Specification of retinotectal connexions during development of the toad *Xenopus laevis*

By S. C. SHARMA¹ and J. G. HOLLYFIELD²

From the Department of Ophthalmology, New York Medical College, and Cullen Eye Institute, Baylor College of Medicine, Houston

**SUMMARY**

The specification of central connexions of retinal ganglion cells was studied in *Xenopus laevis*. In one series of experiments, the right eye primordium was rotated 180° at embryonic stages 24–32. In the other series, the left eye was transplanted into the right orbit, and vice versa, with either 0° or 180° rotation. After metamorphosis the visual projections from the operated eye to the contralateral optic tectum were mapped electrophysiologically and compared with the normal retinotectal map. In all cases the visual projection map was rotated through the same angle as was indicated by the position of the choroidal fissure. The left eye exchanged into the right orbit retained its original axes and projected to the contralateral tectum. These results suggest that retinal ganglion cell connexions are specified before stage 24.

**INTRODUCTION**

Studies of neuronal specificity in the visual system are concerned with understanding how neuronal connexions between the retina and visual centers in the brain are established during development. Although the specific developmental mechanisms which govern the establishment of the precise neuronal circuitry present in the adult visual system remain to be determined, a chemospecificity hypothesis initially proposed by Sperry (1951) suggests that their formation is made possible by the existence of selective affinities between retinal ganglion cells and their target cells in the optic tectum. These properties are thought to vary relative to the position of individual cells within the retina and tectum (Sperry, 1963). Sperry also suggested that the specification of a cell is achieved through a polarized field type of coordinate system within the retina and the tectum, with the alignment of these coordinates parallel to the two main embryonic axes. Evidence for this hypothesis came from behavioral studies of animals whose eye cup had been rotated 180° at various stages of development. Eyes so manipulated developed normal vision if the operations were performed at a sufficiently early stage, whereas inverted visuomotor behavior occurred if

¹ Author's address: Department of Ophthalmology, New York Medical College, Valhalla, New York 10595, U.S.A.
² Author's address: Cullen Eye Institute, Baylor College of Medicine, Houston, Texas 77030, U.S.A.
eyes were rotated at later stages. Furthermore, the nasotemporal axis of the eye was set before the dorsoventral axis (Székely, 1954, in Triturus).

These eye-rotation studies were repeated in *Xenopus laevis* and evaluated using electrophysiological mapping techniques by Jacobson (1968a). Eyes were rotated 180° at and before stage 32 (Nieuwkoop & Faber, 1967) and subsequent electrophysiological maps were performed at late larval stages or after metamorphosis. Jacobson reported that the anteroposterior retinal axis (i.e. nasotemporal) was specified at stage 29/30 whereas specification of the dorsoventral axis was at stage 31. Eyes rotated at or before stage 28 were respecified in both axes so that the eye, though upside down, contained ganglion cells which projected to the optic tectum in a normal orientation. Using [3H]thymidine autoradiography, Jacobson (1968b) also demonstrated that the first ganglion cells in the central retina became post-mitotic shortly before the specification of the ganglion cells' central connexions. Thus, the cessation of DNA synthesis in the differentiating ganglion cell was followed closely by the specification of the cells' central connexion.

In direct contrast to these studies, our experiments exploring the timing of axial specification in the developing retina of *Rana pipiens* showed that ganglion cell central connexions were specified before the first ganglion cells became post-mitotic (Sharma & Hollyfield, 1974a). We observed that specification of the retina had occurred prior to early tail-bud stages while all cells in the retinal neuroepithelium continue to divide. Furthermore, the visual maps in these studies with *Rana* could be predicted prior to actual electrophysiological mapping by the position of the choroidal fissure on the rotated eye. In the normal animal a small notch or cleft is present along the ventral margin of the iris. Closely associated with the fissure position is a large blood vessel on the inferior aspect of the globe. When these morphological markers were present in any position out of register with their normal alignment, the retinotectal map was always shifted through the same angle as was the eye (Sharma & Hollyfield, 1974a). Although this prominent ventral marker is also present in *Xenopus laevis*, none of the earlier studies by Jacobson (1968a, b) or Hunt & Jacobson (1972a, b, 1973a, b) has commented on the relative position of the choroidal fissure in relation to orientation of the retinotectal map.

