

Transplantations of the chick eye anlage reveal an early determination of nasotemporal polarity

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ABSTRACT Cells in the optic vesicle must acquire positional values in order to express the molecular cues necessary for the later establishment of a topographically organized retinotectal projection. It is an open question whether in chick this polarity is determined prior to, or at the onset of retinal neurogenesis. We addressed the issue by constructing compound optic vesicles at Hamburger-Hamilton stages 10 to 11, before the first ganglion cell progenitors differentiate. The retinae developing from such vesicles were examined to find out whether transplanted tissue retained its positional information or whether it was respecified in the host eye anlage. Positional information in embryonic day 6 (E6) retinae was assayed with the stripe assay (Walter *et al.*, *Development* 101: 685-696, 1987). This functional *in vitro* test allows us to distinguish temporal from nasal axons. Populations of ganglion cell axons growing out of retinae from operated embryos behaved as predicted from the position of their progenitors in the donor optic vesicle. Moreover, inversion of the anteroposterior axis of optic vesicles led to the development of retinae with an inverted nasotemporal pattern. In order to investigate the retinotectal projection *in vivo*, a retrograde labeling experiment was employed. In control retinae, most ganglion cells labeled from the caudal tectum were confined to the nasal side on E13. Retinae that had developed from double-anterior optic vesicles, however, were labeled on both the nasal and temporal side. Together, these *in vitro* and *in vivo* results demonstrate that nasotemporal specificity is determined in the optic vesicle at or prior to HH10-11.

KEY WORDS: *optic vesicle, retinotectal, transplantation*

Introduction

The projection from the retina to the tectum is topographically ordered in a highly specific manner. During development, retinal axons interact with guidance cues along their pathway, allowing them to grow to appropriate positions in the tectum. Temporal retinal axons project to targets in the rostral (anterior) tectum and nasal axons to targets in the caudal (posterior) tectum. To accomplish this, retinal and tectal cells have to acquire sets of molecules encoding their position within the tissue (Sperry, 1963; Bonhoeffer and Gierer, 1984). Much effort has been undertaken to find such positional cues, and to determine how they are established in the embryo (Holt and Harris, 1993).

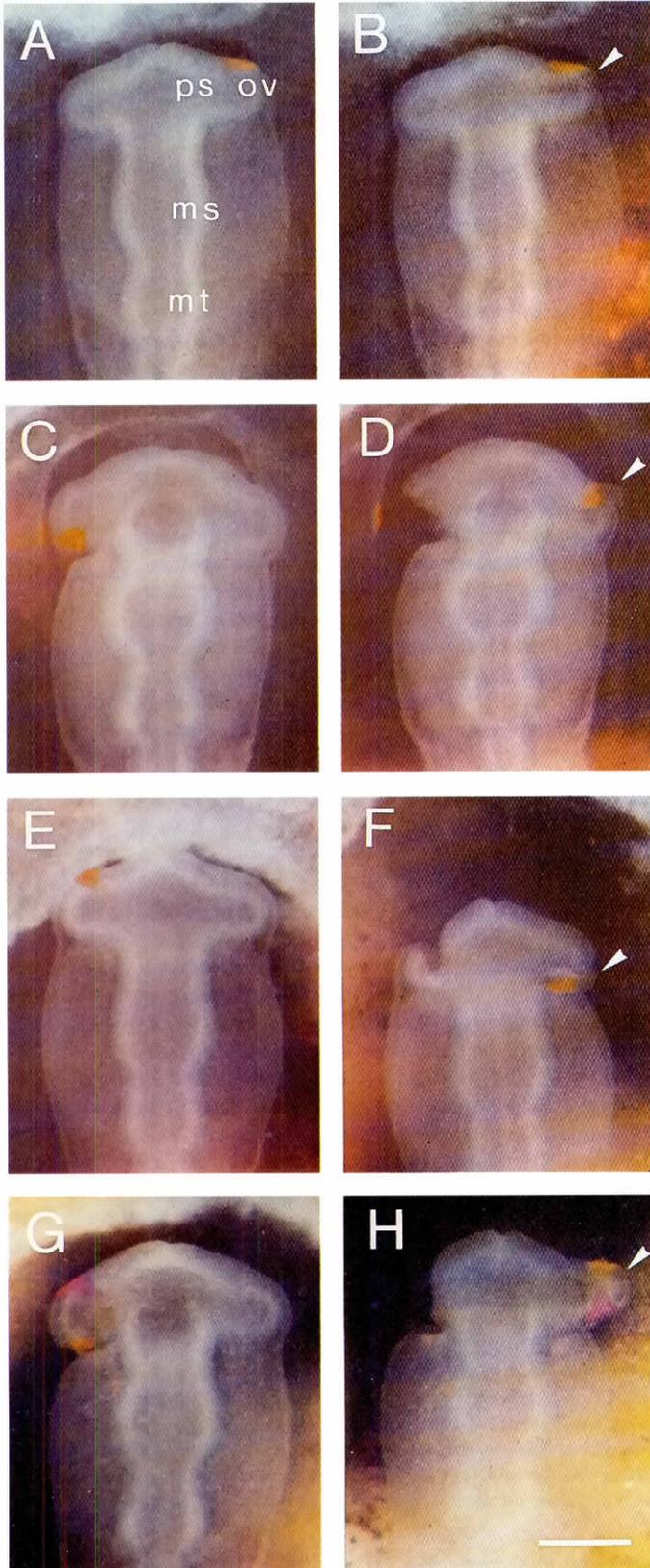
Rotations of the mesencephalic tectal primordium reveal that tectal polarity in the chick is not fixed at Hamburger and Hamilton (1951) stage 10 (HH10) (Ichijo *et al.*, 1990; Nakamura *et al.*, 1994). For the eye anlage it is not clear whether axial polarity is determined at these early embryonic stages. Rotations of the eye anlage in chick between HH11 and HH14 show that some retinal structures (e.g. the location of the optic fissure and retinal fiber patterns) are determined by HH12. Rotations before this stage yield morphologically normal eyes on embryonic day 7-8 (E7-E8)

(Goldberg, 1976). Partial ablations of the optic vesicle at HH12-13 (Crossland *et al.*, 1974) or at HH13-14 (Matsuno *et al.*, 1992) lead to the development of eyes with a projection pattern appropriate to the unexcised part. Together, these experiments suggest that retinal axes are fixed late at HH12, when the first ganglion cells become postmitotic (Kahn, 1973; Prada *et al.*, 1991).

However, recent experiments (Dütting and Thanos, 1995) indicate that anteroposterior polarity may already be established in the eye anlage at early stages (prior to HH11). After ablation of parts of the posterior (temporal) optic vesicle, clusters of cells projecting to the caudal tectum are found within the restored temporal half-retina of the eye. These clusters may arise from nasal progenitor cells, which due to the ablation are displaced into a temporal environment, but retain their already acquired positional value.

Abbreviations used in this paper: HH, Hamburger-Hamilton; E, embryonic day; TT, double-temporal; NN, double-nasal; CoTT, control double-temporal; CoNN, control double-nasal; Rot, rotation; LR, left-right; R, right; DiA, *N*-4-(4-dilinoleylaminostyryl)-*N*-methylpyridinium iodide; DiASP, 4-(4-didecylaminostyryl)-*N*-methylpyridinium iodide; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate.

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Experiments in *Xenopus* also suggest that specification of ganglion cell progenitors occurs early (Fraser, 1991). When labeled undifferentiated cells of the optic vesicle are transplanted into ectopic locations of host eyebuds, the descendent retinal ganglion cells behave as predicted from their position in the donor eyebud. They make an appropriate retinotectal projection independent of the environment in the host vesicle. These experiments, together with earlier rotation and transplantation studies (Gaze *et al.*, 1979; Sharma and Hollyfield, 1980; O'Rourke and Fraser, 1986), suggest that positional values are specified in amphibia before the onset of neural differentiation.

