Alteration of the retinotectal map in *Xenopus* by antibodies to neuronal cell adhesion molecules

(cell–cell adhesion/neural patterning/optic nerve projections/in vivo tissue perturbation/brain development)

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ABSTRACT The neural cell adhesion molecule (N-CAM) mediates neuron–neuron adhesion, is ubiquitous in the nervous system of developing and mature vertebrates, and underlies major alterations in both amount and distribution during development. Perturbation of homophilic (N-CAM to N-CAM) binding by univalent fragments of specific anti-N-CAM antibodies has previously been found to alter neuronal tissue patterns in *vivo*. To show that significant alterations can also occur in vivo, antibodies to *Xenopus* N-CAM were embedded in agarose microcylinders and implanted into the tecta of juvenile *Xenopus* laevis frogs that were undergoing regeneration of their retinotectal projections; 1 week later, the effects of implantation on the projection pattern from the optic nerve were determined. Both polyclonal and monoclonal antibodies to N-CAM distorted the retinotectal projection of the frog. It is ordered in such a fashion that a “map” of the visual field of the eye is conveyed intact to the contralateral optic tectum. This map, cells from neighboring regions of the retina send axons down the optic nerve and terminate at neighboring loci in the tectum. The retinotectal system has served as an important model in the analysis of neural patterning because of its stereotyped pattern of connections, its accessibility to experimental intervention, and the availability of straightforward methods for assaying the order of the projection (for a review, see ref. 1). Both the order and the precision of the retinotectal projection can be determined during development and regeneration by means of anatomical or extracellular electrophysiological techniques.

After damage to the optic nerve, the retinotectal map can regenerate with proper orientation and near-normal precision within weeks (2-4). While some evidence now indicates that an activity-dependent process is involved in refining the order of the projection, its overall topography can be formed in the absence of visual experience or nerve activity (3, 4). Furthermore, experiments on the development of the projection pattern have indicated that a normally oriented projection can form in the absence of the optic tract or nerve activity (5, 6). The overall picture that emerges is that the retinotectal projection is initially patterned by cell interactions independent of the path of arrival of nerve fibers or their neuronal activity; the later refinement of the projection appears, however, to be an activity-dependent process.

Adhesive interactions between cells during development have been assigned a central position in several models of the patterning process (7-9). A detailed understanding of neuronal adhesion requires analysis at the molecular level. New functional assays for cell adhesion based on generally applicable methods of immunological identification have recently led to the isolation and characterization of a number of different cell adhesion molecules (CAMs) (9-11). These include two neuronal molecules: N-CAM, which is responsible for homotypic neuron–neuron adhesion by a homophilic mechanism (N-CAM to N-CAM binding), and Ng-CAM, which mediates heterotypic neuron–glia adhesion by a heterophilic mechanism (Ng-CAM binding to an as yet unidentified glial CAM (11)). Of the two neuronal molecules, N-CAM has been more extensively analyzed and has been shown to undergo cell surface modulation (9) both in its amount and in its carbohydrate structure during development. These findings, along with the observation that neural patterns in cultured chicken retina and dorsal root ganglia are strongly perturbed by univalent fragments of specific anti-N-CAM antibodies, have raised the possibility that N-CAM might play a part in ordering maps in *vivo* (9). The parallels between the known chemistry and distribution of N-CAM and the homophilic adhesive interactions postulated to be important in nerve patterning (7) provoked us to investigate the role of N-CAM in the arrangement of the retinotectal projection.

The experimental design for our study was straightforward. Antibodies to *Xenopus* N-CAM were incorporated into agarose “spikes” and implanted into the optic tectum of young *Xenopus* frogs either undergoing optic nerve regeneration or normal development. One week after implantation, the pattern and precision of the retinotectal projection were assayed using electrophysiological techniques. A distortion in the pattern of the projection and a large decrease in its precision were observed in the presence of specific antibodies to *Xenopus* N-CAM.

