Alterations in the Xenopus Retinotectal Projection by Antibodies to Xenopus N-CAM

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The patterned neural projection from the eye to the optic tectum of lower vertebrates (the retinotectal projection) has been proposed to be ordered by interactions between the optic nerve fibers and their surrounding tissues. To investigate the role of one such defined cell interaction, agarose implants containing antibodies to the neural cell adhesion molecule, N-CAM, were inserted into the tectum of the African clawed frog, Xenopus laevis. Both monoclonal and polyclonal antibodies against N-CAM reversibly and specifically distorted the pattern of the retinotectal projection, decreasing the precision of the projection as determined by electrophysiological techniques as well as decreasing the density of retinal innervation of the tectum and the branching of single axons as determined by horseradish peroxidase tracing. The anatomical effects became maximal at 4 to 6 days after implantation and returned to undetectable levels by 2 weeks, whereas the physiological effects became maximal by 8 to 10 days and a normal physiological map was reestablished within 4 weeks. The results are consistent with the hypothesis that anti-N-CAM antibodies perturb the ongoing growth and retraction of the terminal arbors of the optic nerve fibers, such that a region of the tectum becomes largely demaded of fibers. The physiological defects may then be a consequence both of the initial retraction of optic nerve terminals and of the rapid ingrowth of the perturbed and neighboring optic nerve fibers into the demaded region after the antibodies were cleared from the tectum. These results support the concept of a major role for N-CAM-mediated adhesion during map regeneration and maintenance. © 1988 Academic Press, Inc.

INTRODUCTION

Experimental manipulations of the retinotectal projection from the eye to the optic tectum of lower vertebrates have indicated that position-dependent cell interactions play an important role in the patterning of nerve connections (see review, Fraser, 1985). Adhesive interactions between the optic nerve fibers and their surroundings, and among the optic nerve fibers themselves, could provide a powerful guiding influence for the assembly of the retinotectal projection. Evidence for adhesive interactions between the cells comes from aggregation studies that demonstrate the capacity of retinal cells to adhere strongly following dissociation (cf. Brackenbury et al., 1977), in some cases demonstrating regional differences in binding consistent with the topography of the projection (Barbera, 1973; Gottlieb et al., 1976). Additional support for a role of adhesive interactions comes from perturbation studies using specific antibodies (Buskirk et al., 1980; Hoffman et al., 1986). Furthermore, computer modeling studies show that such interactions between optic nerve fibers could be sufficient to pattern the retinotectal projection (Fraser, 1980, 1985; Whitelaw and Cowan, 1982). While all of these approaches suggest a role of adhesion in the guidance of optic nerve fibers, they have not demonstrated the biochemical basis of the interactions, and hence are limited to consistency arguments.

Studies aimed at determining the molecular species responsible for the adhesive interactions between cells have now demonstrated the importance of a relatively small set of cell adhesion molecules, or CAMs (Edelman, 1983, 1986). CAMs have been shown to undergo changes in character, amount, and distribution during development (Chuong and Edelman, 1984; Crossin et al., 1985; Daniloff et al., 1986), raising several interesting possibilities for the guidance of growing axons (Edelman, 1986). The most extensively studied adhesion molecule, N-CAM (neural CAM), was originally isolated from developing chicken brain (Thiery et al., 1977; Hoffman et al., 1982; Cunningham et al., 1987), but is present in all vertebrates on many neural and non-neural cell types during embryonic development (Thiery et al., 1982; Edelman et al., 1983; Crossin et al., 1985; Daniloff et al., 1986). N-CAM is a cell-surface glycoprotein that mediates intercellular adhesion in a homophilic fashion by interacting with N-CAM molecules on neighboring cells (Hoffman and Edelman, 1988). Antibodies that block N-CAM-mediated adhesion have been used in tissue culture experiments that suggest a
purchased from a commercial supplier (Nasco) and were allowed to metamorphose in the laboratory. Animals were used from 2 to 4 months postmetamorphosis. All animals were maintained in rearing solution, a reconstituted pond water (15% Holtfreter solution, 5% Steinberg solution; diluted in charcoal-filtered deionized water). Following any surgical operation, the rearing solution was supplemented with 20 or 50 μg/ml gentamicin sulfate to minimize the risk of infection. Larvae were fed ground nettle leaves and froglets were fed ground beef heart or tubifex worms.