Here, we describe a comparable approach for testing early specification in the eye anlage of chick embryos. Tissue from the left optic vesicle (HH10-11) was transplanted into the right vesicle to construct double-anterior or double-posterior eye anlagen. When embryos with morphologically normal eyes had developed, the behavior of retinal axons was tested in a choice assay ("stripe assay", Walter *et al.*, 1987) or by retrogradely labeling ganglion cells *in vivo*. The majority of cells behaved according to the position of their progenitor cells in the donor eye anlage. These experiments suggest that in contrast to the tectum anlage, positional information in the chick eye anlage is determined very early at the level of retinal progenitor cells.

Results

Heterotopic and homotopic transplantations of the eye anlage

The eye anlage of the HH10-11 chick embryo, the club-shaped optic vesicle, is an evagination of the neural tube in the forebrain region. Most regions of the vesicle are double-layered, the inner neuroectoderm being covered by surface ectoderm. The posterior wall, which borders at mesenchymal tissue, is free of surface ectoderm. The distal and medial portions of the inner layer develop into retina and pigment epithelium, respectively; the adjacent link of the vesicle with the prosencephalon is the prospective optic stalk (Mangold, 1931).

Heterotopic transplantations of the eye anlage were performed at HH 10-11 to either construct double-posterior or double-anterior optic vesicles that develop into double-temporal (TT)- or double-nasal (NN)-eyes (Fig. 1). In addition, homotopic transplantations were performed as controls. For obtaining TT-eyes, the left optic vesicle was freed from the bordering mesenchyme at the posterior wall and subsequently labeled with DiA

Fig. 1. Transplantation of optic vesicle tissue in HH10-11 embryos.

(A) Control TT (CoTT). The anterior right optic vesicle was labeled with DiA and (B) the anterior half excised and reinserted in the same position (position of transplant is indicated by the arrow). (C) Double temporal (TT). The left posterior optic vesicle half was labeled and (D) transferred into the position of the right anterior half. (E) Double nasal (NN). The left anterior optic vesicle half was labeled and (F) transferred into the position of the right posterior half. (G) Rot LR. The left optic vesicle was labeled anteriorly with DiI, posteriorly with DiA, excised, inverted in the anteroposterior axis and (H) transferred into the position of the right optic vesicle. The age of the embryos varied between 10-11 somites (HH10) (E,F) and 12-13 somites (HH11) (C,D). Abbreviations: ov, optic vesicle; ps, prosencephalon; ms, mesencephalon; mt, metencephalon. Scale bar, 300 μ m.

TABLE 1
EFFECTS OF OPERATION

Experiment	Number of operations	Number of survivors	Normal eyes	Eye impairments
CoTT	9	6	4	2
CoNN	10	6	4	2
TT	58	34	14	20
NN	54	29	13	16
Rot LR	34	21	4	17
Rot R	51	31	4	27

Surviving embryos consist of those having normal-sized eyes with an intact ventral optic fissure and those displaying eye impairments (i. e. a complete loss of the eye or severe microphthalmia, non-integrated retinal tissue or a defective optic fissure).

in a posterior-dorsal position (Fig. 1C). Application of the dye label to such a location was necessary to keep track of the dorsal-ventral orientation during transplantation. Thereafter, the anterior half of the right optic vesicle was excised and discarded. Next, the labeled posterior half of the left optic vesicle was excised, turned by 180°, and immediately inserted into the "free space" of the right eye anlage (Fig. 1D) to create a double-posterior optic vesicle labeled anteriorly. In the control experiments (CoTT), the anterior half of the right optic vesicle was labeled in an anterior-dorsal position with DiA, excised and reinserted into the same position (Fig. 1A and B).

For obtaining NN-eyes on the right side of the embryo, the left optic vesicle was labeled with DiA at an anterior-dorsal position (Fig. 1E). To expose the inner neuroepithelium for labeling, the outer surface ectodermal layer was opened with a tungsten needle. The posterior half of the right optic vesicle was removed. Then the labeled anterior half of the left vesicle was excised, turned by 180°, and inserted into the "free space" of the right vesicle (Fig. 1F) to create a double-anterior optic vesicle labeled posteriorly. In controls (CoNN), the posterior half of the right optic vesicle was labeled in a posterior-dorsal position with DiA, excised and reinserted (not shown).

For retrograde labeling experiments NN-eyes were constructed on the left side of the embryo by transferring tissue from right to left, reciprocal to the procedures stated above.

Rotations of the eye anlage

In the two types of rotations performed, Rot LR and Rot R, the optic vesicle was labeled anteriorly with Dil and posteriorly with DiA. In Rot LR (Fig. 1G and H), the labeled left optic vesicle was excised, inverted in the anteroposterior axis, and transferred into the position of the right vesicle. In this experiment the normal dorsoventral orientation is maintained. In Rot R, the labeled right optic vesicle was excised, inverted in both the anteroposterior and dorsoventral axes, and reattached. Grafted tissue contained the entire prospective neuroretina (Goldberg, 1976). After the rotation, Dil labels the posterior, DiA the anterior margin.

Effect of the operations

Embryos with double-anterior, double-posterior or rotated optic vesicles were incubated *in ovo* until embryonic day 6 (E6).

Morphologically normal eyes developed to a varying degree, depending on the type of operation (details are given in Table 1). Successfully operated eyes resulted when the transplant was transferred rapidly, i.e. before the cut edges of the optic vesicle started to round up, and when its size matched the size of the insertion hole. When transferred pieces were too large or too small, defective eyes with non-integrated retinal tissue or with an eyecup containing two unconnected pieces of retina developed. Occasionally, defects in optic fissure formation, pigmentation and lens development occurred in normal sized eyes. In the two types of rotations, the rotated vesicles often did not reattach properly and developed into microphthalmic eyes. Grafted vesicles developed into normal-sized eyes displaying a ventral optic fissure in 19% of the surviving Rot LR and in 13% of the surviving Rot R embryos. Normal-sized eyes that lacked an optic fissure, or had a defective optic fissure not directed ventrally were obtained only in Rot R cases. We flatmounted retinæ from morphologically normal eyes and investigated them for label position.

Distribution of label in retinæ derived from transplanted optic vesicles

Exposure to fluorescent light of labeled cells in retinal whole-mounts used to prepare explant stripes (see below) had to be brief in order to avoid phototoxic damage. Positions of dye were therefore schematically depicted in whole-mount drawings of such retinæ after quick inspection with a fluorescence microscope (Fig. 2; outgrowth patterns from these retinæ are shown in Fig. 5). In addition, we obtained a fate map of transplanted optic vesicle tissue in specimens operated in the same way, but not used for further experiments (Fig. 3).

Excision and reinsertion of the dye-labeled anterior half of the right optic vesicle resulted in a zone of labeled cells in the nasal half of the E6 retina (Figs. 1A,B and 2A). Excision and reinsertion of the labeled posterior half of the optic vesicle resulted in a zone of labeled cells in the temporal half of the E6 retina (not shown). The success rate of such homotopic transplantations was relatively high, yielding normal eyes (CoNN or CoTT) in 67% of the surviving cases.

In the two types of heterotopic transplantations (Fig. 1C-F), the constructed double-posterior and double-anterior optic vesicles developed into TT (41%) and NN-eyes (45%) of normal size and with an intact ventral optic fissure. In TT-retinæ, labeled cells were found almost exclusively in the nasal half (Fig. 2B). A careful investigation of such retinæ confirmed that Dil or DiA-labeled tissue transplanted to an anterior position in the optic vesicle resulted in labeled cells at various positions within the nasal half of the TT-retina, with only a few cells labeled on the temporal side (Fig. 3A shows a representative example). In NN-retinæ a reverse pattern was found (Fig. 2C). DiA-labeled tissue transplanted to a posterior position in the optic vesicle resulted in labeled cells on the temporal side of the retina (Fig. 3B). As in the unoperated controls, the anterior half-vesicle gave rise to the nasal side and the posterior half-vesicle to the temporal side of the retina. These results are in accordance with a detailed fate map of the optic vesicle (Dütting and Thanos, 1995).

