Absence of topography in precociously innervated tecta

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SUMMARY

The retinotectal map in *Xenopus* forms very early: retinal axons are topographically ordered along the dorsoventral axis of the tectum by stage 39, as they first arrive. To test whether topographic cues are present even earlier, we forced retinal axons to innervate the tectum prematurely by transplanting stage 28 eye primordia into stage 20 hosts, then assayed dorsoventral topography using focal injections of lipophilic dye into dorsal and ventral retina at donor stages 39-40. Unoperated and isochronic control projections showed normal dorsoventral ordering both in the optic tract and in the tectum. In contrast, projections from heterochronically transplanted eyes were ordered in the tract, but spread out upon entering the tectum and did not show significant dorsoventral ordering. Individual axons entering the tectum precociously often made abnormally abrupt and topographically incorrect turns. Thus, the topographical cues normally expressed in the tectum at stage 39 appear to be absent a few hours earlier. However, this lack of cues is only temporary, since heterochronic transplants allowed to survive to donor stages 45-46 showed normal topography. The absence of tectal topography at a stage when retinal axons can navigate to the young tectum strongly suggests that the molecules that provide tectal topographical signals are distinct from those used for pathfinding in the diencephalon and target recognition at the tectum.

Key words: *Xenopus*, heterochronic transplant, retinotectal development, DiI

INTRODUCTION

In the retinotectal system, retinal ganglion cell axons terminate in the optic tectum in a highly ordered pattern. Dorsal retina maps to ventral tectum, ventral retina to dorsal tectum, anterior retina to posterior tectum, and posterior retina to anterior tectum. Since this is the main visual projection in nonmammalian vertebrates (Scalia and Fite, 1974; Steedman et al., 1979), its accurate and timely development is essential for appropriate visually directed behavior. The retinotectal system of *Xenopus* has been an excellent model system for studying the development of topographically specific synaptic connections (reviewed by Cowan and Hunt, 1985; Udin and Fawcett, 1988; Fraser, 1992; Holt and Harris, 1993) and early axonal pathfinding (reviewed by Chien and Harris, 1994). *Xenopus* embryos hatch and swim freely by about 54 hours postfertilization (Nieuwkoop and Faber, 1967), and their retinotectal development is correspondingly rapid. By 54 hours, retinal axons have reached the tectum and begun to arborize topographically (Holt and Harris, 1983; Holt, 1984; Sakaguchi and Murphey, 1985). The speed of this developmental program sets a deadline for the positional cues used by retinal growth cones to navigate to the tectum, recognize it as their target and terminate topographically: they must be expressed by the time the first growth cones arrive.

Sperry (1963) first proposed that retinal axons find their topographically appropriate targets by matching positional markers on the tectum. Many experiments have provided evidence for tectal positional cues and several candidate topography molecules have been found (reviewed by Holt and Harris, 1993; Kaprielian and Patterson, 1994). Retinal axons are also topographically organized in the amphibian optic tract, with circumferential position around the retina represented across the width of the tract (Scalia and Fite, 1974; Fujisawa et al., 1981; Reh et al., 1983). Ventral retinal axons grow through the dorsal part of the tract and dorsal axons grow through the ventral part. Two lines of evidence suggest that this order is due to positional cues in the diencephalon. First, the tract is organized very differently from the optic nerve and chiasm (Fawcett, 1981; Reh et al., 1983). The axons of retinal neighbors do not simply adhere to each other throughout their course, but must undergo an active sorting process as they exit the chiasm and enter the tract. Second, the projection from a double-ventral eye (a compound eye formed by fusing two ventral half-eyes) only occupies the dorsal half of the tract (Fawcett and Gaze, 1982). This shows that ventral axons have a preference for the dorsal tract, even in the absence of dorsal axons. Thus, both the tectum and the diencephalon have topographic cues that can guide retinal axons.

The first *Xenopus* retinal axons enter the optic nerve head at stage 28, reach the tectum at stage 37/38 and start to arborize at stage 39 (Holt, 1984; Harris et al., 1987). Dorsoventral topography is clearly established at stage 39: dorsal retinal axons run in the ventral optic tract and project to ventral tectum, and ventral axons run in the dorsal optic tract and project to dorsal tectum (Holt and Harris, 1983; Holt, 1984;
There is conflicting evidence about the onset of anteroposte-
rior topography, but it seems to be crude at stage 39, then
slowly become refined by stage 49 (Holt and Harris, 1983;
Sakaguchi and Murphey, 1985; O’Rourke and Fraser, 1986).
Eyes develop with a slight dorsoventral gradient: dorsal
axons exit the retina and reach the tectum about 6 hours
before ventral axons. When dorsal axons are made to reach
the tectum after the ventral axons by grafting young dorsal halfl-
eyes into older hosts, both the tectum and the optic tract have
normal dorsoventral topography (Holt, 1984). This shows that
dorsoventral order is not simply a matter of earlier axons
growing more ventrally, and is additional evidence that topo-
graphic cues exist in the tract and tectum.