**Nerve crush.** Some experiments were performed on animals that were regenerating their retinotectal projections following a nerve crush. The skin and muscles dorsal to the eye were severed, permitting the eye to be displaced so that the optic nerve could be visualized. Sharpened forceps (Dumont No. 5) were then used to grasp the optic nerve directly behind the eye and squeeze it three times as described (Fraser et al., 1984). The efficacy of the nerve crush was confirmed in a fraction of the animals by demonstrating the absence of either visually evoked neuronal activity in the tectum or visually directed behavior.

**Introduction of antibodies.** Antibodies were introduced into the tectum in the form of an agarose microcylinder or “spike” as previously described (Fraser et al., 1984). Low-melting-temperature agarose (Seaprep (FMC Corp.), 5% solution) was drawn into polyethylene tubing and chilled to 4°C before being forced from the tubing and cut into 1- to 2-mm lengths. Each length was placed in the bottom of a 10-μl well in a 96-well Terasaki plate, and 4 μl of antibody solution (5 mg/ml, in 0.05 M PBS) was added to the well. The plate was kept at 4°C overnight and then desiccated with silica gel at 4°C. The desiccated spikes were very rigid and could be held easily with watchmakers forceps. The lengths were cut into 300-μm-long, sharpened spikes with iridectomy scissors. In some experiments, lyophilized antibody was added directly to 50 μl of molten agarose solution at 37°C. The agarose was then drawn up into the polyethylene tubing and chilled, before the gelled agarose/antibody was expelled from the tubing, desiccated, and cut into lengths. The two types of implants gave indistinguishable results, and therefore experimental results employing each were pooled together. *Xenopus* froglets were anesthetized in MS-222 (Fpinquel, Ayerst) and the skin and the bone over the optic tectum were deflected. A small puncture wound was made in the tectum with a sharpened metal probe at the insertion site, and the spike was then inserted into the puncture. Within 15 sec, the spike absorbed water and swelled, holding it firmly in place.

**Immunocytochemistry.** Conventional cytochemical techniques were used to follow the dispersal of the immunplanted antibody. The brains were fixed for 8-12 hr in 4% paraformaldehyde, soaked in 15% sucrose, frozen in liquid nitrogen, and cut at 15 μm on a cryostat (AO Reichert Histostat). The sections were stored at −20°C until they were reacted at room temperature with biotin-conjugated goat antibodies specified for rabbit Fab' fragments. Streptavidin–fluorescein (Bethesda Research Laboratories) was then applied, the sections were examined with an image intensifying video camera (SIT; RCA), and the data were stored on videotape. The SIT camera and videotape permitted all of the slides to be quickly recorded (without significant bleaching). Furthermore, the same gain settings were used for recording all of the sections, thereby permitting a direct comparison of intensity of fluorescence between the different sections. The spread of the antibody was quantified by measuring tracings of the fluorescent image from the screen of the video-monitor with a digitizing tablet (Jandel Scientific).

**Anatomy.** The distribution of optic nerve terminals was determined by following the transport of horseradish peroxidase (HRP) along the optic nerve. A 25% solution of HRP (Sigma type IV) was soaked into a small fragment of Gelfoam and applied to the severed optic nerve of an anesthetized animal immediately behind the eye. After 24 hr, the animals were sacrificed by over-anesthesia by immersion in MS-222 and were perfused with MS-222 in 0.1 M PBS by cardiac puncture to clear the red blood cells from the tectum. The MS-222 in the perfusion buffer was to prevent the remote possibility of an underanesthetized animal recovering consciousness during the procedure. The brains were removed, fixed by immersion in 2.5% glutaraldehyde, stained by a cobalt-intensified diaminobenzidine method (Taylor and Gaze, 1985), and viewed in whole mount. Some brains were embedded in glycolmethacrylate (Historesin, LKB) and sectioned with a glass knife at 6 μm to confirm the distribution of the HRP reaction product in the tectum.

