# Retinotectal Projection in Goldfish to an Inappropriate Region with a Reversal in Polarity

Abstract. *An abnormal, ipsilateral projection was formed by deflecting optic fibers that normally innervate the posterior part of one tectum into the anterior end of the opposite tectum. When anterior recipient tectum was simultaneously denervated, the deflected fibers formed a retinotopic map in this region that was reversed with respect to the anterior-posterior tectal axis.*

In the course of neural development, nerve fibers grow, often for considerable distances and past millions of inappropriate nerve cells, to eventually terminate on their appropriate target cells, thereby forming the elaborate and precise "wiring" of the nervous system. Even though the outgrowth of fibers and the origins of target cells are precisely orchestrated in space and time, substantial evidence indicates that orderly timing and spacing are not necessary for selective fiber growth and connection. Instead, locus and time of origin appear to somehow encode intrinsic position information into individual fibers and target cells. These position markers then enable fibers to be directed (guided) to their targets even if the fiber pathways and the normal sequence of fiber outgrowth are surgically or genetically disrupted (*1*, *2*). I now describe a controlled manipulation of pathway and time of growth that consistently resulted in a fiber projection to an abnormal connection site with an orderly exceptional reversal of the normal polarity.

The design relies on the capacity of optic fibers of the adult goldfish to regrow to their original target sites when severed and so to reestablish the original retinotopic projection onto the midbrain tectum, its major projection site (*3*). Normally this projection is strictly contralateral. In this experiment, however, a select fraction of optic fibers, those normally innervating posterior (contralateral) tectum, were deflected into the anterior end of the opposite (ipsilateral) tectum. The deflection technique (*4*-*6*) involved teasing free and cutting optic radiation fibers from one tectum, directing them across the midline, and inserting them into the other tectum (Fig. 1A) (*7*). At the same time, the normal anterior innervation of the recipient (ipsilateral) tectum was permanently eliminated by electrocoagulating the temporal retina (*8*) of its corresponding (contralateral) eye (Fig. 1A). Thus, deflected fibers were made to grow into an inappropriate but denervated region of a tectum in which the appropriate target region for the deflected fibers was already occupied.

The predicted result based on most previous experiments (*6*, *9*, *10*) would be (i) for the deflected fibers to innervate the posterior tectum in the polarity appropriate for the ipsilateral tectum and (ii) for the ipsilateral and contralateral fibers to then expand across the tectum (Fig. 1B). However, evidence that ipsilateral fibers do not intermix with contralateral fibers under certain conditions (*6*, *11*) and can innervate inappropriate denervated areas (*12*) led me to expect that deflected fibers might innervate the anterior tectum. Should deflected fibers do so, as proved to be the case, two alternative predictions could be made about the possible topography of this projection. If topography is determined only on the basis of interactions between fibers and tectal polarity markers, the projection would be appropriately

