. Abbreviations: DVDT, dorsoventral diencephalic tract; In, intersection between the tracts; TPOC, tract of the postoptic commissure.

We did not apply HRP to the dorsal diencephalon at 2 days.

HRP application to the posterior commissure

At 1 day, this procedure labelled axons in the tract of the posterior commissure both anterogradely and retrogradely (n=14).

Anterogradely labelled axons extended ventrally in the tract of the posterior commissure to its intersection with the tract of the postoptic commissure, where they turned either rostrally or caudally (Fig. 17). They extended variable distances, some of the caudal ones as far as the cerebellum (Fig. 17A), and some of the rostral ones as far as the intersection of the tract of the postoptic commissure with the supraoptic tract (not shown).

Two groups of cell bodies were labelled retrogradely, one in the ventral tegmentum and the other along the rostral margin of the midbrain (Fig. 17A), confirming Chitnis and Kuwada (1990). In favourable preparations, in which only a few cells and axons were labelled, it was possible to discern that the labelled cell bodies were unipolar, and sent their axons exclusively dorsally, toward the commissure. Therefore, the tract of the posterior commissure is composed of dorsally coursing axons of ipsilateral origin and ventrally coursing axons that originated either contralaterally or, if ipsilaterally, very near the midline application site. The two retrogradely labelled groups were in regions that reacted positively for both AChE and HNK-1 (see Fig. 2F, 2G, and 4).

HRP was not applied to the posterior commissure at 2 days because of anticipated problems in distinguishing it from all the nearby dorsal midbrain commissures (see Fig. 4).

In summary, HRP labelling revealed a set of nerves, tracts and commissures that matched the pattern seen with immunolabelling and electron microscopy. In addition, it demonstrated the origins and complexities of these connectives, about which several general statements seem warranted. First, except for two tracts not associated with commissures (the supraoptic and the dorsoventral diencephalic), all of the tracts in the 1-day brains contained axons going both directions. Second, the tract of the postoptic commissure was the major longitudinal channel for axons, forming a rostral link in the chain of tracts connecting the brain and spinal cord.