Individual optic nerve terminals were labeled with a modification of the above technique. Instead of severing the entire optic nerve, a small injection of 25% HRP was made through the iris of the eye of an anesthetized animal, and a tungsten needle was inserted through the retina in one location. This small injury lesioned a subset of optic nerve fibers which then picked up and transported the HRP to their terminals. After reacting the tecta with diaminobenzidine as described above, the tecta were split down the midline, cleared, and mounted beneath a coverglass in mounting medium (DPX). Those optic nerve terminals in which the HRP reaction product smoothly and completely filled the cells were traced with a camera lucida attachment on a Zeiss Universal microscope. Optic nerve terminals that were not
N-CAM isolated from embryonic cells (E form) is highly glycosylated, with as much as 30% of its mass due to an unusual polysialic acid (Hoffman et al., 1982; Rothbard et al., 1982; Finne et al., 1983), and it therefore migrates as a diffuse band ranging from 140 to 250 kDa on SDS-polyacrylamide gels (Rothbard et al., 1982). In contrast, the N-CAM isolated from adult tissues (A form) has much less sialic acid and migrates as a distinct set of bands at 120, 140, and 180 kDa (Rothbard et al., 1982; Chuong and Edelman, 1984). Antibodies directed against Xenopus N-CAM (R684) and against the unique carbohydrate of E-form N-CAM (15G8) were used to demonstrate that Xenopus N-CAM undergoes a qualitatively similar transition (Fig. 2). In the tadpole brain (stage 56), approximately 44% of the N-CAM existed in the E-form (Fig. 2, lane a), whereas 22% of N-CAM was in the E-form in the brain from a fully grown metamorphosed frog (Fig. 2, lane b). The different forms of N-CAM appeared to be regionalized in the brain of metamorphosed frogs, with the tectum remaining much higher in the E-form of N-CAM than other regions (44%; Fig. 2, lanes c, f). This persistence of the E-form of N-CAM in the tectum may be related to the continual rearrangement that the retinotectal connections undergo during the growth of Xenopus (Gaze et al., 1979; Fraser, 1988). Previous observations (Chuong and Edelman, 1984) demonstrated that the E-form persists in regions of the brain such as the olfactory bulb in which nerve ingrowth is believed to persist.

The antibodies used in the anatomical and physiological studies were further characterized for their ability to block the aggregation of membrane vesicles derived from Xenopus brain. The anti-N-CAM reagents R684

![Fig. 2. Embryonic to adult form conversion of Xenopus N-CAM. Brain extracts were resolved by electrophoresis in a 7.5% polyacrylamide gel, blotted onto nitrocellulose, reacted with anti-N-CAM antibodies followed by 125I-labeled protein A, and visualized by autoradiography. Extracts reacted with (a–c) polyclonal antibody R684 (which recognizes Xenopus N-CAM) or (d–f) monoclonal antibody 15G8 (which recognizes the unique polysialic acid determinant on E-form N-CAM), followed by rabbit anti-mouse IgG antibodies. (a and d) Tadpole whole brain (stage 56); (b and e) adult whole brain (>2 years old); (c and f) adult optic tectum. Migration positions of molecular weight markers (kDa) are indicated at the left.]

![Fig. 3. Aggregation of Xenopus brain membrane vesicles and its inhibition by anti-N-CAM antibodies. (A) Aggregation proceeds normally in the presence of preimmune antibodies (circles) but is inhibited by increasing amounts of R684 Fab' fragments (triangles, 15 μg; squares, 40 μg; ×, 100 μg; all in a final volume of 400 μl). (B) The inhibition of aggregation is neutralized by increasing amounts of Xenopus N-CAM (40 μg of R684 preincubated with ×, no N-CAM; squares, 0.5 μg of N-CAM; triangles, 1.5 μg of N-CAM; filled circles, 4.5 μg N-CAM). Open circles, 40 μg of nonimmune Fab'; closed triangles, 1.5 μg Xenopus N-CAM alone.]

![Fig. 1. Purification of Xenopus N-CAM. Silver stained gels showing (a) tadpole brain extract, (b) components immunoprecipitated by monoclonal antibody 15G8 (cross-reactive antibody used to isolate Xenopus N-CAM), and (c) components immunoprecipitated by monoclonal antibody 10H4. Migration positions of molecular weight markers (kDa) are indicated at the left.]

![Image 0x0 to 612x792]
showing no measurable effects on the optic projection to the contralateral tectum (Fraser et al., 1984).

