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Retinal Axons Following Overexpression of EphrinA Ligands on Retinal Ganglion Cell Axons

Dieter Dütting, Claudia Handwerker, and Uwe Drescher

Department of Physical Biology, Max-Planck-Institute for Developmental Biology, Spemannstrasse 35, FRG-72076 Tübingen, Germany

In the retinotectal projection, the Eph receptor tyrosine kinase ligands ephrinA2 and ephrinA5 are differentially expressed not only in the tectum, but also in a high-nasal-to-low-temporal pattern in the retina. Recently, we have shown that retrovirally driven overexpression of ephrinA2 on retinal axons leads to topographic targeting errors of temporal axons in that they overshoot their normal termination zones in the rostral tectum and project onto the mid- and caudal tectum. The behavior of nasal axons, however, was only marginally affected. Here, we show that overexpression of ephrinA5 affects the topographic targeting behavior of both temporal and nasal axons. These data reinforce the idea that differential ligand expression on retinal axons contributes to topographic targeting in the retinotectal projection. Additionally, we found that ectopic expression of ephrinA2 and ephrinA5 frequently leads to pathfinding errors at the chiasm, resulting in an increased stable ipsilateral projection. © 1999 Academic Press

Key Words: **retinotectal projection; axon guidance; ephrins; chiasm; pathfinding; topographic targeting; retrovirus; DiI labeling.**

INTRODUCTION

A current focus of interest in developmental neurobiology is the identification and characterization of mechanisms and molecules contributing to the formation of neuronal connections. A number of guidance molecules playing a role in this process have recently been identified. They can be classified tentatively according to some basic mechanisms by which axonal pathfinding to and guidance in the target area are thought to occur: chemoattraction by netrins and secreted semaphorins; chemorepulsion by netrins and secreted semaphorins; contact-mediated attraction by Ig domain-containing cell adhesion molecules, cadherins, and extracellular matrix (ECM) proteins; and, finally, contact-mediated repulsion by transmembrane semaphorins, ECM proteins, and ephrins (Tessier-Lavigne and Goodman, 1996; Müller, 1999).

The Eph family of receptor tyrosine kinases and their ligands contains at present 14 different receptors and 8 ligands, which are subdivided into two classes. The EphA subclass contains GPI-anchored ephrinA ligands interacting with EphA receptors and the EphB subclass transmembrane-anchored ephrinB ligands interacting with a complementary set of EphB receptors. This subdivision is followed relatively strictly, and few exceptions to this classification have been reported. Characteristics of the Eph family are the extensive promiscuity in the interaction of ligands and receptors within each subgroup and the bidirectional signaling of EphB family members, i.e., the capacity of ephrinB ligands to transduce signals. Other typical features of the Eph family are the lack of induction of mitogenic responses and the requirement for membrane anchorage of ephrinA and -B ligands to activate their receptors (for reviews see, e.g., Drescher, 1997; Gale and Yancopoulos, 1997; Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999; O'Leary and Wilkinson, 1999).

Members of the Eph family are predominantly and widely expressed in the developing and adult nervous system. In early development, the embryo appears to be subdivided into domains defined by reciprocal and mutually exclusive expression of the receptor subclasses and their corresponding ligands (Gale *et al.*, 1996). As the Eph family appears to exert its function predominantly by a repellent mechanism, the complementary expression of ligands and receptors indicates a patterning function of the Eph family. For example, the growth of axons (e.g., Cheng *et al.*, 1995;

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Drescher *et al.*, 1995; Nakamoto *et al.*, 1996) or the movement of cells (e.g., Krull *et al.*, 1997; Smith *et al.*, 1997; Wang and Anderson, 1997; Mellitzer *et al.*, 1999; Xu *et al.*, 1999) expressing a certain receptor (ligand) is restricted to regions of the embryo devoid of the corresponding ligand (receptor) class. However, in addition to complementary expressions, overlapping expressions of ligands and receptors have been found (e.g., Flenniken *et al.*, 1996; Hornberger *et al.*, 1999). Additionally, it became clear that, in addition to repellent interactions, members of the Eph family are involved in other processes like attraction of cells (Pandey *et al.*, 1995) and cell adhesion (Böhme *et al.*, 1996; Winning *et al.*, 1996; Holash *et al.*, 1997; Zisch *et al.*, 1997; Jones *et al.*, 1998).

The retinotectal projection is a well-characterized system (Mey and Thanos, 1992; Holt and Harris, 1993) serving as a model for topographic projections which are numerous in the central and peripheral nervous system. In such projections there is a faithful transfer of spatially organized information from one area of the brain to another. This means that cells neighboring in the projecting area are connected to cells neighboring in the target area. In the retinotectal projection temporal retina is connected to rostral/anterior tectum and nasal retina to caudal/posterior tectum. Similarly, dorsal and ventral retina are connected to ventral/lateral and dorsal/medial tectum, respectively. How such precise connections are formed has been a longstanding question. The presently prevailing hypothesis, formulated by Sperry decades ago, is the chemoaffinity hypothesis (Sperry, 1963). He proposed that molecules expressed in gradients in the tectum would provide positional information along which ingrowing retinal axons endowed with receptors expressed in gradients might find their correct retinotopic position.

Both ephrinA2 and ephrinA5 are expressed in overlapping high-caudal-to-low-rostral gradients in the tectum (Monschau *et al.*, 1997), and a corresponding receptor, EphA3, is found in a high-temporal-to-low-nasal expression in the retina (Cheng *et al.*, 1995). This complementary pattern makes these molecules likely candidates for being involved in the topographic mapping of retinal axons, which is further supported by comparably high binding affinities between EphA3 and ephrinA2/ephrinA5 (Monschau *et al.*, 1997). In addition to these three molecules there are at least nine other Eph family members expressed during development of the retinotectal projection, often in dynamic spatially and temporally restricted patterns (for reviews see Drescher, 1997; Pasquale, 1997; Flanagan and Vanderhaeghen, 1998; O'Leary *et al.*, 1999).

In vitro, ephrinA5 repels both nasal and temporal axons, with temporal axons being more sensitive than nasal ones (Monschau *et al.*, 1997). EphrinA2 exhibits a repellent activity only for temporal axons, with no effect on nasal axons (Nakamoto *et al.*, 1996; Monschau *et al.*, 1997). Soluble neutralizing forms of the ephrinAs completely abolished the striped outgrowth of retinal axons growing on alternating lanes of rostral and caudal tectal membranes (Ciossek *et al.*, 1998). *In vivo,* temporal, but not nasal axons avoid patches of ectopically expressed ephrinA2 (Nakamoto *et al.*, 1996). A loss-of-function analysis showed that ephrinA5 is essential for the correct topographic mapping of retinal axons in the mammalian visual system (Frisen *et al.*, 1998). Here temporal axons formed termination zones not only in the rostral, but also in the topographically inappropriate caudal superior colliculus, a structure homologous to the chick optic tectum. Additionally, a transient overshooting of retinal axons into the inferior colliculi in these mutant mice points to a function of ephrinA5 in limiting the growth of retinal axons to the superior colliculi. Additionally, ephrinA5 mutant mice showed severe topographic targeting errors of both temporal and nasal retinal axons into the lateral geniculate nucleus (LGN), from which visual information is further passed on to the visual cortex (Feldheim *et al.*, 1998).

Beyond their expression in the tectum, ephrinA2 and ephrinA5 are differentially expressed in a high-nasal-to-low-temporal pattern on retinal ganglion cell axons projecting onto the tectum (Hornberger *et al.*, 1999; see also Marcus *et al.*, 1996; Brennan *et al.*, 1997; Connor *et al.*, 1998). Toward an understanding of the expression of ephrin molecules on retinal axons, we took advantage of the stripe assay. Here, normally only temporal axons are sensitive to repellent guidance cues of the caudal tectum (Walter *et al.*, 1987). We could show that retrovirally driven overexpression of either ephrinA2 or ephrinA5 on retinal axons led to an abolishment of the striped outgrowth behavior of temporal axons. Conversely, treatment with PI-PLC both removed ephrinA ligands from retinal axons and induced a striped outgrowth of nasal axons formerly insensitive to the repellent guidance cues (Hornberger *et al.*, 1999). These data suggested a prominent role of retinally expressed ephrins in the guidance of RGC axons, possibly by modulating the function of coexpressed Eph receptors. *In vivo,* after retinal overexpression of ephrinA2, temporal axons overshoot into the topographically inappropriate mid- and caudal tectum. Nasal axons, however, were only marginally affected (Hornberger *et al.*, 1999).