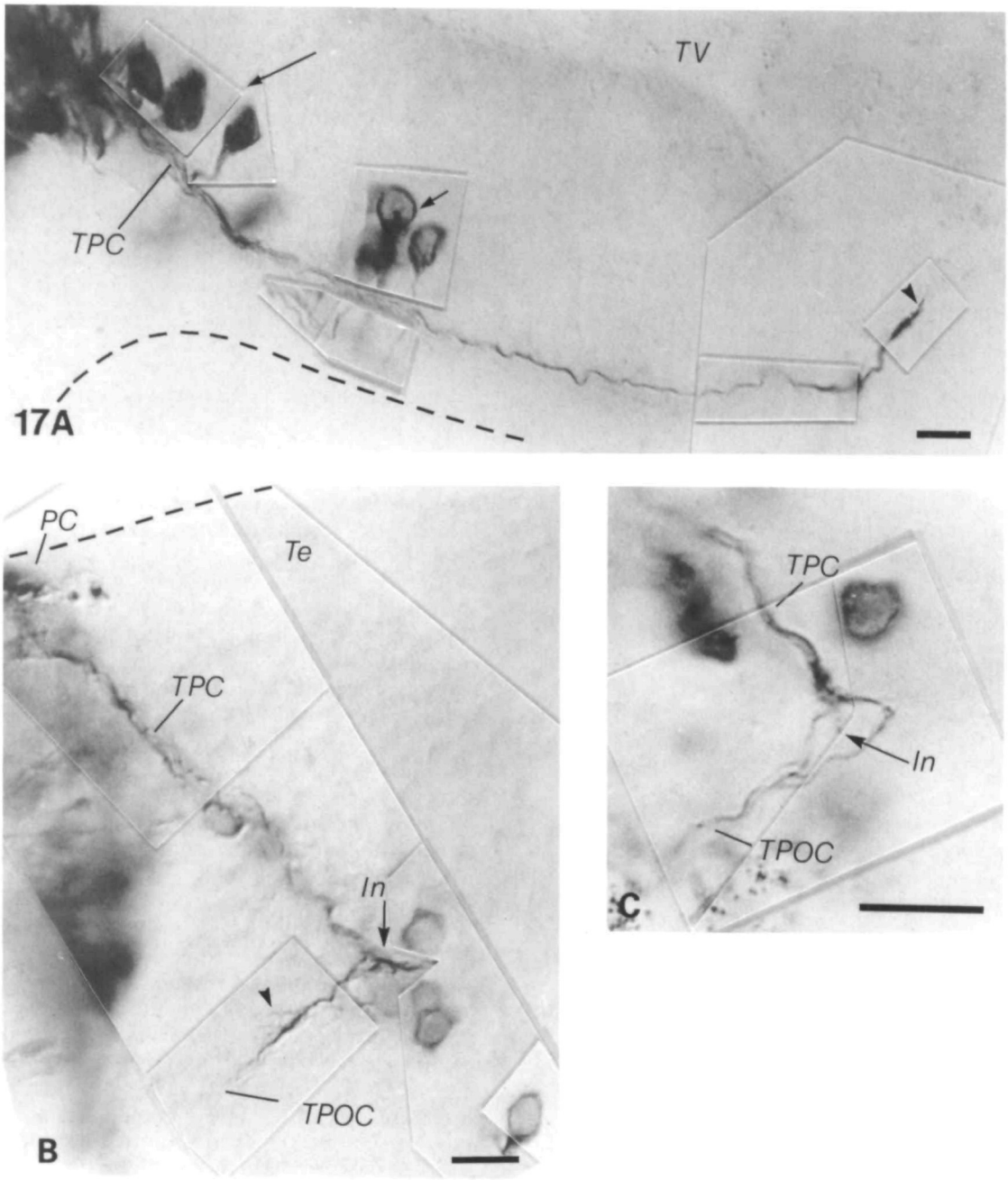

Fig. 17. Axons and cell bodies labelled following HRP application to the posterior commissure at 1 day. Whole-mounted brains. Rostral is to the left, and dorsal is up. Dashes outline the brain. (A) Labelled cell bodies in the tegmentum (short arrow) and along the rostral margin of the developing tectum (long arrow) and axons. Most anterogradely labelled axons turn caudally and one (arrowhead) extends to the tectal/cerebellar fold. (B) Photomosaic of a large lamellipodial growth cone (arrowhead) turning rostrally into the tract of the postoptic commissure, from the tract of the posterior commissure. (C) Photomosaic of axons turning rostrally into the tract of the postoptic commissure. Preparations in B and C are printed in reverse orientation to match A. Scale bars, 10 μm. Abbreviations: In, intersection between tracts; PC, posterior commissure; Te, tectum; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure; TV, tectal ventricle.

It assumed that role very early; by 1 day, it contained axons both ipsilateral and contralateral in origin, and it received axons from all but one of the tracts (that of the anterior commissure). Third, all tracts were straight or gently curved; the axons turned abruptly (up to 90 degrees) only at the intersections. Fourth, at the intersections, incoming axons were not generally funnelled into one branch, but in most cases, all branches received axons.

Discussion

Although several recent papers have described the very

early development of axons in the vertebrate CNS (Harris, 1986; 1989; Kuwada, 1986; Foster and Roberts, 1987; Easter and Taylor, 1989), this is the first to attempt to describe the system of tracts and commissures in the brain (see also Chitnis and Kuwada, 1990). Such an approach resembles that of the earlier neuroembryologists (Bergquist, 1932; Herrick, 1937; 1938*a*,*b*,*c*) and we have, with only a few exceptions, confirmed their findings.

Comments on the experimental methods

We have also advanced these early findings in ways made possible by methods developed since the earlier work. First, we have visualised the axons in intact, whole-mounted brains rather than in wax reconstructions from sections. Second, we have used newer techniques (immunolabelling of axonal molecules expressed very early, electron microscopy, and HRP tracing) that visualise axons very early in development. (Much of the earlier neuroembryology was based on reduced silver stains that depend on neurofilaments, absent from the earliest axons.) Third, the microapplication of HRP successfully labelled small sets of neurons from origin to termination, thereby giving a more selective view of parts of the system, and an indication of directionality of axonal outgrowth in the pathways. Fourth, electron microscopy permitted axon counting, which is impossible to do light microscopically when axons are very small.

It was essential to use several methods, because none was completely reliable alone. The antibodies labelled an array of axons in 1-day embryos that was consistent with other reports, but one can not be sure that the antibodies labelled all axonal bundles. The electron microscopy and the HRP labelling confirmed that there were no others, so we concluded that the relatively small number of tracts and commissures constitute all the connectives. The antibodies labelled fewer somata than axons, and therefore did not show the origins of the tracts, but retrogradely transported HRP did.

