Proc. Nat. Acad. Sci. USA
Vol. 69, No. 10, pp. 2860–2864, October 1972

Specification of Positional Information in Retinal Ganglion Cells of *Xenopus*: Stability of the Specified State

(neuronal specificity/retinotectal connections/eye transplantation)

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Communicated by Eliot Stellar, July 28, 1972

ABSTRACT **In the normal development of retinotectal connections, the site in the tectum at which an optic fiber synapses is related to the position of its ganglion-cell body in the retina. How and when the ganglion cells acquire information about their positions is unknown, but the positional information that each ganglion cell will ultimately act upon is determined or specified at embryonic stages 28–32 in the clawed frog, *Xenopus laevis*. Here we report that once positional information has been so specified, it remains stable when the eye is "back-grafted" into the orbit of a stage-28 host, or cultured *in vitro* for up to 10 days before grafting into the orbit of a stage-38 host. Thus, the ganglion cells of these eyes form tectal connections appropriate to their *original* positions in the donor orbits and independent of their final positions in the host orbits. We conclude that specification of positional information involves stable changes in the phenotypic properties of the differentiating retinal cells that (*i*) render the cells refractory to information about changes in their positions after stage 32 and (*ii*) commit each ganglion cell to the development of a unique property (locus specificity) that predisposes its axon to synapse at a particular locus in the retinotectal map.**

Retinal ganglion cells, whose axons make up the optic nerve, form precise point-to-point connections with visual relay cells in the optic tectum, such that a "map" of the retina is projected onto the tectal surface. Preliminary observations on several vertebrate species (1–6) suggested that this pattern of connectivity is determined in early embryonic life, before the outgrowth of optic nerve fibers to the brain. In *Xenopus laevis* for example (6), a normal pattern of retinotectal connections developed after 180° rotation of the eye-cup at early tail-bud stages 28–29, but the same operation performed a few hours later produced inversion of the retinotectal map in the anteroposterior axis of the tectum (stage 30 operation) or in both tectal axes (operation at stages 31–32 or later). Although physiochemical analysis of this system has not yet been undertaken, a wealth of indirect evidence (7–9) suggests that each ganglion cell possesses a unique biochemical indentity, termed *neuronal specificity*, which determines the synaptic relations it will entertain with other neural cells.

We are presently concerned with only one aspect of neuronal specificity, that which we call *locus specificity*, which enables the axon of each ganglion cell to reach its proper locus in the retinotectal map. In a recent report (10), we found that the development and expression of locus specificity do not depend upon (*i*) induction by substances unique to the ocular orbit, or (*ii*) the absolute position of the ganglion cell on the body surface, or (*iii*) a precise timetable of arrival of different ganglion cell axons into the tectum. Instead, locus specificity apparently derives from *positional information* (10), that is, information that the ganglion cell acquired concerning its position in relation to the other cells in the retinal field, and to the major body axes. Without making assumptions about the exact timing of intercellular communication or information processing in the retina, we concluded (10) that the positional information that each ganglion cell will ultimately act upon is determined or *specified* at stages 28 to 31–32.

The present experiments further explore the transition from the "unspecified" state at stage 28 to the "specified" state at stage 32. Particular attention is paid to the reversibility or irreversibility of the transition, and to the stability of the specified state.

METHODS

Embryos of the African clawed frog, *Xenopus laevis*, were obtained and staged as described by Nieuwkoop and Faber (12), anesthetized for surgery in 0.01% MS-222 (Sandoz), and reared through metamorphosis on nettle powder in 10% amphibian saline at 20–26°. Six experimental designs *A–F* were used (Fig. 1). In two of these (*A* and *D*), the right eyes from stage 31–32 embryos were cultured *in vitro* for 6–10 days, and then reimplanted into the enucleated right orbits of stage-38 siblings. The remaining four procedures (*B*, *C*, *E*, and *F*) involved "back-grafting" eyes from stage-30 embryos or from stage 31–32 embryos into the enucleated right orbits of stage-28 siblings. Detailed descriptions of the individual experiments are given in the *Results*.

A control experiment was done in order to assure the "unspecified state" of the host embryos in the back-grafting experiment. Hosts were selected when the first criteria for stage 28 appeared. In each series, when the *host's* right eye was preserved and transferred directly into another stage-28 right orbit, these animals subsequently showed normal vision through the transplanted eye.