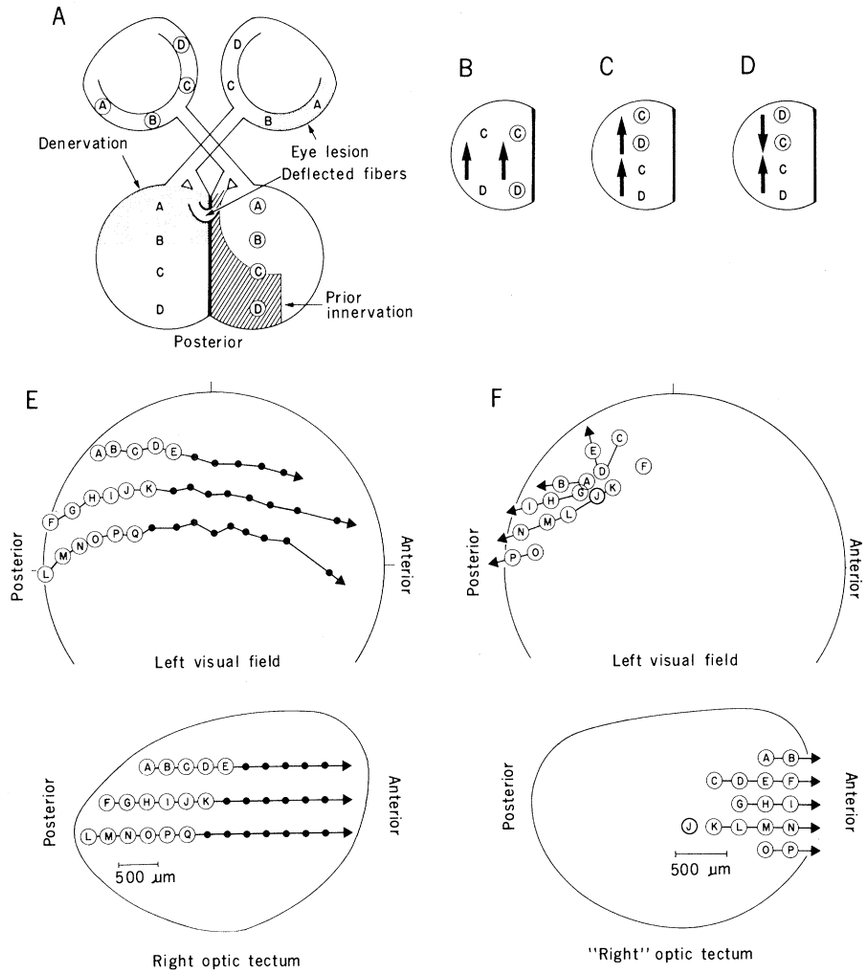


Fig. 1. (A) Schematic representation of the retinotectal projection showing the surgical procedure. Various retinal positions are designated with the letters *A* to *D*, with those in the left eye circled. The normal projection is shown by the corresponding letters on tectum. Shading indicates the lesioned area of the right retina and the corresponding zone of denervation in the left tectum. The small loop bridging both tecta at their anterior end represents the deflected fibers, and the hatching shows the normal area of tectum that these deflected fibers previously innervated. (B to D) Diagrams of the left or recipient tectum showing the three possible projections. Again the projection from the normal or undeflected fibers (from the right eye) is shown as letters without circles and that from the deflected or ipsilateral fibers (from the left eye) as circled letters. (E and F) Maps obtained by recording the terminals of optic fibers at the tectum with microelectrodes. For each recorded tectal position (not all of which are presented) the corresponding receptive field was plotted. Correspondence is indicated by letters and polarity by arrows. (E) Normal fish. Tectal area designated with letters is roughly equivalent to that normally supplied by the fibers deflected in the experimental animals. (F) Experimental fish with the most recorded ipsilateral units 286 days after surgery. The left tectum has been transcribed as a "right" tectum for easy comparison with the normal map. Polarity is reversed along the anterior-posterior axis.


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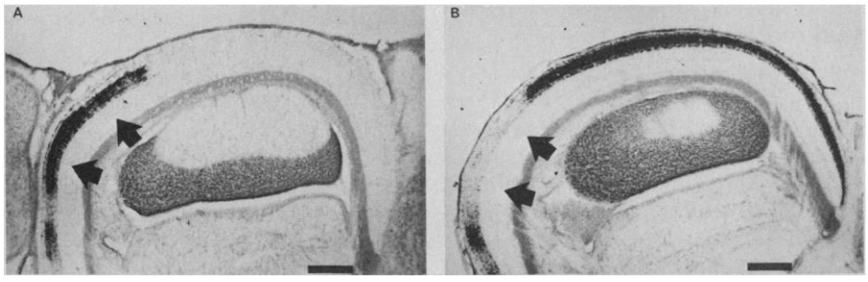