**MATERIALS AND METHODS**

**Antibodies.** N-CAM was prepared from *Xenopus* tadpoles and antibodies were raised essentially as described (12). These antibodies react specifically with *Xenopus* N-CAM present in extracts of *Xenopus* brain membrane separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Antibodies against the chicken liver cell adhesion molecule, L-CAM, have been described (10). The antibodies used in each experiment are indicated along with the data in Table 1.

**Optic Nerve Crushes.** To induce regeneration of the retino-

Abbreviations: N-CAM, neural cell adhesion molecule; Ng-CAM, neuron–glia cell adhesion molecule; L-CAM, liver cell adhesion molecule.

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Antibody implants were inserted into the tecta of 1-3 month postmetamorphic Xenopus. In the majority of the studies, the optic nerve was crushed 3 weeks before the antibody was implanted; in others, the implant was placed into normal frogs. One week later, the order of the projection was assayed by extracellular electrophysiology. Some of these animals were reassayed to determine whether the effects of the antibodies were reversible.

In the majority of studies, the optic nerve projection, the animal was anesthetized with MS-222, and the nerve was gently grasped with jewelers forceps (Dumont type 5) and squeezed 3 times. Animals so treated reform a crudely ordered retinotectal projection within 2 weeks, which slowly refines over the next 3 weeks to appear near normal; receptive field sizes remain slightly greater than normal for several additional weeks.

Preparation of Antibody Implants. All antibodies were introduced into the frog in the form of an agarose implant (Sea-prep, FMC Biocolloids). A 6% agarose solution was drawn into a length of polyethylene tubing (180 µm, inside diameter), chilled at 4°C for several hours, and then forced from the tubing by pressure. One 2- to 3-mm length of agarose gel was placed in each well of a 10-µl multwell plate (Falcon), to which 4 µl of Fab' or monoclonal antibody solution (3-6 mg/ml) was added. The plate was sealed and refrigerated for 24 hr; internal humidity was regulated by a 1 M salt solution placed inside the multwell plate. After 24 hr, the antibody solution in the wells had been concentrated by evaporation so that most of it was associated with the agarose cylinder. The agarose cylinder was then removed from the well, allowed to dry further at room humidity, and then cut into sharpened "spikes" =500 µm long. This partially drying further concentrated both the agarose and the antibody, producing a spike with enough inherent strength to be handled with the jewelers forceps used to implant it into the tectum. The concentrating and drying steps of this procedure resulted in a spike of about 100 µm diameter made of ≈12% agarose and containing ≈0.5 µg of antibody.

Insertion of the Implants. The antibody implants were introduced into the tectum 3 weeks after the nerve crush. The Xenopus froglets (1-3 months postmetamorphosis) were anesthetized in MS-222 (Finquel: Ayerst Laboratories, New York), and the skin and bone overlying the tectum was deflected. The implant site was ≈350 µm posterior to the anterior edge of the tectum and near the midpoint of the medio-lateral extent of the tectum. A small puncture wound was made in the tectum with a sharpened metal probe, and the agarose/antibody spike was then inserted point first into the puncture wound, leaving ≈100 µm of the spike projecting out from the surface of the tectum. Within seconds, the spike rehydrated and expanded in girth, which contributed to holding the implant firmly in place. After implantation was complete, the deflected bone and flap of skin were pulled back into place. The frogs were revived and maintained in a solution of gentamicin sulfate (50 µg/ml, Sigma) to minimize the chances of infection. The positions of the implants were stable for at least 1 week after implantation and often were stable for more than 3 weeks. Most implants were into the left tectum; no dependence on sidedness of the implant was observed in this study. The tectum contralateral to the antibody implant was left unimplanted or was implanted with a different antibody to act as a control within the same animal.