After a left-to-right-rotation (Rot LR), DiA was present in an anterior location and Dil in a posterior location of the healed optic vesicle (Fig. 1G,H). On E6, retinæ developing from such vesicles

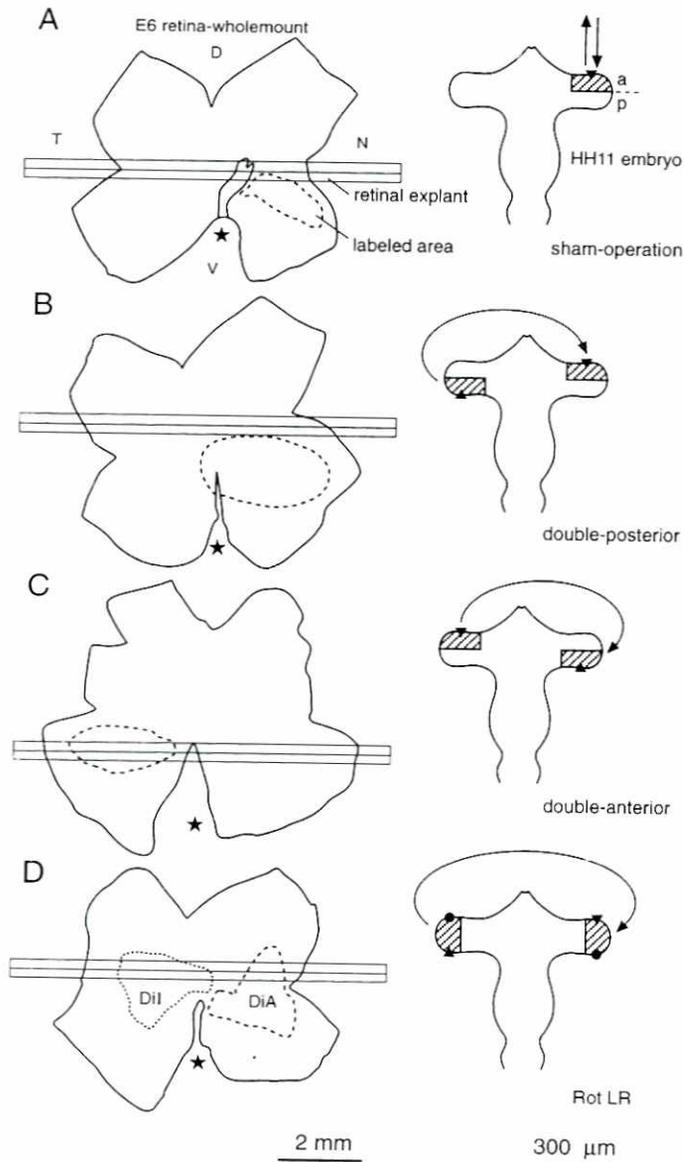


Fig. 2. Positions of dye label in retinal whole-mounts from operated embryos used for stripe assay. The hatched part of the optic vesicle in HH10-11 embryos was dye labeled (triangle: DiA; dot: DiI) and transplanted as indicated (see Fig. 1 for details). On E6, the outline of retina whole-mounts was drawn with a camera lucida device and the area containing labeled cells was marked. The position from which retinal explants for the stripe assay were taken is indicated (outgrowth patterns from these retinæ are shown in Fig. 5). Abbreviations: a, anterior; p, posterior; D, dorsal; V, ventral; T, temporal; N, nasal. Asterisk indicates position of optic fissure. (A) Control TT (CoTT). Removal and subsequent reinsertion of the anterior half of the optic vesicle results in dye label in the nasal retina. (B) Double-temporal (TT). To construct TT-eyes, the anterior half of the right optic vesicle (presumptive nasal) was replaced by the posterior half of the left optic vesicle (presumptive temporal). Dye label is found in the nasal retina. (C) Double-nasal (NN). The posterior half of the right optic vesicle (presumptive temporal) was replaced by the anterior half of the left optic vesicle (presumptive nasal). Dye label is found in the temporal retina. (D) Rot LR. The right optic vesicle was replaced by the left optic vesicle, inverted in the anteroposterior axis. DiA (triangle) is found in the nasal and DiI (dot) in the temporal retina.

cles displayed DiA-labeled cells in the nasal and DiI-labeled cells in the temporal half (Fig. 2D). In this type of rotation the dorsoventral axis is maintained. This was confirmed by labeling the optic vesicle dorsally with DiI and posteriorly with DiA (Fig. 3C). After the rotation, DiI remained in a dorsal position whereas DiA was now in an anterior position. On E6, DiI-labeled cells were found in the dorsal retina and DiA-labeled cells in the nasal retina (Fig. 3C). Finally, we rotated the optic vesicle on the right side (Rot R). By this procedure, the anteroposterior and the dorsoventral axis is inverted (Fig. 3D).

Analysis of axonal behaviors in vitro

When retinal explants from unoperated embryos are analyzed in the stripe assay (Walter *et al.*, 1987), axon outgrowth on the nasal side of the explant is uniform (axons do not distinguish between membranes from anterior and posterior tectum), whereas outgrowth on the temporal side is striped (axons behave temporal-like and grow preferentially on anterior lanes).

For quantitative analysis of compound eyes, two adjacent explants with good outgrowth from each retina were chosen (the approximate position of such explants is indicated in Fig. 2; categories according to which outgrowth from the explants was scored, are shown in Fig. 4). On average, explants extended over 60 stripes or 30 anterior/posterior stripe pairs (smaller or larger explants covered less or more stripe pairs). Outgrowth on pairs of stripes corresponding to the same region within the two explants was compared and scored as *striped*, *uniform* or *mixed*. Axons were said to display striped behavior when the majority of them was growing only on the anterior lanes (Fig. 4A). Uniform behavior meant that outgrowth was not oriented along the membrane lanes, that is axons behaved as if growing on a homogeneous substrate (Fig. 4B). Those stripe pairs which showed both features, i.e. some axons growing preferentially on anterior lanes while others grew unrestricted, were scored as mixed behavior (Fig. 4C). Stripe pairs with no outgrowth or with behaviors which could not be identified unambiguously were omitted. In each experiment, the total number of behaviors for each category was added (temporal and nasal side separately) (Table 2).

Behavior of axons extending from control and compound retinæ

Outgrowth from retinæ of sham-operated control embryos in which the anterior or posterior half-vesicle was removed and reinserted in the same position (Figs. 1A,B and 2A) was similar to that of unoperated controls (Fig. 5A). Occurrence of striped behaviors on the nasal side was rare (0% in unoperated and 4.8% in operated controls). Similarly, uniform behaviors on the temporal side were rare (2.1% in unoperated and 3.3% in operated controls).

Double-posterior optic vesicles were constructed to test whether at HH10-11 the posterior part of the optic vesicle is determined to generate temporal specificity. On average 71.4% of the behaviors scored on the nasal side of these retinæ were found to be striped (temporal-like), compared to less than 5% in the controls (Figs. 5B and 6A). To test whether nasal specificity is determined at HH10-11, double-anterior optic vesicles were constructed. The percentage of uniform (nasal-like) behaviors in the temporal portion of retinæ derived from the transplanted anterior part of the optic vesicle amounted to 70.5%, compared

to less than 5% in the controls (Figs. 5C and 6B). These results demonstrate that the majority of behaviors displayed in the operated retinal half were determined by the original position of the grafted optic vesicle tissue.