Anatomical assays of the projection. Nerve tracing using HRP was used to label all of the optic nerve terminals in 44 animals implanted with either R645 or R684. The distribution of the dark HRP reaction product was then examined in whole-mounted brains to determine the density of the retinal innervation of the tectum. The density of optic nerve terminals was not noticeably altered by the presence of the control antibody (R645) when assayed 2, 4, 6, and 13 days after implantation (Fig. 5). In contrast, the distribution of optic nerve terminals was disrupted in tecta implanted with R684 anti-N-CAM (Fig. 6). The pattern remained relatively normal for the first 2 days after R684 was implanted (Fig. 6A), but was noticeably distorted by 4 days, with a region of decreased labeling near the implant (Fig. 6B). Groups of fibers near the caudal margin of the tectum were well labeled with HRP, making it unlikely that the bald region was due to incomplete filling of the optic nerve fibers. At 6 days after the implant the anatomical defect was beginning to regress; the region of decreased labeling was present in all animals but varied in its severity. To demonstrate that this variation was not due to some systematic animal-to-animal differences, individual animals were implanted.
TABLE 2

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<th>Antibody</th>
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Note. Paucity refers to animals with decreased density of HRP reaction product near the antibody implant; thickening refers to animals with HRP reaction product extending deeper into the tectal neuropil than in normal animals.

" Single animal with very slight evidence of both defects.

amples of these defects are shown in Fig. 7. These defects first appeared at 2 days after the implant was inserted and largely regressed by 13 days (see Table 2).

To define better the means by which the anti-N-CAM disrupted the retinotectal projection, the trajectories of the individual optic nerve terminals were examined in animals 6 days after the antibody implantation. Small lesions in the retina were used to introduce HRP into only a few optic nerve fibers in each animal. Tracings of unambiguous single optic nerve terminals demonstrate that terminal arbors in animals implanted with control antibody R645 are normal in morphology, even when they were quite close to the antibody implant (Fig. 8A).

In contrast, terminal arbors in animals implanted with anti-N-CAM R684 were much more sparsely branched, even when they were more than 100 µm from the implant (Fig. 8B). The significantly fewer terminal branches found in the anti-N-CAM-treated animals in comparison to the animals treated with R645 (see legend Fig. 8) suggests that an extensive pruning of terminal arbor branches has taken place. Only one arbor with multiple branches was observed in the R684 animals; however, even in this case, the pattern of branching was unusual (Fig. 8B, right). The concentrated branching pattern of this one arbor may suggest that it was in the process of renervating the central area of the tectum as the antibody was cleared from the tectum.

Electrophysiological assay of antibody effects on the retinotectal projection. As in our previous experiments, extracellular electrophysiology was used to assay both the pattern and the precision of the projection by noting the positions and the sizes of the receptive fields, respectively (see Schmidt and Edwards, 1983; Fraser et al., 1984). Antibodies directed against N-CAM distorted the pattern of the projection and enlarged the receptive field sizes of normal frogs (Figs. 9D, E). In contrast, control polyclonal antibody R645, which binds to cell membranes but not to N-CAM, did not distort the projection appreciably (Figs. 9B, C). Previous experiments also indicated that irrelevant monoclonal antibodies did not significantly affect the projection (Fraser et al., 1984). To permit a quantitative comparison of the effects, the receptive field diameters were measured for a set of animals one week after implantation (Table 3). All of the antibodies directed against N-CAM significantly increased the receptive field size when compared with either preimmune antibodies or R645. Rabbit anti-N-CAM R684 and mouse monoclonal antibody 7C8 had the largest effects; mouse monoclonal antibody 10H4 had a somewhat smaller, but significant, effect (Table 3). Similar, statistically significant increases in receptive field sizes were obtained when antibodies were implanted into animals undergoing optic nerve regeneration (after a nerve crush 3 weeks before the antibody implant; Table 3). The average receptive field sizes reported in Table 3 probably represent lower estimates of the effects of the antibodies because they average data from tectal positions near the implant that are generally more affected by the antibody with data from more distant and less affected regions.

In a few cases, implants with a very high concentration of the antibody 7C8 were used, and the multi-unit receptive fields became extremely weak and disrupted (data not shown). Only a few weak single units responding to disparate regions of the visual field could be recorded clearly by the electrode. The size of the signals recorded by our low resistance extracellular electrodes is thought to depend on the total current flowing through the terminal arbor and, hence, the surface area of the terminal arbors. Therefore, the weakened responses probably indicate that individual optic nerve terminals had a dramatically reduced arborization. The disproportionate positioning of the single units that make up each multi-unit receptive field is also consistent with a dramatic "pruning" of the terminal arbors, resulting in the displacement of the terminal arbors. These animals were not analyzed in detail because the limits of the multi-unit receptive fields were difficult to assess reliably.