Electrophysiological Assay of the Retinotectal Projection. The pattern and precision of the retinotectal projection were assayed 1 week after implantation of the agarose spike. After the animal was anesthetized with MS-222 and paralyzed with curare, the skin and skull were deflected to expose the dorsal surface of the optic tectum. An iridium electrode was lowered into the superficial neuropil at successive positions in a regular grid (150 × 150 µm). The resistance and tip geometry of the electrode were selected so that the electrode would record from the terminal branches of the optic nerve fibers. The depth of the electrode was adjusted to maximize the responses to the stimuli, spots of light provided by an Aimark projection perimeter. Signals from the electrode were amplified (×1000) and filtered (100-10 kHz band pass; 60 Hz rejection filters) before being displayed on an oscilloscope and played over a loudspeaker. For each electrode position, the region of the visual field that elicited any activity at the electrode tip was determined (the multiunit receptive field). Any signal above the background noise of the equipment was taken as a positive response. The receptive field size offers a measure of the precision of the retinotectal projection pattern (4). This is the case because, in the more imprecise patterns, the multiunit receptive fields will be made up of a more diverse group of single-unit receptive fields (single optic nerve fiber responses). For this reason, the same physiological techniques can be used to measure both the pattern and the precision of the projection. To control for experimenter bias in determining the extent of the responsive areas, a subset of the frogs was assayed double-blind by another experimenter. The results obtained from these double-blind experiments were indistinguishable from the others, and, therefore, in the presentation here, all of the experimental results have been combined. A global protocol for the entire implantation and recording sequence appears in Fig. 1.

RESULTS

Both normal frogs and frogs in the midst of regenerating their retinotectal projections were used in the studies. The normal (nonregenerating) animals provided the advantage of a well-ordered and refined retinotectal projection against which to test the effects of the anti-N-CAM implants. Regenerating animals were more frequently used, however, because they were clearly undergoing larger synaptic rearrangements at the time that specific antibodies to N-CAM were introduced into their tecta. At the time of implantation (3 weeks after a nerve crush), the regenerating animals had already formed an ordered but somewhat imprecise projection to the tectum, which normally would refine to near-normal order over the next few weeks. Although similar results were obtained for both normal and regenerating animals, the description below centers mainly on the regenerating animals, because more data were collected for them.

One week after implantation of the antibody, both the order of the retinotectal projection and the size of the receptive fields for each electrode position were assayed using extra-
cellular electrophysiology. Representative patterns are displayed in Fig. 2. The retinotectal maps observed can be classified into three categories: class 1, indistinguishable from normal; class 2, correct overall orientation of the map but with distortions in the pattern of the projection; class 3, jumbled orientation with a large central blind spot. Animals with class 1 and class 2 projection patterns always showed responses from all regions of the visual field. The effects of different antibodies on the pattern of the projection and the average receptive field sizes in the treated animals are listed for comparison in Table 1.

Class 1 patterns were obtained from animals implanted with antibodies not directed against N-CAM. The pattern was quite normal, and demonstrated near-normal receptive fields (Fig. 2B). Receptive field sizes for these animals continued to refine with time, just as they did in regenerating control animals with no implants.

Class 2 patterns resulted from implants containing antibodies 684 and 7C8 directed against Xenopus N-CAM. Animals with implants of antibody 10H4, also directed against Xenopus N-CAM, showed class 2 patterns in one-half of the cases. In the class 2 patterns, a complete set of optic nerve fibers projected to the tectum, but the pattern of the projection was distorted. The size of the receptive fields was increased in class 2 animals, indicating a parallel decrease in the precision of the projection. The distortions found in class 2 projection patterns were most noticeable for positions near the implant, and they appeared to be somewhat larger along the anteroposterior dimension of the tectum than along the medio-lateral dimension (Fig. 2C). Single unit receptive fields, representing the activity of single optic nerve fibers, that were recorded in animals with both class 1 and class 2 patterns were always of normal size. This indicates that the physiology of the retina and the optics of the eye were unaltered by the treatments, and it supports the argument that the increases in the size of multiunit receptive fields are the result of decreased precision in the projection pattern. Consistent with these findings, preliminary experiments using autoradiographic tracing of the optic nerve fibers suggested that the fiber density in the central region of the tectum was lower in antibody-treated animals with class 2 patterns than in untreated animals or animals with class 1 patterns (unpublished data). Detailed correlation of these anatomical findings with the physiological findings remains to be done.