Here we show that retinal overexpression of another Eph-receptor ligand, ephrinA5, led to topographic targeting errors of both temporal and nasal axons, that is, an overshooting of their normal termination zones into more caudal positions of the tectum. In addition to these targeting errors, overexpression of both molecules led to pathfinding errors at the chiasm, resulting in an increased ipsilateral projection.

MATERIALS AND METHODS

Cloning of Retroviral Constructs

A fragment from the cDNA encoding ephrinA5 was amplified by PCR using the primers 5′-ATA TAC CAT GGC GCA CGT GGA G-3′ and 5′-GGA GCA TAC TGT GCT ATA ATA TC-3′, intro-

ducing an *Nco*I site at the initiation codon. This fragment was cloned into a *Nco*I/*Sma*I vector of the pCla12Nco helper plasmid (Hughes *et al.*, 1987), from which the corresponding *Cla*I fragment containing the 5′ UTR of v-src was cloned into pRCAS(B). High-titer stocks were generated by transfection of these plasmids into line zero chicken embryo fibroblasts according to published protocols (Fekete and Cepko, 1993; Morgan and Fekete, 1996). Titers used for injection into the eye of chick embryos were $>10^8$ IU/ml. The generation of an ephrinA2-expressing virus has been described in Hornberger *et al.* (1999).

Injection of Retroviral Liquid

Fertilized chicken eggs (White Leghorn) obtained from a local supplier (A. Weiss) or from Lohmann Tierzucht GmbH, Cuxhaven (virus-free SPF eggs), were incubated at 38°C and 60% humidity with occasional turning for 40 to 45 h and windowed. At Hamburger–Hamilton stages 11–12 (10- to 14-somite embryos; embryological day E1.5), black ink was injected into the region under the head of the embryo for a better visualization of the injection area. Through a hole in the membrane above the right part of the forebrain, concentrated retroviral solution (titer $>10^8$ IU/ml) was injected into the right optic vesicle (OV) by means of a pulled-out 25-μl microcapillary (see also Morgan and Fekete, 1996). The tip of the capillary was introduced into the OV through its single-layered posterior wall (see Dütting and Thanos, 1995; Fig. 1). By adding Fast green (Raymond A. Lamb, Laboratory Supplies, London) to the viral solution, its flow from the right OV into the ventricle of the forebrain (prosencephalon) through the optic stalk region was followed. The liquid leaves the embryo through the anterior neuropore and frequently in addition through a hole produced in the anterior wall of the OV. Most likely, the whole pathway from the presumptive right eye to the future optic chiasm gets infected by the virus. On the basis of later immunofluorescence analysis, infections of the mesencephalon/tectum occurred only rarely and if so, contained only few patches.

Labeling the Retinotectal Projection of a Chick Embryo

The trajectories of retinal axons in the final retinotectal projection of the chick were studied after inserting a small number of crystals of the recrystallized fluorescent dye DiI (D282) or the so-called "large crystal" DiI (D3911; Molecular Probes) on E14.5/E15.5 into the temporodorsal or nasodorsal periphery of the right retina by means of fine forceps (Thanos and Bonhoeffer, 1987). These locations were chosen to get the mature projection completely onto the ventral tectal half and to enhance the chance of visualizing mistargeting. Two days after insertion, on E16.5 or 17.5, the local anterograde labeling of temporal or nasal axons was terminated by isolating the midbrain of the chick (both optic tecta were freed of the pia) and the right retina. The tissues were fixed overnight in 4% paraformaldehyde–PBS and then kept in PBS. Whole mounts of both tecta were prepared by cutting the tectal lobes along the rostrocaudal axis into a ventral (lateral) and a dorsal (medial) half. The tectal halves and the retinas were mounted on cleaned glass slides with a few drops of *n*-propylgallate in glycerol (2 g *n*-propylgallate (Sigma), 8 ml 0.1 M phosphate buffer (pH 7.2), 90 g glycerol) under cover dishes. Thereafter, drawings of the tectal and retinal outlines were made using a camera lucida attachment to a dissecting microscope. Subsequently the projections of retinal axons on retina and tectal halves were added free-hand under a fluorescence microscope. Photographs were taken with a Zeiss (Axiophot) fluorescence microscope, equipped with a 100-W mercury lamp and a Cy3 filter, and subsequently reassembled using PhotoShop 5.0 (Adobe).

Recrystallization of DiI and the Evaluation of Termination Zones (TZ)

DiI (D282, 100 mg; Molecular Probes), dissolved in 5 ml 100% ethanol, was distributed into five 1-ml portions. These portions were further diluted with 1 ml 100% ethanol each and heated to 40°C. After adding 50 μl 40 mM NaCl to each tube, the five 2-ml portions were distributed after heating (40°C) into 16 Eppendorf tubes (0.6 ml portions) which were kept open at room temperature for 2–3 days. DiI crystallized along the inner wall of the tubes during the slow evaporation of the ethanol.

The appearance of a TZ depends on the number of axons labeled and on the intensity of axonal labeling. Previously (Dütting and Thanos, 1995; Müller *et al.*, 1998), the large crystal DiI (D3911) gave a bright, intense labeling of single axons. If enough axons were labeled, the TZ appeared as a bright fluorescent circular or elliptic zone. More recently, crystals of this quality have ceased to be available. We therefore did most experiments with recrystallized D282 DiI or, since the beginning of 1999, with crystalline D3911 DiI (Lot 4591), the crystals of which label retinal axons less intensively than formerly. Depending on the number of nasodorsal axons labeled, a TZ in the caudoventral tectum can lose its compactness and may look more diffuse. Few axons labeled lead to single axon termini in the target zone. Frequently the axons projecting to the TZ were only barely visible on a highly red fluorescing background. Obviously, the photographic evaluation of such preparations provided less detail than formerly.