In a previous study, we forced retinal axons to enter the brain
earlier than usual by transplanting older eyes into younger
embryos. Precocious axons navigated correctly to the tectum
even in brains young enough that no other axons were present
(Cornel and Holt, 1992). This showed that pathfinding cues are
present in the neuroepithelium at least 10 hours before retinal
axons normally enter the tract. Precocious projections spread
abnormally in the tectum and seemed disorganized (Cornel and
Holt, 1992), suggesting that topography might be disrupted. In
the present study, we asked whether topography cues are also
expressed before retinal axons arrive. We used two methods to
assay the dorsoventral topography of precocious projections:
first, focal injections of diI and diA into dorsal and ventral
retina; and, second, focal injection of diI into dorsal retina
combined with whole-eye labelling using fluorescein dextran (FlDx; O’Rourke and Fraser, 1986).

**MATERIALS AND METHODS**

**Embryos and transplants**

Adult *Xenopus laevis* housed in our colony at the University of Cal-
ifornia, San Diego were injected with human chorionic gonadotropin
(HCG; United States Biochemical, Cleveland, OH) for hormonally
induced matings and in vitro fertilizations according to standard pro-
cedures. Embryos were raised in 10% Holtfreter’s solution (Holt-
freter, 1943), varying the temperature between 14 and 27°C to control
their speed of development, and staged according to the normal tables
of Nieuwkoop and Faber (1967). Times in the text are from these
stages (approximately 21°C): stage 20, 22 h; stage 26, 30 h; stage 28,
33 h; stage 29/30, 35 h; stage 32, 40 h; stage 33/34, 45 h; stage 35/36,
50 h; stage 37/38, 54 h, stage 39, 57 h (hours postfertilization).

Isotopic eye transplants were performed as described previously
(Cornel and Holt, 1992). Briefly, embryos were dejellied with 2%
cysteine, anesthetized with tricaine (ethyl 3-aminobenzoate methane-
sulfonic acid, Aldrich) in 100% modified Ringer’s (MR; Gimlich and
Gerhart, 1984), placed in a depression in a clay dish and held down
with a L-shaped pin. Right eye primordia were dissected out from
donors, then placed in the vacated orbit of the host, taking care to
align the cut surfaces of the optic stalks. After holding the transplanted
eye in place with a coverslip shard for 20 minutes, the embryo was
transferred to 100% MR for an hour, then to 10% Holtfreter’s for
rearing. Fig. 1A schematizes the two types of transplants: isochronic,
in which stage 28 eyes were transplanted into stage 28 hosts, and het-
erochronic, in which stage 28 eyes were transplanted into stage 20
hosts, so that retinal axons entered the brain approximately 11 hours
earlier than normal. Embryos were usually reared until the donor eye
was stage 39-40 (judged both by eye morphology and by unoperated
controls reared in parallel), the stage when retinal axons have
normally begun to arborize in the tectum. In a few cases, embryos
were allowed to survive for two more days (to stage 45-46) after the
usual labelling. All heterochronic transplants were from stage 28 to
20, except for that shown in Fig. 2C, which was from stage 26 to 19.

**Labelling techniques**

Horse radish peroxidase (HRP) was used as previously described
(Harris et al., 1985; Cornel and Holt, 1992) to label the entire retinal
projection (Fig. 1B). Briefly, the lens was removed and replaced with
a plug of partially dried HRP (type VI, Sigma; approximately 30% HRP
in 1% lyssolecithin). After waiting 20-30 minutes for the HRP to be
anterogradely transported, embryos were fixed in parafomalde-
hyde (4% PF in 0.1 M sodium phosphate buffer, pH 7.4, 1 hour),
reacted using diaminobenzidine (Sigma) histochemistry and postfixed
in glutaraldehyde (0.5% in 0.1 M buffer, 30 minutes). Brains were
photographed with epi-illumination before clearing (Fig. 2A,B), or
after clearing with benzyl benzoate and mounting in Permount (Fig. 2C).

Two methods were used to assay topography: diI/diA labelling, in
which small areas of dorsal and ventral retina were labelled with
different dyes, and diI/FlDx labelling, in which the entire eye was
labelled with one fluorophore, and a small area of dorsal retina with
another. For diI/diA labelling (Fig. 1C), dye was pressure-injected into
the retina (Picospritzer, General Valve; 50 ms pulse, about 30 psi). DiI
(DiIC18(3), Molecular Probes; Honig and Hume, 1986) and
diA (4-Dis-16-ASP; Molecular Probes) were made up as 2% stocks in
DMSO, then diluted 1:10 in 70% EtOH just before use. The tip of a
glass micropipette was filled with this working solution, then pro-
gressively broken off with forceps until the ejected drop was roughly
20 μm in diameter. After using a minutien pin to prepare a channel
that passed through the overlying skin and beside the lens, the pipette
was inserted into dorsal or ventral retina and a single drop of dye was
ejected, precipitating as it left the pipette. Embryos were allowed to
recover for 2-4 hours to allow dye to be actively transported, and fixed
in 4% PF for 3-4 days at 4°C before dissecting out the brains. DiI
gave better labelling efficiency than diA, but showed more lateral
spread. In order to minimize labelling of dorsal axons as they passed
through the optic nerve head, diA was used as the ventral marker and
diI as the dorsal marker (Fig. 1C). The rate of successfully diI/diA
double-labelled embryos was quite low (a few percent), so for later
experiments we used diI/FlDx labelling (success rate about 50%).