Because the receptive field sizes were most affected near the antibody implant, the average receptive field sizes that are listed in Table 1, based on the whole visual field of the animal, are likely to underestimate the receptive field sizes in the most affected region of a class 2 projection pattern. This averaging procedure also increases the standard deviation of the measurement by including both more- and less-affected regions. Nevertheless, the average receptive field size for these animals was significantly increased over that found in normal or class 1 animals.

As indicated above, not all antibodies had similar efficacies in producing class 2 patterns: monoclonal antibody 10H4 directed against Xenopus N-CAM had a variable effect on patterning and field size (Table 1). The ability of antibodies to perturb the projection pattern appeared to be correlated with their ability to block adhesive interactions in vitro. In preliminary experiments, antibody 10H4 was less effective at blocking the aggregation of Xenopus membrane vesicles than either monoclonal antibody 7C8 or Fab' fragments of polyclonal rabbit antibody 684 (unpublished data).

Normal animals that were implanted with antibodies 684 and 10H4 showed class 2 patterns, which were affected to a lesser degree than their counterparts in the regenerating animals. These normal animals also showed similar, though less...
A large fraction of the optic nerve fibers had been killed or blocked the re-establishment of an ordered projection but, in contrast to the patterns described so far, class 3 projections were very similar. This indicates that the retinotectal projection need not be in the midst of massive synaptic reorganization and refinement for the antibodies to have severe, effects on the receptive field sizes. This suggests that the comparable but more extensive changes described for regenerating animals were not solely the result of special states induced only in regenerating optic nerve fibers. In contrast to the patterns described so far, class 3 projection patterns consisted of a large central blind spot, exemplified in Fig. 3, which shows the position of the implant and the general good health of the tectum; after 4 weeks, the tectum had returned to essentially normal gross morphology. It is important to note that within 4 weeks after the implantations, the projection pattern and the size of the receptive fields had returned to values much closer to normal in all cases assayed showing class 2 changes. This suggests that the induced perturbations were reversible. More precise correlation of this effect with the time-dependent depletion of antibodies within the implanted tectum will require both extensive histochemical analysis and radioactive tracer studies.

**DISCUSSION**

The results presented here show that the order of the retinotectal projection is altered by introducing antibodies against N-CAM into the tectal neuropil. Anti-N-CAM causes both a local distortion in the patterning of the optic nerve fibers also showed that the innervation of the optic tectum was greatly decreased in class 3 animals, consistent with the lack of a projection from the central retina. Gross examination failed to show other anomalies that might be attributed to the experimental manipulation. This is exemplified in Fig. 3, which shows the position of the implant and the general good health of the tectum; after 4 weeks, the tectum had returned to essentially normal gross morphology. It is important to note that within 4 weeks after the implantations, the projection pattern and the size of the receptive fields had returned to values much closer to normal in all cases assayed showing class 2 changes. This suggests that the induced perturbations were reversible. More precise correlation of this effect with the time-dependent depletion of antibodies within the implanted tectum will require both extensive histochemical analysis and radioactive tracer studies.

**Table 1. Disruption of projections by anti-N-CAM antibodies**

<table>
<thead>
<tr>
<th>Antibody*</th>
<th>Regenerating animals</th>
<th>Normal animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Classes of maps found</td>
<td>Classes of maps found</td>
</tr>
<tr>
<td>None (agarose only)</td>
<td>4 1</td>
<td>4 1</td>
</tr>
<tr>
<td>Control antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 684 (preimmune)</td>
<td>6 1</td>
<td>4 2</td>
</tr>
<tr>
<td>Rabbit 623</td>
<td>4 1</td>
<td>2 2†</td>
</tr>
<tr>
<td>Monoclonal 7C5</td>
<td>4 1†</td>
<td>4 1</td>
</tr>
<tr>
<td>Monoclonal 12G4</td>
<td>4 1</td>
<td>2 2†</td>
</tr>
<tr>
<td>Anti-N-CAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 684</td>
<td>7 2†</td>
<td>6 2</td>
</tr>
<tr>
<td>Monoclonal 7C8</td>
<td>6 2</td>
<td>4 1</td>
</tr>
<tr>
<td>Monoclonal 10H4</td>
<td>4 1, 2§</td>
<td>4 1</td>
</tr>
</tbody>
</table>

*Rabbit polyclonal antibodies were used as Fab' fragments and mouse monoclonal antibodies were used as IgG. Control antibodies were the following: (i) preimmune IgG from the same animal (rabbit 684) used to raise anti-Xenopus N-CAM antibodies and (ii) polyclonal (rabbit 623) and monoclonal (7C5 and 12G4) antibodies directed against chicken L-CAM (10). None of these antibodies reacted detectably with Xenopus brain.