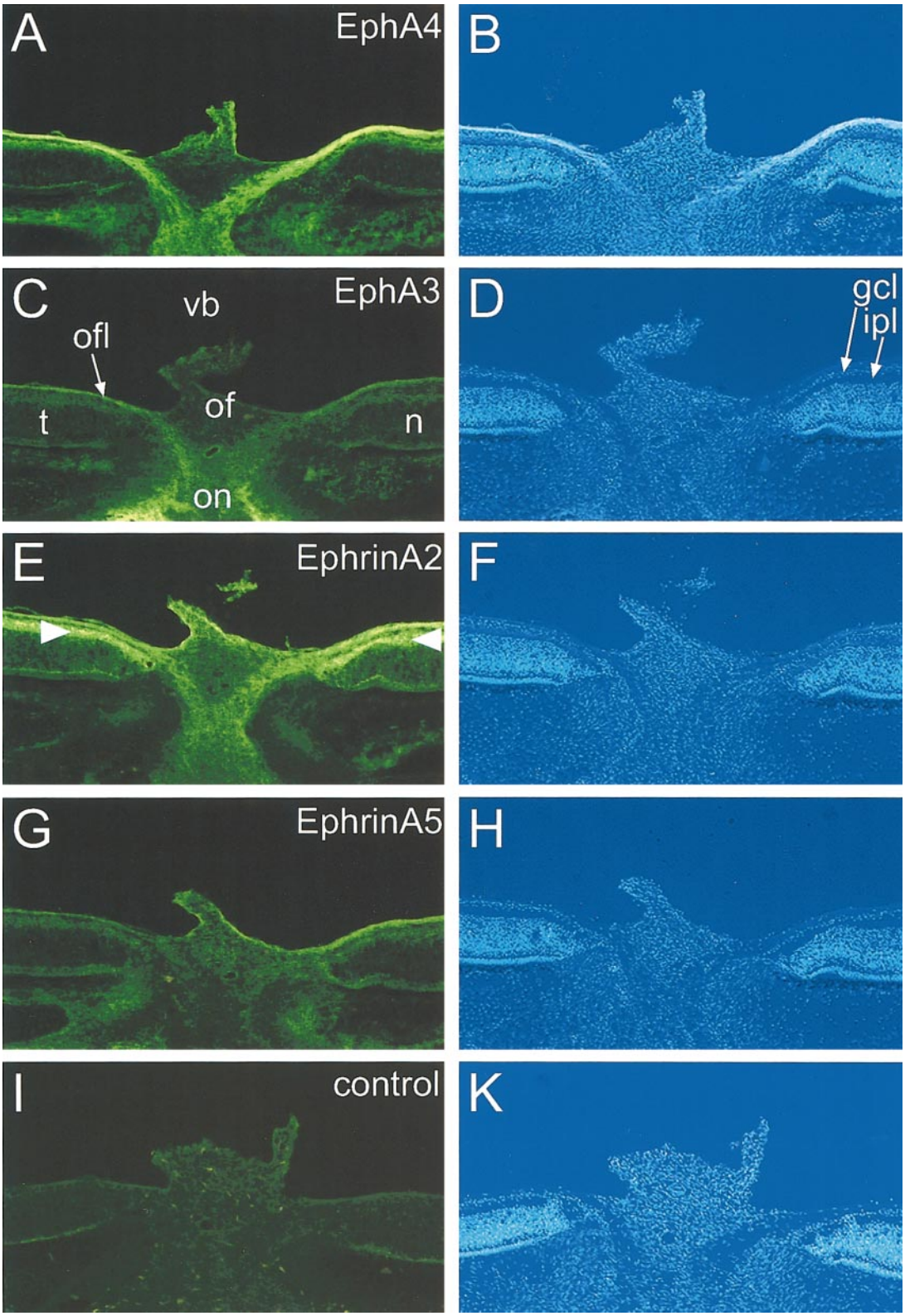
RESULTS

Expression Pattern of EphA Family Members during Development of the Retinotectal Projection

We investigated, by using specific monoclonal antibodies (Hornberger *et al.*, 1999), whether members of the EphA family are expressed not only at the onset of the formation of the retinotectal projection (E6; Hornberger *et al.*, 1999), but also at later stages (E12). Figure 1 summarizes the expression of the EphA receptors EphA3 and EphA4 and their ligands ephrinA2 and ephrinA5 in E12 horizontal sections, cut at the level of the exit point of retinal axons into the optic nerve.

In general, the expression patterns of these molecules at E12 are similar to those at E6, but some small changes have occurred. EphA4 appears to be slightly more highly expressed on nasal than on temporal retinal axons. EphA3 is expressed on temporal axons and, to some extent, also on nasal axons. In more dorsal parts of the retina, this differential expression is more pronounced.

EphrinA2 and ephrinA5 are expressed more strongly on nasal than on temporal retinal axons, whereby the differ-



ence in expression is more distinct for ephrinA5. As is true for EphA3, the differences in expression level between nasal and temporal retina are more pronounced in dorsal than in ventral parts of the retina. Notably, there is prominent staining of the inner plexiform layer by the anti-ephrinA2 antibody, which is equally strong in nasal and temporal retina (Fig. 1E). The inner plexiform layer contains no cell bodies and consists of the dendrites of retinal ganglion cell axons and neurites of amacrine and bipolar cells.

The strength of staining for EphA4 and ephrinA2 on retinal axons at E12 appears to be similar or even stronger than the corresponding expression at E6. It has to be kept in mind, however, that the number of retinal axons at the exit point of the eye is also increased by E12. EphA3 and ephrinA5 seem to be downregulated compared to E6.

In sum, these data indicate that EphA receptors and ligands are expressed on retinal axons throughout the major phase of ingrowth of these axons into the tectum.

Pathfinding Errors at the Chiasm after Retinal Overexpression of EphrinA2 and EphrinA5

To further characterize the function of ephrinA ligand expression in the retina, an RCAS(B)-type based retrovirus (Hughes *et al.*, 1987) containing the cDNA for chick ephrinA5 or ephrinA2 was injected into the optic vesicle of chick embryos of developmental age day E1.5 (stage 11–12; Hamburger and Hamilton, 1951). At E14.5 or E15.5, small populations of retinal axons were labeled by inserting DiI crystals into small peripheral areas of the temporodorsal or nasodorsal retina. Two days later, at E16.5 or E17.5, the growth behavior of retinal fibers was investigated. The great sensitivity of this labeling method even allows the path of single fibers to be followed from the retina to the tectum.

Normally, during development of the chick retinotectal projection, axons in the retina grow straight to the optic fissure, the exit point of retinal axons out of the eye into the optic nerve (Halfter and Deiss, 1984; Halfter *et al.*, 1985). Subsequently, these axons migrate in a highly ordered manner within the optic nerve toward the chiasm and cross at this midline choice point over to the contralateral side. Then they travel through the optic tract to the tectum, which they invade from the rostroventral pole (Ehrlich and Mark, 1984; McLoon, 1985).

Crossing of retinal axons at the chiasm to the contralateral side is not complete in chick: during development there is a transient small fraction of retinal axons (less than 10–15% of the total number of fibers) which do not cross at the chiasm but grow to the ipsilateral tectum. This projection, however, is entirely eliminated during the period of morphogenetic ganglion cell death between E12 and E16 (O'Leary *et al.*, 1983; Thanos and Bonhoeffer, 1984; Ernst *et al.*, 1998).

Interestingly, in ephrinA5-infected embryos we often found a strongly increased number of retinal ganglion cell axons projecting onto the ipsilateral tectum apparently reflecting severe pathfinding errors at the chiasm. Also, in contrast to the normal ipsilateral projection, these aberrant projections appear to be stable as the analyses were done at E16.5 to E17.5. Similar data were obtained after retrovirally driven overexpression of ephrinA2 (Tables 1 and 2).

An example of a pathfinding error at the chiasm after ephrinA5 overexpression is illustrated in Fig. 2. In this embryo, a number of retinal axons were DiI labeled at E14.5–E16.5 in a peripheral nasodorsal location of the retina. The analysis showed an apparently undisturbed growth of retinal fibers in the eye (not shown) and optic nerve (Fig. 2); however, proximal to the midline, the axons diverged and projected into both the contralateral and, in higher numbers, the ipsilateral optic tract.

Overall, pathfinding errors at the chiasm were found for both nasal and temporal retinal axons with a similar frequency. In Tables 1 and 2, the extent of pathfinding errors of retinal axons at the chiasm has been summed up and classified according to the strength of the ipsilateral projection.

To investigate the cause of this pathfinding error in more detail, the chiasm itself and the region around the chiasm were analyzed by immunofluorescence staining in uninfected and ephrinA-infected embryos. Figure 3 shows the asymmetric expression of EphA3 (Fig. 3A) and the uniform expression of EphA4 (Fig. 3B) on retinal axons within the optic fiber layer and the optic nerve. Interestingly, EphA3 and, to a lesser extent, EphA4 additionally are expressed ensheathing the optic nerve. Figures 3C and 3D show a comparably weaker staining of ephrinA2 and ephrinA5 in the optic nerve and in the ventral diencephalon. Specifically, both molecules are also expressed at the border of the

FIG. 1. Expression pattern of EphA family members at E12 in the chick retinotectal projection. Cryostat sections of E12 embryos cut horizontally at the level of the optic fissure where retinal axons leave the eye into the optic nerve are shown. The fissure is located in the ventral region of the eye. Sections were stained with monoclonal antibodies against EphA4 (A), EphA3 (C), ephrinA2 (E), and ephrinA5 (G). In (I) an analysis in which the first antibody has been omitted is shown. The corresponding DAPI staining to (A, C, E, G, I) is shown in (B, D, F, H, K) to highlight the localization of the optic fiber layer (ofl), ganglion cell layer (gcl), and inner plexiform layer (ipl) labeled in (C) and (D). EphA4 is slightly more highly expressed on nasal than on temporal axons. In this ventral region of the eye, EphA3 is expressed on temporal axons, but to a considerable degree also on nasal axons. EphrinA2 and ephrinA5 are expressed more highly in the nasal than in the temporal optic fiber layer. This difference in expression is more pronounced for ephrinA5. A very strong expression of ephrinA2 in the inner plexiform layer is marked by arrowheads. of, optic fissure; on, optic nerve; n, nasal; t, temporal; vb, vitreous body.