In the culture experiments (*A* and *D*), only eyes that remained perfectly intact throughout the culture period were reimplanted, since damaged embryonic eyes are usually rejected by most larvae. The eyes cultured under our conditions (modified Steinberg's medium, ref. 13, 3–4 eyes in 4 ml of medium in 10-ml petri dishes, at 21 ± 1°C) showed excellent

This is paper No. 2 on "Specification of Positional Information in Retinal Ganglion Cells of *Xenopus*." Paper 1 is ref. 10.

ocular and retinal differentiation, including extensive optic-nerve fiber growth in older cultures.* The percentage of eyes that remain intact varied widely with matings, ranging from 0–50% for naked eye cups and 0–95% for eyes cultured in a small piece of epithelium. Further information on the culture system will be published later.

Visually guided strike responses to a lure placed in the visual field of the experimental eye were tested in the host animals at metamorphosis (14). These responses (scored as normal, misdirected, or absent) assay for the existence and organization of *functional* visual connections in the brain. Most of the animals were tested during metamorphosis, before the visual fields of the two eyes come to overlap (15); occasionally, however, the animal could only be tested later, and this required crushing the optic nerve from the normal eye.

Shortly after metamorphosis, we used electrophysiological methods to map the pattern of termination of the ganglion-cell axons in the tectum (6, 10). A 100-nm grid was superimposed on a 50 × magnified photograph of the frog's tectum, surgically exposed after anesthesia (0.05% MS-222) and paralyzation (0.01% Tubocurarine). At each grid position, we penetrated the superficial layers of the tectum with a platinum–iridium microelectrode (tip diameter about 2 nm) and then determined the position on an opthalmic perimeter (radius 33 cm, centered about the frog's right eye) at which a small spot of light (10′–5°) evoked the maximum response. Responses typically consisted of spikes or spike trains from 1 to 5 units, and were monitored on oscilloscopes and over a loudspeaker. To confirm that *functional* retinotectal synapses developed from the experimental eye, recordings from intertectal visual fibers were also made in about half the animals. Where possible, the projection from the normal eye to the right tectum was also mapped and is included in the figures; however, since the pattern of connections from the normal eye was always normal, further discussion concerns only the experimental right eye. Although agenesis of the right optic nerve occurred in several animals, clear and consistent results were obtained from the 34 host animals (4–7 of each experimental type) in which the right optic nerve reached the tectum.

RESULTS

All type *A* and type *B* host animals showed normal visually guided strike responses and normal retinotectal maps (Fig. 2*a* and *b*). In type *A*, the stage-31–32 right eye was cultured for periods of 6–10 days and reimplanted *with normal orientation* into the stage-38 right orbit; in type *B*, a stage-30 right eye was transplanted directly into a stage-28 right orbit, again maintaining the normal anatomical orientation of the eye (Fig. 1). That both groups showed normal vision and normal retinotectal connections indicates that (*i*) transplantation of a stage-30 eye or (*ii*) culture and transplantation of a stage-31–32 eye do not inherently alter the pattern of retinotectal connections the eye will establish with the brain. Thus, these type *A* and *B* animals serve as controls for the other four experiments in which the orientation of the eye was changed during transplantation to the host embryo.

In type *C*, a stage-31–32 right eye was transplanted, with 180° rotation from its normal orientation, into a stage-28 embryo. In type *D*, a similar eye was cultured for 6–13 days *in vitro* and reimplanted, with 180° rotation from its normal orientation, into a stage-38 embryo. Hosts of type *C* and type *D* all showed misdirected visually-guided strike responses and a pattern of retinotectal connections that was inverted in both tectal axes (Fig. 3*a* and *b*). This means that in both groups, locus specificities derived from positional information that was specified in the *donor* embryo, before surgical intervention.