The use of AChE histochemistry to indicate differentiation is a relatively recent addition to the neuroembryological arsenal. Layer (1983) first noted a correlation between AChE expression and the state of differentiation in the chick's nervous system. He offered no explanation for the correlation, and noted that non-cholinergic neurons were involved, so the appearance of AChE seemed not to be related to its synaptic function. The possibility remained that it was only an epiphenomenon, that AChE actually had no role in development. A recent report by Wolfgang and Forte (1989) investigated this issue in *Drosophila*, which has only one gene for AChE. In mutants that lacked the gene, neural development was abnormal, strongly suggesting that AChE does in fact have a role. We have not elucidated what that might be, but our observation that the first neurons to send out axons were in AChE-positive regions strengthens the correlation and confirms a recent prediction of Layer *et al.* (1988). (The only AChE-positive region that was not associated with an axonal projection was the pituitary. We do not know if there are axons associated with it at 1 day. At 2 days, electron microscopy revealed a few axons nearby, but their origins were uncertain.)

Axonal choices: new tracts or old

We believe that we have described all the tracts and commissures, and many of the somata of origin, in the 1-day forebrain and midbrain. The description at 2 days is less complete because the brain is so much more complex. The tracts are all considerably larger than at 1 day, and many of the new axons in them probably originate from places other than the ones that had initially formed the tracts. We know this to be true for the tract of the postoptic commissure, which originates from a small number of somata adjacent to it in the diencephalon, and then acquires axons from the retina, among other places, by 2 days. We assume it is true for other tracts, too.

In spite of the daunting complexity of the 2-day brain, the preparations still revealed much about the development of pathways. The few tracts and commissures present at 1 day were augmented by even fewer new ones at 2 days. Instead of forming new tracts, the new axons tended to join pre-existing ones. Again, the clearest example was the postoptic commissure. Between 1 and 2 days, the number of axons increased nearly one hundred fold, and most of the new ones were from the retina.

Comparisons with earlier work

Tracts and commissures

The most useful guides for the interpretation of these data were the old papers by Bergquist, Herrick and others, who worked on a variety of vertebrate species. Our observations conformed so readily to theirs that we used their nomenclature for the commissures (Bergquist, 1932; Herrick, 1937; Ariens Kappers *et al.* 1967). We avoided much of their nomenclature of the tracts, however, because it often anticipated an adult structure. We profess greater ignorance of what these tracts will become later in life, apart from a few obvious ones like the olfactory nerve and optic tract. Thus we give them accurate, but unrestricted names such as 'the tract of the posterior commissure'. Nomenclature aside, our descriptions of the tracts are consistent with others (Baker and Graves, 1932; Bergquist, 1932; Windle and Baxter, 1935; Herrick, 1937; 1938*a*,*b*,*c*; Rhines and Windle, 1941.)

The dorsoventral diencephalic tract has not been described before (see also Chitnis and Kuwada, 1990). It does not correspond to projections from either the pineal organ (Hafeez and Zerihun, 1974; Ekstrom and van Veen, 1983) or the habenular nucleus (Clairambault and Pairault Fasolo, 1986) that have been described in adult lower vertebrates. It is somewhat similar to a projection described by Foster and Roberts (1982) in larval *Xenopus*. If the projections we have labelled are from pineal ganglion cells, then they are projecting axons long before retinal axons enter the brain (at about 36 h: Stuermer, 1988). A similar developmental disparity between the two photoreceptive

organs has been reported in another teleost, the stickleback (Ostholm *et al.* 1988) and in *Xenopus* (Roberts, 1978).

The projection of hindbrain neurons into the postoptic commissure has not previously been reported in lower vertebrates. We are currently using longer survival times following HRP application in an attempt to label more of these neurons and to follow their development in greater detail.

Hypotheses of tract formation

The blueprint hypothesis (Singer *et al.* 1979) and the labelled pathways hypothesis (Goodman *et al.* 1982; Raper *et al.* 1983) both provide explanations of what underlies the formation of tracts. How does this report relate to them?

According to the blueprint hypothesis, axons grow where an enlarged extracellular space is provided. We have noted (see also Easter and Taylor, 1989) that the neuroepithelial nuclei have withdrawn from the subpial zone through which the axons grow, and that there is more extracellular space surrounding axons at the earliest stages of tract formation. However, we have not examined those same regions prior to the arrival of axons, to see if either the spaces have appeared or the nuclei have withdrawn in advance of the axons. So the results are not inconsistent with the blueprint hypothesis, but neither do they support it with any positive results.