TABLE 1

Pathfinding Errors of Temporodorsal (A) and Nasodorsal (B) Retinal Axons at the Chiasm and/or Topographic Targeting Errors after Retinal Overexpression of EphrinA5

↓ Chiasm effect / → Topographic effects	Strong to medium	Medium to weak	No to weak	Total
(A) Temporodorsal projections				
Contra- and mainly contralateral	1	3	8	12 (41.4%)
Contra- and ipsilateral	2	3	1	6 (20.7%)
Ipsi- and mainly ipsilateral	—	5	6	11 (37.9%)
Total	3 (10.4%)	11 (37.9%)	15 (51.7%)	Σ29
(B) Nasodorsal projections				
Contra- and mainly contralateral	—	1	10	11 (35.5%)
Contra- and ipsilateral	—	4	2	6 (19.3%)
Ipsi- and mainly ipsilateral	—	11	3	14 (45.2%)
Total	—	16 (51.6%)	15 (48.4%)	Σ31

Note. (A) For temporodorsal axons a topographic effect is indicated by axons passing over the rostroventral termination zone (TZ) toward the mid- and, rarely, caudal ventral tectum. Strong-to-medium defect, TZ poorly or not developed, many overshooting axons; medium-to-weak effect, TZ more developed, fewer overshooting axons. In three cases, a normal TZ appeared on the contralateral tectum, but diffusely distributed single axon termini on the ipsilateral tectum. (B) For nasodorsal axons a diffuse, extended TZ in the caudoventral tectum with axons overshooting to the caudal rim was considered a medium-to-weak defect. Only projections which have been reasonably well labeled with DiI have been taken into account.

ventral diencephalon in direct proximity to the optic nerves/chiasm.

The normally observed crossed-finger-like structure (Figs. 3A–3D) can be taken as an indication of the ordered crossing of retinal axons at the chiasm (Mark and Ehrlich, 1984). However, this structure apparently is lost after retinal ephrinA5 overexpression, indicating a structurally disorganized chiasm (Figs. 3E and 3F). Here, the infected retina and the corresponding optic nerve are strongly stained by an anti-ephrinA5 antibody (Fig. 3E). Lack of ordering in the optic nerve and the chiasm is evident from staining with an anti-EphA3 antibody (Fig. 3F).

Generally, there were also patches of ectopic ephrinA in the ventral diencephalon, indicating viral infection outside the optic pathway. Analysis of sections of tecta from infected embryos, however, showed no or only very few patches of ephrinA infection (data not shown).

TABLE 2

Pathfinding Errors of Temporodorsal (A) and Nasodorsal (B) Retinal Axons at the Chiasm and/or Topographic Targeting Errors after Retinal Overexpression of EphrinA2

↓ Chiasm effect / → Topographic effects	Strong to medium	Medium to weak	No to weak	Total
(A) Temporodorsal projections				
Contra- and mainly contralateral	5	6	4	15 (37.5%)
Contra- and ipsilateral	7	6	2	15 (37.5%)
Ipsi- and mainly ipsilateral	5	2	3	10 (25%)
Total	17 (42.5%)	14 (35%)	9 (22.5%)	Σ40
(B) Nasodorsal projections				
Contra- and mainly contralateral	—	0	5	5 (38.5%)
Contra- and ipsilateral	—	1	2	3 (23.0%)
Ipsi- and mainly ipsilateral	—	1	4	5 (38.5%)
Total	—	2 (15.4%)	11 (84.6%)	Σ13

Note. (A) The strong-to-medium topographic mistargeting of temporal axons after ephrinA2 overexpression occurs with higher frequency (42.5%) compared to that after ephrinA5 (10.4%). Temporal axons consistently passed over the rostroventral termination zone (sometimes represented as very diffuse TZ) to the mid- and, frequently, caudal ventral tectum without forming there a TZ. In three cases the spread was not only along the rostrocaudal, but also along the dorsoventral tectal axis. Medium-to-weak topographic effects frequently showed diffuse TZs on the rostroventral tectum and axons overshooting to the mid-, but rarely to the caudal, ventral zone. No-to-weak effects are those with compact TZs in the rostroventral tectum and few overshooting axons. Part of the data concerning the topographic targeting errors after EphrinA2 overexpression have been shown already in Hornberger *et al.* (1999). In this paper, emphasis was placed on a discussion of the topographic effects after overexpression of ephrinA2 omitting the effects on axon pathfinding at the chiasm. In the meantime, more temporodorsal projections were obtained and have been added to the tables. (B) Only two projections were classified as medium-to-weak topographic effect (in one case, single axons terminated on the ventral and dorsal halves of both tecta at the far caudal rim with side branching also in the midtectum; in the other case a diffuse termination zone was formed with a number of axons found in the most caudal region of the tectum). All others showed more or less compact TZs in the caudoventral tectum with few overshooting axons.

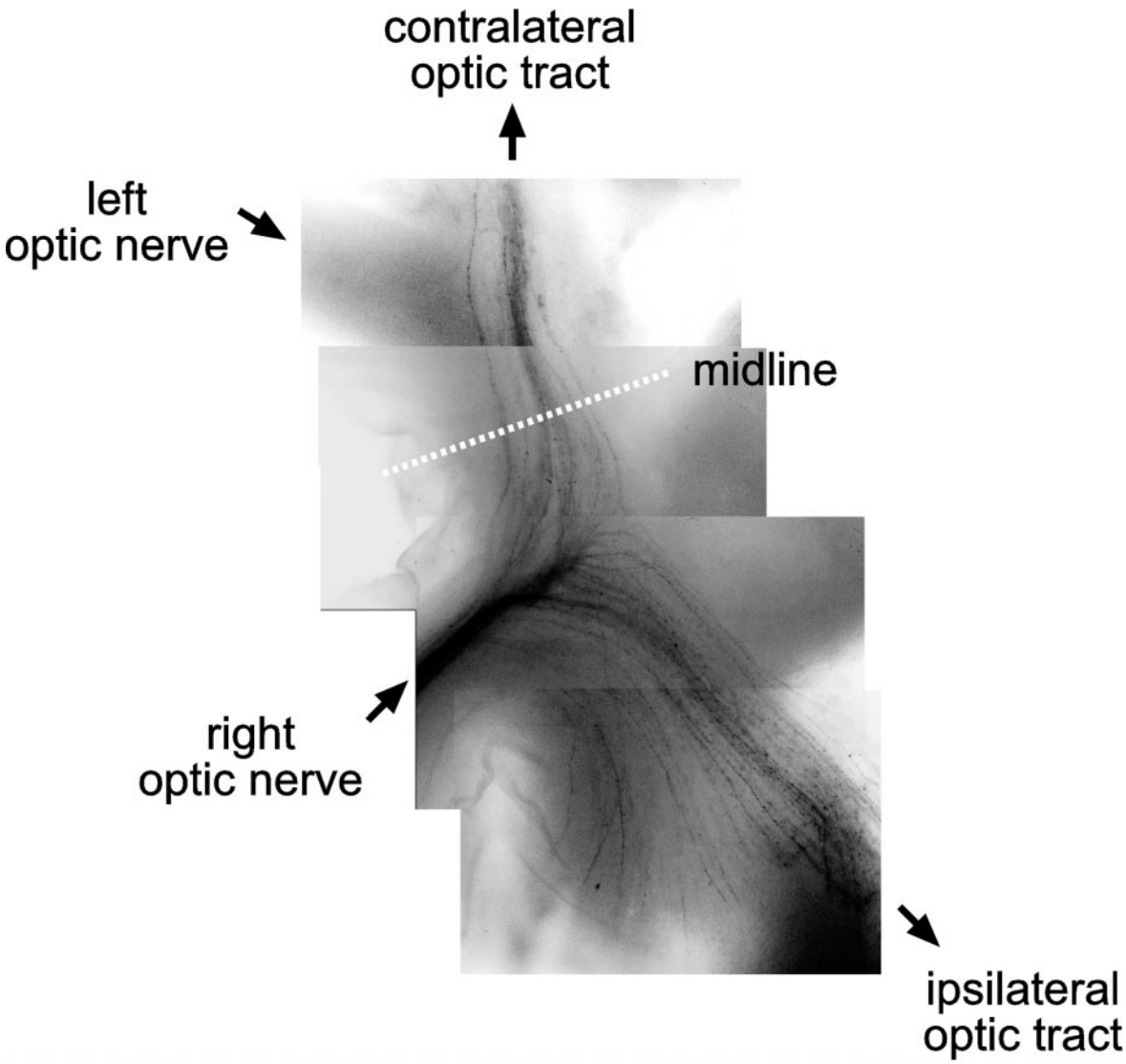


FIG. 2. Overexpression of ephrinA5 leads to pathfinding defects at the chiasm. After infection of the optic vesicle with an ephrinA5-expressing retrovirus at E1.5, retinal axons from the nasodorsal periphery of the retina were DiI labeled at E14.5. Two days later, at E16.5, the tecta were isolated together with the optic tract, chiasm, and optic nerve. Labeled retinal axons originating from the right eye (right optic nerve) split up proximal to the midline and invaded both the ipsi- and the contralateral optic tract. The axons projected on both tecta to terminal zones in the caudoventral tectum. From E16 on, there is normally only a contralateral projection in the chick.

