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The early development of retinal ganglion cells with uncrossed axons in the mouse: retinal position and axonal course

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Summary

The carbocyanine dye, DiI, has been used to study the retinal origin of the uncrossed retinofugal component of the mouse and to show the course taken by these fibres through the optic nerve and chiasm during development. Optic axons first arrive at the chiasm at embryonic day 13 (E13) but do not cross the midline until E14. After this stage, fibres taking an uncrossed course can be selectively labelled by unilateral tract implants of DiI. The earliest ipsilaterally projecting ganglion cells are located in the dorsal central retina. The first sign of the adult pattern of distribution of ganglion cells with uncrossed axons located mainly in the ventrotemporal retina is seen on embryonic day 16.5, thus showing that the adult line of decussation forms early in development. A small number of labelled cells continue to be found in nasal and dorsal retina at all later stages.

At early stages (E14–15), retrogradely labelled uncrossed fibres are found in virtually all fascicles of the developing nerve, intermingling with crossed axons throughout the length of the nerve. At later stages of development (E16–17), although uncrossed fibres pass predominantly within the temporal part of the stalk, they remain intermingled with crossed axons. A significant number of uncrossed axons also lie within the nasal part of the optic stalk. The position of uncrossed fibres throughout the nerve in the later developmental stages is comparable to that seen in the adult rodent (Baker and Jeffery, 1989). The distribution of uncrossed axons thus indicates that positional cues are not sufficient to account for the choice made by axons when they reach the optic chiasm.

Key words: optic chiasm, axonal pathway, optic axons, DiI.

Introduction

In mammals, the adult optic chiasm serves to segregate retinofugal axons according to the retinal locus of their ganglion cells; many temporal cells have an uncrossed axon whereas virtually all nasal cells have a crossed axon. The developmental processes that lead to this chiasmatic segregation are of considerable interest because the pathway choice is a simple one that can be readily identified once it is made and because there are known mutants in which the segregation is abnormal (Lund, 1965; Guillery, 1974; LaVail *et al.* 1978).

In the adult optic nerve, one finds a rough retinotopic order behind the eye (Horton *et al.* 1979; Naito, 1986; Baker and Jeffery, 1989) and a simple view might lead to the speculation that the fibres from the temporal retina could maintain their lateral position and be guided into the ipsilateral tract by virtue of their position (Silver and Shapiro, 1981; Webster *et al.* 1988). However, there are a number of difficulties about such a proposal. One is that a significant number of ganglion cells in the temporal retina have crossed axons, and these tend to be born later than ganglion cells with uncrossed axons (Dräger, 1985). Their chiasmatic course would appear not to be guided by their position. A second problem is that there is a significant change in fibre order between the eye and the chiasm (Walsh, 1986; Guillery and Walsh, 1987), which brings the fibres into an age-related order as they approach the chiasm. To some extent, this prechiasmatic reordering also involves a change in the relative position in the nerve of the uncrossed component. Recent studies (Naito, 1986; Baker and Jeffery, 1989) have shown that in the adult nerve the uncrossed fibres lie in the temporal part of the nerve behind the eye but become more scattered in their further course so that a significant number lie in the nasal part of the nerve close to the chiasm, scattered among the crossed fibres. Clearly, if this mingling of crossed and uncrossed fibres characterizes the developing as well as the adult system, then local guidance cues could play no more than a secondary role in producing the segregation of crossed from the uncrossed axons. Each group of axons would have to have the capacity to react in a characteristic way to the same local cues.

The present study was undertaken in order to define how distinctive the course of the uncrossed axons is at early stages when pathway choices are made, and to define the extent to which the retinal position of a ganglion cell is related to the course that its axon takes through the nerve and chiasm.

Materials and methods

Fixed tissue was obtained from four mice (C57bl6J) at each of the following ages: embryonic day (E)12, 13, 14.5, 15.5, 16.5, and 17.5, and studied by the use of the carbocyanine dye, DiI (Honig and Hume, 1986; Godement *et al.* 1987). The plug date of a mated female was taken as day 0. At the required stage of pregnancy, the mouse was killed by cervical dislocation and the fetuses removed and transcardially perfused with 4 % paraformaldehyde in 0.1 M phosphate buffer at room temperature and pH 7.2. Some fetuses were also prepared for electron microscopy using standard fixing and embedding techniques (Guillery and Walsh, 1987).

After overnight immersion in the fixative, the heads were washed in phosphate buffer and the lower jaw and roof of the mouth dissected free to expose the optic tract. A crystal of DiI was then embedded into the tract well caudal to the chiasm. It should be noted that this placement avoided labelling of an early uncrossed component described by Guillery and Walsh (1987) in ferrets. This component, which passes to the anterior hypothalamus from the optic nerve and may also exist in mouse, represents a very early uncrossed retinofugal pathway but was left unlabelled in this study. For anterograde labelling of fibres, a crystal of DiI was embedded into the optic disc. Following all dye implants, the whole preparation was then immersed in 2 % buffered formalin and left in the dark at room temperature for the following periods:

E12–13 – <2 weeks

E14.5 – 3 weeks

E15.5 – 4 weeks

E16.5 – 5–6 weeks

E17.5 – 6–8 weeks.