The consistency of our results in *Rana pipiens* prompted us to reinvestigate in *Xenopus* the orientation of retinotectal maps relative to choroidal fissure position in eyes rotated at various stages of development or reciprocally exchanged between left and right orbit. A preliminary report of this study was presented previously (Sharma & Hollyfield, 1974b).
MATERIALS AND METHODS

*Xenopus laevis* embryos used in the present study were laboratory spawned. The embryos were removed from the jelly mass and washed through ten changes of sterile 10% Holtfreter solution (Hamburger, 1960). Embryos were staged according to Nieuwkoop & Faber (1967). The embryos were then transferred to 100% Holtfreter solution. Using finely sharpened iridectomy scissors or tungsten microelectrodes, incisions were made in the area surrounding the right eye rudiment. The mesodermal cells, which were occasionally attached to the eye rudiment, were removed using a hair loop (Hamburger, 1960). The eye rudiment was then returned to the orbit with 180° rotation and was held in place with a small glass bridge until healing commenced. Operated embryos were transferred to 50% Holtfreter solution until healing was completed (usually in 30 min to 1 h) and were then transferred to 10% Holtfreter solution. All operations were performed at room temperature (21–23 °C). Right-eye rotations were performed on 73 embryos from stage 24 through to stage 32.

In a second series of experiments the positions of right and left eyes were exchanged. Using identical surgical procedures described above, after removal of both eyes the left eye rudiment was transferred to the right orbit and held in place until healing commenced. This procedure was repeated for the right eye. The left-to-right eye exchanges, and vice versa, were done either with no rotation or with 180° rotation on embryos at stages 26–31. Some of these procedures (ten animals in each series) were also performed in 25% Steinberg solution pH 7.2. Tadpoles were fed nettle powder or ‘Beechnut’ brand baby food until metamorphosis. Five to eight tadpoles were reared in 10 gal tanks. After metamorphosis, *Xenopus* were fed minced beef heart.

The technique for mapping the visuotopic projection was similar to that described earlier (Sharma & Hollyfield, 1974a), with the exception that the electrodes used were glass-coated platinum-iridium wires with a tip diameter of 4–6 μm. The tips were coated with platinum black. The electrodes were placed 200 μm apart on the tectum. Following electrophysiological mapping, the brain was fixed in Heidenhein’s Susa and sections were stained with Holmes’s silver method.

RESULTS

The position of choirodal fissures in *Xenopus laevis* is prominent at tadpole stages as well as in postmetamorphic animals when all our electrophysiological mapping was performed. A number of examples of choroidal fissures and ventral blood vessels in normal and rotated positions are illustrated in Fig. 1. The choroidal fissure was prominent in each of the experimental animals used in these studies.

The normal visual field of each eye in *Xenopus* projects to the contralateral optic tectum. The nasal visual field to the rostral tectum; temporal field to the
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caudal tectum; dorsal field to the medial tectum; and inferior field to the ventral tectum. The projection of a normal map onto the optic tectum in *Xenopus* is presented in Fig. 2a. Electrophysiological maps obtained in the experimental series are described below.

180° eye rotations. A total of 59 animals were mapped, with six giving no results. Histology of these six animals revealed that the optic nerve failed to exit from the orbit. A summary of the visual field mapping results from the 53 remaining animals utilized in this series is presented in Table 1. In animals operated upon at early stages, the choroidal fissure most frequently appeared on the dorsal surface (35 of 53), indicating that the eye was rotated 180° out of register from its normal position. In each of these animals the visuotopic projection was also rotated 180°. This result was consistent regardless of the stage of eye rotation. A map from an animal which had 180° rotation at stage 24 of development is presented in Fig. 2b. In five animals the choroidal fissure was in a temporal location, 90° out of register with its normal location. In each of these animals the visuotectal map was shifted approximately 90° (Fig. 3). In eleven animals the position of the choroidal fissure was ventral, suggesting that rotation had not occurred. In all of these animals the maps were normal.