According to the labelled pathways hypothesis, after an initial set of pathways have formed, later axons grow along them, in some cases jumping from one to another. The various pathways are labelled differentially, and the follower axons read the different labels and navigate accordingly. This hypothesis, originally proposed to explain pathfinding in grasshoppers, now has considerable experimental evidence to back it up both in invertebrates (Raper *et al.* 1983; Bastiani *et al.* 1984; Harrelson and Goodman, 1988) and vertebrates (Kuwada, 1986; Dodd *et al.* 1988). While we do not know how axons navigate within the tracts we have described, we are impressed by the fact that most axons generated after 1 day tended to grow along pre-existing tracts, exactly as the hypothesis predicts. Our demonstration that axons tend to grow along other axons is strong support for the labelled pathways hypothesis, or at least for that part that posits axons as a favored substrate for other axons.

Growth cones

The growth cones that we have labelled in isolation resemble those seen elsewhere by others (e.g. Tosney and Landmesser, 1985; Bovolenta and Mason, 1987; Harris *et al.* 1987; Nordlander, 1987). They are up to 10 μm wide with their filopodial and lamellipodial extensions and, therefore, in some cases are broader than the tracts they form or join. Note that most of the tracts that we have identified are separated from one another by considerably more than a growth cone diameter. Therefore, most growth cones will not be within physical reach of more than one tract except at intersections.

At these intersections, growth cones encounter two or more pathway options. For example, axons from the dorsal diencephalon (see Fig. 16) could turn either rostrally or caudally at the intersection of the dorsoventral diencephalic tract with the tract of the postoptic commissure, however, they almost all turned rostrally. The appearance of the tract suggests that the environment changed abruptly at the intersection; in the proximal part of the tract, the axons were unbranched and fasciculated together, but at the intersection they separated and some of them branched. It seems likely that there are guidance cues at these intersections. The rearrangement of axons (see for example Fig. 13) is reminiscent of axon behaviour in peripheral nerve plexi (Tosney and Landmesser, 1985; Wilson and Holder, 1988). In these plexi axon behaviour (Myers *et al.* 1986; Eisen *et al.* 1986) and growth cone morphology (Tosney and Landmesser, 1985) change. Such changes have been associated with axons reading guidance cues.

Primary and secondary neurons

Two populations of neurons, primary and secondary, have been described in the spinal cord and hindbrain of lower vertebrates (Coghill, 1913; Forehand and Farel, 1982; Roberts and Clarke, 1982; Fetcho, 1987). Primary neurons are characterised by their small number, early development, characteristic location and large size. They generally pioneer axon tracts (see Myers *et al.* 1986) and are believed to be responsible for establishing the first functional circuits within the nervous system (Sillar and Roberts, 1980). Secondary neurons develop later and are produced in far greater numbers (Myers, 1985; Myers *et al.* 1986). It is possible that the relatively few neurons with long axons described at 1 day are primary, but they must be characterised further to see if they meet the criteria suggested for primary neurons in other regions of the zebrafish nervous system (Grunwald *et al.* 1988). Along this line, the genetic mutation that spares primary neurons in zebrafish does not spare any retinal neurons, implying that none of them are primary (Grunwald *et al.* 1988). This is consistent with the results of this study, in which we have shown that retinal axons do not pioneer their pathway, but follow a pre-existing tract.

Segmentation

Our results suggest that there are islands of early neuronal maturation and axon outgrowth in the zebrafish brain, but does their pattern suggest a segmental organisation to the brain? The hindbrain can be divided into neuromeres in both zebrafish (Hanneman *et al.* 1988) and chick (Lumsden and Keynes, 1989). A cluster of segmentally homologous cells differentiates relatively early within each of these repeating units and, in chicks, a transverse ventral commissure separates each unit (Lumsden and Keynes, 1989). Applying such criteria, we have attempted to evaluate the segmentation of the forebrain and midbrain. The ventral commissures (the anterior and postoptic, and the com-