After sufficient time had passed for the dye to diffuse along the axons, the eyes were dissected from the block and the retinae flat mounted. Care was taken while excising the eyes not to stretch or distort the optic nerve. The retinal flatmount was coverslipped using an anti-quenching mounting medium, PPD (Johnson *et al.* 1982), and viewed with a Leitz microscope using a rhodamine filter set.

The remaining preparation containing the optic nerves was embedded in a gelatin/albumin solution and cut on a vibratome (200–400 μm sections) either as horizontal sections or as parasagittal sections perpendicular to the path of fibre outgrowth. The sections were mounted, coverslipped with PPD and viewed with either the Leitz fluorescent microscope or the MRC 500 confocal imaging system.

Results

The distribution of ganglion cells with uncrossed axons

To label the early uncrossed pathway to the optic tract selectively, it was necessary to establish when ganglion cell fibres first reach the chiasm. This was done by labelling the fibre projection anterogradely, from the retinae of embryonic mice aged 12 to 14 days with DiI and defining the leading edge of fibre outgrowth. During embryonic day 12, optic fibres penetrate the stalk, reaching to about half its length. 24 h later, as more fibres are added to the projection, the leading fibres reach the chiasm (Fig. 1A,B). At this point, a few fibres could be seen crossing in the chiasm but most of the fibres appeared to group at the midline. At embryonic day 14, optic fibres were first clearly entering the optic tract (Fig. 2A,B). This confirms previous electron microscopic observations. (Colello and Guillery, 1987).

Labelling one optic tract with DiI at E14.5 showed retrogradely labelled ganglion cells predominantly in a dorsal central position in the ipsilateral retina (Fig. 3A). The mean of ipsilaterally projecting ganglion cells from a sample of 4 retinae at this age group was 64 (S.E.=7.0). There were only a few labelled ganglion cells in ventral retina and none were observed in the periphery of the retina. The total number of labelled ganglion cells varied slightly between litter mates but this may be due to the variation in developmental maturity within a litter. The eye contralateral to the dye placement site was labelled evenly around the optic disc within an area roughly two-thirds the radius of the retina (Fig. 3B). Ganglion cells in the far periphery of the contralateral retina remained unlabelled.

24 h later, at E15.5, labelled ganglion cells on the side of the dye placement were seen in each quadrant surrounding the optic disc, with a slight preference for the dorsal central retina (Fig. 4). The mean of ipsilaterally projecting ganglion cells from a sample of 4 retinae at this age group was 110 (S.E.=11.1). Labelled cells continued to be absent from the retinal periphery. The area containing labelled ganglion cells in the contralateral eye had now enlarged to roughly three-quarters the radius of the retina. Each quadrant of the retina appeared to be evenly labelled with no quadrant containing labelled cells in the far periphery.

The adult pattern of distribution of ganglion cells with uncrossed axons starts to appear on embryonic day 16.5. At this age, the majority of labelled ipsilaterally projecting ganglion cells were found in the ventrotemporal crescent of the retina (Fig. 5A). Labelled cells were also found scattered throughout nasal and dorsal retina. However, no cells were labelled in the extreme periphery. The mean of ipsilaterally projecting ganglion cells from 4 retinae at this age group had increased to 325 (S.E.=26.3). The labelling of the contralateral retina was almost complete, with only the far periphery remaining devoid of fluorescent cells (Fig. 5B).

24 h later (E17.5), the overall distribution of labelled cells in the ipsilateral retina remained essentially unchanged, except for some addition to the labelled population in the ventrotemporal crescent. The boundary of labelled cells in the contralateral retina extended to all but the most peripheral margin.

The distribution of uncrossed fibres in the developing optic stalk

The nerves from the above retinae were used to define the course taken by the uncrossed fibres as they run through the optic stalk. The gross arrangement of retinofugal axons has been described earlier (Silver and Sapiro, 1981), and the distribution of the axon bundles will be summarized only briefly to provide the background on which the distribution of the uncrossed fibres must be based.