For diI/FlDx labelling, the entire donor embryo was pressure-
injected (Picospritzer, 100 ms pulse at 50 psi) at the 2- or 4-cell stage
with FlDx (lysinated fluorescein dextran, 10,000 MW, Molecular Probes).
After in vitro fertilization, embryos were dejellied with 2%
cysteine, transferred to 6% Ficoll in 10% Holtfreter’s and placed on
a nylon grid which held them in place. Glass micropipettes were filled
with 50 mg/ml FlDx in 10 mM potassium phosphate buffer, and the
tips broken off to give 50 μm drops. All two or four blastomeres were
injected with several drops each, for a total injection volume of
roughly 1 nl per embryo. Injected embryos were checked 2 days later
under a fluorescence microscope; only those that showed bright,
uniform labelling and had gastrulated and neurulated normally were
used. After transplanting eyes at donor stage 28 and rearing the
embryos to donor stage 39-40, the dorsal retina was labelled with diI
desicribed above.

**Sample selection and brain mounting**

Order in the tract and in the tectum was assessed from fluorescent
images of lateral views of whole-mounted brains. Since the eye at
these stages is very small (lens diameter about 125 μm at stage 37/38),
accurate placement of dye in the retina was critical. Therefore, before
dissecting out the brains, dye placement was always checked by
taking double-label images of the filled eye. The only exceptions were
the long-survival heterochronic transplants (Figs 4D, 5), in which the
diI fluorescence was obscured by the strongly refracting stage 45-46
lens. However, these old transplants all projected to the ventral half
of the tectum, suggesting that the dye placement was indeed correct (see Results). FlDx transplants were checked to see that the entire eye was labelled. DiI placement was scored on a scale from −2 to 2, where −2=extreme ventral, 0=central or widely spread, and 2=extreme dorsal; diA placement was scored on the same scale, only inverted. The figures shown here include all individuals that had diI and diA scores of 1 or greater, that is, completely within the dorsal (diI) or ventral (diA) hemispheres. This sample set is conservative: questionable specimens in which dye was well-placed within the retina, but had spread in the lens or on the surface of the embryo, were excluded. Brains were dissected out and mounted in phosphate buffer within two stacked clear plastic reinforcement rings (Avery), coveredslipped and the coverslip sealed with nail polish. Care was taken to avoid rolling the brains so that they could be viewed laterally.

Since we were concerned only with the retinotectal projection, it was necessary to distinguish from the minor retinofugal projection to the basal optic nucleus (BON), which has also begun to develop by stage 39 (Holt and Harris, 1983; Easter and Taylor, 1989). BON fibers are part of the tract of the postoptic commissure (TPOC, Easter and Taylor, 1989). They may be distinguished from retinotectal fibers on the basis of two criteria: first, they are sparsely arrayed, in contrast with the dense retinotectal tract; second, they usually pass ventral to the retinotectal tract, though occasionally a long fiber is seen to enter the tectum, turn ventrally and then pass out of the tectum towards the BON. For analysis of topography, we included only those axons that coursed within the body of the retinotectal tract and ended in the tectum.

**Imaging and data analysis**

Digital fluorescence images were taken with a silicon-intensified-target (SIT) camera and with a laser scanning confocal microscope. SIT images were taken with a SIT-650 (Dage-MTI) camera on a Nikon Optiphot-2 upright microscope using 10×/0.45 and 20×/0.75 PlanApo objectives (Nikon), and digitized with an LG-3 framegrabber card (Scion) in a Macintosh IIfx (Apple Computer). The microscope was equipped with rhodamine (for diI) and fluorescein (for diA and FlDx) cubes. The rhodamine cube effectively excluded diA and FlDx fluorescence, but for some brightly labelled samples, diI fluorescence bled through with the fluorescein cube. During analysis, those fibers that were bright with the rhodamine cube and dim with the fluorescein were counted as diI fibers; a fiber that was bright in both images, presumably truly double-labelled, was counted once as a diI fiber and again as a diA fiber. Images were digitized using the NIH Image program, version 1.55 (public domain software written by Wayne Rasband at the US National Institutes of Health, available from the Internet by anonymous ftp from zippy.nimh.nih.gov). Since labelled fibers often ranged over several planes of focus, montages were made by manually combining the in-focus areas from different planes.