missure of the posterior tuberculum) can be taken as the interneuromeric boundaries. This generates four neuromeres approximately equivalent to the telencephalon; the optic stalk, supraoptic region and retina; the diencephalon; and the midbrain. They correspond quite closely to those proposed by Bergquist (1952) on the basis of external topography. They all contain clusters of AChE-positive cells at 1 day (Fig. 2A). However, the clustering is very uneven. The cluster in the supraoptic region (Fig. 2B) is tiny compared to the others, and there are several isolated groups of labelled cells in other neuromeres that do not fit very well into this scheme. The cells in the epiphysis and those along the rostral margin of the presumptive tectum are examples. A further problem with the neuromeric hypothesis arises with a consideration of tracts and axonal projections, which show little apparent segmental homology (Fig. 10B, C). Although there is a continuous rostrocaudal pathway (supraoptic tract, tract of the postoptic commissure and the ventral longitudinal tract), the dorsal inputs into this pathway are not segmentally repeated. For instance, the dorsoventral diencephalic tract is unique to the diencephalic 'neuromere'. Perhaps the rostral brain was primitively segmented, and subsequent evolution has obscured this pattern. If so, then segmentation may be more evident at earlier stages. We do not think that our results strengthen the segmental interpretation of regions rostral to the hindbrain.

Visual system

Easter and Taylor (1989) have described the early development of the retinofugal pathway in *Xenopus laevis*. Their description is very like this one; that is, the postoptic commissure and its associated tracts are present before the optic axons reach the brain. When they do, they join this commissure and advance in close association with it, along its anterior boundary up the side of the diencephalon, and onto the presumptive tectum. However, the optic chiasm is quite unlike that of *Xenopus*, in which the axons from both eyes are completely intermingled (Wilson *et al.* 1988). In zebrafish, the two nerves are adjacent, but quite separate from each other, and from the postoptic commissure. The basis for these two very different examples of axonal segregation is unknown.

Terminal nerve

This is the name given to a very early projection from the olfactory placode to sites beyond the olfactory bulb (Herrick, 1937). Our applications of HRP to the nasal placode never revealed such a projection. This failure can not be attributed to axons' inability to transport the HRP that far, because much longer projections were seen in other preparations. The zebrafish has not been shown to have a terminal nerve, but it is so well documented in other teleost fish (von Bartheld, 1987) that it is very probably present in the zebrafish, too. We suggest that it develops after 2 days.

Endnote

This work has only begun to describe some of the aspects of brain development, so the questions that remain are countless. Even so, several observations seem particularly suitable for further inquiry, and they are given below.

What are the cues that draw the axons into the small number of particular pathways? Whatever their nature, they must be restricted to the most superficial lamina of the brain, since all tracts form in that region, just deep to the pia and superficial to the cellular core. Now that we know where the first tracts are, we are better equipped to find what molecules or structures presage them. A recent report (Jacobs and Goodman, 1989) has implicated glial cells in this role.

To what extent are the later axons dependent on the pre-existing tract that they join? This question will undoubtedly have multiple answers, as it has had in the insect nervous system, both peripheral (Keshishian and Bentley, 1983) and central (Raper *et al.* 1984) and in the fish spinal cord (Kuwada, 1986; Eisen *et al.* 1989). These earlier papers have shown a successful experimental strategy for answering the question; that is, abolish the pioneers and see if the followers can manage on their own.

How do deep tracts form? If all the early tracts form superficially, and yet the adult brain has abundant deep tracts (tracts separated from the pia by cell nuclei), how did they get there? Were they originally superficial and secondarily enveloped by nuclei migrating outward from the periventricular zone, as Easter and Taylor (1989) have suggested for *Xenopus*? Or do later tracts form in the deeper layers without a pre-existing scaffold of axons? This question can be answered best by picking an adult deep tract, and describing its development through embryogenesis.

How does the brain with relatively few tracts at 1 day acquire so many later on? The comparison between 1 and 2 days suggests that most axons added during the second day join pre-existing tracts rather than form new ones. If this trend continues, then how are the few tracts parcellated into many? The postoptic commissure and its associated tract would seem to provide a good substrate for this question. We know already that it gives rise to the optic tract and chiasm, and it seems likely that the transverse, horizontal and minor commissures identified in the adult (Franz, 1912; Easter *et al.* 1978) must have originated in the embryonic postoptic commissure. This should be traceable through development.

In summary, the zebrafish forebrain and midbrain consists of a surprisingly simple set of connectives: at 1 day, the olfactory nerve, four commissures, their associated tracts and three other tracts are present; at 2 days, the optic nerve, three more commissures and their associated tracts have been added. We have described a developmentally intermediate stage. Future studies will be directed at both earlier and later stages.

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