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Fiber–fiber interaction and tectal cues influence the development of the chicken retinotectal projection

(axon guidance/positional markers/optic fissure/neural cell adhesion molecule)

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ABSTRACT **The development of the retinotectal projection has been studied by a new experimental approach combining antibodies against the nerve cell adhesion molecule (NCAM), and techniques for mapping neuronal pathways using rhodamine B isothiocyanate (RITC) crystals. Anti-NCAM Fab', which specifically inhibits neurite fasciculation, was injected into the eye cup of 4-day-old chicken embryos. After 4–6 days of development, a small RITC crystal was placed on the neural retina to stain selectively axons arising from a localized region. One day later the retina, optic nerve, and tectum were examined and the paths of the fluorescent retinal ganglion cell axons were traced. These studies have led to four observations: (*i*) The presence of anti-NCAM Fab' causes the axons to form a disordered nerve bundle at the optic fissure. (*ii*) Disorder produced in the optic nerve persists throughout the optic pathway up to the tectum. (*iii*) Many of the misrouted fibers growing on or near the tectal surface can at least partially correct their position. (*iv*) Late axons grow in straight tracks along other fibers and do not correct their position. Together the results suggest that formation of the retinotectal projection involves both reading of positional cues on the tectum by growth cones of early arriving retinal axons and the tracking of growth cones along preexisting fibers that normally belong to neighboring retinal ganglion cells.**

The innervation of the optic tectum by retinal ganglion cell axons occurs in a topographically ordered manner that preserves the spatial relationships of the visual field. The developmental events by which this neural projection is generated have been the subject of theoretical and experimental work for nearly a century. Proposed mechanisms include, for example, the recognition of positional markers on tectal cells by retinal axons, and the preservation of ganglion cell body topography ("neighbor" relationships) in the projection of their axons (for review see refs. 1 and 2).

Several experimental approaches have been used to test these models and to characterize the retinotectal map and its genesis. These usually have involved experimental perturbation of various tissues before, during, or after innervation, and an analysis of the resulting map. The perturbation in most studies has been a displacement, grafting, removal, or lesioning of the retina, tectum, or optic nerve (for review see refs. 3 and 4). The literature on this subject is vast and includes a number of important observations that must be considered in any model. However, the complexities of analyzing surgical experiments that combine aspects of both development and regeneration often have made it difficult to evaluate the relationship between these studies and to resolve apparent contradictions.

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This report describes experiments designed to circumvent some of the problems encountered with surgical manipulations. It represents the combination of two recent developments in the analysis of nerve fibers: the use of rhodamine B isothiocyanate (RITC) crystals for tracing axons from small groups of neurons over their entire length (5), and the ability to perturb neurite fasciculation with antibody against the neural cell adhesion molecule (NCAM) (6). Injection of anti-NCAM Fab' into the eyecup of chicken embryos and localized staining of retinal ganglion cell axons with RITC have provided a relatively simple system to evaluate the properties and behavior of individual axons during development of the retinotectal projection.

MATERIALS AND METHODS

Antibodies to NCAM. Specific rabbit antisera against purified chicken NCAM were produced by three intraperitoneal injections of 50 μg of protein in complete Freund's adjuvant at 3-week intervals. Fab' fragments were prepared as described (7), lyophilized, and dissolved in phosphate-buffered saline (pH 7.4) to a concentration of 50 mg/ml. The Fab' from one rabbit was more effective in inhibiting neurite fasciculation in culture than others, and this preparation was also found to give the best results *in vivo*. In some experiments the anti-NCAM Fab' was neutralized prior to injection of embryos by incubation of 1 mg of Fab' with an excess (100 μg) of purified NCAM.