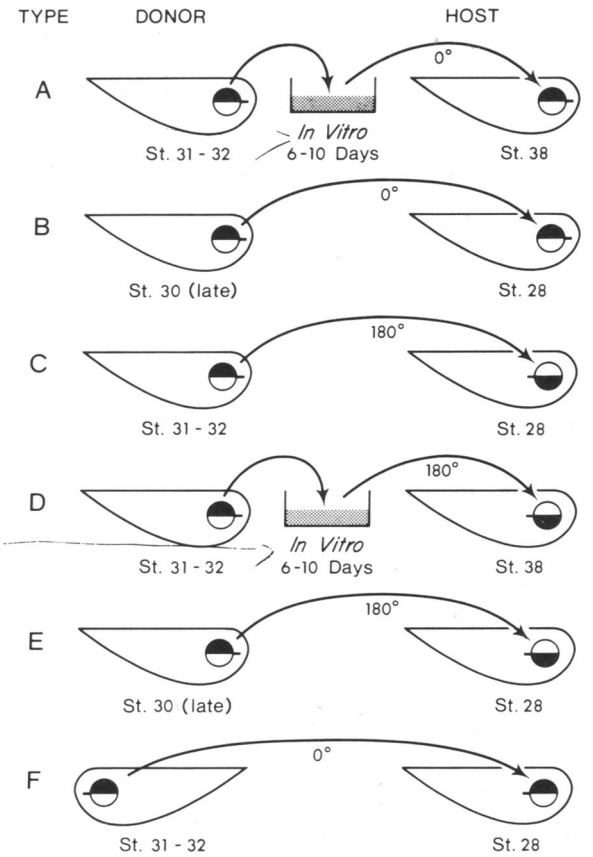

FIG. 1. Design of all experiments showing the stages of the operations and the orientation of the eye on the donor and on the host. The anatomical dorsal half of the eye is *shaded*, and *a key* projects anteriorly. *St*, stage.

In type *E*, late stage-30 right eyes were transplanted with 180° rotation into stage-28 embryos. As had been observed for stage-30 eyes rotated *in situ* (6), two type-*E* hosts showed misdirected, visually-guided strike responses and a pattern of retinotectal connections that was inverted in the anteroposterior axis of the tectum, but not in the mediolateral axis (Fig. 4*a*). The remaining two type-*E* hosts showed complete inversion of the connections in both axes.

In type *F*, a stage-31–32 *left* eye was transplanted with normal dorsoventral orientation (but inverted anteroposterior orientation) into the right orbit of a stage-28 embryo. All type-*F* hosts showed misdirected, visually-guided strike responses and a pattern of retinotectal connections that was inverted in the anteroposterior axis, but not in the mediolateral axis, of the tectum (Fig. 4*b*). Thus, in groups *E* and *F*, the anteroposterior axial component of positional information, specified on the donor, was not altered by conditions present on the host after eye transplantation.

* It was this extensive ocular growth and differentiation *in vitro* that necessitated the use of stage-38 hosts for the cultured eyes (types *A* and *D*), since stage-28 embryos could not accommodate these eyes.