It is important to note that on average the effect of the heterotopic transplantations resulted in some 70% striped or uniform behaviors. Most of the other behaviors displayed on the operated side of the retina were mixed behaviors. In fact, 28.0% mixed behaviors in the nasal half of explants from TT-eyes (Fig. 6C) and 22.8% mixed behaviors in the temporal half of explants from NN-eyes were found (Fig. 6D). The increase was not due to the manipulation of the eye-anlage as such, because in sham-operated embryos the percentage of mixed behaviors amounted to only 4.8% in CoTT-eyes and 10% in CoNN-eyes. The finding that more than 20% of the behaviors are mixed behaviors in the operated half of the eye indicates that both temporal-like and nasal-like behaving ganglion cells can appear together in some regions of a retina after tissue transplantation.

Behavior of axons extending from retinæ developed from rotated optic vesicles

We performed rotation experiments in addition to heterotopic transplantations to investigate whether retinæ with an inverted nasotemporal axis were generated. Compared with the unrotated control (Fig. 5A), both types of rotation (Rot LR and Rot R) yielded retinæ with striped behaviors predominantly displayed in the nasal and uniform behaviors displayed in the temporal half (Table 2). The inversion of axonal behavior in a Rot LR retina is shown in Fig. 5D. It results in a high percentage of striped behaviors on the nasal side (Fig. 6A) and a high percentage of uniform behaviors on the temporal side (Fig. 6B). Interestingly, less than 10% mixed behaviors were found in such retinæ (Fig. 6C and D).

Retrograde labeling of ganglion cells in NN-eyes

Most axons from both the temporal and the nasal side of a NN-retina grow uniformly in the stripe assay – they do not seem to be responsive to repulsive guidance molecules present in caudal tectal membranes. Do such axons also grow into the caudal tectum *in vivo*? We retrogradely labeled ganglion cells by inserting Dil crystals into the dorsal aspect of the right caudal tectum on E11.5 in both embryos with left NN-eyes and in unoperated control embryos growing in Petri dishes (for approximate positions of dye in the tectum see Fig. 7). In control embryos we observed a strong labeling of ganglion cells in the nasoventral sector of the contralateral retina but not in the temporoventral sector (Fig. 8A). In the ventral sector of NN-retinæ, comparable strong labeling was found on both the temporal and the nasal side (Fig. 8B).

We quantified the intensity of label on both sides of the retina for control and operated embryos and calculated a specificity index (Fig. 9). The difference between control retinæ (n= 5, specificity index: 4.3±4.6) and NN-retinæ (n= 3, specificity index: 50.9±4.9) was highly significant (unpaired t-test, p<0.001). These results demonstrate that the contralateral caudal tectum of embryos with operated NN-eyes receives a projection of axons from ganglion cells residing in both the nasal as well as in the temporal side of the retina.

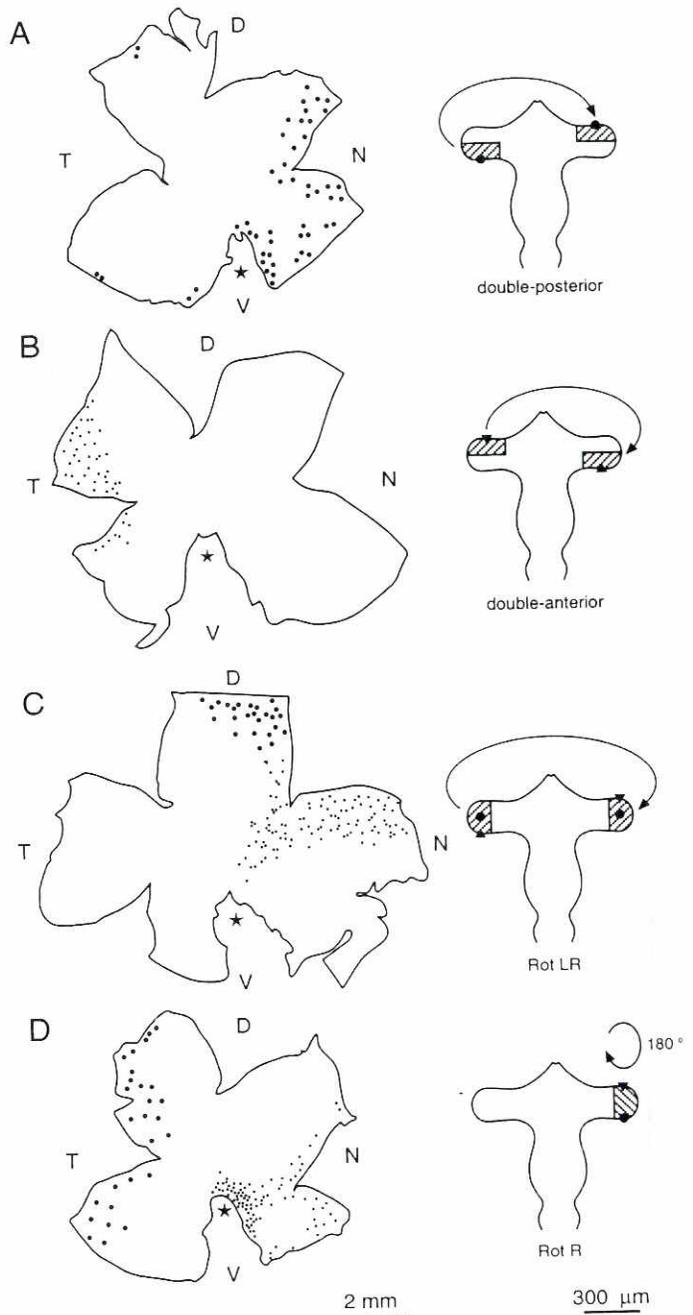


Fig. 3. Fate map of transplanted optic vesicle tissue in operated embryos. Dil (dot) or DiA (triangle) was used to label vesicle tissue (compare with Figs. 1 and 2). The density of labeled cells in the retinæ was indicated by bold dots (Dil) and fine dots (DiA). (A) Vesicle tissue transplanted to an anterior position resulted in labeled cells on the nasal side of the retina. (B) Transplantation to a posterior position resulted in labeled cells on the temporal side. (C) Rot LR. Label from the dorsal side of the vesicle was found in a dorsal portion of the retina, label from the anterior side in a nasal position. (D) Rot R. The right optic vesicle was rotated *in situ* (final position is shown). Label at the anterior portion of the vesicle ended up on the nasal side, label at the posterior portion ended up on the temporal side of the retina. Abbreviations: D, dorsal; V, ventral; T, temporal; N, nasal. Asterisk indicates position of optic fissure.