The remaining two animals gave duplicate maps and are labelled as compound maps. In one of these maps (Fig. 4) the dorsal visual hemifield projected to the full extent of the dorsal tectum (dashed lines). However, the ventral visual half field also projected to the entire extent of the dorsal tectum but was rotated 180° (solid lines). Two choroidal fissures were present in this eye, one directed dorsally and the other ventrally. A double fissure had been previously observed in this animal at stage 40 (Fig. 1). The two eyes from which compound maps were obtained each contained a single lens and otherwise appeared normal. The presence of direct ipsilateral projections was not tested in any animal.

Reciprocal eye exchange. Retinotectal projections were mapped in 40 animals in which the left eye had been transplanted into the right orbit and vice versa with either 0° or 180° rotation. The number of animals, time of surgery and

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**Figure 1**

(a) The right eye of a normal two-week post-metamorphic *Xenopus*. The choroidal fissure is located at the 6 o’clock position. The visuotopic map of this eye is normal (see Fig. 2a).
(b) The right eye of an animal rotated 180° at embryonic stage 24. The choroidal fissure is located at the 12 o’clock position, indicating the rotation of the eye by 180°. (For visuotopic map, see Fig. 2b.)
(c) The right eye indicating the position of the choroidal fissure at the 9 o’clock position. The eye was rotated 180° at stage 25.
(d) Both eyes showing the position of the choroidal fissure at 180°. In this animal the right eye was transplanted into the left orbit (and vice versa) with 180° rotation.
(e) The right eye of an embryo photographed at stage 40. In this animal the right eye was rotated 180° at stage 26. Two fissures, one at the 12 o’clock position and the other at the 6 o’clock position, appeared at stage 34. The resultant map is shown in Fig. 4.
resultant visuotectal projections are summarized in Table 2. In all cases the transplanted eye projected to the contralateral optic tectum.

Whenever the eye exchanges were done with 0° rotation, the choroidal fissure appeared at its normal ventral position. In these cases the nasotemporal axis of the eye was reversed as compared to the body axis, and the dorsoventral axis was aligned with the body axis. In cases where eye exchange was done with 180° rotation, thereby reversing the dorsoventral axis of the eye, the nasotemporal axis of the eye would remain normal with respect to the nasotemporal axis of the host orbit. The visuotectal projection of the left eye placed in the right orbit with 0° rotation showed a reversal of the nasotemporal axis, but the dorsoventral axis was normal. Such projections were consistent whether the eye exchange was done as early as stage 27 or later. A map from one of these animals is shown in Fig. 5.

However, when the eye exchange was performed with 180° rotation, the choroidal fissures were positioned at altered locations. The positions of the
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Fig. 2. (a) The visual projection map from a normal right eye to the left optic tectum. Each number in the visual field shows the position of the stimulus that evoked maximum potentials recorded by an electrode at the position indicated by the same number on the tectum. Electrodes were placed 200 μm apart on the tectum. The visual field extends 100° from the center to the periphery. The small circle on the right side shows the eye diagrammatically with the choroidal fissure pointing ventrally. d, Dorsal; v, ventral; n, nasal; and t, temporal. The conventions are the same for subsequent figures. (b) Right visual projection map to the left tectum in an animal whose eye was rotated 180° at embryonic stage 24. The position of the choroidal fissure is dorsal. The map is rotated 180°.

Fissures at the time of recordings are shown in Table 2. When the fissure appeared at the dorsal position, the nasotemporal axis of the electrophysiological maps was normal but the dorsoventral axis was rotated (Fig. 6). In two animals the choroidal fissure in the left eye transplanted into the right orbit was directed temporally. The resulting map was rotated in a 90° clockwise orientation. In these cases both nasotemporal and dorsoventral axes were rotated 90° clockwise. The maps were similar to Fig. 3 except the dorsoventral axes were reversed.
Table 1. 180° right eye rotations only