In the E14.5 mouse, as axons leave the eye through

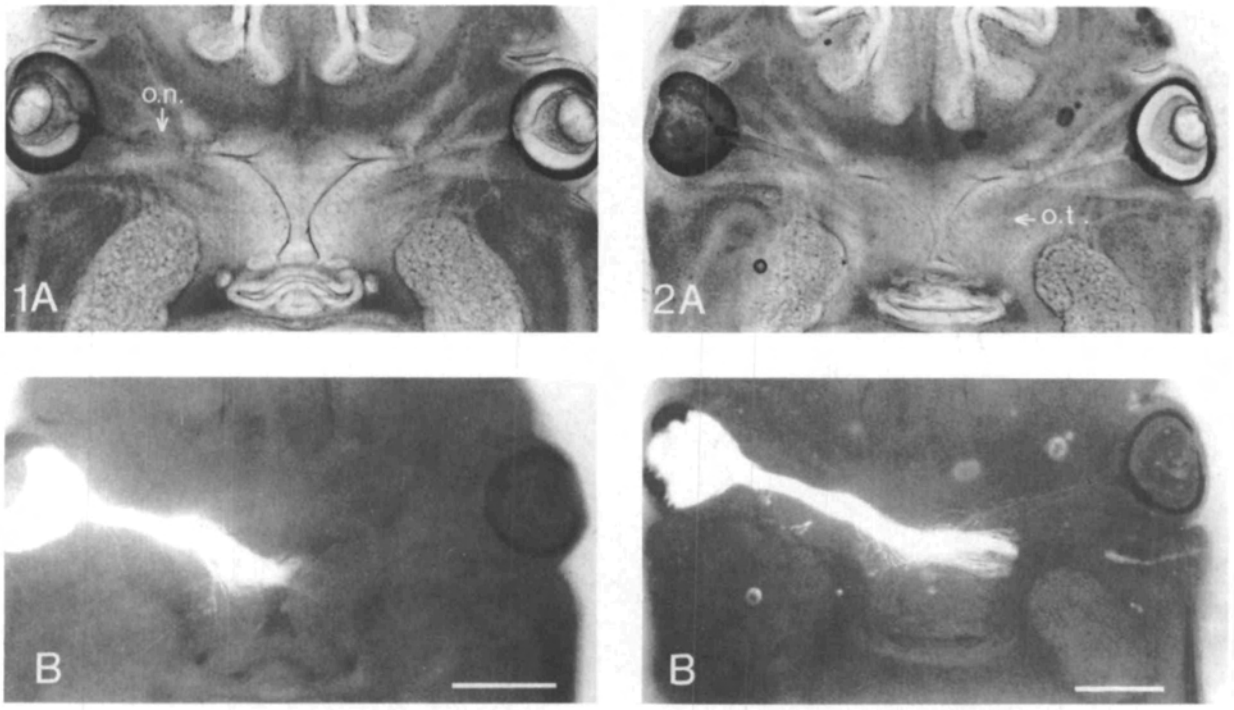

Fig. 1. Bright-field (A) and fluorescent (B) micrographs showing the extent of axon outgrowth through the optic nerve (o.n.) and chiasm of an E13 mouse after labelling with an intraocular fill of DiI. Photomicrographs were taken from a 300 μm horizontal section. Scale bar is 500 μm.

Fig. 2. Same preparation as in Fig. 1 but prepared from an E14 mouse embryo. Note axon outgrowth extends across the midline of the chiasm and into the optic tract (o.t.). Fibre outgrowth into the optic tract continues through sections dorsal to the one presented. Scale bar is 500 μm.

the optic disc, they become grouped into fascicles that are restricted to the ventral three-quarters of the optic stalk (Fig. 6A). Approximately 200–300 μm nearer the brain, the fascicles form a ring around the central lumen (Fig. 6C), and just rostral to the chiasm the fascicles are once again restricted to the ventral portion of the stalk (Fig. 6E).

At E14.5, virtually all fascicles at the retinal end of the optic stalk contained some axons originating from ipsilaterally projecting ganglion cells (Fig. 6B). Although it is difficult to ascertain the position of these axons within an individual fascicle, it is clear that the positional preference of these fibres does not seem to be restricted to a particular fascicle or group of fascicles within the nerve. Nearer the brain, at approximately 300 μm from the eye, labelled fibres continued to be found in virtually all fascicles (Fig. 6D). The lack of a discernable pattern of distribution of uncrossed fibres in the nerve continued to the level of the chiasm (Fig. 6F). Further caudally, the course taken by uncrossed fibres was obscured by the intense labelling of the contralateral projection. In the nerve contralateral to the dye placement, each fascicle appeared to be completely filled with labelled axons (Fig. 6G). The fluorescence was restricted to the fibre bundles and absent from the non-fascicular portions of the nerve.

At E16.5, uncrossed axons were present mainly on the temporal side of the nerve. Near the eye cup-nerve junction, the majority but not all of labelled axons were located within the temporal half of the nerve. There was also some scatter of labelled axons in the nasal portion of the nerve (Fig. 7A). At a point midway between the eye and the brain (450 μm from the eye), labelled axons continued to be found predominantly in the ventrotemporal portion of the nerve (Fig. 7B), but a number of uncrossed axons were still dispersed throughout the cross-sectional area of the nerve. Near the chiasm, at approximately 1 mm from the eye, the uncrossed fibres became more dispersed through the cross-section of the nerve (Fig. 7C). Although there was a preferential distribution of labelled axons to the temporal half of the nerve, the scattering of uncrossed axons to all parts of the nerve increased as they approached the chiasm. In the contralateral nerve, the intensity of fluorescence made it difficult to ascertain if the density of labelled axons favoured any particular location. What remained clear, however, was that at any point along the course of the nerve virtually all fascicles contained labelled crossed axons.