The NCAM used in these studies was prepared by immunoaffinity chromatography using monoclonal anti-NCAM antibody (8). The monoclonal antibody was obtained from a hybridoma generated from spleens of BALB/c mice (9) that had been immunized twice with retinal cells prepared by mechanical trituration of tissue from 9-day-old chicken embryos. Hybrid cells from two fusions were mixed and distributed into 96 wells. After 10 days, the culture medium from each well was tested for antibody binding to retinal cell and fibroblast monolayers, and 19 retina-positive, fibroblast-negative wells were identified. The screening for anti-NCAM activity was carried out by fractionation of a Nonidet P-40 extract of 10-day-old brain tissue by gel electrophoresis in sodium dodecyl sulfate, transfer of the proteins to nitrocellulose sheets (10), incubation of equivalent strips of the nitrocellulose with the hybridoma culture supernatants, and staining for bound antibody by the peroxidase-antiperoxidase procedure (11). Three of the 19 prescreened wells produced staining patterns displaying the diffuse band of apparent molecular weight 200,000–250,000, which is characteristic of NCAM from embryonic brain (8).

Injection and Incubation of Embryos. The results reported

Abbreviations: NCAM, neural cell adhesion molecule; RITC, rhodamine B isothiocyanate.

here were confirmed in three separate experiments, each with the following protocol. Embryos were incubated for 3 days and then cultivated without shells in 100-mm Petri dishes as described (5, 12). Such conditions produce embryos with normal development through late embryonic age. On developmental day 4, more than 50 embryos were divided into four experimental groups, each receiving an intravitreal injection into the right eye of 1–2 μl of either Fab′ from unimmunized rabbits, anti-(NCAM) Fab′, anti-NCAM Fab′ neutralized with NCAM, or mouse monoclonal anti-rabbit-NCAM Fab′ at 10 mg/ml. In each study, the embryos were incubated for an additional 4–8 days, with approximately half the embryos in each group surviving without obvious developmental defects or abnormally small injected eyes.

Staining of Axons with RITC. Localized anterograde staining of retinal ganglion cell axons was carried out by placing RITC crystals at central or peripheral sites along the dorsal half of the dorsoventral axis of the retina (5). The dye is taken up by ganglion cells and transported to the axon tip within 24 hr. This results in the staining of a few hundred fibers arising from adjacent cells lying beneath the crystal as well as more peripheral cells whose axons pass near the crystal.

Fluorescence Microscopic Analysis of Fiber Paths. One day after application of RITC, the embryos were sacrificed, their heads were fixed overnight with 4% paraformaldehyde in phosphate buffer, pH 7.1, and the tissue was soaked in several changes of the buffer to remove the fixative. Retinae were examined by fluorescence microscopy as whole mounts, and optic nerves and tracts were examined as frozen sections. Tecta were observed as whole mounts after being cut in half along the anteroposterior axis. While the paths of labeled fibers were readily traced by direct viewing with the microscope, the photographic recording of the result was complicated by the combination of continuous changes in the focal plane and bleaching of the rhodamine. Consequently, the data are presented most effectively as camera lucida representations and numerical evaluations.

RESULTS

Injection of 50–100 μg of anti-NCAM Fab′ into the eyecup of a 4-day-old chicken embryo caused a localized perturbation in the spatial relationships and pathways of retinal ganglion cell axons, as visualized 4–8 days later by RITC staining. Control animals for these experiments were prepared by injection of equivalent amounts of Fab′ from serum of unimmunized rabbits, of anti-NCAM Fab′ that had been neutralized by NCAM or of a monoclonal anti-NCAM Fab′ that binds to NCAM but has only a weak adhesion-blocking activity. In each control, the fiber paths were indistinguishable from uninjected embryos. Injection of 100 μg of NCAM also did not alter development of the optic nerve. The perturbation caused by rabbit anti-NCAM Fab′, and its effect on the route and projection of the misdirected neurites, is described below.

Axon Tracts Within the Retina. In the chicken, the topographical arrangement of the retinal ganglion cells is preserved in the nerve bundle containing their axons, and this results in the formation of a highly ordered optic nerve (2, 5).