Confocal images (except for Fig. 3D, taken with a Biorad MRC-600) were taken with a Noran Odyssey confocal unit on a Nikon Diaphot 200 inverted microscope, using 10×/0.45 and 20×/0.75 PlanApo lenses (Nikon). This unit was controlled by a Macintosh Quadra 950 running OdysseyW software written by Daniel Chin of the Agouron Institute. There was no detectable bleedthrough between the FlDx images, taken with 488 nm excitation, 500-530 nm emission, and the diI images, taken with 529 nm excitation, 575-645 nm emission. Z-series were taken at 20× (50 μm confocal slit, 3 μm steps) and combined into a single image using the brightest-point projection of NIH Image. Images were stored on magneto-optical disks (PMO-650, Pinnacle Micro) and analyzed using NIH Image macros written by C.-B. C. Data reduction was done using the spreadsheet program Excel 4.0 (Microsoft), and statistics (histograms and t-test) using Statview 4.0 (Abacus Concepts). Double-label images of selected samples were combined in Photoshop 2.5 (Adobe Systems), where they were contrast-enhanced and color-balanced, and color prints (Figs 3, 5) were made using a Fujix Pictrography 3000 silver halide printer.

**Topographical axes**

We refer to the topographical axes of the embryonic retina and tectum as dorsoventral and anteroposterior. The tectum expands laterally during growth and the eyes move during metamorphosis; therefore in the eye, embryonic anteroposterior = adult nasotemporal; in the tectum, embryonic anteroposterior = adult rostrocaudal and embryonic
Table 1. Tectal behavior of transplant and control projections

<table>
<thead>
<tr>
<th>Condition</th>
<th>Unplayed</th>
<th>Partially splayed</th>
<th>Splayed</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Unoperated controls</td>
<td>30</td>
<td>4</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>(88%)</td>
<td>(12%)</td>
<td>(0%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Isochronic transplants</td>
<td>21</td>
<td>5</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>(81%)</td>
<td>(19%)</td>
<td>(0%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Heterochronic transplants</td>
<td>5</td>
<td>2</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>(23%)</td>
<td>(9%)</td>
<td>(68%)</td>
<td>(100%)</td>
</tr>
</tbody>
</table>

Number of optic projections showing different tectal behaviors in unoperated controls, isochronic (stage 28 to 28) transplants, and heterochronic (stage 28 to 20) transplants. ‘Unplayed’ projections showed only normal widening; ‘partially splayed’ projections had many fibers making abnormal ventral turns at the ventral edge of the tract, but at most a few fibers doing so at the dorsal edge of the tract; ‘splayed’ projections showed abnormal turns both ventrally and dorsally. Only samples with well-labelled tracts were included.

RESULTS

Precocious optic tracts terminate abnormally in the tectum

Fig. 2A shows the normal retinal projection at stage 39, visualized by filling the entire contralateral retina with HRP. After crossing contralaterally at the optic chiasm, the retinotectal projection proceeds dorsally to the mid-diencephalon, where it turns posteriorly. It then continues to the optic tectum, where it widens and terminates.

When eyes were forced to innervate the brain precociously by means of a stage 28 to 20 heterochronic transplant, the retinal projections navigated correctly to the tectum (Fig. 2C; Cornel and Holt, 1992), as did retinal projections in isochronic transplants (Fig. 2B). Most transplants showed full contralateral projections, though some projections were absent or had fewer fibers than normal. In both isochronic and heterochronic transplants, those retinal axons that reached the chiasm navigated normally to the tectum. This confirms that pathfinding cues are present in the neuroepithelium well before the retinal axons are normally present (Cornel and Holt, 1992).

In controls and isochronic transplants (Fig. 2A,B), the typical projection widened from 60 μm to 100 μm when entering the tectum. The widening was more marked at the ventral edge, where the retinal projection adjoins the TPOC (Easter and Taylor, 1989; Cornel and Holt, 1992). The typical heterochronic transplant showed much more dramatic widening, with both the ventral and dorsal edges of the tectal projection abnormally splayed and tectal widths as great as 200 μm (Fig. 2C). Whereas control projections had the shape of a hand with fingers closed (the wrist being the presumptive tectal border), the heterochronic projections had the shape of a hand with fingers splayed. Table 1 summarizes the tectal behavior of HRP-filled optic projections in a set of 34 unoperated controls, 26 isochronic transplants and 22 heterochronic transplants. The isochronic transplants were indistinguishable from controls, while the majority of the heterochronic transplants were splayed.

Precocious fibers must somehow recognize the young tectum, since heterochronic projections spread out when crossing the tectal border and terminate there rather than continuing to grow through. However, the abnormal splaying of heterochronic projections suggests that precocious fibers take aberrant paths once in the tectum and raises the possibility that tectal topography might be disrupted.