The time course of the effects of anti-N-CAM and control reagents on the precision of the retinotectal projection of normal animals was determined in animals 2 months after metamorphosis. Examples of the distortion in the map created by antibody 7C8 are shown in Fig. 10 and the time course of the effects of R684 antibodies on receptive field sizes are plotted in Fig. 11. The multi-unit receptive field size of animals implanted with any of the reagents remained normal for 3 days following the implant (Figs. 10 B, C). A difference between the control and R684-implanted animals became apparent beginning at 4 to 5 days after the implantation (Fig. 11). The receptive field sizes of the 7C8 or R684-implanted animals continued to increase,
peaking at 8-10 days (Figs. 10D, E) and then slowly decreasing to normal values over the next few weeks. Animals implanted with preimmune antibodies showed no such increase, and animals implanted with the R645 showed only a slight (not statistically significant) increase when compared with either preimmune treated or untreated animals.

**DISCUSSION**

The present studies extend an experimental paradigm in which N-CAM-mediated adhesion is disrupted by implanting N-CAM-specific antibodies in vivo. Both anatomical and electrophysiological techniques demonstrated a disruption in the pattern of retinotectal innervation following the introduction of anti-N-CAM but not following the introduction of control reagents. Qualitatively similar distortions were noted in the projections of normal developing animals and those in the midst of regenerating their retinotectal projections (Table 3). Thus, the results demonstrate that N-CAM-mediated interactions are important for both the regeneration and maintenance of the retinotectal projection in *Xenopus*.

Both the overall time course of the antibody effects and the nature of the distortions suggest that the experimental results are the product of disrupting some component of the dynamic process involved in retinotectal patterning and are not due to some experimentally induced pathological state. Although our data indicates that the antibody spreads to fill much of the experimen-
namic sprouting and retracting of terminal arbors (Gaze et al., 1979; Fraser, 1983; O’Rourke and Fraser, 1986), biasing the process toward retraction and thereby leading to a decreased density of optic nerve terminals near the implant. This would account for both the observed anatomical defects and the increased incidence of weak receptive fields. As the antibody is depleted from the tectum, both the displaced fibers and the fibers neighboring the denuded region begin to invade the region around the antibody implant. This invasion of the implant region would both distort and decrease the precision of the projection. The distortion of the projection would result from the implant region being invaded by optic nerve fibers that normally terminate elsewhere; the decreased precision would result from the rapid ingrowth of optic fibers into a largely denervated zone of the tectum, similar to the crude topography formed by optic nerve fibers as they initially renerate the tectum following a nerve crush (Meyer, 1983; Schmidt and Edwards, 1983). The antibodies disappear from the tectum within 6–8 days, whereas the return to normal topography requires 2 to 3 weeks and follows a time course similar to the return of topography following an optic nerve crush (Fraser, unpublish observations). Thus, it appears that the decay of the antibody effects on the precision of the projection is a reflection, not of the time required for the antibody to be depleted from the tectum, but instead, of the time required for refined topography to reappear following its disruption.

This study supports the view that nerve growth and patterning involves a dynamic process of neurite outgrowth and retraction. Evidence now indicates that outwardly static patterns such as the normal retinotectal projection are driven by ongoing rearrangements in neural connectivity (cf. Easter, 1983; Fraser, 1983; O’Rourke and Fraser, 1986; O’Rourke et al., 1987). These dynamic rearrangements are manifest in the refinement of the early retinotectal projection and the shifting of optic nerve terminals over the tectum during development. The results presented here indicate that N-CAM function is important for at least some of these processes and are consistent with the hypothesis that local cell-surface modulation of N-CAM prevalence, distribution, or chemical structure plays a critical role in the control of neural rearrangements (Edelman, 1984). Further research will be required to determine whether N-CAM has a general role in the maintenance of either synaptic morphology or tissue architecture, whose disruption indirectly affects the map, or a more direct role in establishing and maintaining adhesive gradients that might provide positional cues within the tectum (cf. Fraser, 1980, 1985).

Although N-CAM is a logical candidate for playing a major role in adhesive interactions that might underlie the patterning of neuronal maps, it is unlikely that it is the only adhesive molecule important in these processes. In fact, experiments using antibodies to other adhesion molecules have demonstrated distinct changes in the retinotectal projection (Fraser and Edelman, unpublished observations). The experimental approach characterized above offers a direct approach for determining the relative contributions of N-CAM and other adhesive species in the patterning of nerve connections and opens the possibility of relating molecular to cellular mechanisms in map formation.

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REFERENCES