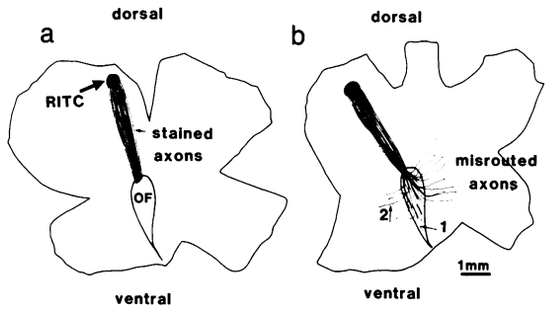

FIG. 1. Semi-schematic drawings showing the intraretinal routes of fibers locally labeled with RITC in a control embryo (*a*) and in an anti-NCAM-injected embryo (*b*) on day 10. In controls, fibers arising from a distinct retinal region (RITC) maintained within the retina their neighbor interrelationships and left the retina at a distinct position in the optic fissure (OF). If anti-NCAM was injected, fibers arising from a quite similar retinal position displayed a normal pattern between the RITC application site and the optic fissure. At the fissure, however, perturbation of the fiber order occurred, producing misrouted axons that either left the optic fissure at ectopic positions (1) or did not leave the eye and remained in the optic fiber layer of the retina (2). See Table 1 for quantitation.

In the present experiments, anti-NCAM Fab′ had no detectable effect on the path that ganglion cell axons normally take toward the fissure (Fig. 1*a*). At the fissure, however, the antibody caused obvious misrouting of an average of 18% of the fibers that grew to the fissure after the injection (Table 1). Looking at fibers arising from a localized group of RITC-labeled dorsal cells, about two-thirds of the misrouted axons spread over the fissure and left the retina in ectopic positions, and about one-third failed to exit at the fissure (Fig. 1*b*). The majority of fibers that were not detectably affected by the antibody left the retina as a compact bundle at the expected position for dorsal cell axons (Table 1; Fig. 1*b*).

Fiber Distribution in the Optic Nerve and Optic Tract. In the anti-NCAM-injected embryos, a substantial proportion of the stained fibers in the optic nerve was found to occupy diffuse ectopic positions (Fig. 2*b*), and these abnormal fibers decreased in frequency with increasing distance from the expected position in the nerve. The ratio of ectopic to normal fibers in the nerve appeared to be greater than at the nerve head, suggesting that there is a continuing loss of retinotopic order along the length of the optic stalk. Beyond the stalk, serial frozen sections of the optic nerve and tract displayed a similar amount of fiber disorder. Thus, about two-thirds of the labeled axons that reached the tectum occupied their appropriate positions, and the remaining fibers were dispersed over the width of the optic tract (Fig. 3).

Routes of Retinal Axons on the Tectum. In normal embryos, fibers arrive at the tectum with retinotopic order, and the projection of RITC-stained dorsal axons is a localized area of the ventral tectum (Fig. 3*a*). Observations of the tectum in our experiments focused on the fate of the dispersed ectopic fibers on encountering this tissue.

In contrast to the optic nerve, a significant fraction (about 15%) of the ectopic axons displayed a capacity for dramatic

Table 1. Average number of labeled fibers in retina of control and anti-NCAM-treated embryos

Injected with	Labeled fibers per embryo	Fibers leaving retina at normal position	Misrouted fibers: In optic fissure	Misrouted fibers: Failing to exit at fissure
Control antibody	206	206	0	0
Anti-NCAM antibody	305	249	33	23

Average number of labeled fibers in control embryos (n = 5) and in anti-NCAM-treated embryos (n = 8). Thirty-three of 305 fibers in the second group leave the retina at ectopic positions, and 23 fibers remain in the retina, whereas in the control group all fibers (206) leave the retina correctly.