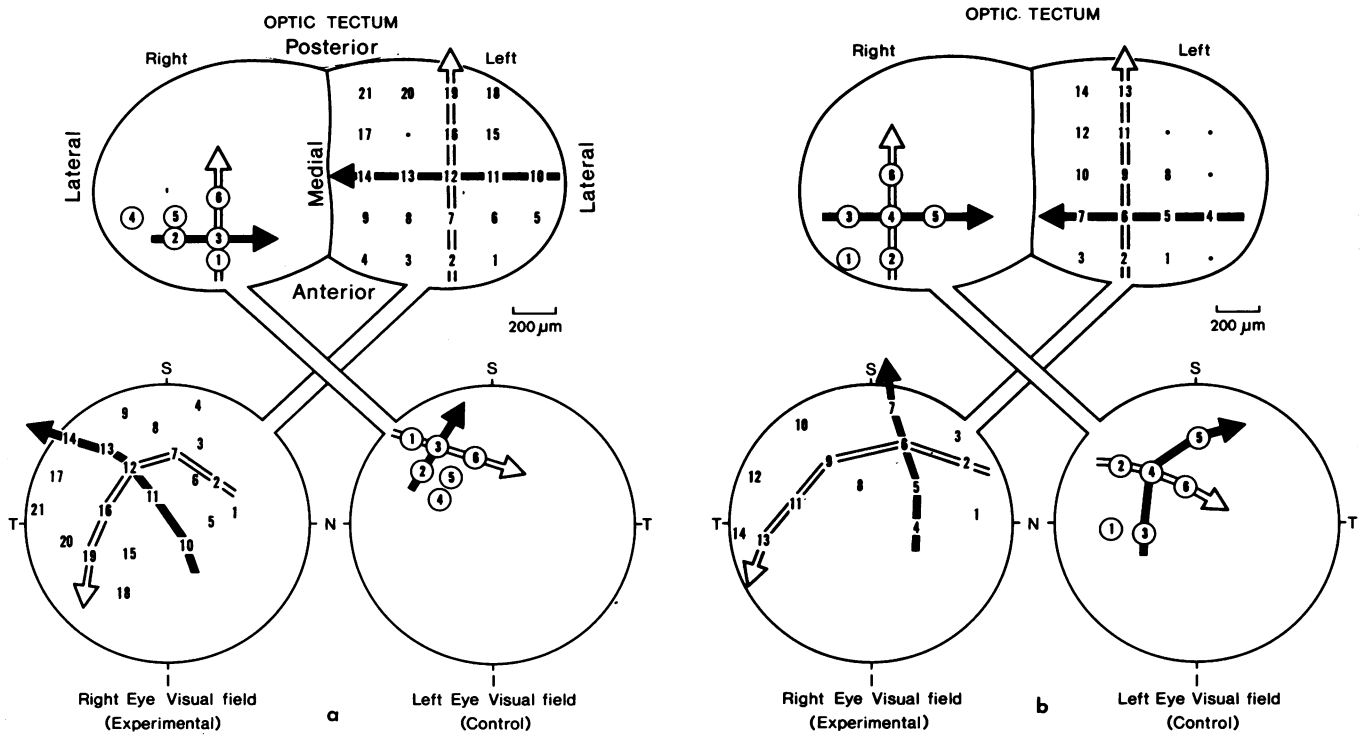

FIG. 2. (*a*) The projection from the visual field of each eye to the optic tectum in a *type-A host* (the culture period in this case was 10 days). The right eye of the animal was centered, which permits complete mapping of the left tectum, but limits mapping of the right tectum to its anterior half and requires geometrical transformation of the data recorded for the normal left eye onto its own visual field. Each number in the visual field shows the position of the stimulus that optimally evoked potentials recorded by an electrode at the position shown by the same number on the tectum. The distance between tectal electrode positions is shown by the *bar*. The visual field extends 100° from the center to periphery. The conventions are the same for the other figures. (*b*) The projection from the visual field of each eye to the optic tectum in a *type-B host*.