Individual axons make abnormal tectal turns in heterochronic transplants

In order to examine the tectal behavior of precocious projections at the level of single axons and, in particular, to determine if dorsoventral topography was present, we used two labelling procedures. First, small areas of retina in untreated controls and heterochronic transplants were labelled using injections of the lipophilic dyes dI (dorsal retina) and dA (ventral retina). When successful, this procedure gave good visualization of individual axons with terminal branches and growth cones and made it easy to compare the paths taken by dorsal and ventral axons. Isochronic and heterochronic transplants were compared using a second method. Transplanting the eye from an embryo injected with FIDx at the two- or four-blastomere stage gave excellent, reliable labelling of the optic projection. Dil was then injected into dorsal retina to show the position of the dorsal axons relative to the overall FIDx-labelled projection.
The behavior of individually labelled axons in the tectum correlated with the HRP-filled tracts: fibers in heterochronic transplants made abrupt turns rarely seen in isochronic or unoperated controls. In controls, diA fibers entered the tectum without turning (Fig. 3A), while diI fibers either went straight (Fig. 3C) or turned ventrally (Fig. 3A). Control axons had often begun to branch, making small, immature arbors (Fig. 3A). In heterochronic transplants, individual axons made many more wide turns than in controls, turning both dorsally and ventrally (Fig. 3B,D). This was especially noticeable for diA-labelled (ventral) fibers, which rarely turned in controls, but made wide turns in both directions (Fig. 3B) in precocious projections. Fig. 3E shows an example of especially deranged behavior: after this precocious fiber entered the tectum and branched, one of its branches turned 180° and headed back towards the tectal border. Such axons were seen several times in heterochronic transplants, but never in controls. In some heterochronic transplants, dorsally turning fibers travelled nearly to the dorsal midline of the brain (Fig. 3D). Precocious fibers sometimes branched in the tectum, though more rarely than control fibers did.

Control projections were generally topographic in the dorsoventral dimension; diA-labelled fibers from ventral retina projected to dorsal tectum (Fig. 3A), while diI-labelled fibers from dorsal retina projected to ventral tectum (Fig. 3A,C). Heterochronic projections were often less topographic, as in Fig. 3B where diI and diA fibers overlap significantly; many were nontopographic, as in Fig. 3D where the diI fibers span the FIDx fibers in the tectum; and a few were antitopographic, with dorsal fibers projecting dorsally in the tectum. Thus, the abnormal turning of heterochronic fibers in the tectum correlated with reduced dorsoventral topography. To describe topography more precisely, we measured the dorsoventral positions of fibers in the optic tract and branch tips in the tectum.

**Heterochronic transplants are ordered in the tract, but lack dorsoventral topography in the tectum**

To quantify topography in the tectum and the optic tract, we used the system diagrammed in Fig. 4A. Identified landmarks on each brain were used to draw a standard transect across the optic tract in the middle of its turn and to estimate the direction of the dorsoventral tectal axis. This axis was chosen to be perpendicular to the entering optic fibers, as it is in the adult tectum (see Methods for details). In the optic tract, the positions where diI and diA fibers crossed the transect were marked and measured. In the tectum, the tip of each axonal branch or growth cone was marked and its dorsoventral position was measured.

**Fig. 2.** Heterochronic projections show abnormal splaying in the tectum. Left lateral view of whole-mounted brains after the right eye was filled with HRP. Arrowheads indicate the landmarks used later for quantitative analysis: the anterior border of the chiasm (AC) and the posterior edge of the tectum, or isthmus (PT). The estimated dorsal and posterior axes of the tectum are indicated by the arrows D and P. Dashed lines indicate the presumptive anterior border of the tectum; the square brackets indicate the width of the optic projection in the tectum. tel, telencephalon; di, diencephalon; rh, rhombencephalon; p, pineal; tec, tectum; hy, hypothalamus; bon, basal optic nucleus. (A) Unoperated control, stage 39. The retinotectal tract turns in the mid-diencephalon, spreads slightly upon reaching the tectum, then terminates. Basal optic nucleus fibers are sparse and more ventral than retinotectal fibers. (B) Isochronic transplant (stage 28 to 28), shown at stage 39. The tract appears normal. (C) Heterochronic transplant (stage 26 to 19), shown at donor stage 35/36, host stage 37/38. The retinal fibers successfully find the tectum, but spread widely both dorsally and ventrally.
Fig. 3. Tectal behavior of axons in controls and heterochronic transplants. (A-D) Double-label images of control and transplant tracts and (B,D, insets) the eyes from which they came. DiI always labels dorsal retina and is shown in red; diA, shown in green (A,B), labels ventral retina; FlDx, also shown in green (C,D), labels the entire eye. Overlap between diI and diA or FlDx appears yellow. All images have been oriented with body dorsal (+30°) straight up; dashed lines indicate the approximate border of the tectum. A-B are SIT images with the same scale; C-E are confocal images with the same scale; eyes (insets in B,D) are roughly 350 μm across. (A) Unoperated control, stage 39, diI/diA labelled. DiI and diA fibers are ordered both in the optic tract and in the tectum, where some fibers have begun to arborize (arrowheads). (B) Heterochronic transplant, donor stage 39, host stage 37/38, diI/diA labelled. DiI and diA fibers are separated in the optic tract, but upon entering the tectum, they both make wide turns and overlap extensively. Inset: labelled eye of the same embryo. (C) Isochronic transplant, stage 39, diI/FlDx labelled. In the tectum, diI fibers are in the ventral half of the tract (compare with FlDx labelling). (D) Heterochronic transplant, donor stage 40, host stage 37/38, diI/FlDx labelled. DiI fibers are ventral in the optic tract, but branch dorsally (arrowhead) to cover the entire dorsoventral extent of the tectum. A few fibers turn dorsally at the border of the tectum and travel nearly to the dorsal midline of the brain. (E) DiI-labelled fibers from heterochronic transplant, donor stage 40, host stage 37/38. One axon is tipped by a growth cone; the other branches at the point marked Y. The right branch terminates in a growth cone at R, while the left branch turns 180° at R and terminates at L. (Branching pattern determined by examining individual confocal sections.)
Timing of topographic cues