Discussion

We have studied the retinal ganglion cells that have uncrossed axons at early stages of the development of the retinofugal pathway (E14–17). The advantage of the dye used in this study lies in its ability to travel both anterogradely and retrogradely in fixed tissue, its com-

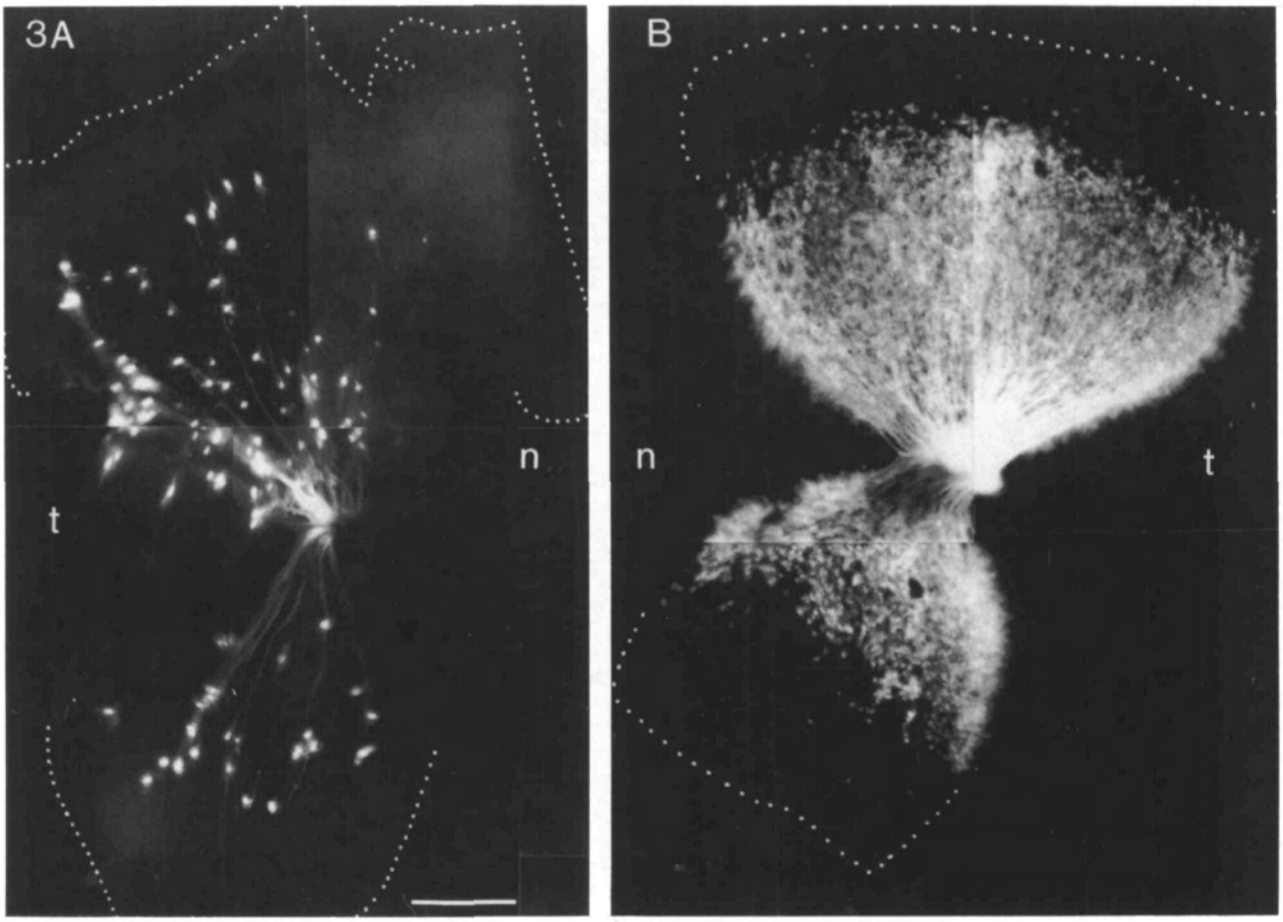

Fig. 3. E14.5 mouse retinae retrogradely labelled by a unilateral optic tract fill of DiI. The margins of both retinae ipsilateral (A) and contralateral (B) to the tract fills are outlined by dots. All retinae shown in this and subsequent figures are orientated dorsal up, with nasal (n) and temporal (t) marked. Scale bar is 200 μm.

plete staining of individual neuronal processes and its negligible spread to unlabelled areas (Godement *et al.* 1987). We have shown that the uncrossed axons mingle with crossed axons in all parts of their course and at all periods studied. We have confirmed the earlier observations of Godement *et al.* (1987) that the earliest uncrossed axons present in the optic tract have their cell bodies in the central retina, not in the ventrotemporal retina from where the uncrossed component originates at later stages. Further, we have shown that when the axons of cells of the more peripheral retinal sectors first reach the optic tracts there is already a clear retinal line defining the border of the region that contains the majority of ganglion cells with uncrossed axons; that is, the ventrotemporal ipsilaterally projecting sector of the retina is established.

The earliest uncrossed axons are scattered throughout the cross-sectional area of the stalk. The later uncrossed axons lie in the ventrotemporal quadrant of the stalk just behind the eye but then come to occupy the whole cross-sectional area of the stalk as they approach the chiasm. No part of the stalk is free of uncrossed axons at any stage and even the ventrotemporal region, containing most of the uncrossed axons near the eye, is never free of a dense population of crossed axons.

The position of the uncrossed axons relative to the crossed axons in the optic stalk is important for defining the developmental mechanisms that may be producing the segregation of the two groups at the chiasm. It may also prove relevant for understanding the development of the abnormal pathways found in several mutant forms (LaVail *et al.* 1978).