Fig. 2. Autoradiograms of recipient tectum of the two fish having the heaviest labeling of each group. The plane of section is sagittal, with anterior to the left. The anterior region shown here corresponds to the anterior region recorded. The bar represents 300 $\mu$m. (A) Deflected fibers labeled by injection of ipsilateral eye. Arrowheads point to anterior labeling. (B) Contralateral ("normal") fibers labeled by injection of contralateral eye. Arrowheads indicate the anterior gap in tectal label.

polarized for that tectum (Fig. 1C). On the other hand, if topography is determined by interfiber interactions (*1*), in particular a tendency for fibers from neighboring retinal areas to aggregate (*5, 6, 12*), the anterior-posterior axis of the ipsilateral projection would be reversed with respect to the tectal axis (Fig. 1D). Such reversal would allow the deflected fibers to be the nearest retinal neighbors to the remaining contralateral fibers along the region where the two groups of fibers touch.

At 279 to 293 days after the operation, the terminal arborizations of optic axons were mapped electrophysiologically in 13 fish according to the eye-in-water mapping technique (*13*). The deflection was successful in nine animals, all of which showed the same pattern (*14*). Ipsilateral (deflected) units were confined to the anterior tectum, while contralateral units were obtained only from the rest of the tectum, with very little overlap between the two (Fig. 2). Of 404 penetrations, 151 were ipsilateral only, 239 contralateral only, and just 14 contained units from both eyes, most of which were at or near the border between ipsilateral and contralateral regions. In five of these nine fish, one eye was injected with 50 $\mu$Ci of tritiated proline and, 12 to 24 hours later, fixed for autoradiography of serial sections (*15*). Label from the ipsilateral eye (three fish) was almost exclusively confined to the anterior third of the tectum, whereas label from the contralateral eye (two fish) was distributed through the remaining tectum (Fig. 2).

As for topography, orderliness was good, comparable to that recorded in other regeneration experiments (*6, 13*), but the anterior-posterior axis of the ipsilateral projection was reversed from the tectal axis in all animals (Fig. 2B). As an index of reversal and orderliness, the position of the receptive field at each penetration was compared with the receptive field obtained 250 $\mu$m anterior in the tectum (*16*). Of the 92 pairs of fields that could be so compared, 76 (83 percent) were reversed from normal. This is actually a considerable underestimate of the overall reversal since the presence of some topographic disorder reduced the number of reversed pairs computed in such a strict fashion. Also, most of the nonreversed pairs were found near the tectal edges where, because of some variability in the location and size of retinal lesions, the occasional persistence of contralateral innervation might be expected to prevent reversal or cause disorder. A comparison made between each of the most posterior units with one recorded 500 $\mu$m anterior showed 37 (92 percent) of the 40 pairs of receptive fields to be reversed.

The data demonstrate that in the central nervous system certain select altered conditions of position and timing of fiber ingrowth can produce an apparently permanent, inappropriate projection pattern despite the availability of appropriate targets and the capacity of fibers to reach these targets. These same deflected fibers will grow into the posterior tectum when the entire contralateral innervation is temporarily or permanently disrupted (*5, 6*). Even if the normal optic innervation is left intact, uncrossed fibers can still innervate posterior tectum (*10, 17*). Apparently, the preference by growing fibers for denervated rather than innervated tissue, in this case, succeeded in overriding the predisposition of fibers for their normal target sites.

This result also represents the first (to my knowledge) experimental case of a projection that is reversed with respect to the intrinsic polarity of retina and tectum under conditions in which a surgically induced change of these polarity markers can be ruled out (*18*). The reversal is not a consequence of the deflection procedure itself, since in other similar deflection experiments the polarity was normal (*5-7*). Previous work also makes any explanation of this in terms of fiber-tectum interactions implausible. Fibers from a surgically formed nasal hemi-retina (comparable to the fibers deflected here) have been shown to expand across an intact tectum or translocate into a surgically formed, anterior half tectum (*19*). In both cases no other fibers were present and polarity was normal. The preexistence of the normal posterior innervation forming a boundary across tectum appears critical for the reversal. This result is perhaps the most direct and least ambiguous evidence yet for the regulative role of highly selective interfiber interactions in the topographic ordering of axonal connections. Within the fiber pathways, sequential outgrowth of fibers during development would allow late-growing fibers to exhibit a similar preference for existing fiber shafts, thereby generating topographic organization within nerve bundles and fiber tracts and together with postsynaptic interactions guiding fibers to their appropriate target cells.