<table>
<thead>
<tr>
<th>Stage of operation</th>
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<th>Fissure position at time of recording</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>4</td>
<td>Dorsal – 2 animals Temporal – 1 animal Ventral – 1 animal</td>
<td>Map rotated 180°</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Rotated 90° counterclockwise</td>
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<td></td>
<td></td>
<td></td>
<td>Normal map</td>
</tr>
<tr>
<td>25</td>
<td>7</td>
<td>Dorsal – 4 animals Temporal – 1 animal Ventral – 2 animals</td>
<td>Rotated 180°</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rotated 90° counterclockwise</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Normal map</td>
</tr>
<tr>
<td>26</td>
<td>4</td>
<td>Dorsal – 1 animal Temporal – 1 animal Ventral – 1 animal Ventral and dorsal – 1 animal</td>
<td>Rotated 180°</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Rotated 90° counterclockwise</td>
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<td>Normal map</td>
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<td></td>
<td></td>
<td></td>
<td>Compound map</td>
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<tr>
<td>27</td>
<td>8</td>
<td>Dorsal – 4 animals Temporal – 1 animal Ventral – 3 animals</td>
<td>Rotated 180°</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Normal map</td>
</tr>
<tr>
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<td>10</td>
<td>Dorsal – 5 animals Temporal – 1 animal Ventral – 3 animals Ventral and dorsal – 1 animal</td>
<td>Rotated 180°</td>
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<tr>
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<td>Normal map</td>
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<td></td>
<td></td>
<td>Compound map</td>
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<tr>
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<td>12</td>
<td>Dorsal – 11 animals Ventral – 1 animal</td>
<td>Rotated 180°</td>
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<td></td>
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<td>Normal map</td>
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<tr>
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<td>6</td>
<td>Dorsal – 6 animals Ventral – 1 animal</td>
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</tr>
<tr>
<td>32</td>
<td>2</td>
<td>Dorsal – 2 animals</td>
<td>Rotated 180°</td>
</tr>
</tbody>
</table>

Some delay may occur in the complete spread of ganglion cell axons on to the tectum following the surgery employed in these studies. The retinotectal projection in a normally developing frog is initially confined to the rostral portion of the tectum and then proceeds to fill the caudal regions. Normally the entire tectum has received input by the time of metamorphosis. In the present series of experiments a few animals were found to have visual responses confined to the rostral three-fourths of the tectum. In some of these animals, even at 1 month beyond metamorphosis, the retinal projection still had not extended to the caudalmost tectum. All animals mapped 6–8 weeks after metamorphosis showed extension of the visual projection over the entire tectum. These findings suggest a temporary delay in the course of development of the retinotectal projection, possibly caused by the prior surgical manipulations of the eye. The caudal tectal areas in which we failed to show any visually stimulated responses also were free of melanocytes within the covering meninges. However, in the rostral tectum, where we were able to record visually evoked responses, the meningeal melanocyte population was normal. Animals showing the complete coverage of the tectum by optic input also contained meningeal melanocytes throughout the entire tectal expanse. These results may indicate some interaction between optic nerve fibers and invasion of melanoblasts into the meninges. Alternatively
the absence of prominent melanocytes in the meninges in this location may reflect some interdependence of optic nerve fibers interaction for normal melanogenesis.

**DISCUSSION**

From this study we make the following conclusions: (a) the degree of rotation of the visuotectal map always corresponds to the degree of rotation of the choroidal fissure; (b) the reciprocal eye exchange, with or without eye rotation, expressed retinotectal connexions according to the original axes of the eye and were never respecified by axial cues from the new orbit; (c) eyes reciprocally exchanged to contralateral orbit projected invariably to the contralateral optic tectum indicating the absence of side specificity.