Earlier accounts, based on sections in which the axon populations had not been differentially labelled, suggested that the axons destined for an uncrossed course occupy a distinct position in the cross section of the optic stalk (Silver and Sapiro, 1981; Horsburgh and Sefton, 1986). On this basis, it was possible to suggest that a specialized cell group, such as a patch of pigmented cells near the retinal end of the eyestalk (Silver and Sapiro, 1981; Strongin and Guillery, 1981), or a specialized zone of glial cells at the cerebral end of the stalk, the 'glial knot', (Silver, 1984) might play a local, mechanical role in guiding axons towards an appropriate course on their way to one or other optic tract. Further, the suggestion that the abnormal pathways formed in mutants might develop because the

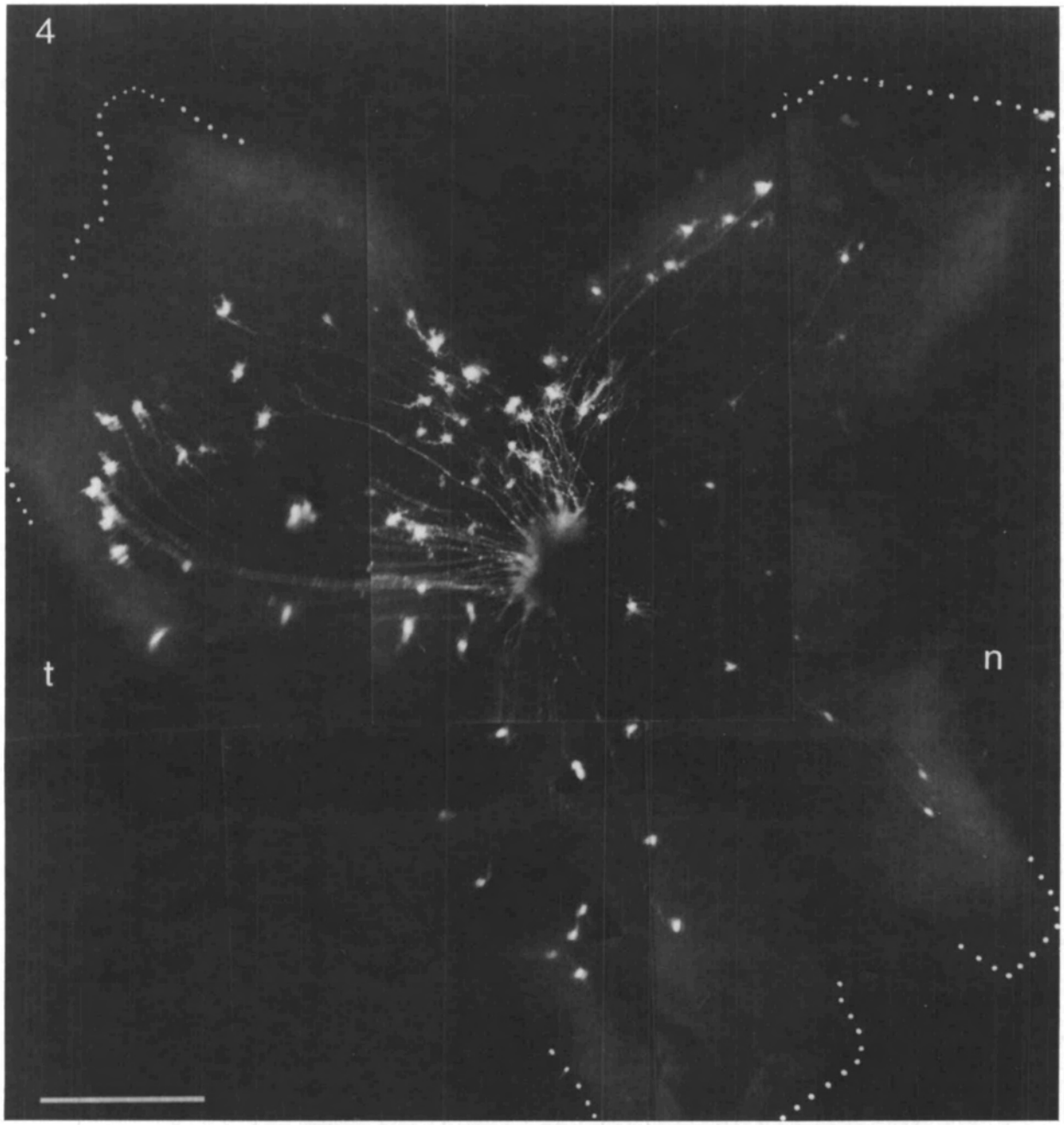

Fig. 4. The retina of an E15.5 mouse prepared as in Fig. 3, ipsilateral to a tract fill showing the location of ganglion cells with uncrossed axons that project to the site of the dye placement. Scale bar is 300 μm.

patch of pigmented cells is not present in the mutants seemed to grow naturally out of this view of a mechanical guidance acting selectively upon the uncrossed component (Silver and Sapiro, 1981; Webster *et al.* 1988).

Investigations of the adult retinofugal projection (Horton *et al.* 1979; Naito, 1986; Baker and Jeffery, 1989) have suggested that, in cat and rat, the crossed and uncrossed axons are not clearly segregated in the optic nerve as they approach the optic chiasm, and the observations reported here now confirm that there is no clear segregation at any stage of development. The forces that act to send one group of axons ipsilaterally and another contralaterally cannot be dependent upon the position of the axons in the pathway; other factors must play a role.