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### References and Notes

1. See reviews: R. W. Sperry, *Growth* **10**, 63 (1951); *Proc. Natl. Acad. Sci. U.S.A.* **50**, 703 (1963); R. K. Hunt, in *Cell, Tissue and Organ Culture*, S. Fedoroff and L. Hortz, Eds. (Academic Press, New York, 1977), p. 369; M. Jacobson, *Developmental Neurobiology* (Holt, Rinehart & Winston, New York, 1978); R. D. Lund, *Development and Plasticity of the Brain* (Oxford Univ. Press, New York, 1978); V. S. Caviness and P. Rakic, *Annu. Rev. Neurosci.* **1**, 297 (1978); A. Ghysen, *Nature (London)* **274**, 869 (1978).
2. R. L. Meyer and R. W. Sperry, in *Studies on the Development of Behavior and the Nervous System*, G. Gottlieb, Ed. (Academic Press, New York, 1976), p. 111.
3. R. W. Sperry, *Physiol. Zool.* **28**, 351 (1948); M. Jacobson and R. M. Gaze, *Exp. Neurol.* **13**, 418 (1965).
4. H. L. Arora, *Anat. Rec.* **154**, 311 (1966).
5. R. L. Meyer, *Brain Res.* **155**, 213 (1978).
6. ———, *J. Comp. Neurol.* **183**, 883 (1979).
7. To avoid fibers of passage to posterior tectum, deflected fibers were inserted into the recipient tectum several hundred micrometers lateral to the medial edge. It might be noted that deflected fibers were oriented in such a way so as to favor correct polarity along the anterior-posterior axis; that is, fibers normally posterior were posterior at the insertion point and that autoradiography and histology indicated considerable disorder in the organization of the deflected fiber pathway at the point of insertion.
8. Lesioning was by surgical diathermy modified from standard ophthalmological procedures. About a third of the retina was destroyed [R. L. Meyer, in *Developmental Biology, Pattern Formation, Gene Regulation*, D. McMahon and C. F. Fox, Eds. (Benjamin, Palo Alto, Calif., 1975), p. 257].
9. Y. L. Tung and S. C. Sharma, *Neurosci. Abstr.* **1**, 101 (1975); R. K. Hunt, *Biophys. J.* **17**, 128A (1977); for earlier work see (*2*).
10. J. T. Schmidt, *J. Comp. Neurol.* **177**, 279 (1978).
11. For examples of segregation during ontogenesis see G. E. Schneider, *Brain Behav. Evol.* **8**, 73 (1973); R. D. Lund and J. S. Lund, *J. Comp.*