Marking branch tips was a convenient method for describing the extent of branches and turns that axons made once they entered the tectum. To discount differences in how individual brains were mounted, fiber and tip positions were measured relative to the estimated midpoints of the optic tract and the tectum. The diI/diA sample set comprised 11 heterochronic transplants and 14 normal (unoperated) controls; the diI/FlDx set comprised 21 heterochronic transplants and 16 isochronic transplants. We included all positions measured. Marking branch tips was a convenient method for describing the extent of branches and turns that axons made once they entered the tectum. To discount differences in how individual brains were mounted, fiber and tip positions were measured relative to the estimated midpoints of the optic tract and the tectum. The diI/diA sample set comprised 11 heterochronic transplants and 14 normal (unoperated) controls; the diI/FlDx set comprised 21 heterochronic transplants and 16 isochronic transplants. We included all positions measured.

**Fig. 4.** Topography in the optic tract and tectum. (A) Coordinate systems for quantitation. Placing AC at the origin of the polar coordinate system and PT at (1.0, 0°), a transect (labelled *tract*; see Methods) was drawn at 90° through the optic tract, and the intersection with each fiber was measured. In the tectum, the dorsoventral axis (labelled *tec*) was drawn at 70°. The dorsoventral position of each branch tip was measured by projecting it onto this axis. (B,C) Histograms showing positions of fibers in the optic tract (B) and branch tips in the tectum (C) of diI/diA-labelled samples. Dashed lines indicate midpoints; the number of fibers or branch tips for each histogram is shown in parentheses as (ndiI /n diA). Data compiled from 14 unoperated controls and 11 heterochronic transplants. (B) Both unoperated controls and heterochronic transplants show topographic order in the optic tract, with diI fibers ventral of the midpoint on average, and diA fibers dorsal. Positive is dorsal. (C) Controls, but not heterochronic transplants, show significant dorsoventral topography in the tectum. In controls, diI branch tips are on average ventral and diA branches are dorsal; in heterochronic transplants, diI and diA distributions are spread out and not significantly different. Positive is dorsal. (D) Average positions of diI-labelled fibers in the tract and tectum of diI/FlDx-labelled transplants. Heterochronic transplants show dorsoventral topography in the tract but not the tectum, while isochronic transplants and old heterochronic transplants show topography both in the tract and in the tectum. ISO, isochronic transplants; HET, heterochronic transplants; OLD, heterochronic transplants grown to stage 45-46. Error bars indicate mean±s.e.m. Asterisks show significant differences from the midline by a one-tailed t-test: * = P<0.02, *** = P<0.0001. Bracket indicates comparisons between isochronic and heterochronic transplants using a one-tailed t test.

**Fig. 5.** Tectal topography in an old heterochronic transplant. Double-label confocal images of the optic projection in a heterochronic transplant (stage 28 to 20), allowed to survive to stage 45 after labelling the dorsal retina with diI at stage 39. The dorsal midline of the brain is shown in blue. (A) FlDx labelling shows the entire retinal projection, which forms an oval in the tectum. The estimated tectal border is shown as a dashed line. (B) DiI labelling shows projections from dorsal retina which are confined to ventral tectum. The dashed curve shows the outline of the FlDx labelling.
brains for which the operation (for transplants) had been successful, fibers labelled with both dyes had reached the tectum and dye placement in the eye was verified (see Methods).

Dil/diA heterochronic transplants

Both unoperated brains and heterochronic transplants were ordered in the optic tract (Fig. 4B). Although the dil and diA distributions overlap, dil fibers were on average ventral to the midpoint (unoperated: \(-4.7\pm1.8\,\mu m\), heterochronic: \(-3.2\pm1.8\,\mu m\); mean±s.e.m.) and diA fibers were dorsal (unoperated: \(5.4\pm1.8\,\mu m\), heterochronic: \(2.3\pm1.9\,\mu m\)). The dil distribution was significantly ventral to the diA distribution both in unoperated brains (\(P<0.001\), two-tailed \(t\)-test) and in heterochronic transplants (\(P<0.05\)). The overlap between dil and diA distributions indicates that the tracts were not perfectly ordered.