Retinal Overexpression of EphrinA5 Leads to Topographic Targeting Errors of Nasal and Temporal Axons in the Tectum

The mature retinotectal projection with compact, well-defined TZ on the tectum is established in chick by E16 (Nakamura and O'Leary, 1989). At that time, DiI labeling shows retinal axons projecting directly and without branching to the TZ, and any significant overshooting of axons is no longer apparent (see for example Fig. 4). Early in development of the retinotectal projection, between E10 and E14, retinal fibers in chick "overshoot" the zone of final termination (Nakamura and O'Leary, 1989; Dütting and Thanos, 1995). Collaterals formed along the axons will eventually give rise to TZ at topographically appropriate sites; overshooting axon segments are degraded (Nakamura and O'Leary, 1989).

After retinal overexpression of ephrinA5, in total 29 temporodorsal and 31 nasodorsal projections were obtained, for which the guidance behavior on the tectum could be analyzed. As summarized in Table 1A, clear retinotopic targeting errors of temporodorsal axons were found in about half of the cases (48.3%). They were further classified into those with strong-to-medium targeting defects (10.4%) and

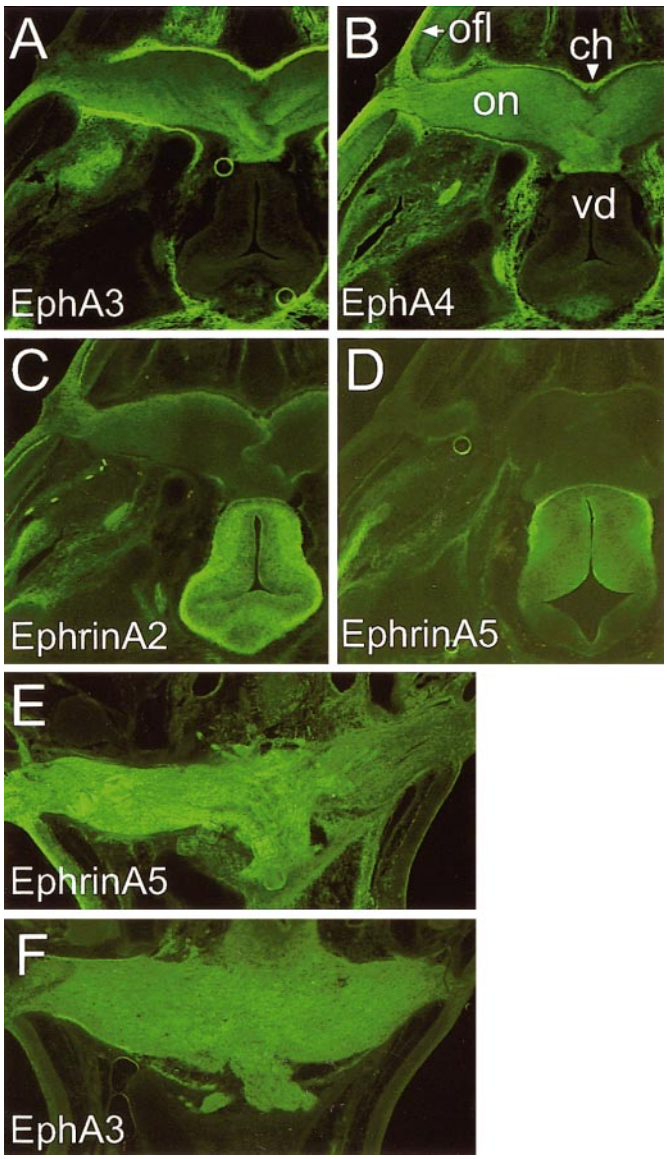


FIG. 3. Expression pattern of Eph family members at the chiasm. The expression of EphA3 (A), EphA4 (B), ephrinA2 (C), and ephrinA5 (D) at E8 in horizontal sections cut at the level of the chiasm is shown. (E) Staining of a section cut at a comparable level derived from an embryo infected in the retina with an ephrinA5-expressing retrovirus. (F) A section from the same embryo as in (E) stained with an antibody against EphA3. The location of the optic fiber layer (ofl), optic nerve (on), chiasm (ch), and ventral diencephalon (vd) is indicated.

those with medium-to-weak defects (37.9%). The basis for this classification was the presence or absence of a faint diffuse or compact TZ and the approximate number of axons overshooting this zone of normal termination.

An example of a strong-to-medium targeting defect of retinal axons DiI labeled in the temporodorsal periphery after ephrinA5 overexpression is shown in Fig. 5. A TZ is formed only diffusely on the rostroventral tectum, axons overshoot this zone and project onto the topographically inappropriate mid- and, to a lesser extent, caudal tectum. The retinotectal projection of this embryo was found on both the contra- and the ipsilateral tectum and looked alike on both tecta (Fig. 5 shows the ipsilateral projection). Medium-to-weak topographic effects after ephrinA5 overexpression frequently consisted of normal-looking termination zones on the rostroventral tectum, with several axons overshooting the TZ toward midtectal areas.

For nasodorsal axons as well, about half of the projections were affected after ephrinA5 overexpression (51.6%), but the strength of the effect was weaker than that seen for temporodorsal projections. As in normal projections, nasodorsal axons project to the caudoventral tectum, but form diffuse elongated termination zones with axons overshooting the TZ to a far caudal terminal position. Such phenotypes were classified as medium-to-weak effects (Table 1B).

The projection shown in Fig. 6 was exclusively to the ipsilateral tectum (DiI labeling in nasodorsal retinal periphery E15.5 to 17.5). In fact, the topographic disturbances in nasodorsal projections after ephrinA5 overexpression were largely restricted to embryos with strong ipsilateral projections (Table 1B). Embryos with projections to the contralateral tectum alone showed usually no effect (no-to-weak category).

Infections with control viruses did not affect the retinotectal projection. Normal compact TZ of temporodorsal and less compact TZ of nasodorsal axons with few overshooting axons were observed (Hornberger *et al.*, 1999). Either viruses expressing alkaline phosphatase (RCAS(B)AP) or those containing no insert (RCAS(B)) were used as a control (Fig. 4). Immunofluorescence analyses of infected embryos to check for the spread of retroviral infection showed little or no expression in the tectum.

A comparison of the strength of the topographic targeting defects seen after ephrinA5 vs ephrinA2 overexpression (Tables 1 and 2, see also Hornberger *et al.*, 1999) showed obvious differences: compared to the ephrinA2 effect, the ephrinA5 effect on temporal axons is weaker and less consistently observed, i.e., axons overshoot the rostral termination zone to termini in the mid- and caudal tectum less frequently after ephrinA5 overexpression than after ephrinA2 overexpression (but see Discussion). On the other hand, a significant effect on nasal axons is apparent after ephrinA5, but only rarely after ephrinA2 overexpression. In these cases, compact termination zones in the caudal tectum become diffuse with axons overshooting to far caudal positions.