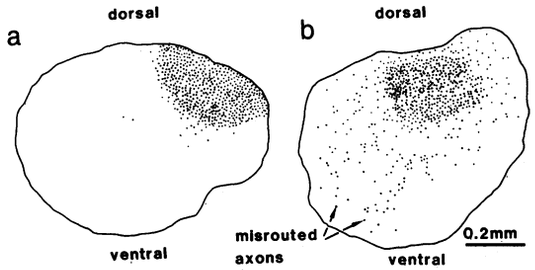

FIG. 2. Camera lucida drawings from cross sections through the optic nerve of a control embryo (*a*) and an anti-NCAM-injected embryo (*b*) on day 10. In controls, fibers arising from a localized region in the retina occupy a distinct region in the optic nerve (black dots; ≈420 fibers). In the antibody-treated embryo, misrouted fibers occupy ectopic positions (≈170 fibers) within the optic nerve, whereas the majority of fibers (≈390 fibers) lie within the expected normal region (high-density region).

changes in their relative dorsal–ventral position on the tectal surface (Figs. 3 and 4; Table 2). It is striking that all of these belonged to the population of stained fibers whose focal plane was very near the tectal surface. On the other hand, fibers lying further from the tectal surface, including those growing in incorrect positions, grew in straight paths as observed for normal axons in control embryos. The frequency of misrouted fibers that displayed a dorsal–ventral displacement was found to be greater if they arose from cells in the central retina rather than in the later-developing peripheral retina (Table 2). These observations are consistent with the fact that, in the chicken, axons from central retina arrive first at and lie closer to the tectal surface (5).

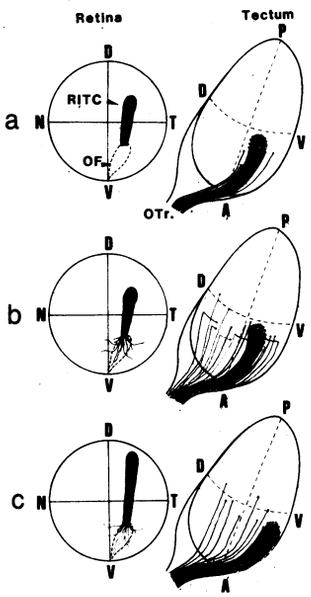

FIG. 3. Schematic representation of the fiber routes on the tectum in control and in anti-NCAM-treated embryos on day 10. In control embryos (*a*), fibers originating from a RITC-stained region of dorsal retina grew parallel to each other and terminated at their projection site on the ventral tectum. In anti-NCAM-treated embryos (*b* and *c*), the majority of stained fibers followed the normal route, and the remainder grew in ectopic pathways. If cells in the central retina were stained (*b*), the ectopic fibers grew near the tectal surface and corrected their position along the dorsoventral axis by a right-angle turn to their appropriate target region. Fibers turning to directions other than the target region were relatively infrequent and exhibited smooth curved pathways. If cells of peripheral retina were stained (*c*), the ectopic fibers followed straight routes on top of fibers and did not correct their dorsoventral position. A, anterior; D, dorsal; N, nasal; P, posterior; T, temporal; V, ventral; OF, optical fissure; OTr, optic tract.

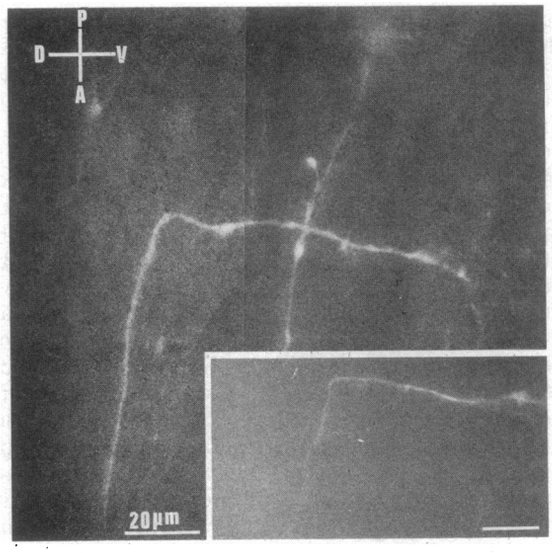

FIG. 4. Fluorescence photomicrograph showing the right-angle correcting routes of two axons to the ventral tectum (target region). A third axon grows straight in the anteroposterior direction. A, anterior; P, posterior; D, dorsal; V, ventral.