In the tectum, in contrast, unoperated brains showed significant dorsoventral topography, but heterochronic transplants did not (Fig. 4C). In unoperated brains, the dil branch tips were significantly ventral to the diA branch tips (dil: \(-2.0\pm3.0\,\mu m\), diA: \(15.2\pm3.1\,\mu m\); \(P<0.0001\), two-tailed \(t\)-test). In heterochronic transplants, the dil and diA distributions showed greater overlap and did not differ significantly (dil: \(0.5\pm4.6\,\mu m\), diA: \(10.7\pm5.9\,\mu m\); \(P=0.2\)). Heterochronic fibers were spread more widely in the tectum than fibers in unoperated projections; this difference was statistically significant (unoperated s.d.=31.7 \(\mu m\), heterochronic s.d.=40.2 \(\mu m\); \(P<0.01\), two-tailed \(F\)-test). This spreading corresponds to the splaying seen with HRP injected into the diencephalon. Precocious projections showed significant order in the tract, but were not detectably ordered once they entered the tectum. Is this reduction in tectal topography due to the immaturity of the host brain or to the transplantation itself? For instance, the transplant operation could disorder the projection as it enters the brain. This is unlikely because the heterochronic projections are ordered in the optic tract and only lose this order once they reach the tectum. Such a distal effect is unlikely to be fibers that splayed across the tectum after innervating it precociously, then later reorganized themselves. We have two reasons for believing this: first, the dil was placed at the same time and in the same place as for normal heterochronic transplants and, second, the final tectal projection is generally not ventroperipheral but ventrocentral, just as one would predict for fibers originating from the early born dorsocentral retina. However, since new retinal axons are continually arriving at the tectum, we cannot rule out the possibility that some of these fibers are late-generated axons that picked up dil and projected correctly simply because they arrived at an already-mature tectum. In any case, these results clearly show that the once-young tectum can direct proper retinal projections after it has matured.

DISCUSSION

We have used heterochronic transplants to test when pathfinding and topographic cues appear in the brain, by confronting the diencephalon and tectum with retinal axons earlier than usual and seeing if the axons are properly directed and organized. Unoperated controls and isochronic transplants showed dorsoventral order in the optic tract and tectum by stage 39. This confirms previous results (Holt and Harris, 1983; Holt, 1984; Sakaguchi and Murphey, 1985) and shows that isochronic transplants do not perturb the development of dorsoventral order. In contrast, heterochronic transplants show dorsoventral order in the optic tract, but not in the tectum, as shown by three observations. First, individual precocious axons made unusual turns upon entering the tectum. Second, dil/diA-labelled projections showed significant dorsoventral separation in the optic tract, but not in the tectum. Finally, in dil/diA-labelled projections, diI fibers were significantly ventral of the FIDx midpoint in the optic tract, but dil branch tips straddled the FIDx midpoint in the tectum. Heterochronic transplants allowed to survive for 2 days longer had significant dorsoventral topography, showing that the initial lack of topography is due to the immaturity of the tectum and not some long-lasting effect of the transplantation. Thus, topographic cues are present in the young diencephalon, but absent from the young tectum. The diencephalic and tectal dorsoventral topography cues could be molecularly distinct or, if they are the same molecules, must be temporally distinct (expressed much earlier in the diencephalon).
Timing of topographic cues

Retinal axon growth can be divided into three phases: pathfinding through the diencephalon, target recognition at the tectum and arborization in the tectum. In response to different cues encountered during each phase, retinal axons must grow, turn, stop and branch correctly, and also order themselves topographically. Since the first axons navigate correctly and are topographically ordered, molecular cues for pathfinding and topography must already be present when they normally arrive.

The heterochronic transplant experiments performed here and previously (Cornel and Holt, 1992) further define when these cues are first expressed. In stage 28 to 20 transplants, axons enter the brain approximately 11 hours earlier than usual, and arrive at the tectum by stage 32 to 33/34, 9-13 hours early (Cornel and Holt, 1992). Pathfinding cues in the diencephalon must therefore be present by stage 26, 10 hours before retinal axons usually reach the chiasm. Dorsoventral topography cues in the diencephalon must similarly be present at least 10 hours early. Tectal target recognition cues must be present by stage 32, since precocious projections stop forward growth and spread out upon entering the tectum. Dorsoventral topography molecules must appear in the tectum in the 9 hours between stage 33/34 and stage 37/38 because axons entering the tectum at stage 37/38 find their correct target positions immediately, while axons entering earlier make wide turns and lack topography.