Additionally, these topographic defects were compared with pathfinding errors at the chiasm. The alignment shows in most cases no correlation between pathfinding errors at the chiasm and topographic mistargeting, which is most clearly evident for infections with the ephrinA2-expressing retrovirus: some ephrinA2-overexpressing embryos showing a predominantly ipsilateral projection formed normal TZ at correct topographic positions; con-

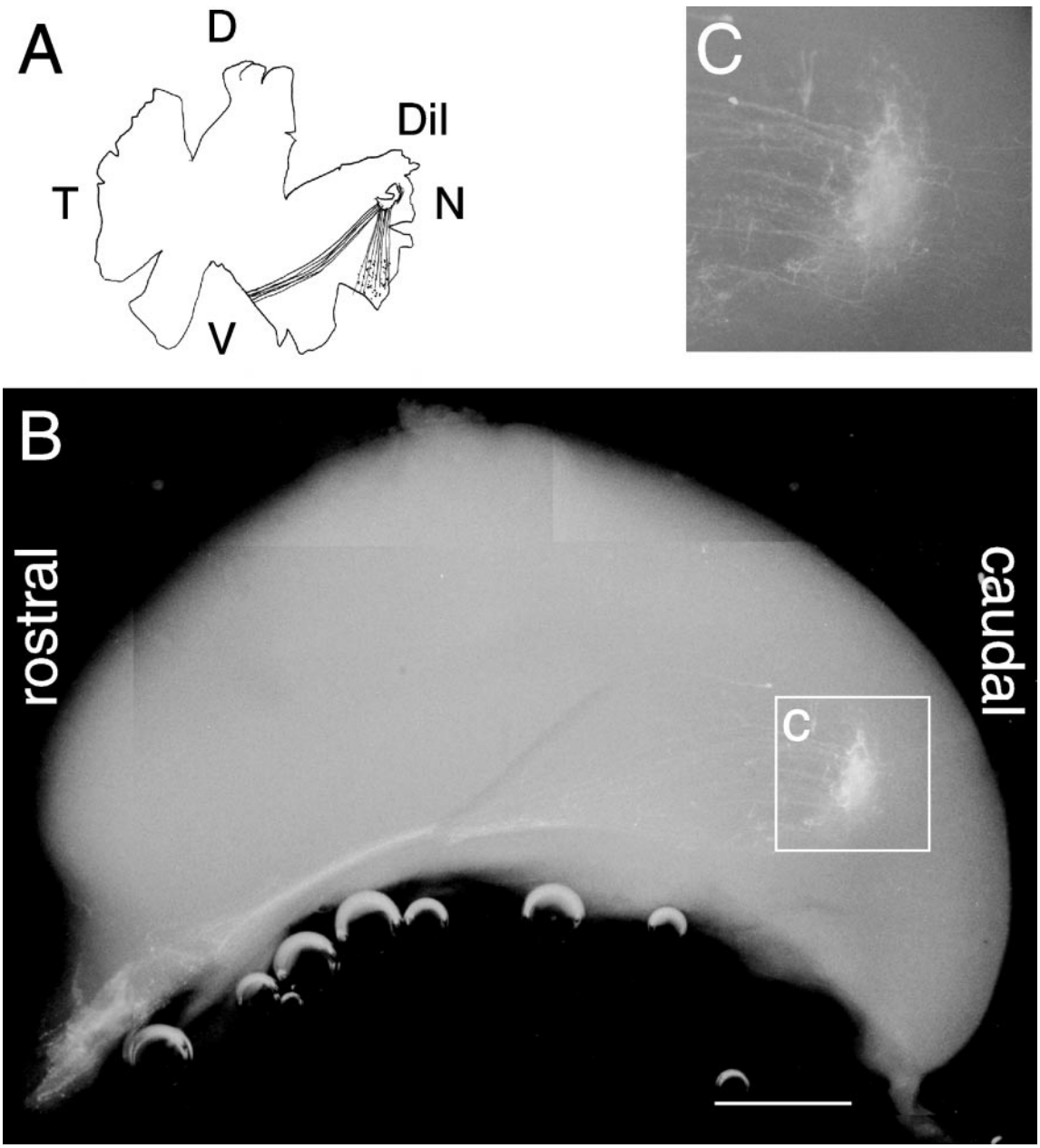


FIG. 4. Development of a compact TZ of nasal axons at E16.5. Normal uninfected eggs were windowed on E2 or E3 for later manipulations. At E14.5, a subpopulation of retinal axons was DiI labeled; tecta and retina were isolated at E16.5. (A) Camera lucida drawing of the flat-mounted retina. The location of the DiI crystals in a nasodorsal position of the retina and the path of labeled axons toward the optic fissure are indicated. (B) On the contralateral ventral half-tectum of this embryo, axons project in a pathway which follows in the rostral half the isthmo-optic tract and spreads then toward a compact, well-developed TZ, which is located in the topographically appropriate caudoventral position. (C) Enlargement of the area covering the TZ shown in (B). Very few axons overshoot the TZ. D, dorsal; N, nasal; T, temporal; V, ventral. Scale bar, 1 mm.

versely, there were embryos with normal contralateral projections but strong topographic targeting errors (Table 2A). Only in the case of nasodorsal projections after ephrinA5 overexpression do data seem to suggest a correlation between pathfinding and targeting errors.

DISCUSSION

Effects of Retinal EphrinA5 Overexpression in Vivo

Gain-of-function and loss-of-function experiments, performed *in vitro,* have provided evidence that ephrinAs

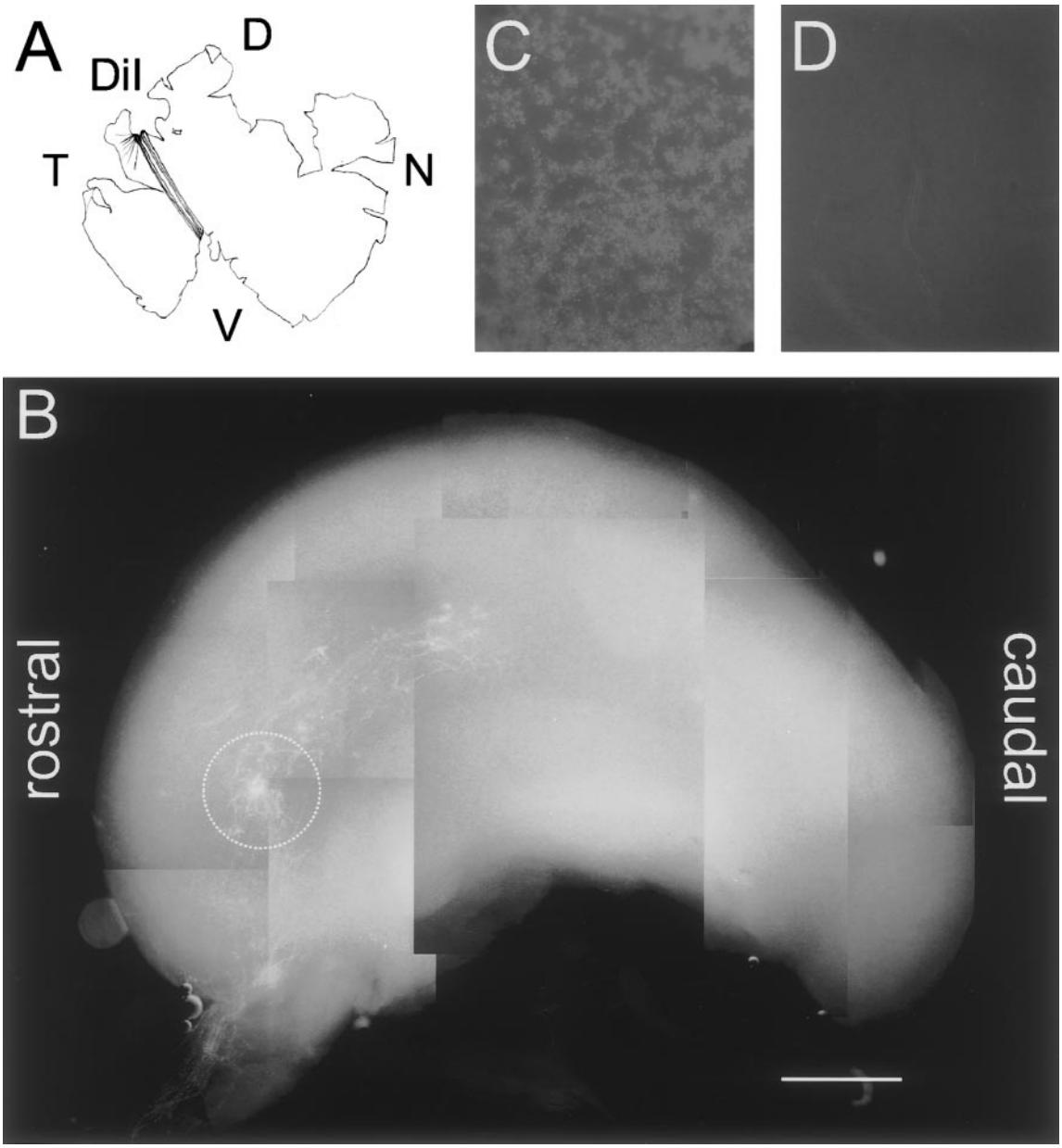


FIG. 5. Topographic targeting errors of temporodorsal axons after infection with a retrovirus expressing ephrinA5. After infection of the optic vesicle at E1.5 with an ephrinA5-expressing RCAS-retrovirus and DiI labeling at E14.5, the retina and tecta were analyzed at E16.5. (A) Camera lucida drawing of the flat-mounted retina; the position of the DiI crystal in the temporodorsal periphery and the path of labeled retinal axons to the optic fissure is indicated. (B) In the rostral part of the contralateral ventral tectum a faint diffuse TZ (marked by a dotted-lined circle) is formed. Numerous retinal axons overshoot the rostroventral target zone and form single axon termini on the topographically inappropriate midtectum. (C) Rate of infection of an E16.5 retina derived from virus-free SPF eggs as determined by immunohistochemical analysis using the 3C2 antibody directed against a retroviral epitope. (D) Staining of an uninfected retina using the same protocol. Staining rates of cells usually range between 30 and 70%. D, dorsal; N, nasal; T, temporal; V, ventral. Scale bar, 1 mm.