*Neurol.* **169**, 133 (1976); M. Constantine-Paton and M. I. Law, *Science* **202**, 639 (1978).
12. R. L. Meyer, *Diss. Abstr. Int. B* **35**, 1510 (1975). Fibers deflected into inappropriate lateral positions of the opposite tectum would innervate these regions when the normal optic fibers to these regions were cut by a tectal incision.
13. R. L. Meyer, *Exp. Neurol.* **56**, 23 (1977).
14. In three of the unsuccessful cases, no ipsilateral units or only a few weak ones were recordable. In the fourth fish, the deflected fibers were inadvertently inserted at the medial edge of tectum thereby cutting fibers of passage to the posterior tectum (*7*). The map was comparable to that of previous experiments (*6*) in which these fibers were intentionally cut.
15. Paraffin embedding, 30-$\mu$m sections, and 1 week's exposure were specified after the method of W. M. Cowan, D. I. Gottlieb, A. E. Hendrickson, J. L. Price, and T. A. Woolsey [*Brain Res.* **37**, 21 (1972)]; for details see (*5*, *6*, *11*).
16. Pairs of field positions were classified as clearly reversed or nonreversed (normal polarity or confused). Each pair was unique so that fields from the most anterior tectal positions were included only as the object of a comparison. Occasionally a comparative unit could not be obtained at exactly 250 $\mu$m anterior along the row of recording points. In this case the closest anterior units > 250 $\mu$m were compared. Rarely, one tectal penetration produced two separated receptive fields; a position midway between was used for such comparisons.
17. S. C. Sharma, *Exp. Neurol.* **41**, 661 (1973); R. L. Levine and M. Jacobson, *Brain Res.* **98**, 172 (1975).
18. In a number of previous studies in which a part of retina or tectum has been surgically rotated or otherwise transplanted, the resulting projection has sometimes been contrary to the original polarity of the transplanted tissue. The problem has been that the surgery itself can lead to an alteration or regulation of the original polarity so that the projection may actually be in accordance with the existing polarity of the transplanted tissue. Through the work of Hunt, Jacobson, and others, such regulation of polarity has, in fact, been shown to be the case at least when the surgery is performed at embryonic stages. For juvenile and adult stages, the question has remained unanswered. In the present experiment, this kind of regulative change could not occur because the integrity of both retina and tectum were preserved. This demonstration that selective interfiber interactions can predominate over intrinsic polarity suggests that regulation need not be the explanation for previous transplantation experiments in juvenile and adult animals. A recent preliminary report by J. Rho [*Biophys. J.* **21**, 137A (1978)] indicates, in fact, that regulation is not occurring. In an exhaustive and ingenious series of tectal transplantations, Rho observed elements of both appropriate and inappropriate polarity in one graft. This means that either regulation is exceedingly complex and varied or that it is not the explanation. Taken together, Rho's results and the present findings suggest that polarity of a projection onto a transplanted piece of tectum can be appropriate or inappropriate depending on the degree to which fibers invading the transplant can communicate with fibers in surrounding tectum. Variability in healing of the transplanted tissue or in the rapidity with which fibers invade it would then account for the variability of previous results. For detailed discussion of this literature, see R. K. Hunt and M. Jacobson [*Curr. Top. Dev. Biol.* **8**, 203 (1974)]; R. L. Meyer and R. W. Sperry [in *Plasticity and Recovery of Function in the Central Nervous System*, D. G. Stein, J. J. Rosen, N. Butters, Eds. (Academic Press, New York, 1974), p. 45]; and R. Levine and M. Jacobson [*Exp. Neurol.* **43**, 527 (1974)].
19. T. J. Horder, *J. Physiol. (London)* **216**, 53P (1971); M. Yoon, *Am. Zool.* **12**, 106 (1972); *Exp. Neurol.* **37**, 451 (1972).
20. I thank R. W. Sperry, C. R. Hamilton, K. Gaston, and L. Wolcott for their comments on the manuscript; J. Macenka for histology; and L. Wolcott for illustrations. This work was supported by PHS grant MH-03372 to R. W. Sperry.