When changing their position, the misplaced fibers usually grew toward their normal site of tectal innervation. That is, axons whose growth cones lay ventral to their normal position grew in a dorsal direction on the tectum, and conversely, axons lying dorsal to their target corrected ventrally (Table 2; Fig. 3*b*). The correction is best illustrated by the stained fibers that were found on the dorsal tectum and therefore must all be ectopic. Nineteen percent of these fibers grew on or near the tectal surface, and of this population 85% turned ventrally and 15% turned dorsally. The remainder of the fibers on dorsal tectum were located further away from the tectal surface and all grew in straight paths along the anteroposterior axis. Ectopic fibers on the ventral tectum were more difficult to observe because of their proximity to normal routes of innervation. However, they also displayed a clear preference for turning toward dorsal (154 fibers) rather than ventral positions (45 fibers). An interesting feature of the correcting fibers was that the turning of their growth cones toward their appropriate dorsal–ventral position appeared to occur as a single concerted movement without anteroposterior displacement, thereby producing a "right-angle" turn (Fig. 4). In contrast, those few fibers that were observed to turn away from their target followed a path of relatively gentle curvature. Right-angle turns were observed only in that part of the tectum where such a correction would lead the axon to its normal projection site. Thus, right-angle turns were not found in either the anterior or the posterior third of the tectum.

DISCUSSION

These studies show, under the conditions used in our experiments, that the development of the retinotectal system in the chicken involves at least two factors: the reading of positional information on the tectum by retinal growth cones and the tendency of these growth cones to grow along a preexisting fiber or fiber bundle. The specific conclusions leading to this proposal are discussed below, beginning with the likely mechanism behind the perturbation induced by anti-NCAM Fab′.

As shown by experiments with labeled antibodies, injection of Fab′ into the eye cup on day 4 can produce antibody concentrations in the milligram per milliliter range that persist for at least 2 days and remain largely confined to the

Table 2. Quantitative analysis of the experiments with control antisera and with anti-NCAM Fab'

Origin of stained axons	Total stained axons: Retina	Total stained axons: Tectum	Stained axons on Dorsal tectum: Total	Dorsal tectum: Turning to Dorsal (gentle curvature)	Dorsal tectum: Turning to Ventral (right angle)	Stained axons on Ventral tectum: Total	Ventral tectum: Turning to Dorsal (right angle)	Ventral tectum: Turning to Ventral (gentle curvature)
			Control antiserum					
Dorsal retina	250	30	0	0	0	30	0	0
	100	0	0	0	0	0	0	0
	180	0	0	0	0	0	0	0
	>200	≈82	0	0	0	≈80	2	0
	≈300	≈150	0	0	0	≈150	0	0
Total	≈1030	≈262	0	0	0	≈260	2	0
			Anti-NCAM-Fab' treated					
Dorsal retina	>400	≈334	>40	4	6	≈250	30	4
	≈500	≈390	≈40	5	5	≈300	≈30	10
	>600	≈412	85	2	13	≈300	10	2
	≈300	≈213	5	0	0	≈200	5	3
	≈400	≈314	>100	0	9	≈200	5	0
	>700	≈311	80	1	30	≈150	30	20
	>500	≈119	4	0	0	≈100	15	0
	>550	≈168	≈30	0	0	≈130	6	2
	≈200	≈188	≈20	0	3	≈150	13	2
	>300	≈112	0	0	0	>100	10	2
Total	≈4450	≈2811	≈404	12	66	≈1880	≈154	45
Dorsal retina (periphery)	200	45	0	0	0	40	2	2
	140	0	0	0	0	0	0	0
	≈300	20	0	0	0	20	0	0
	≈300	5	0	0	0	5	0	0
	200	≈115	15	0	0	≈100	0	0
	>600	≈245	70	0	0	≈150	15	10
	≈400	≈209	0	0	0	≈200	6	3
	>300	8	2	0	0	6	0	0
	>500	0	0	0	0	0	0	0
Total	≈2940	≈647	87	0	0	≈521	23	16