†Two of the animals treated with antibody 684 and two of the animals treated with antibody 7C5 showed class 3 patterns. These animals were not used in calculating the mean diameters of receptive fields (see text).

‡P value (using Student's t test) compared with the aggregate mean of the values shown for the regenerating animals treated with control antibodies.

§Two animals were in class 1 and two animals were in class 2.

Fig. 3. Photographs of the tectum 1 week (A) and 4 weeks (B) after antibody implantation. Typically, within 4 weeks, the implant had been extruded from the tectum. The gross appearance of the tectum is normal at both stages, showing healthy tissue and normal vasculature. The size of the implant (arrow in A) appears somewhat larger than was actually the case because of the withdrawal of the pial pigment from the site of the implant. (×10.)
projection in normal frogs may therefore have occurred as a result of interference with this normal ongoing dynamic rearrangement of the retinotectal projection. The reversibility of the effects of perturbation by antibodies in class 2 animals after prolonged times of implantation is also consistent with the idea that the rearrangement is a dynamic process.

On the basis of early experiments on the lower vertebrate visual system, Sperry (2) proposed that the retinotectal projection pattern is ordered by detailed chemospecific interactions between the optic nerve fibers and the tectal cells. Although certain aspects of this proposal, such as the existence of large numbers of prespecified markers responsible for the microscopic details of mapped patterns (14), appear incompatible with the results of more recent experiments, the basic premise of chemical cues in the retina and tectum has formed the backdrop for a great body of experimental work and for models in which cell adhesion plays a central role. Two such models (7, 8) propose a dominant position-independent adhesion between neurons, which is supplemented by one or more minor quantitative gradients of adhesion to provide positional information. The previously demonstrated dominant role of homophilic N-CAM to N-CAM binding in neuron–neuron adhesion (9, 15), when combined with the results of the present experiments, suggests that N-CAM may mediate the dominant adhesive interaction called for by these models.

N-CAM has been shown to be differentially modulated in prevalence at the cell surface during early embryonic development and during histogenesis, consistent with a dynamic view of the early establishment of projections. During development it undergoes a conversion from a heterogeneous form containing very large amounts of polysialic acid (E form) to several less heterogeneous forms (A form) with lesser amounts of this sugar. Both the prevalence modulation and the later chemical modulation [called E to A conversion (9)] are accompanied by changes in the rates of homophilic binding (15). The existence of these modulation mechanisms raises the possibility that the minor positional variations in adhesion proposed by the neural patterning models might also be provided by alterations in the relative amounts or chemical properties of N-CAM.

Although the concordance between these findings and certain predictions of neural patterning models is generally satisfactory, it does not preclude the possibility of other mechanisms coming into play. For example, the results of dynamic alterations of the amounts as well as the forms of N-CAM on individual neurites and growth cones remain to be determined. The contribution to neural patterning of Ng-CAM (11) also needs to be assessed in view of the possibility that coordinate variations in neuron–neuron and neuron–glia interactions might contribute to patterning. The existence of such local or interactive contributions would not be directly detected by the perturbation methods used in the experiments described here. Nonetheless, the present studies clearly show that disrupting the cell–cell adhesion mediated by N-CAM is sufficient to distort the pattern of the retinotectal projection as well as to decrease its precision; the results support the hypothesis that N-CAM plays a central role in the formation of neuronal projections (9). Quantitative refinement of the experimental approach described here and its execution at a more microscopic level may help to ascertain the role of defined molecular interactions in the in vivo patterning of neurons and their interconnections.

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