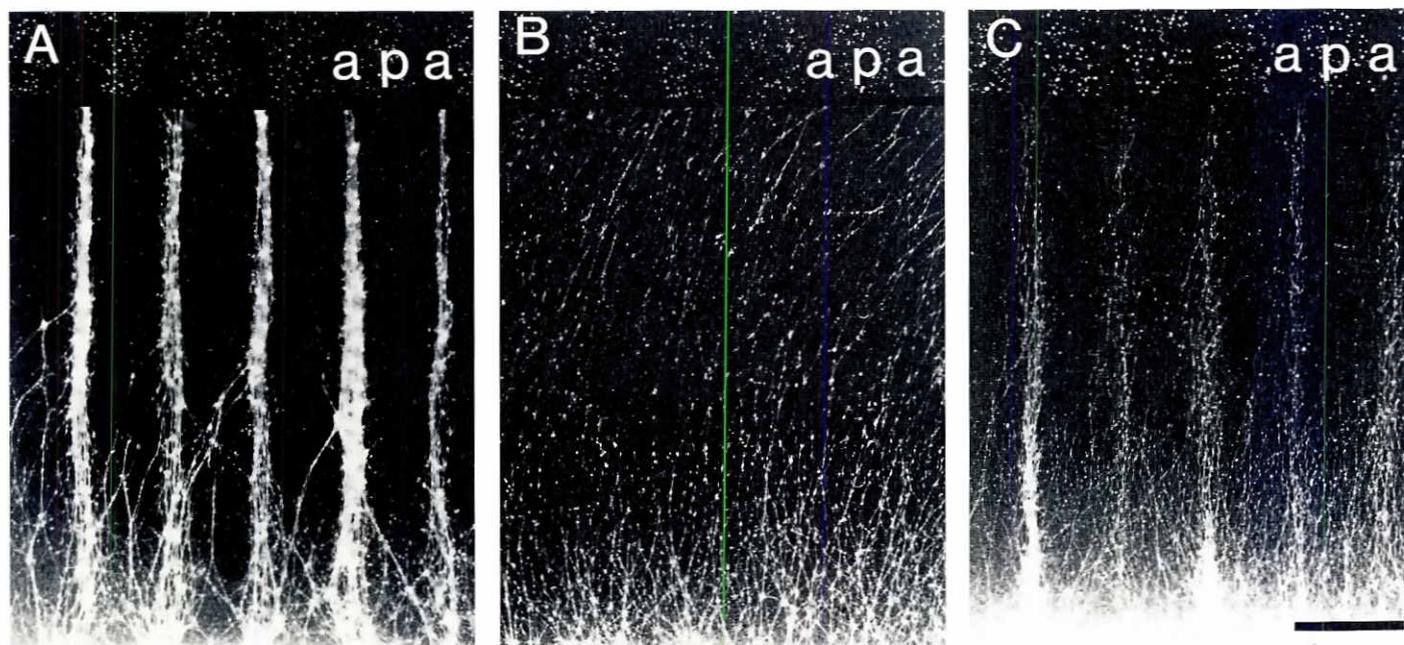


Fig. 4. Categories of axonal behavior in the stripe assay. (A) *Striped behavior.* Axons grew preferentially on stripes consisting of anterior tectal membranes and avoid stripes of posterior tectal membranes. (B) *Uniform behavior.* Axons show no preference for either membrane type. (C) *Mixed behavior.* Some axons prefer anterior stripes, while others show no preference. In each panel (A-C) axon outgrowth is seen on 5 pairs of ap stripes. For example, quantification of behaviors in this region of the retina would yield 5 striped behaviors in (A), 5 uniform behaviors in (B) and 5 mixed behaviors in (C). Abbreviations: a, anterior; p, posterior. Scale bar, 200 μ m.

Discussion

According to Crossland *et al.* (1974), the acquisition of positional information in the chick eye anlage coincides with the onset of neurogenesis (late HH12). At this stage, the first ganglion cell progenitors withdraw from the cell cycle and start to differentiate (Kahn, 1973; Prada *et al.*, 1991). Studies in amphibia challenged such a view by demonstrating that progenitor cells can transmit positional values to their progeny (Fraser, 1991). Here, we present evidence from transplantation experiments in chicks suggesting that positional specification along the antero-posterior axis of the eye anlage, the later nasotemporal axis of the retina, is established at or prior to HH10, i.e. before ganglion cell production starts.

Analysis of double-temporal and double-nasal compound eyes

At HH10-11 double-posterior and double-anterior optic vesicles were constructed by transplanting the posterior or anterior half of the left donor vesicle into the position of the anterior or posterior half of the right host vesicle. The vesicles developed into eyes with an intact ventral optic fissure in many of the surviving, successfully transplanted embryos. We analyzed compound eyes from such embryos at E6 with the stripe assay developed by Bonhoeffer and colleagues (Walter *et al.*, 1987). This *in vitro* assay allows temporal axons to be discriminated from nasal retinal axons. Either axons behave temporal-like, that is, they prefer anterior (rostral) over posterior (caudal) tectal membrane lanes, or they behave nasal-like and grow equally

well on both lanes. No graded behavior of temporal or nasal axons has been observed in the stripe assay or in any modified version (Baier and Bonhoeffer, 1992). Guidance of axons toward appropriate tectal positions, not only with respect to the rostral and caudal tectal halves, but also within the halves themselves, are likely to require graded positional cues in retina and tectum (Gierer, 1987). It remains undecided whether the binary nasotemporal distinction demonstrated in this study is due to the limited resolution of the stripe assay, or it indicates a simple subdivision of the early optic vesicle into two specified halves.

The stripe assay analysis revealed that the majority of ganglion cells in retinae derived from double-posterior vesicles behaved temporal-like, whereas the majority of cells from double-anterior vesicles behaved nasal-like. In both cases, the position of progenitor cells in the donor optic vesicle determined the subsequent behavior of their descendent ganglion cells in the host retina. These results suggest that progenitor cells are committed for positional values in HH10 optic vesicles and express adequate markers later in development. We could not extend the study to developmental stages prior to HH10 due to technical constraints. Therefore, commitment may occur earlier, for example already in the early neural plate (Altshuler *et al.*, 1991).

The fate of regions in the optic vesicle has been mapped by injection of lipophilic dyes (Dütting and Thanos, 1995). The resolution of this method did not allow us to delineate a sharp border between a putative anterior and a putative posterior compartment in the vesicle. It is therefore likely that transplanted anterior tissue contains some cells from the posterior compartment and *vice versa*. In that case, an anterior graft, which con-