Stained axons were counted on whole-mount preparations, both in the retina and on the dorsal half and ventral half of the tectum. The numbers of counted fibers in the retina and on the tectum are unequal, because, at the time of evaluating these experiments (days 8–10), a number of retinal fibers had not yet arrived at the tectum. In cases in which the density of stained fibers was high, an exact count of fibers was impossible. In such cases, we have relied on estimates, which are probably accurate to within 5% of the total number of fibers.

inside of the eye and the optic fissure (13). This concentration of anti-NCAM Fab' is known to cause a substantial decrease in adhesion among nerve cells and neurites *in vitro* (14, 6) and results in an intermixing of fibers both within and between fascicles (6). The effect of the Fab' is believed to reflect a specific inactivation of the function of NCAM as a ligand (15) and does not appear to alter the rate of outgrowth of neurites on a collagen substrate or to affect cell viability and differentiation (14, 16). Although the growth of fibers along other fibers can be affected (16), in the present experiments the antibody did not cause a noticeable delay in innervation of the tectum relative to control embryos.

On this basis, it is not surprising that perturbation of the retinotectal projection by intraocular injections of anti-NCAM Fab' largely reflects an inhibition of interactions among retinal ganglion cell axons in the vicinity of the eye during the time when they leave the retina. That is, the primary effect of anti-NCAM was found to be a disruption of the normal axon paths and bundling pattern during the formation of the optic nerve, and all other abnormalities appear to reflect the consequences of this localized perturbation. Therefore, the subsequent fate of the disordered fibers should reflect development in an otherwise normal environment and represents a valuable system in which to observe the visual pathway.

It has been suggested that retinal ganglion cell axons can use positional cues within the optic nerve to establish order when they grow from the retina to the tectum (17). However, an obvious topographical reorganization of the misplaced fibers in the optic pathway was not observed in our experiments, although subtle changes might not have been detected. In sharp contrast to this situation was the dramatic change in dorsal–retinal position of early fibers that grow on or near the tectal surface. A variety of models and studies (17–21) have suggested that the tectum provides information to help incoming retinal fibers reach their appropriate target site, and the present experiments provide direct support for a contribution by such a mechanism. The apparent right-angle correction exhibited by single growth cones of early fibers was striking and might suggest that their final anterior–posterior position is established prior to corrections along the dorsal–ventral axis. It is conceivable that, in the absence of any preexisting fibers on the tectum, deviated fibers would curve smoothly toward their targets and that the mis-

routed fibers observed in our experiments are following the paths of preexisting straight fibers until the correct anteroposterior position is reached and only then leave the track and grow along the dorsoventral axis. In any case, the observations suggest that fibers are capable of sensing two spatial cues on the tectum: where to stop growing in the anteroposterior direction, and in which direction to leave the original track with respect to the dorsoventral axis. Other explanations for the right-angle turn are also possible. For example, displacement, stretching, or branch elimination subsequent to the correction could have altered the original pathway to the observed right-angle configuration.

In contrast to the early fibers, the dorsal–ventral position of both normal and misplaced axons that arrived at the tectum later in development was relatively insensitive to tectal influences, as previously observed for mechanically misrouted axons (22). Their straight paths appeared to be dominated by "tracking" of their growth cones along other fibers that normally would belong to neighboring cells. This phenomenon suggests that an additional guidance mechanism, based on fiber–fiber interactions, may contribute to normal development of the retinotectal projection by maintenance of retinotopic order among optic nerve axons (2).

In summary, the simplest model to explain the present results would be that the axons from the central retina, which arrive first at the tectum, are able to interact directly with positional markers on the tectal surface and thereby reach a more or less correct site. Then, as more and more fibers leave the eye and the tectal surface becomes hidden by an increasing fiber layer, there is a progressive shift to a second guidance mechanism based on tracking of neurites along preexisting fibers. Exactly when or to what degree this shift takes place has not been determined. In addition, the relative contributions of either mechanism to the ultimate precision of the projection cannot as yet be evaluated, and it would seem likely that other factors, such as the elimination or shifting of connections by activity-dependent properties (for review, see ref. 23), also operate. In any case, the experimental paradigm represented by this study can be extended to gain more detailed information on this problem as well as to study a variety of other neuronal projections.

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