At the earliest stages of development, when axons from central retina are first reaching the optic tract, it appears that the retinal position of a ganglion cell is unrelated to the chiasmatic course of its axon, and it may be that entry into one or other tract is random. Shortly thereafter, however, when axons from the more peripheral parts of the retina reach the optic tracts, retinal position becomes crucial. The segregation of ipsilaterally projecting ganglion cells is well established as soon as the first axons from the ventrotemporal

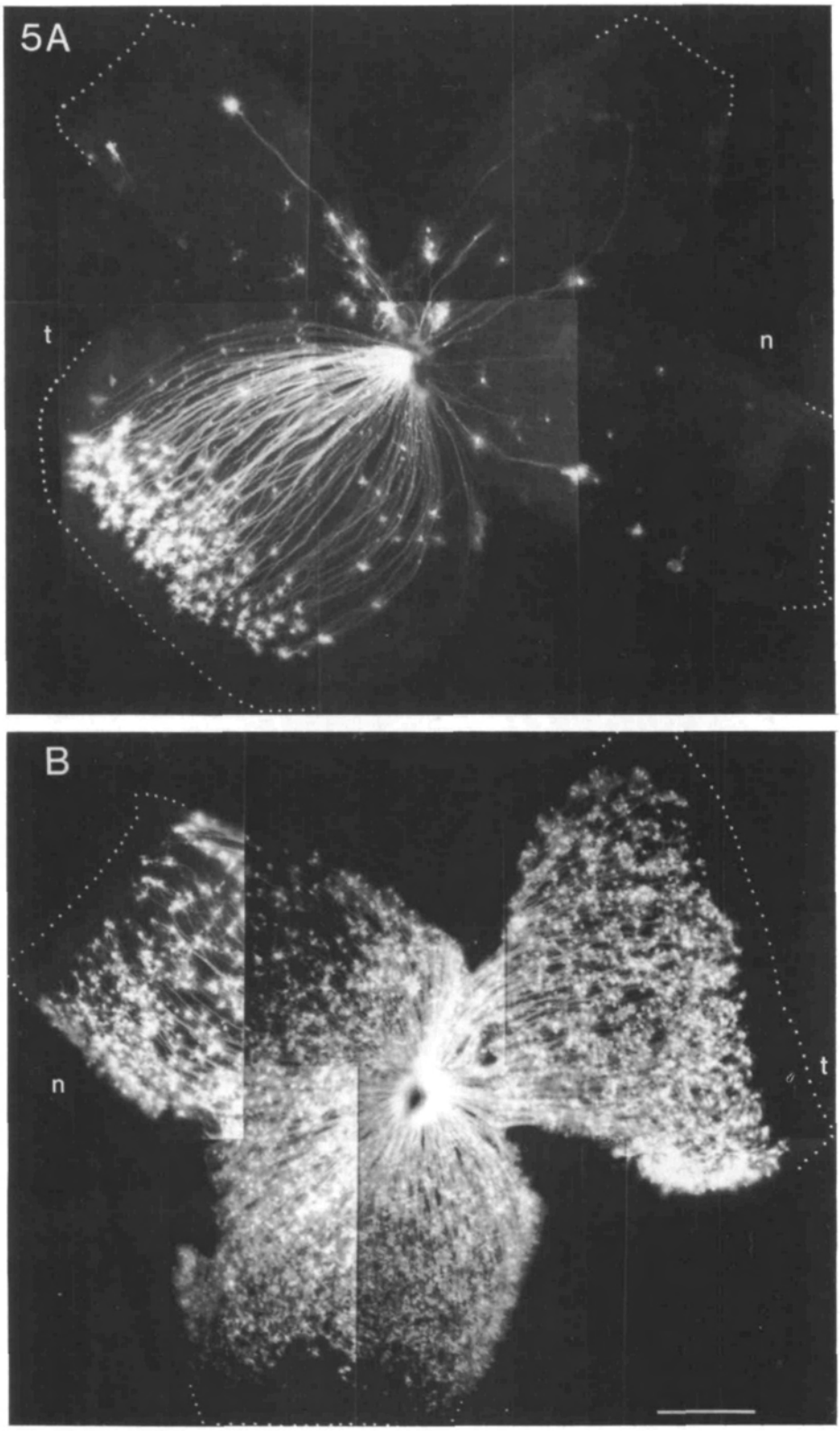

Fig. 5. E16.5 mouse retinae ipsilateral (A) and contralateral (B) to tract fill prepared as in Fig. 3. In the ipsilateral retina, ganglion cells with uncrossed axons are mainly found in the ventrotemporal crescent. Scale bar is 300 μm.

retina reach the optic tracts and it would appear that any later role of cell death in defining the line of decussation need be no more than a minor tidying operation (Martin *et al.* 1983; Jeffery, 1984; Insausti *et al.* 1984).