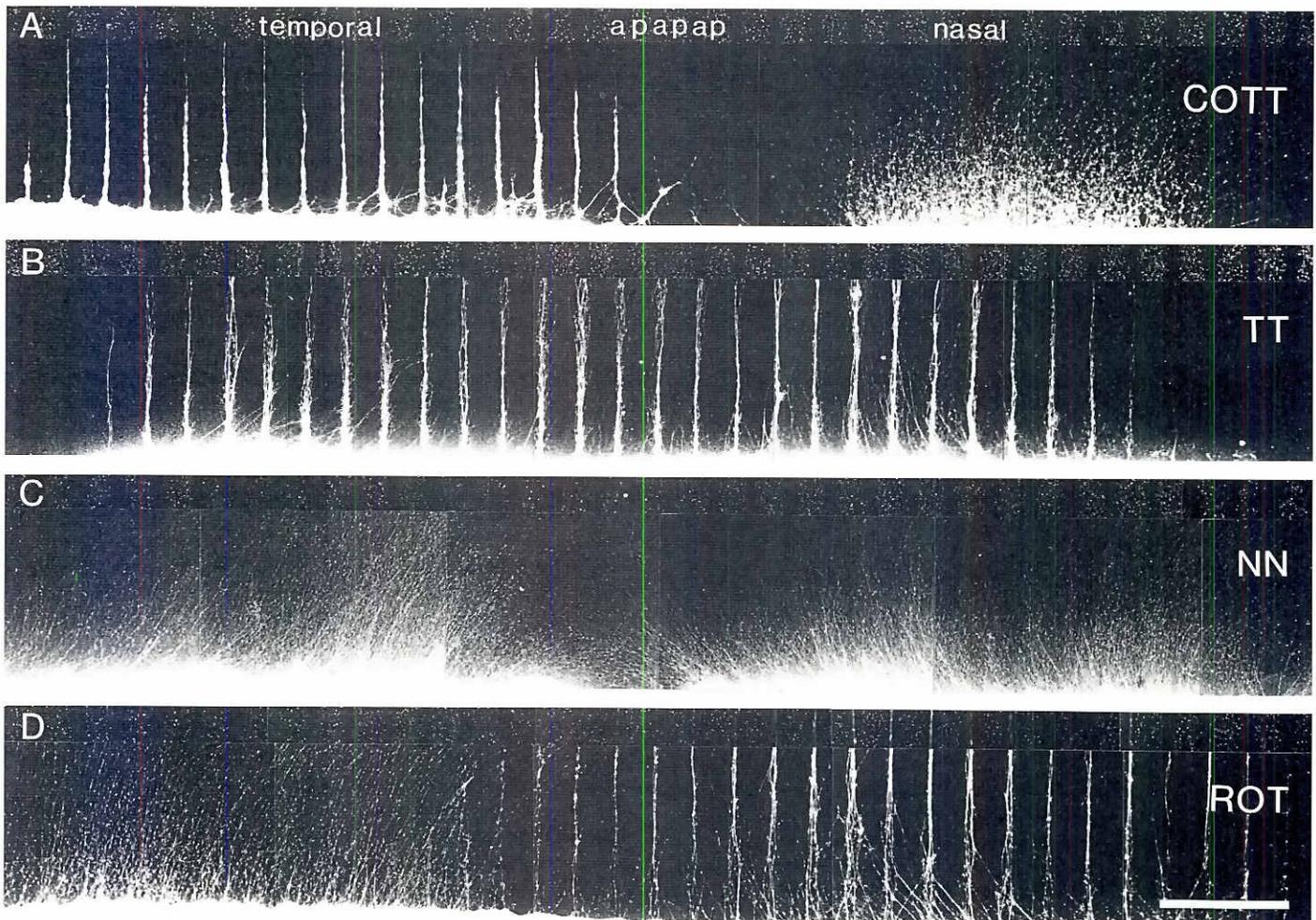


Fig. 5. Outgrowth behavior of axons from E6 retinae after experimental manipulation of optic vesicles at HH10-11. Operations were performed as described in Figure 1A-H. Retinal explants were taken from whole-mounts indicated in Figure 2A-D. Temporal is to the left, nasal to the right. **(A)** Control TT (CoTT). Axons display striped behavior in the temporal half and uniform behaviors in the nasal half. **(B)**, Double temporal (TT). Axons in the temporal and nasal half prefer stripes of anterior membranes. **(C)** Double nasal (NN). Axons in both retinal halves show no preference for stripes. **(D)** Rotation LR (Rot). Axons in the nasal half display striped behaviors whereas axons in the temporal half show uniform behaviors. Scale bar, 600 μ m.

tains mostly progenitors of nasal cells, would also contain some progenitors of temporal cells. Only if progenitors are axially committed and maintain their fate, such a graft would be expected to give rise to retinal regions which contain mostly nasal-like behaving ganglion cells and in addition some areas in which nasal-like and temporal-like cells appear together. In the stripe assay outgrowth from the latter areas would appear "mixed".

Indeed, we found areas in the compound retinae displaying such mixed behaviors (Fig. 6C and D). The production of heterogeneous grafts may occur to varying degrees, sometimes yielding only pure (striped or uniform) and sometimes yielding additional mixed behaviors within a retina (Table 2). Sham-operated embryos showed more variation than unoperated ones, but considerably less amounts of mixed behaviors than the heterotopic transplantations. This argues that the operation can cause some mixing of cells, but the transfer of a heterogeneous popu-

lation of cells with the graft is most likely to account for the higher numbers of mixed behaviors observed in compound retinae. The observed outgrowth patterns are best explained, if one assumes that irreversibly committed progenitor cells mix after the transplantation and their progeny differentiate appropriately.

Colocalization of nasal and temporal ganglion cells has also been observed by Dütting and Thanos (1995). After partially ablating the posterior (temporal) optic vesicle often a morphologically normal retina developed. When a retrograde tracer was applied to the caudal tectum, clusters of cells with nasal-like projection patterns were displayed in a temporal retinal background. These cells may stem from determined nasal progenitors which were displaced into an environment of temporal progenitors after the ablation.

Together, these studies suggest that the nasal or temporal fate of progenitor cells is not altered by environmental factors

TABLE 2

BEHAVIOR OF RETINAL AXONS FROM TRANSPLANTED EYES IN THE STRIPE ASSAY

Operation	experiment	Temporal side			Nasal side		
		SB _T	UB _T	MB _T	SB _N	UB _N	MB _N
unoperated	Co 1	11	0	2	0	11	0
	Co 2	15	1	0	0	17	0
	Co 3	16	0	0	0	14	2
sham-operated	CoTT1	11	2	4	0	16	0
	CoTT 2	16	0	0	0	12	0
	CoTT 7	15	0	0	2	10	2
sham-operated	CoNN7	12	1	2	0	15	0
	CoNN 9	14	0	1	0	12	3
double post. (TT-eye)	TT 1	15	0	0	14	0	0
	TT 16	12	0	0	2	0	7
	TT 20	13	0	0	9	0	3
	TT 35	14	0	0	15	0	0
	TT 37	18	0	0	18	0	0
	TT 45	15	0	0	5	0	11
double ant. (NN-eye)	NN 8	0	6	10	0	17	0
	NN 10	5	10	0	0	13	1
	NN 12	0	12	0	0	14	0
	NN 27	0	7	3	0	10	0
	NN 45	0	11	3	0	13	2
ap rotation (Rot LR)	Rot LR 17	4	11	2	17	0	0
	Rot LR 26	0	17	0	14	1	0
ap+dv rotation (Rot R)	Rot R 16	0	6	0	13	0	0
	Rot R 23	0	11	4	15	0	0

In each experiment, axonal growth patterns across the nasotemporal axis of the retina were analyzed on all pairs of membrane stripes covered by the retinal explant. Axon growth on each stripe pair was assigned to one of three possible categories ([SB] striped-, [UB] uniform-, [MB] mixed behavior) (compare with Fig. 4). A stripe pair consisted of one anterior and one posterior lane of tectal membranes and its evaluation yielded one behavior. On average, a retinal explant extended over 30 stripe pairs and accounted for 30 behaviors (variations occurred due to the size of individual explants). Co, Control; TT, double-temporal; NN, double-nasal; ap, anteroposterior; dv, dorsoventral; Rot, Rotation.

after HH10. Our results are in agreement with a model proposed by Altshuler *et al.* (1991). According to this model, positional information is established early in mitotic progenitor cells and can be passed to their progeny independently of later commitment and differentiation processes leading to the different cell types.

In contrast to this model, Crossland *et al.* (1974) have argued that the chick optic vesicle is not yet axially polarized at HH11. Their conclusion is based on a single case (CC29), described first in Cowan and Wenger (1968). In the experiment large parts of the optic vesicle were removed at HH11 and no intact eye developed. From the small amount of retinal tissue that remained in the optic tract few fibers projected to all regions of the tectum. It is possible that the residual part of the operated vesicle contained progenitor cells each determined to give rise to neurons projecting to one of the quadrants of the target. These could have been mixed and then resulted in a largely reduced projection including all topographic regions.

Analysis of rotation experiments

Both types of rotations yielded retinae with an inverted nasotemporal axis. These results are consistent with the heterotopic transplantation experiments, suggesting that nasotemporal polarity is determined early. A lower amount of mixed behaviors was found in some rotations (Rot LR) as compared to the heterotopic transplantations. This may be explained by the fact that in a rotation the complete vesicle is transplanted, leaving the anterior and posterior halves together. Such a type of manipulation may be less likely to result in mixing of anterior and posterior progenitor cells.

In addition, the rotations demonstrate that the inversion of the dorsoventral axis does not interfere with the inversion of the anteroposterior axis. Unfortunately, the stripe assay does not allow us to distinguish dorsal from ventral axons. Thus, we can not deduce from these experiments whether dorsoventral polarity is also established at HH10-11.