downregulate the activity of coexpressed EphA receptors, possibly explaining why retinal axons expressing both receptors and ligands are relatively insensitive to repellent tectal guidance cues (Hornberger *et al.*, 1999). In particular, nasal axons, known to express high(er) amounts of ephrinA ligands and strongly tyrosine-phosphorylated EphA receptors (Connor *et al.*, 1998), are insensitive to the repellent. Conversely, temporal axons, which express low(er)

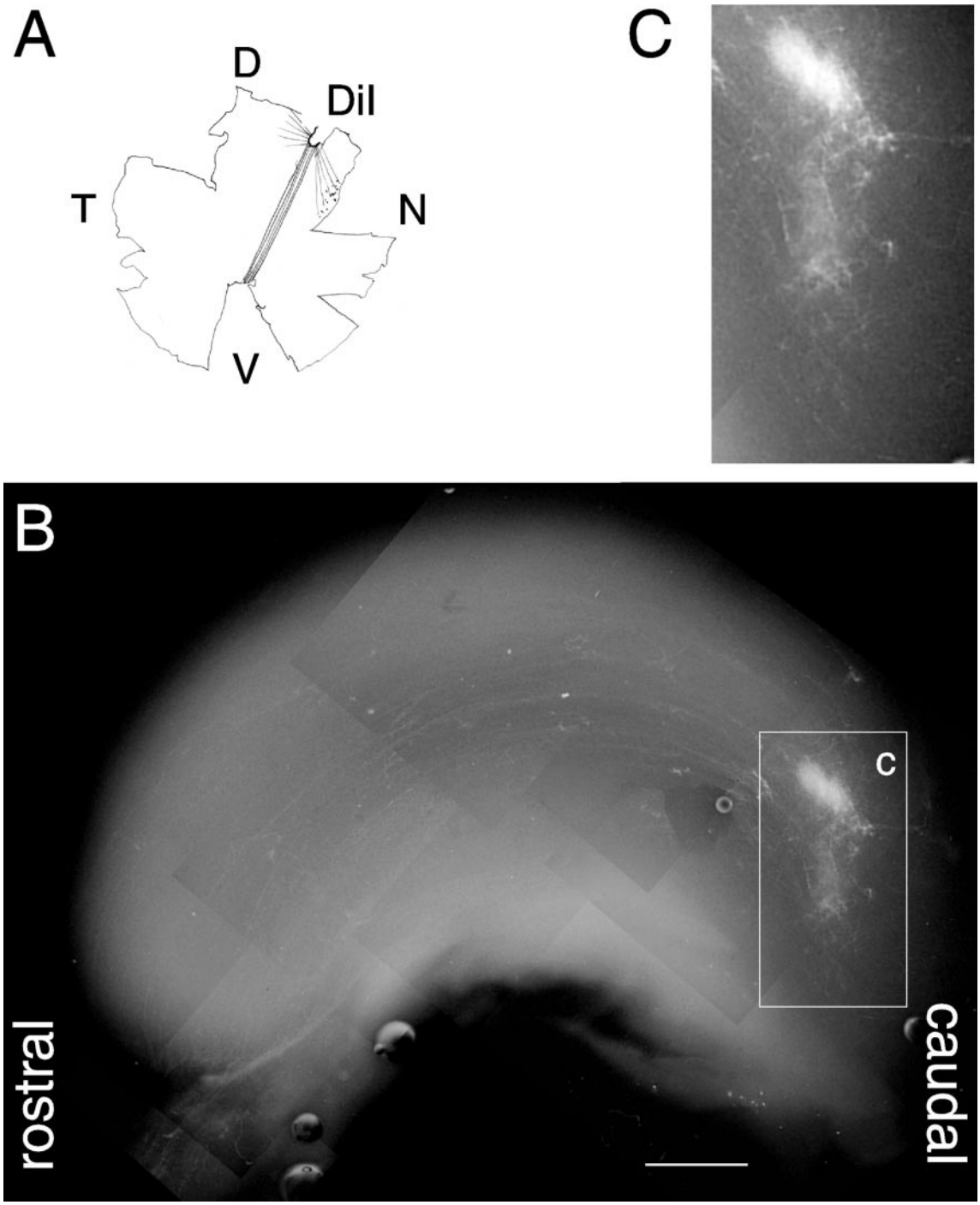


FIG. 6. Retinal expression of ephrinA5 results in topographic projection errors of nasal axons. After infection of the optic vesicle with an ephrinA5-expressing retrovirus at E1.5, at E15.5 DiI crystals were inserted into a nasodorsal position of the retina. Two days later at E17.5, labeled axons were analyzed. (A) Camera lucida drawing of the flat-mounted retina showing the location of the DiI crystals and path of retinal axons. As shown in (B) and enlarged in (C), in addition to a more or less compact TZ in the topographically appropriate region of the caudoventral tectum, numerous axons overshoot this zone and project into more caudal regions. The strength of the effect was classified as "medium-to-weak" (see Table 1B). The embryo analyzed here had only an ipsilateral projection. Contrary to Fig. 4, axons did not follow the isthmo-optic tract, but spread over a broad part of the ventral tectum.

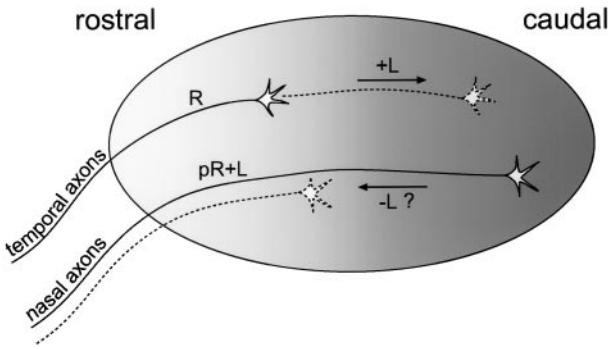


FIG. 7. Differential ephrinA ligand expression on retinal axons contributes to the topographic patterning of retinal axons in the tectum: a model. The model concentrates on the regulation of EphA receptor activity (like EphA4 and EphA5) uniformly expressed on retinal axons. Nasal axons express high(er) amounts of ephrinAs and strongly phosphorylated EphA receptors (pR+L), indicative of a reduced level of *functional* EphA receptors. In this way, nasal axons are *less* sensitive to the gradedly expressed repellent of the tectum (shaded in gray) only at higher concentrations and project to more caudal positions. Temporal axons, on the other hand, are *more* sensitive to the repellent, possibly because of low(er) levels of ephrinA expression, leading in turn to EphA receptors which are hardly phosphorylated (R). Thus, temporal axons are repelled at lower concentrations of repellent and project onto the rostral tectum. As indicated by dotted lines, overexpression of ephrinA ligands on temporal retinal axons (+L) results in an increased phosphorylation of EphA receptors with a concomitant reduction in the level of functional EphA receptors. In turn, these axons invade more caudal regions of the tectum (containing higher concentrations of the repellent). Conversely, inactivation of ephrinA ligands on nasal axons (−L) should lead to an earlier stopping of these axons within the repellent gradient and, correspondingly, to a projection onto more rostral positions of the tectum. Such a shift in the projection pattern of nasal axons indeed has been reported for the behavior of retinal axons projecting onto the dLGN in ephrinA5-mutant mice (Feldheim *et al.*, 1998). In addition to a number of receptors which are uniformly expressed in the retina, there is one EphA receptor expressed in a high-temporal-to-low-nasal pattern, which appears also to be involved in the setup of a graded EphA receptor activity, especially on temporal axons (see Discussion for details).

amounts of ephrinA ligands, and EphA receptors, which are only slightly tyrosine-phosphorylated, are sensitive to the repellent. This concept was supported by results from retinal ephrinA2 overexpression experiments *in vivo*, which led to targeting defects of temporal axons (Hornberger *et al.*, 1999). However, nasal axons were barely affected.