In the mouse, thymidine studies have shown that retinal ganglion cells are generated in a rough central-to-peripheral gradient with oldest cells in central retina and the younger cells peripherally (Dräger, 1985). Our findings in labelled contralateral retinae extend this observation to the fibre outgrowth of these cells. That is, fibres from the earliest generated cells cross the

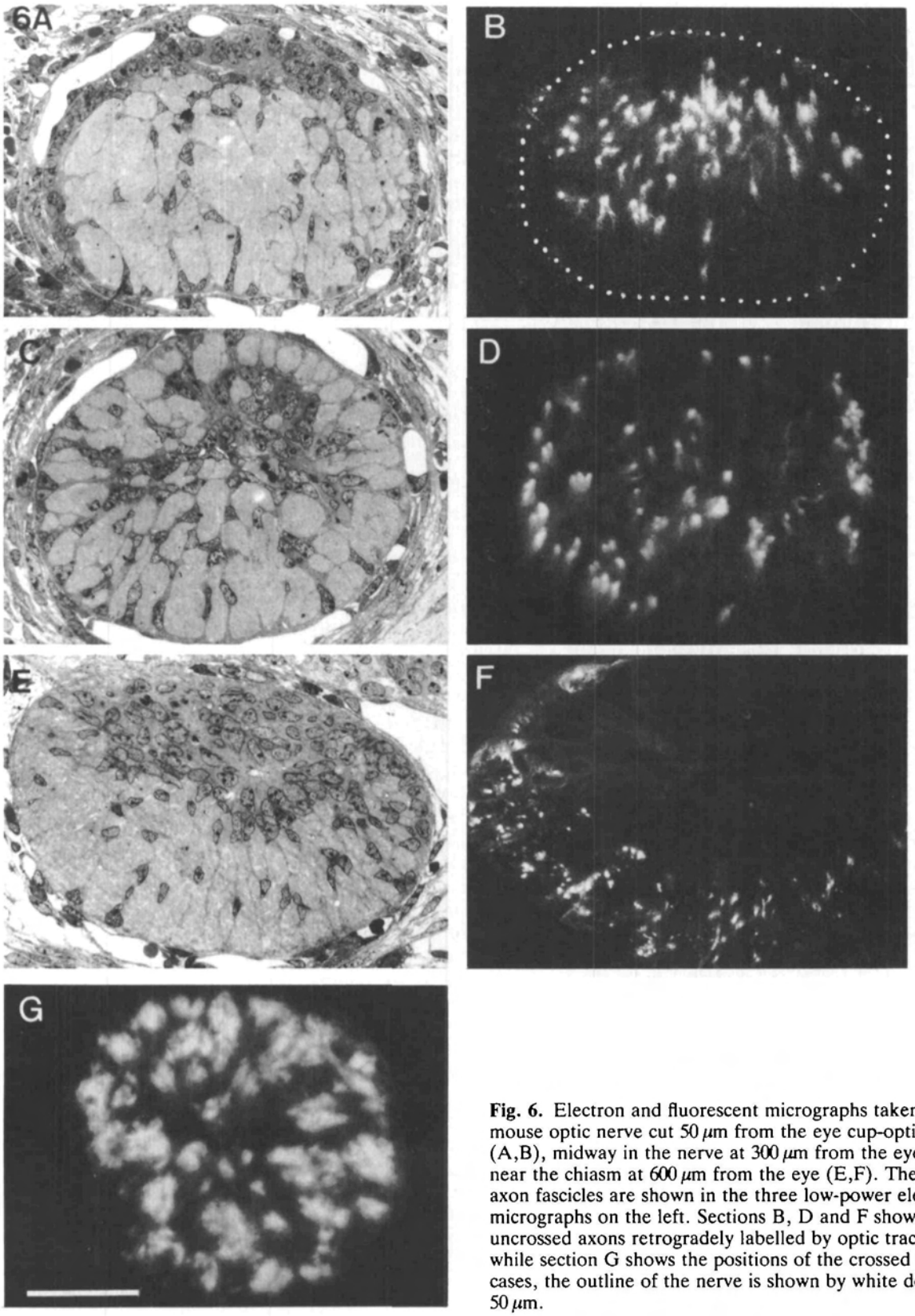

Fig. 6. Electron and fluorescent micrographs taken of an E14.5 mouse optic nerve cut 50 μm from the eye cup-optic nerve junction (A,B), midway in the nerve at 300 μm from the eye (C,D,G), and near the chiasm at 600 μm from the eye (E,F). The positions of all axon fascicles are shown in the three low-power electron micrographs on the left. Sections B, D and F show the position of uncrossed axons retrogradely labelled by optic tract fills of DiI while section G shows the positions of the crossed axons. In some cases, the outline of the nerve is shown by white dots. Scale bar is 50 μm.

chiasm first followed by the fibres of later generated ganglion cells. However, our methods cannot define whether fibres from peripheral retina reach the tract later because they need to course further or some other reason (see below).