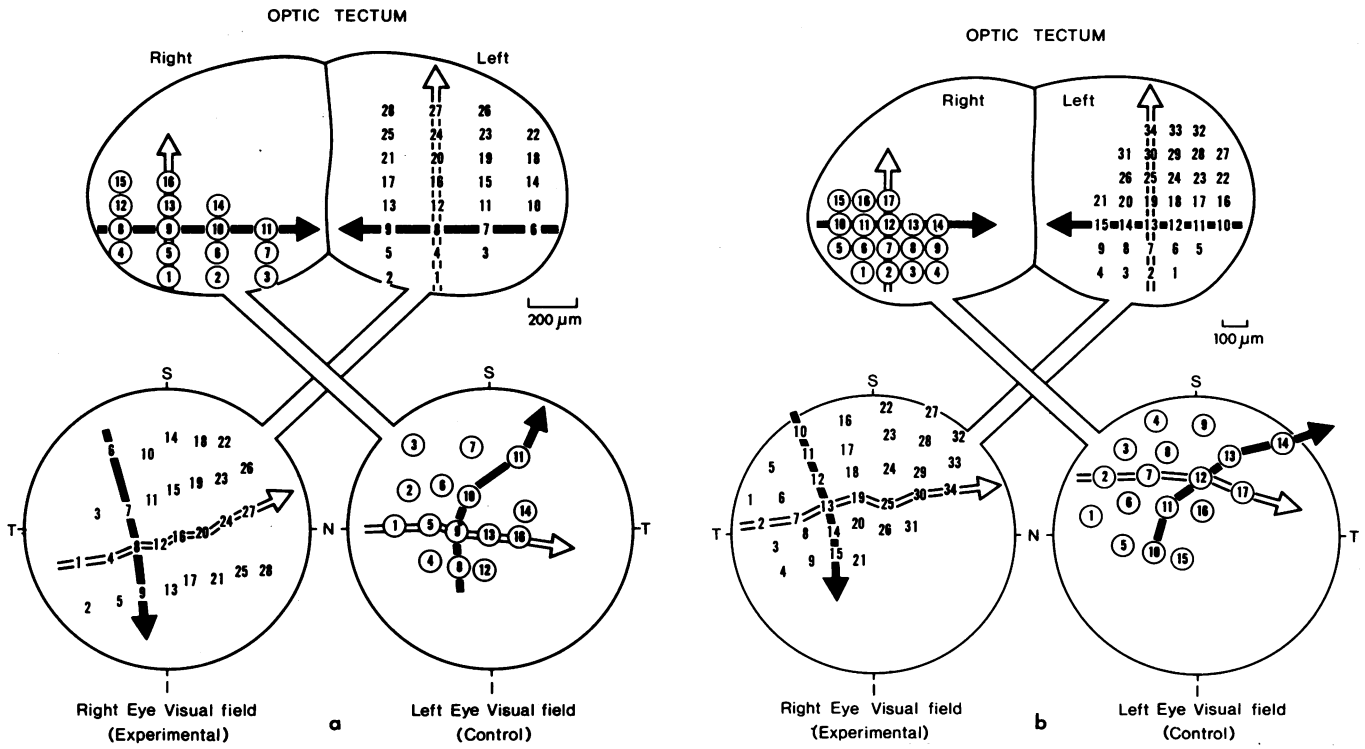

FIG. 3. (*a*) The projection from the visual field of each eye to the optic tectum in a *type-C host*. (*b*) The projection from the visual field of each eye to the optic tectum in a *type-D host*.

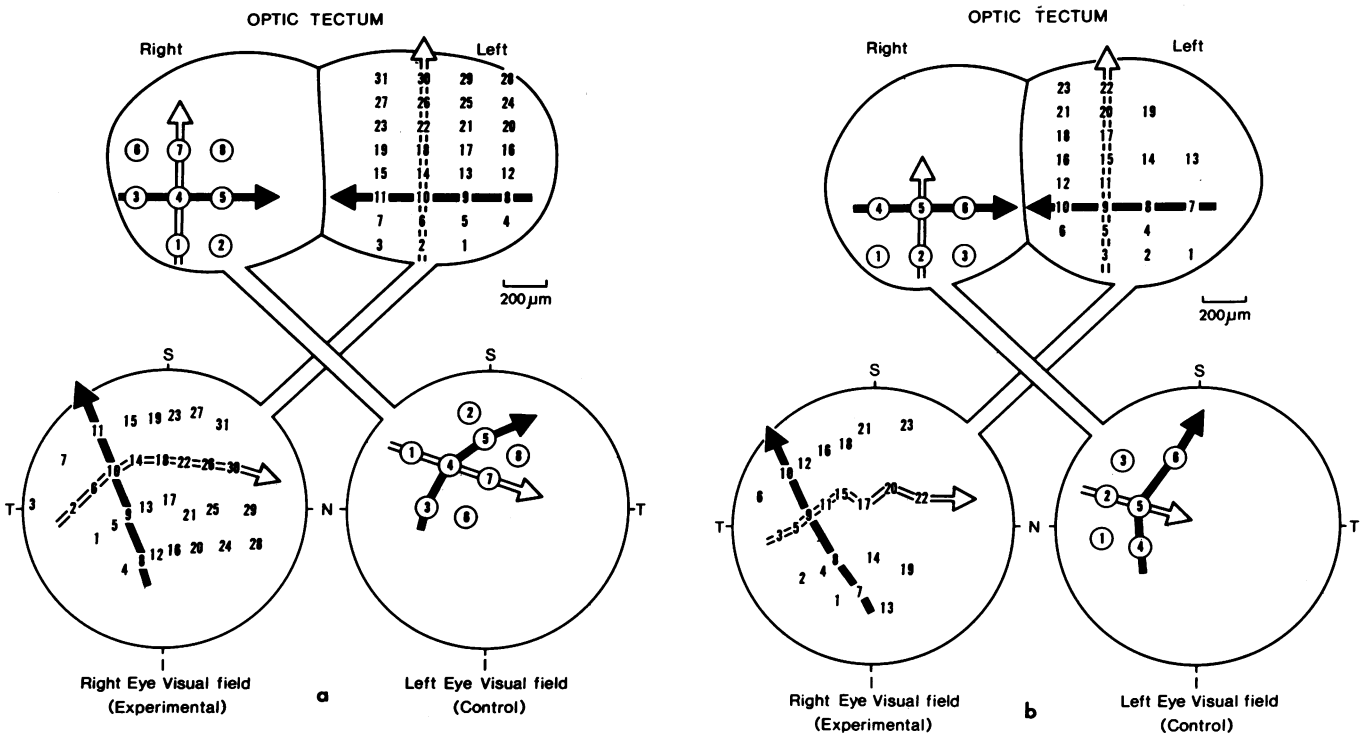

FIG. 4. (*a*) The projection from the visual field of each eye to the optic tectum in a *type-E host*. (*b*) The projection from the visual field of each eye to the optic tectum in a *type-F host*.