Retrograde labeling of ganglion cells in double-nasal retinae

By using an *in vitro* test, we found that retinal polarity is established as early as HH10 in the optic vesicle. In addition, we investigated the projection pattern of operated eyes *in vivo*. On E13/14 the caudal tectum is invaded predominantly by nasal axons and only by a small portion of temporal axons (6.4% on E13, as measured by retrograde labeling; Dütting and Thanos, 1995). Although a large population of temporal axons overshoot their final target area on E13, the majority of these axons does not invade the caudal tectum (Nakamura and O'Leary, 1989). Therefore retrograde tracing from the caudal tectum is an appropriate method to selectively label ganglion cells of nasal specificity. In retinae from unoperated embryos, label was predominantly found on the nasal side. In NN-retinae, however, large labeled areas were present on both the nasal and the temporal side. These data indicate that the anterior progenitor cells, transplanted to a posterior position in the optic vesicle (HH10-11), developed into ganglion cells of nasal retinotopic specificity on the temporal side of the compound retinae. They also underline the validity of the stripe assay as a reliable test for positional information on chick retinal axons: axons growing uniformly in the stripe assay grow into the posterior part of the tectum *in vivo*.

Our results extend those of Goldberg (1976), who demonstrated that when the axes in the optic vesicle are only defined by morphological criteria (i.e. orientation of the fissure), they do not appear to be fixed before HH12. When functional criteria (i.e. axonal behaviors) are used to evaluate the effect of optic vesicle manipulations *in vitro* or *in vivo*, as was done in this study, they reveal that nasotemporal polarity is present before HH12. The same may hold true for dorsoventral polarity. Although the optic vesicle is unrestricted in its morphological development before HH12, this does not imply that progenitor cells are not committed as dorsal or ventral at these stages. Insight into the determination of dorsoventral polarity requires assay systems which are sensible for these differences. Classical transplantation experiments combined with the investigation of expression patterns of regulatory genes in the optic vesicle and manipulation of their expression are promising means to further elucidate the events contributing to the very early determination of polarity in the vertebrate eye anlage.

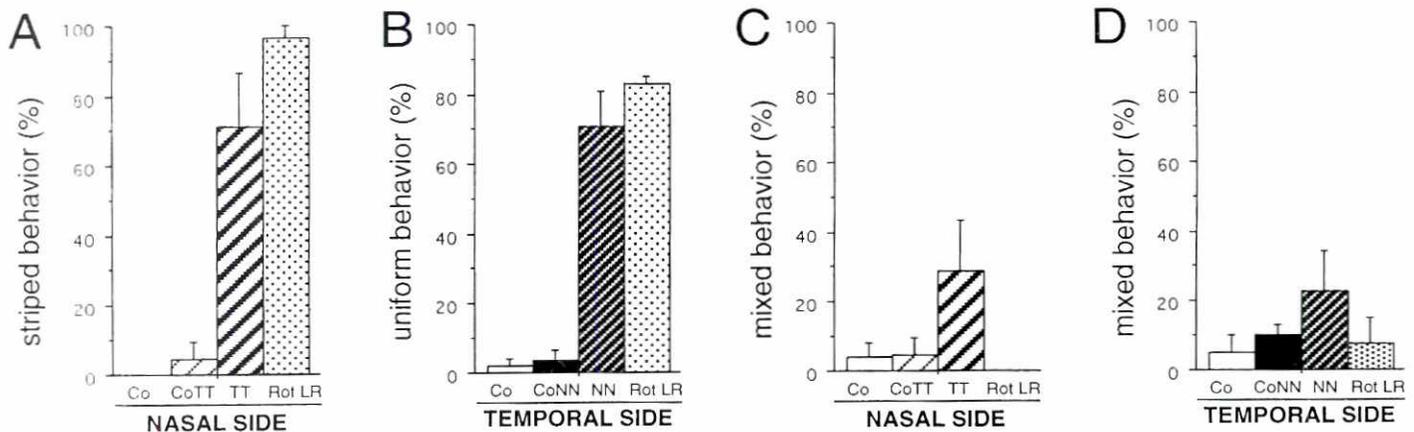


Fig. 6. Quantification of axonal behaviors from retinæ of E6 embryos. The percentage of behaviors on either the temporal or nasal side of a retina was calculated from the values given in Table 2 (e.g. % striped behavior = $100 \times$ number of striped behaviors/number of total behaviors). Mean behaviors for all retinæ with the same type of operation are shown (error bar indicates standard error). **(A)** On the nasal side the percentage of striped behaviors was only high (>70%) when it developed from posterior (presumptive temporal) optic vesicle tissue (TT, double-temporal; Rot LR) but not in controls when it was derived from the anterior (presumptive nasal) optic vesicle (Co, unoperated; CoTT, sham-operated). **(B)** On the temporal side more than 70% of the behaviors were uniform, when the retina was derived from anterior optic vesicle tissue (NN, double-nasal; RotLR) but less than 5% when it was derived from posterior optic vesicle tissue (Co, unoperated; CoNN, sham-operated). **(C)** The percentage of mixed behaviors on the nasal side was increased in TT-embryos. **(D)** The percentage of mixed behaviors on the temporal side was increased in NN-embryos.

Materials and Methods

General procedures and terminology

Fertilized chicken eggs (White Leghorn) were incubated at 38°C and 60% humidity with occasional turning for 36 to 40 h and windowed. Between HH10 and HH11 (10 to 13 somite embryos), the embryo's head was visualized by injecting a solution of black drawing ink (Pelikan) diluted in phosphate buffer under the blastoderm. The microsurgery of the optic vesicles was performed with electrolytically sharpened tungsten needles. After the operation, the windows were covered with a plastic lid and the eggs returned to the incubator until they reached E6 (HH29).

To relate regions of the compound and rotated optic vesicles to those of E6 retinæ, we locally injected one of the water-insoluble fluorescent dyes, Dil (D3911) or DiA (Fast DiA oil, D3897; Molecular Probes Inc., Eugene, USA) dissolved in ethanol (2 mg/0.1 ml).

A fate-map of the eye anlage (Dütting and Thanos, 1995) demonstrated a correspondence between the anteroposterior axis of the optic vesicle and the nasotemporal axis of the retina. The anterior part of the optic vesicle develops into nasal retina, the posterior part into temporal retina. Therefore, we use the terms double-anterior and double-posterior when referring to compound optic vesicles, and the terms double-nasal (NN) and double-temporal (TT) when referring to the retinæ derived from them. This terminology is not meant to imply any presumptions about the distribution of positional information in the optic vesicle and retina, or the time of its determination.

Stripe assay

We prepared E6 retinæ as described previously (Walter *et al.*, 1987). Camera lucida drawings of retina whole-mounts were made and positions of dye label marked under epifluorescence illumination. A low power objective (5x) was used to avoid phototoxic damage. Next, we stained the retinæ with DiASP (D-291, Molecular Probes). Fine crystals of this dye (1% DiASP in dimethylformamide) were centrifuged (15 min at 2000 rpm) onto the retinal surface. Retinal explants (275 µm) were cut along the nasotemporal axis. The position of each explant was marked in the camera lucida drawing of the retina. The explants were then cultured on tectal membrane carpets in 3 ml of supplemented F12 medium contain-

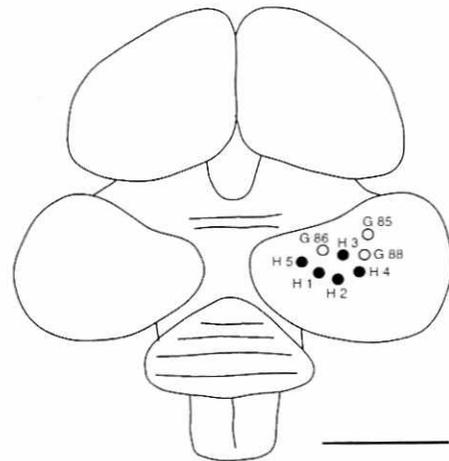


Fig. 7. Positions of Dil crystals in the tecta of embryos used for retrograde labeling. A camera lucida drawing of a representative embryonic chick brain (dorsal view, E13.5) is shown. Crystals were inserted into the caudal part of tecta in control embryos (H1-H5) and in embryos with NN-retinæ (G85, G86, G88) on E11.5 and are shown in their final positions on E13.5. Scale bar, 3 mm.

ing 0.4% methyl cellulose at 37°C and 4% CO₂. Carpets with alternating stripes of anterior (rostral) and posterior (caudal) membranes were prepared according to Walter *et al.* (1987). After 48 h, cultures were fixed (4% paraformaldehyde containing 0.33 M sucrose) and examined with a Zeiss Axiophot microscope.