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## GABA Receptors in Clonal Cell Lines: A Model for Study of Benzodiazepine Action at Molecular Level

Abstract. *A "receptor unit" for γ-aminobutyric acid (GABA), which includes brainlike receptor binding sites for tritium-labeled GABA and benzodiazepines (diazepam, clonazepam, and flunitrazepam) and a thermostable endogenous protein (GABA modulin) that inhibits both GABA and benzodiazepine binding, has been demonstrated in membranes prepared from $NB_{2a}$ neuroblastoma and C6 glioma clonal cell lines. In these cells, as in brain, diazepam (1 micromolar) prevents the effect of GABA modulin, and in turn GABA (0.1 millimolar) increases the binding of [$^3$H]diazepam. The neuroblastoma and, to a lesser extent, the glioma cells represent a suitable model to study the interactions and the sequence of membrane and intracellular events triggered by the stimulation of benzodiazepine and GABA receptors.*

In crude synaptic membranes prepared from brain, benzodiazepines increase the affinity of γ-aminobutyric acid (GABA) receptors by competing with an endogenous thermostable protein. This protein has been termed "GABA modulin" because it allosterically modulates the high-affinity binding of GABA to its postjunctional receptor sites (*1*, *2*). The potency of several benzodiazepines in competing with GABA modulin correlates with their binding affinity for specific sites in crude synaptic membrane preparations and with their in vivo ability to relieve anxiety (*3*). This relationship has suggested that the action of benzodiazepines on GABA modulin represents a possible molecular mechanism for their well-documented facilitatory action on GABA transmission in vivo (*4*). Since GABA modulin could be released from its storage sites in brain and bind to synaptic membranes during homogenization (*1*), it could not be concluded from experiments with crude brain synaptic membranes whether interaction with GABA modulin accounts for benzodiazepine modification of GABA receptors in vivo.

We now present evidence that mouse neuroblastoma $NB_{2a}$ (NB) cells and rat C6 glioma (C6) cells are an adequate model for studying the supramolecular organization of GABA receptors and for monitoring the action of benzodiazepines at the molecular level. For such studies, an ideal cell line should contain on its membrane: (i) the benzodiazepine receptor, (ii) the GABA receptor regulating a $Cl^-$ channel, and (iii) GABA modulin. Moreover, to satisfy the postulates that derive from experiments with brain tissue, it should be shown that these three processes interact with each other in the following ways: (i) GABA modulin should inhibit the high-affinity binding of GABA and benzodiazepines (*1*, *2*); (ii) the activation of GABA receptors should change the flux of $Cl^-$ across the membrane (*5*); (iii) the $K_d$ values for binding of GABA (*6*) and benzodiazepines (*7*) should be similar to those in brain membranes (*8*); and (iv) the addition of benzodiazepines should change the membrane binding sites for GABA from a homogeneous class with a $K_d$ of 200 n$M$ to two classes ($K_{d1}$ = 200 n$M$, $K_{d2}$ = 20 n$M$) (*1*, *2*), and vice versa: the addition of GABA should lower the $K_d$ for benzodiazepine binding (*8*).

The high-affinity receptors for the benzodiazepines and GABA located on the membranes of NB and C6 cells have properties similar to those of receptors located on brain membranes (Table 1). The $K_d$'s of diazepam, clonazepam, and flunitrazepam are approximately equal in C6 (5 to 6 n$M$), NB (3 to 9 n$M$), and rat brain cortex (3 to 7 n$M$) membranes (*9*). In the membranes of NB cells, as in brain membranes, the affinity of [$^3$H]-clonazepam and [$^3$H]flunitrazepam for the receptor is higher than that for [$^3$H]diazepam (Table 1).

The density of binding sites is three- to fourfold higher in brain synaptic membranes than in the membranes of NB cells. The $B_{max}$ values for the three [$^3$H]benzodiazepines are similar in membranes of NB cells; however, in C6 cells, [$^3$H]clonazepam labeled only half as many binding sites as did [$^3$H]diazepam (Table 1).

The membranes prepared from brain and those prepared from clonal cell lines have similar kinetics for the high-affinity binding of GABA to receptors. Scatchard analysis of the saturation curve obtained with freshly prepared membranes from NB revealed only one receptor component. When these membranes were frozen, thawed, and treated with Triton X-100 (*1*), Scatchard analysis revealed two populations of GABA receptors. The binding of GABA to the high-affinity site of both NB and C6 cells was saturable; GABA was displaced by


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