Szekély (1954) performed reciprocal exchanges between left and right eye anlage in embryonic urodeles and showed that in some animals the optic fibers grow into the ipsilateral tectum while in other cases the growing optic nerve
Fig. 4. The right visual projection to the left optic tectum. The right eye shows two fissures – dorsal and ventral. The temporal half of this eye projected normally, whereas the nasal eye map was rotated 180°. Although this eye had a duplicate map, it has only one lens (see Fig. 1e).

decussated to form a chiasm and innervated both tecta. The author proposed that guiding structures in the brain and optic stalk led to the crossing of fibers. Recently, Beazley (1975) transplanted a host eye from the opposite side of a donor *Xenopus* (stage 30–38) with either 0° or 180° rotation. In a majority of cases, the transplanted eye projected to the contralateral side. Beazley concluded a lack of ‘side specificity’ for the growing optic axons and suggested that a complete decussation depended upon the ability of growing optic axons to enter the optic stalk and that the stalk itself was responsible for guiding the direction of the growing axons. In the present experiments the bilateral exchange of the eye anlage from stage 24–32 showed that transplanted eyes had no preference to connect with the tectum normally appropriate to that eye but invariably proceeded to the contralateral tectum. These experiments further support the notion that the optic stalk plays a major role in guiding the fibers to the opposite side of the brain.
Sperry (1945) predicted the possibility of separate specification of the naso-temporal and dorsoventral axes of the retina. Later Székely (1954) rotated the eyes by 180° at neural-plate stage and the resulting animals showed normal behavior, whereas eyes rotated at neural-tube stage developed reversed vision. Furthermore, by transplanting the right eye to the left orbit and by rotation of the nasotemporal and dorsoventral axes separately, Székely (1957) found that the nasotemporal axes were determined first, followed by dorsoventral. However, optokinetic behavior of these animals was reported as confused and, as pointed out by Székely, these studies warranted further investigation. Similarly in the studies utilizing *Amblystoma*, the behavioral paradigm employed was likewise not conclusive, since eyes rotated before the so-called critical stage of specification showed aberrant behavior (Stone, 1960).

Comparatively strong electrophysiological evidence for the separate axial specification was reported by Jacobson (1968a) and Hunt & Jacobson (1972a, b, 1973a, b), but in none of these studies is there any indication that the position of the choroidal fissure was utilized to verify whether the test eye had been successfully rotated. In the present study the visuotectal maps we obtained always correlated with the anatomical markers of the eye. There was no indication that the rotated eyes or eyes exchanged between left and right orbit were respecified even when the operations were performed before the so-called critical period at stage 29/30. In each of these animals the degree of rotation of the resultant map always shifted to the same degree as indicated by the position of the choroidal...
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Fig. 5. Visual projection of a right eye on to the left tectum. This was originally a left eye which was transplanted into the right orbit at stage 27 without any rotation. The nasotemporal axis of the visual projection is reversed, but the dorsoventral axis remains the same as compared to the normal right visual projection. The eye diagram indicates the choroidal fissure at the ventral position. n and t in the parentheses represent the original pole of the eye.

Recently published studies by Gaze et al. (1979) have also found that the results of visuotectal map orientation always corresponded to the orientation of the eye at the time of mapping except for a few cases in which 'compound' maps were recorded. In the latter cases, the portion of the map which was oriented like the eye came from the originally operative eye tissue, while the other portion of the map came from eye tissue newly grown from the optic stalk.

The question of the position of the choroidal fissure is an important aspect of the conclusions in this study. From the time of its appearance in the ventral rim of the early optic cup its location is evident throughout the life of the animal. Its position was utilized in this study as an index to the degree of altered orienta-
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Fig. 6. The right visual projection map on to the left tectum. Initially the left eye was transplanted into the right orbit with 180° rotation at embryonic stage 27. The choroidal fissure at the time of recording was at the 12 o'clock position. v and d in parentheses indicate the original poles of the left eye. The dorsoventral axis of the projection was inverted, but the nasal temporal axis was normal.

tion of the eyes at the time of recording. Stone (1966) studied the timing of the determination of the location of choroidal fissure by rotating eyes in Amblystoma in a manner similar to the techniques employed in all the studies dealing with determination of retinotectal specificity. Stone rotated eye rudiments at progressively later stages of development (before the morphological appearance of the choroidal fissure) and then assessed for a subsequent determination of choroidal fissure position by recording the fissure location following its appearance at subsequent stages. Using these criteria he reported that the choroidal fissure position was not permanently established until around stage 34 (late optic-vesicle stage). The critical stage for retinal polarization in Amblystoma is stage 36 (Stone, 1960). We can conclude from these two studies that choroidal fissure location is determined before retinal polarization. Because of this
sequence of developmental events in *Amblystoma*, we would expect that an eye rotated after stage 34 could be respecified and have a normal map but have dorsally directed choroidal fissure.