Retrograde labeling of ganglion cells in vivo

The whole content of windowed eggs with operated and control embryos was transferred into Petri dishes on E3 (Dütting and Thanos, 1995). Most embryos come to lie on their left side, rendering the right side most easily accessible for experimental manipulation. On E11.5 a

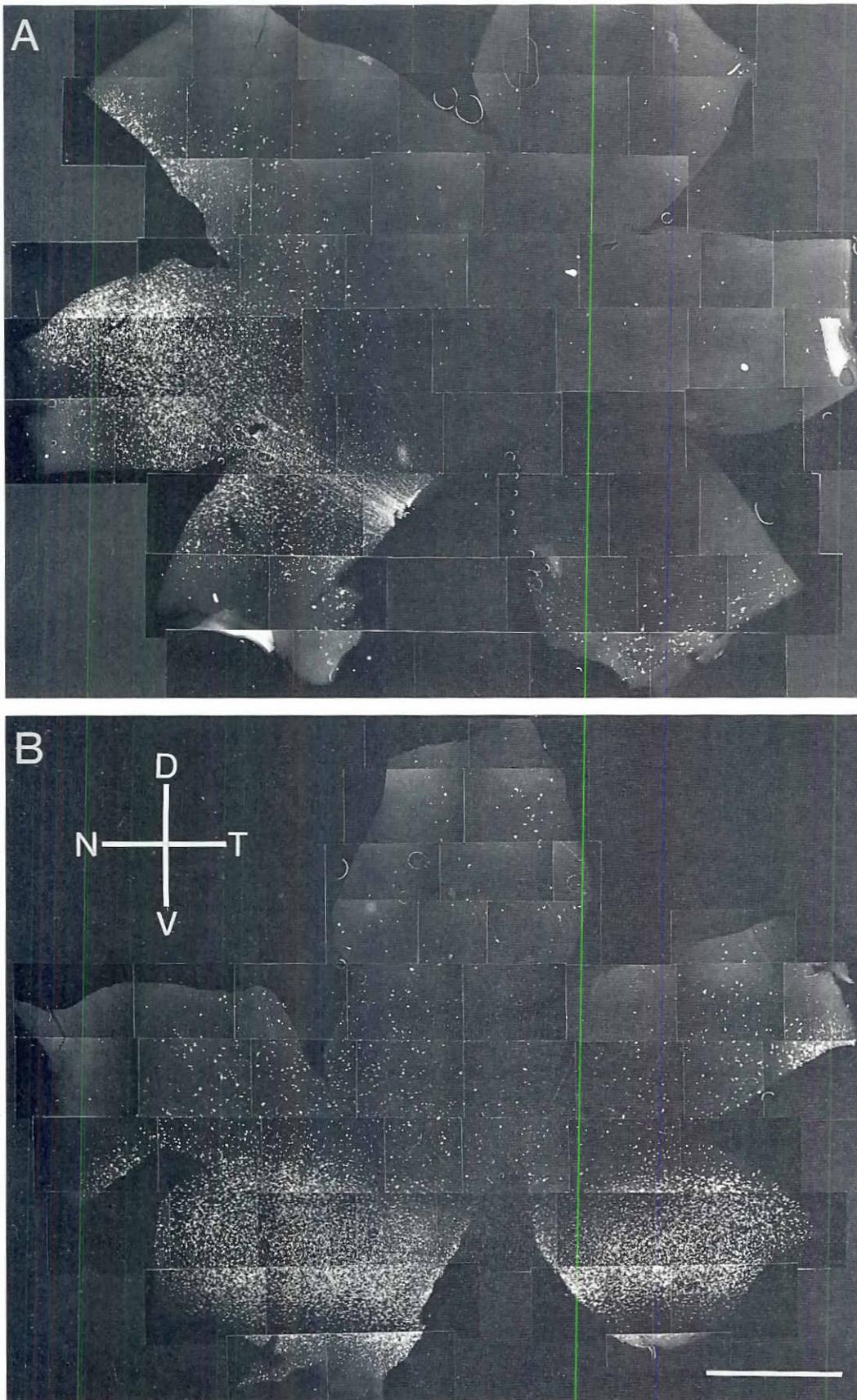


Fig. 8. Retrograde labeling of ganglion cells in control and NN-retinae. (A) Unoperated control retina (H1). Label is predominantly found in the nasoventral sector. **(B)** NN-retina (G86). Ganglion cells in the nasoventral and in the temporoventral sector are labeled with comparable density. Abbreviations: N, nasal; T, temporal; D, dorsal; V, ventral. Scale bar, 3 mm.

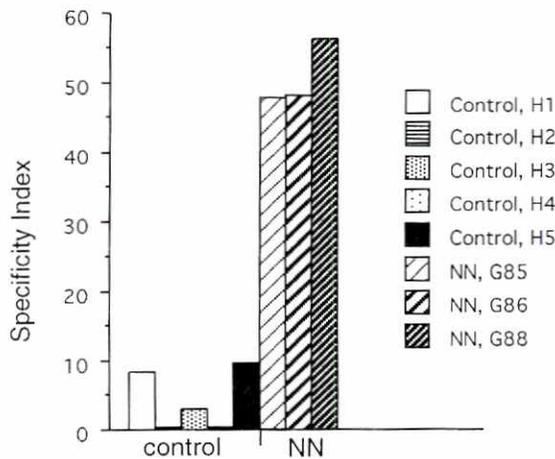


Fig. 9. Quantification of labeling intensity in control and NN-retinae. Retinae were retrogradely labeled from the caudal tectum (compare Fig. 7) and analyzed with a CCD camera in temporal and nasal regions with highest staining intensity (compare Fig. 8). A specificity index (SI) was calculated (values can range from zero to 100; a value of zero indicates that all label is in the measured nasal field; a value of 50 indicates identical amounts in the nasal and temporal fields). In controls ($n=5$, $SI=4.3\pm4.6$) label is predominant on the nasal side. In NN-retinae ($n=3$; $SI=50.9\pm4.9$) comparable amounts of label are found on the nasal and on the temporal side.

crystal of Dil (D3911; Molecular Probes) was inserted into the right optic tectum, as far caudally as possible. After 48 h further incubation, the left retina and the brain were removed from embryos and fixed in 4% paraformaldehyde. Retinae were flatmounted on glass slides, covered with 2% n-propyl gallate (Sigma) in phosphate buffered glycerol and photographed with an Axiophot (Zeiss) fluorescence microscope (5x objective, rhodamine filter).

Analysis of retrograde labeling experiments

The distribution of label in retinae obtained after retrograde tracing from the caudal tectum was analyzed by a first observer (D.D.) and depicted in a camera lucida drawing of the retinal whole-mount. Then, a second observer (S.U.M.) used these drawings to locate the regions of highest staining intensity in the nasal and the temporal half of the retina. In both half-retinae, 4 areas ($400\ \mu\text{m}^2$ each) were selected from these regions for quantitative analysis. Furthermore, 4 areas of the same size within regions of low staining intensity were selected to determine background staining.

Images from all selected areas within each retina were obtained by using a CCD video camera and Adobe Photoshop software. NIH Image software was used to calculate the mean pixel intensity in each image. The values were averaged for the temporal, nasal and background areas in each retina. Background intensity was subtracted from temporal intensity (yielding I_t) and from nasal intensity (yielding I_n). For individual retinae, a specificity index ($100 \times I_t / (I_t + I_n)$) was calculated. Values can range between zero (all measured intensity is in the nasal half) and 100 (all measured intensity is in the temporal half).

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