DISCUSSION

These experiments are addressed to a distinction, adapted from Holtzer (16–18), between two alternative mechanisms for the emergence of what appears *in situ* to be a fixed developmental program or specified state. In the first mechanism, stable or irreversible changes in the phenotypic traits of the differentiating retinal cells may have occurred between stages 28 and 31, which render the cells refractory to information about changes in their positions after stage 31. In the second mechanism, changes in the embryonic environment of the retinal cells (that is, changes in the embryo between stages 28 and 31) may select for certain retinal cell behaviors and suppress others—in the absence of an irreversible change in their phenotypic properties. Examples in the latter category include (*i*) the disappearance of cues involved in organization of the retinal axes, or (*ii*) a change in concentration of some effector molecule whose concentration at any point in time reversibly controls the behavior of cells at that time.

The present results indicate that the first of the above mechanisms, rather than the second, underlies the specification of positional information in *Xenopus* retinal ganglion cells. Since a normal retinotectal map develops after *in situ* rotation of the eye at stage 28 (6), the stage-28 embryo clearly contains all the conditions necessary for providing retinal-ganglion cells with information about their new positions after translocation. Yet when a stage-31–32 eye is transplanted into a stage-28 embryo, its retinal ganglion cells do not show the influence of any such new positional information. Rather they develop locus specificities (and form retinotectal connections) based on positional information that was specified in the donor embryo before transplantation (type *C*).

Moreover, the anteroposterior axial component of positional information in a stage-31–32 *left* eye is independently stable in a stage-28 *right* orbit (type *F*), even though such a transfer does not invert the eye dorsoventrally, and the dorsoventral axial component would not have to be modified to produce a normal pattern of retinotectal connections in the host. This independent stability in the anteroposterior axis was also exhibited in the two type-*E* experiments, in which the donor eye was "caught" during the intermediate stage between specification in the two retinal axes. Thus, the type-*E* and type-*F* experiments confirm and extend the earlier observations that contralateral transplantation of adult eyes leads to nasotemporal misdirection of visuomotor responses (19), and that *in situ* rotation of a stage-30 *Xenopus* eye may independently invert the anteroposterior axis of the resulting retinotectal map (6).

Finally, the stability of the specified state is further illustrated by the results of the culture experiments. Despite a protracted stay *in vitro* from stage 31–32, retinal ganglion cells develop locus specificities (and form appropriate tectal connections in a host embryo) based on positional information that was specified before explantation (type *D*). Recently, we adapted classical serial-grafting methods (20) to this system and showed that the specified state is stable under conditions that deprive the retina of tectal connections for 30 days (10); we may now add that this stability is manifest for at least 10 days in total isolation from the rest of the embryo.

It is important to recognize, in considering these results, that indirect analyses permit only limited inferences about the biochemical changes occurring during differentiation of retinal ganglion cells. We can only speculate, for example, on the timing and mechanisms of acquisition of positional information, and the translation of this information into definitive locus specificities. Many more data are required on the cell-fiber relations in the normal tectum (21), the timetables and mechanisms by which retinal and tectal cell

types are generated (22–24), the significance of the cell deaths observed in retinal and tectal neurogenesis (25, 26), and the possible role of non-neural elements in the eye primordium.

For the present, we may conclude that the specification of positional information in the *Xenopus* retina at stages 28–32 involves stable changes in the phenotypic properties of some or all of the differentiating retinal cells that leave the cells refractory to new information about changes in their positions after stage 31. The ultimate results of these changes is to render individual ganglion cells committed to evolving particular locus specificities that predispose their axons to synapse at particular loci in the retinotectal map. Whether the transition from the unspecified to the specified state, between stages 28 and 32, *additionally* includes changes in the extraretinal conditions important to the specification process remains to be investigated. But in light of the present results, changes in the embryo as a milieu for specifying positional information are neither necessary nor sufficient to explain the emergence of the specified state in retinal ganglion cells.

Howard Holtzer provided advice in the culture experiments and helped support the work in Philadelphia (National Science Foundation Grant GB 5047X). Jean Piatt's suggestions improved the surgery and management of the embryos. Additional support was from MSTP Grant GM-02046-03 from the National Institutes of Health (R.K.H.) and Grant GB 24900X from the National Science Foundation (M.J.).

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