We show now that retinal overexpression of ephrinA5 perturbs the targeting behavior of both temporal and nasal axons. These data reinforce a model in which differential ephrinA ligand expression on retinal axons contributes to topographic targeting in the retinotectal projection (see Fig. 7).

Interestingly, the different activities of ephrinA2 vs ephrinA5 after retinal overexpression correlate to some extent, but apparently not completely, with their activities in stripe assays when used there as repellent substrates for retinal axons. In the stripe assay, ephrinA2 exerts an effect on temporal, but not on nasal axons, whereas ephrinA5 repels both nasal and temporal axons with temporal axons being slightly more sensitive than nasal axons (Monschau *et al.*, 1997). Apparently, this behavior fits well with the functional characteristics of ephrinA2 and ephrinA5 after retinal overexpression.

Comparing the strength of activities of both ligands to each other, ephrinA5 exerts in the stripe assay a stronger effect on temporal axons than ephrinA2 (Monschau *et al.*, 1997). This behavior was proposed to be associated with higher binding affinities of ephrinA5 vs ephrinA2 to EphA receptors expressed on temporal axons (Gale *et al.*, 1996; Monschau *et al.*, 1997; Connor *et al.*, 1998). In contrast, however, this interrelationship was not found in the experiments reported in this paper. Here temporal axons were more strongly affected after retinal ephrinA2 overexpression than after ephrinA5 overexpression. The meaning of this apparent functional discrepancy is not known, but the following possible explanations have to be considered: first, of course, both assays are not strictly comparable, that is, the stripe assay represents an *in vitro* assay done with E6–E7 retinae, whereas the retinal overexpression results are derived from *in vivo* experiments in which the analysis was done about a week later in development (E15–E17). Possibly other factors contribute to the guidance of retinal axons *in vivo*, which are not present in the stripe assay. Second, in the two assays the ephrinA ligands are presented in different surroundings, i.e., on retinal axons colocalizing with EphA receptors vs on membranes of COS/HEK293 cells. This might affect to some extent their activities/specificities. Experiments are under way to investigate whether EphA receptors can be tyrosine-phosphorylated when ligands are presented only *in cis*, i.e., within the same membrane. Third, simply the amount of ligand produced by the two retroviruses *in ovo* might be different. Although we have used a number of different retroviral stocks, and batches with similar concentrations of virus, *in ovo* the ephrinA2-expressing virus might be more stable or might be more effectively propagated, finally resulting in higher concentrations of ephrinA2 protein. Thus, from this last point, which we favor at the moment, the stronger effect of ephrinA2 on temporal axons compared to ephrinA5 might simply be due to a higher expression level of this protein *in vivo*, which would imply that the activities of ephrinA2 and ephrinA5 in both assays are well comparable.

With respect to the proposed correlation between level of tyrosine phosphorylation of EphA receptors and insensitivity of retinal axons for repellent guidance cues (Hornberger *et al.*, 1999), one might speculate that targeting errors of nasal axons can be achieved only by ephrinA ligands with comparably high binding affinities for these receptors. This would mean that only ephrinA ligands like ephrinA5, but

not ephrinA2, might cause an effective increase of the already high level of tyrosine phosphorylation of nasally expressed EphA receptors which then leads to an overshooting of retinal axons.

Pathfinding Errors at the Chiasm

We also had a close look at the pathway of retinal axons to the tectum, as we found that retrovirally driven ephrinA2 or ephrinA5 overexpression frequently led to pathfinding errors of both nasal and temporal axons at the chiasm. This was revealed by a partial or complete ipsilateral projection that persists beyond the period of morphogenetic cell death.

The cause of the pathfinding errors is not understood. Possibly ordering in the optic nerve is necessary for an ordered pathfinding at the chiasm and the differential expression patterns of EphA receptors and ephrinA ligands on retinal axons might normally contribute to this process (Fig. 3). Thus, a deregulated misexpression of ephrinAs might have caused a crucial loss of ordering of retinal axons, which might have resulted in erroneous pathfinding at the chiasm.

However, pathfinding errors at the chiasm typically are not correlated with topographic targeting errors on the tectum, as has been shown in a number of investigations. For example, surgical removal of one optic vesicle during the very early development in chick leads to a significant ipsilateral projection, indicating disturbed pathfinding at the chiasm, yet still a normal topographic projection is formed at later stages of development (O'Leary *et al.*, 1983). Also, in another investigation, retinal axons were challenged to grow to their targets along abnormal pathways, but still retinal projections were basically normal (Harris, 1984). Similarly, in an analysis of zebrafish mutants with varying extents of ipsilateral projection, it became evident that misrouted axons, when reaching the ipsilateral tectum, were able to find their correct retinotopic position (Karlström *et al.*, 1996).

Likewise, our data indicate that the topographic targeting errors are not caused by pathfinding errors at the chiasm. As mentioned, this is evident from projections in which the pathfinding is disturbed (ipsilateral), but normal TZ are formed, and from other cases in which pathfinding is normal at the chiasm (contralateral), but the topographic patterning is affected. The exception from this is the behavior of nasal axons after ephrinA5 overexpression, which show a bias for topographic mistargeting in cases in which the pathfinding is affected. However, as this link has been observed only in this combination, a causal relationship appears to be rather unlikely.

Ideally, one would have expected that after retinal overexpression of ephrinA ligands corresponding compact termination zones would develop at positions in more caudal regions of the tectum. However, the effects observed instead, i.e., the overshooting of numerous axons without formation of TZ, might be due to the inhomogeneous retroviral infection and thus to varying levels of expression of ephrins on retinal axons.

At the moment there is no hard and fast answer to the question of why these aberrant projections survive at all the refinement processes acting later on mistargeted axons. Possibly, ephrins are normally involved in later stages of topographic patterning and ephrinA misexpressions interfere with this process. Interestingly, aberrantly projecting axons are remodeled to a certain extent in an activity-independent refinement process (O'Leary *et al.*, 1986; Simon *et al.*, 1994).

Also, an additional function of nasally expressed ephrins might be to defend their target region, the caudal tectum, from invasion by temporal axons which otherwise might evade the caudally expressed repellent activity by fasciculation with nasal axons (Raper and Grunewald, 1990).

Different Ways to Generate Functional EphA Receptor Gradients

Results presented in this paper and the paper by Hornberger *et al.* (1999) suggest a model in which the differential expression of ephrinA ligands on retinal axons contributes to topographic targeting during development of the retinotectal projection in chick. The sensitivity of retinal axons for the repellent guidance cues of the caudal tectum strongly correlates with the level of the axonal expression of ephrinA ligands (Fig. 7). Here ephrinA2 and ephrinA5 might exert partially overlapping activities.

Thus, a gradient of EphA receptor activity in the retina might be built up by two mechanisms: first, by a graded expression of EphA3 in a high-temporal-to-low-nasal pattern and second, by a modulation of the activity of uniformly expressed EphA receptors like EphA4 and EphA5 through gradedly expressed ephrinAs. This would result in a second gradient of EphA receptor activity with the same orientation as the EphA3 gradient. Thus, expression of ephrinA ligands might lead to a sharpening of the already existing EphA receptor gradient in the retina (see also McLaughlin and O'Leary, 1999).

The proposed modulatory function of ephrinA ligands fits data derived from an analysis of ephrinA5 knockout mice, in which the projection pattern of retinal axons onto the dorsal LGN, another target of retinal axons, has been analyzed (Feldheim *et al.*, 1998). No longer did nasal axons in these mice, now devoid of ephrinA5 expression, project into regions of the dorsal LGN containing high concentrations of ephrinA ligands, as in wild-type mice. Instead, here now nasal axons project to sites of lower ephrinA concentrations (see also Fig. 7), just as if they had acquired an increased sensitivity toward the repellent ephrinA gradient.

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