It is not known when choroidal fissure position is determined in *Xenopus laevis*. In histological preparation of *Xenopus* retinas (Hollyfield, unpublished) the fissure can be identified as early as stage 27, but it is not readily apparent in the living embryo until the appearance of melanin in the pigment epithelium at around stages 31 and 32. In all the eyes either rotated in the orbit or exchanged between left and right orbit from which maps were obtained in this study, the map orientation always corresponded with the orientation of the choroidal fissure in the test eye. This result suggests that if fissure location and retinotectal polarity are independent events in *Xenopus*, they are established at about the same stage of development.

In a number of cases, although eyes were clearly rotated when reimplanted into the orbit, the choroidal fissure later appeared in a normal ventral position. According to the criteria of Stone (1966) we should conclude that although the ventral margin of the developing eye was placed in a dorsal position, a new fissure was established in the dorsal aspect of the rotated eye which was not located ventrally. If this indeed did occur in our experiment, since the retinotectal map we obtained from these animals showed normal orientation with respect to the fissure, we might conclude that the retinotectal projection had been respecified following rotation of the developing eye rudiment in the orbit. However, in the absence of a clear morphological marker indicating that the eye remained in a rotated position and did not rerotate to its original orientation, it is not possible to make firm conclusions regarding the repolarization or respecification of the developing retina in these animals.

In the chick embryo, Goldberg (1976) reported that following eye rotation prior to stage 12, the choroidal fissure always appeared at ventral (normal) position. However, beyond stage 12 there was a gradual shift of the development of the fissure to the 180° position. In the present study the fissure appeared at either 90° or 0° positions following 180° eye rotations in 40% of the animals operated on or before stage 29/30. However, the fissure always appeared at 180° rotated position after stage 30. It is possible that the fissure position in *Xenopus* is established around stage 31. If this is the case, since all our maps corresponded to the position of the fissure, it is possible that retinal polarization and fissure location are established at the same developmental stage. Still remaining, however, is the fact that we failed to demonstrate any stage in which the eye could be rotated or exchanged to the opposite orbit and have the independent specification of either nasotemporal or dorsoventral axes as observed by Jacobson (1968a). In eyes rotated as early as stage 24 or thereafter, the visual field maps indicated that both axes of the developing retina were fixed at the time of rotation.

The question of timing of cessation of DNA synthesis in the ganglion cells
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of the central retina in relation to the specification of their central connexions also deserves comment. Jacobson (1968b) reports that all cells in the retinal neuroepithelium incorporate [3H]thymidine until stage 28, at which time a few cells drop out of the mitotic cycle and subsequently differentiate (become specified). Cima & Grant (1978) have recently reported that a bundle of axons, which they interpret as of ganglion cell origin, is present in the optic nerve head of Xenopus as early as stage 28. Since it is usual for axonal development to occur only in post-mitotic cells (see Jacobson, 1978, for review), it is unlikely that axons would be elaborated prior to withdrawal of these cells from the mitotic cycle. These observations of Cima and Grant suggest that some cells stop dividing much earlier than the stage reported by Jacobson (1968b). Our results reported in this paper indicate that polarization of the developing retina also occurs earlier than previously reported (Jacobson, 1968a). The exact relationship with respect to the relative timing of these two events must await further re-evaluation.

In conclusion, in our experiments retinotectal projections from eyes either rotated in the orbit or exchanged between left and right orbit were expressed according to the original axis of the eye at the time of the manipulations. In no case was a normally oriented map obtained from a rotated eye when the choroidal fissure position indicated that the eye was indeed rotated. We are left with no evidence indicating that orbital cues can influence or respecify the axial coordinates within the retinal rudiment. All our data indicate that specification of the retinal map is determined prior to the earliest stages used in this study (stage 24). Furthermore, there is no evidence from these studies that anterior–posterior and dorsal–ventral axes are specified as separate developmental events.

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