Comparison with other species

Accurate retinotopy seems to be universally present by the onset of visual function in vertebrates, i.e. eye opening for mammals, and hatching for amphibians, fish and birds. However, there are two distinct styles of retinotopic development: single-stage in amphibians and fish, and two-stage in birds and mammals. In *Xenopus* (Holt and Harris, 1983; Sakaguchi and Murphey, 1985), zebrafish (Stuermer, 1988a) and goldfish (Stuermer and Raymond, 1989), retinal axons navigate directly to their correct tectal destination and arborize. Dorsoventral order is apparent at the time of hatching and anteroposterior order soon after. The tectum and retina both continue to grow throughout life; retinal axons slowly enlarge and shift across the tectum in order to maintain correct topography. In contrast, rat (Simon and O’Leary, 1992a), wallaby (Mark et al., 1993) and chick (Nakamura and O’Leary, 1989) develop in two stages. Retinal axons initially make a diffuse projection that overlaps their tectal destination and is then drastically refined by a process of selective branching and elimination of incorrect axons and branches. By the time the eyes open, axonal arbors are precisely localized to the appropriate terminal zone.

Does retinotectal development in birds and mammals differ fundamentally from that in *Xenopus*? Consider when topographic cues first appear in the tectum. One model for two-stage development is that topographic cues are absent or weak when axons first arrive and expressed strongly only later. The heterochronic transplants described here mimic this situation: precocious projections target diffusely, then become topographically refined as the tectum matures. Similarly, late expression of tectal cues in mammals and birds would explain the initial diffuse projection followed by later refinement. A good test of this model would be to delay the arrival of axons at the rat or chick tectum using transplants, but this is technically daunting. Another test is the membrane stripe assay, in which cultured retinal axons are grown on lanes of membrane from anterior or posterior tectum. Temporal retinal axons are able to distinguish between anterior and posterior tectal membranes taken as early as E6 (embryonic day 6) in chicks and E18-19 in rats (Walter et al., 1987; Simon and O’Leary, 1992b). Although in rats it is unclear why axons do not respond well to these anteroposterior cues in vivo (Simon and O’Leary, 1992b), these results show that at least some topographical signals are expressed on chick and rat tecta during the period of diffuse innervation, arguing against this model.

A second model for two-stage retinotopy is that coarse tectal cues are present when axons arrive, but activity-dependent refinement or a second set of cues are necessary for precise targeting. In fact, if we take tectal size into account, one-stage and two-stage development both fit this model. *Xenopus*, goldfish and zebrafish have small tecta (100-200 μm across at hatching), and rats, chicks and wallabies have large tecta (2-8 mm at eye-opening), but all of these species have similar mature arbor dimensions (50-300 μm; Sakaguchi and Murphey, 1985; Thanos and Bonhoeffer, 1987; Stuermer, 1988a). Expressed as a percentage of tectal size, a single arbor in a stage 39 *Xenopus* tectum (~30 μm/100 μm=30%; Sakaguchi and Murphey, 1985) covers the same tectal fraction.
as the projection from a small area of retina in an E9 chick (~3 mm/10 mm=30%; Nakamura and O’Leary, 1989). On the larger chick tectum, however, the projection must be refined to match its mature terminal zone (~5% of tectal width). Two-stage refinement occurs in amphibians and fish during regeneration, when retinal axons reinnervate a (large) adult tectum (Fujisawa et al., 1982, Stuermer, 1988b); the second stage of precise refinement is activity-dependent (Meyer, 1983). Thus it is possible that all vertebrates use the same fundamental mechanisms for establishing retinotopy, with the apparent difference between one-stage and two-stage development arising from differences in tectal size.

The molecules responsible for tectal topography are likely to be highly conserved across vertebrates. In cross-species membrane stripe assays, retinal axons from mouse, chick and goldfish can respond to tectal membranes from other species in a topographically specific manner (Godement and Bonhoeffer, 1989; Vielmetter et al., 1991). Of the plausible candidates for topography molecules (reviewed by Holt and Harris, 1993; Kaprielian and Patterson, 1994), TOPDV is the only one that shows differential dorsoventral expression in the tectum. This 47x10^{-3} M_r membrane glycoprotein is expressed in a tenfold ventrodorsal gradient in the chick tectum and in a reversed gradient in the retina (Moskal et al., 1986; Trisler and Collins, 1987). The tectal gradient is established by E5, just before retinal axons arrive and downregulated by E9, when they begin to arborize in the tectum (Thanos and Bonhoeffer, 1987); this interval roughly corresponds with the initial period of coarse retinotopy. Though antibodies to this molecule can delay synapse formation in the retina (Trisler et al., 1986), no functional studies have yet been done in the tectum and no Xenopus homolog has been reported. The molecules responsible for topography in the diencephalon could be the same as the tectal cues, only expressed much earlier.

Although little is known about the molecular cues for pathfinding, diencephalic topography and target recognition, our results allow us to make two conclusions about how they relate to tectal topographic cues. First, topography in the tectum and topography in the tract are separate phenomena. Order in the tract is not necessary for tectal order, since regenerating axons terminate topographically in the tectum despite taking highly aberrant paths in the tract (Fujisawa, 1981). Neither is tract order sufficient for order in the tectum, since precocious projections are ordered in the optic tract but not in the tectum. Second, since precocious projections change their behavior upon entering the tectum, but do not find their appropriate dorsoventral positions, tectal topography is distinct from target recognition.

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