Dräger (1985) has also shown that ganglion cells that

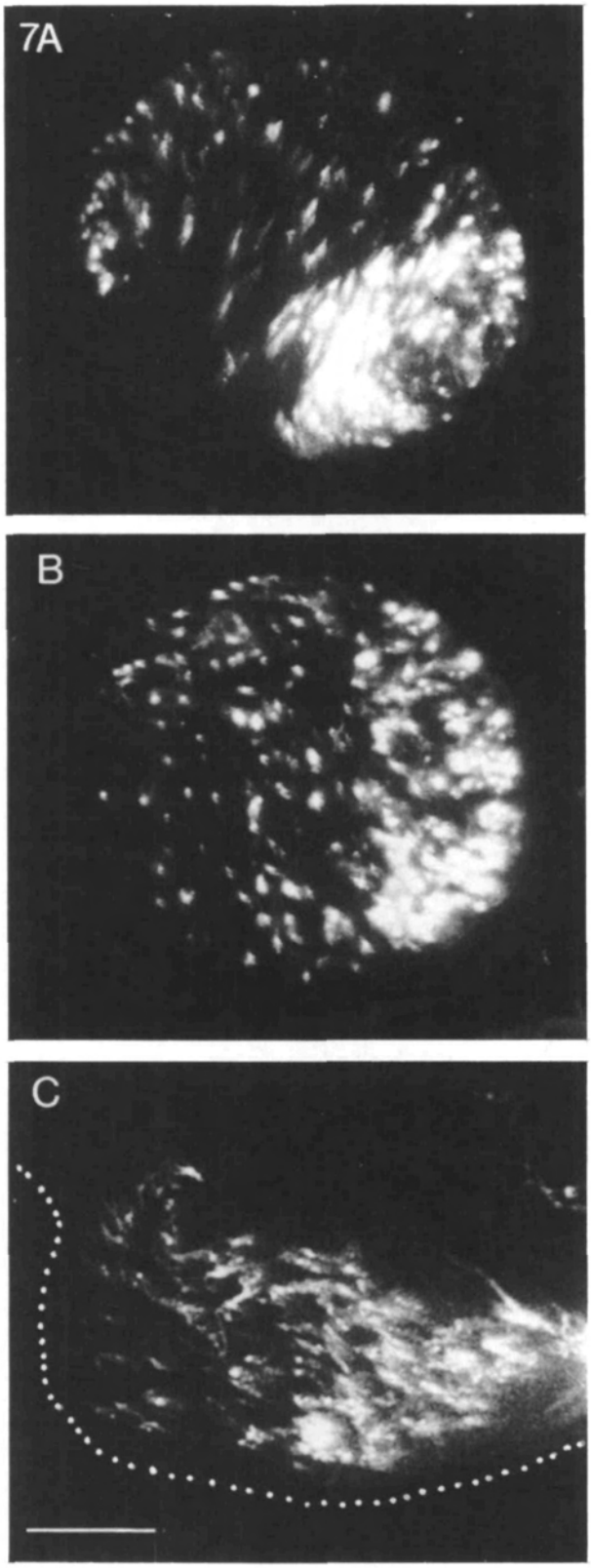

Fig. 7. Fluorescent micrographs of an E16.5 mouse optic nerve retrogradely labelled with DiI from the tract ipsilateral to the nerve. The sections were 50 μm from the eye cup-optic nerve junction (A), approximately midway along the course of the nerve at 450 μm from the eye cup (B), and near the chiasm at approximately 900 μm from the eye cup (C). Scale bar is 50 μm.

survive to adulthood are generated in mouse from E11 onwards and that the earliest population of cells shows a clear segregation, with cells in the ventrotemporal retina projecting ipsilaterally only and cells in the rest of the retina projecting contralaterally only. In view of this, it is surprising to find that the ganglion cells having the first ipsilaterally projecting axons in the optic tract at E14 lie in central retina (Godement *et al.* 1987) not in ventrotemporal retina as might have been expected from Dräger's results. These centrally placed early ipsilaterally projecting ganglion cells may represent the small population of ipsilateral cells present in the nasal retina of new born and even of adult rodents (Land and Lund, 1979; Dräger and Olsen, 1980), or possibly the early central ganglion cells were not seen in Dräger's material because they had all died during subsequent stages (Jeffery and Perry, 1982; Martin *et al.* 1983).

The discrepancies between Dräger's results and ours are difficult to explain. One solution would ascribe them to strain differences but at present there is no evidence to support such a view. The cells of the uncrossed component in the ventrotemporal retina that have, according to Dräger's evidence, already been generated by E11 remain unlabelled in our material until E16.5 even though cells in central retina, also generated at E11 can be labelled retrogradely with DiI at E14.5. This could be because axons, coming from peripheral ventrotemporal retina, take longer to reach the optic tract. However, the proposal that axons from peripheral sectors of the retina take this much longer to reach the tract (two days) is not very appealing because the distance between the ventrotemporal cells and the central cells is of the order of 300–500 μm. Preliminary (unpublished) estimates of axon growth in the eye stalk of E13 mouse embryos suggest a growth rate of about 1 mm per day. This is comparable to the rate of axon elongation by embryonic retinal growth cones *in vivo* of other species (Harris *et al.* 1985). The relatively late appearance of the retrogradely labelled cells in the ventrotemporal crescent may, therefore, have to be explained by extra time taken for axonal growth of the uncrossed as compared to the crossed component. Possibly the uncrossed axons pause longer at the chiasmatic choice point. The only other way of interpreting the present observations in accordance with Dräger's data, would be to consider that the ipsilaterally projecting cells originally present in central retina may migrate across the retinal surface as the retina grows. Although, at present, there is no evidence for such a phenomenon and evidence for its occurrence would not be easily obtained, the possibility cannot be ignored.

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