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Review Article

Target recognition and dynamics of axonal growth in the retinotectal system of fish

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SUMMARY

Embryonic and regenerating retinal axons in fish are able to seek out their retinotopic target sites in the tectum. Neither a specific preordering of axons in the retinotectal pathway nor activity-dependent axon–target interactions are required for appropriate axonal targeting. Axon–target recognition appears to be predominantly mediated by positional cell surface markers. The discrimination of position-dependent differences by retinal axons in a special in vitro assay is consistent with this concept.

To understand retinal axonal regeneration we have analyzed the glial cells of the fish optic nerve and the expression of growth-associated cell surface molecules on the regenerating axons. The surfaces of the glial cells identified as oligodendrocytes are excellent substrates for the elongation of regenerating axons. Raising monoclonal antibodies we have found 3 cell surface proteins specific for growing axons. In the normal adult goldfish optic nerve, these proteins are only expressed by the few new axons from the newborn ganglion cells at the retinal margin. They are re-expressed on all axons during regeneration. A known cell surface molecule, NCAM, is expressed in a similar, specific spatiotemporal pattern on the fish retinal axons and may — in normal nerves — contribute to the establishment of the age-related fiber association. Whether the re-expression of NCAM and the antigens detected by the novel monoclonal antibodies are functionally involved in axonal growth and regeneration remains to be investigated.

INTRODUCTION

The search for mechanisms that enable axons to grow along specific pathways, to regenerate and to establish specific connections with their targets is still a challenge in neurobiology. These questions have been addressed most intensively in the retinotectal system of lower vertebrates (reviewed in Ref. 16). The axons from the retinal ganglion cells terminate in the contralateral tectum opticum in a geometrically simple and precise order, forming the retinotopic map. After optic nerve injury the retinal axons in fish and frogs regenerate and return to their appropriate target sites in the tectum. In the following we present a brief review (1) on experiments that investigated how embryonic and regenerated retinal axons in fish find their retinotopic target sites in the tectum opticum and (2) on recent approaches designed to understand axonal regeneration in fish.

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TARGET RECOGNITION OF RETINAL AXONS IN FISH

To explain appropriate targeting of the regenerating axons, Sperry²¹ formulated his famous chemospecificity theory; 'growing axons recognize their target cells by individual cytochemical identification tags'. A more realistic version of chemospecification are gradient theories^{9,11} suggesting that a few different molecules suffice to specify positions if these molecules are distributed in a graded fashion over the axes of the retina and the tectum. Guidance along gradients guarantees that axons can navigate directed to their target over long distances and reach their target from any, even from abnormal, entrance points into the tectum.

Other theories deny the existence or the importance of positional markers and their influence on axonal guidance. Instead, they try to account for the formation of orderly terminal arrays by preordering of axons, preservation of axonal neighborhood relations and a special timing of target innervation¹⁴ or have suggested that activity-driven axon-axon and axon-target interactions contribute essentially to the order of the axon terminal arborizations^{6,18}. More recent experiments in the fish retinotectal system, using sensitive anatomical fiber tracing techniques and in vitro studies have allowed us to re-evaluate these theories. Moreover, they have brought new insights into the organization of the retinotectal pathway and have led to a revision of some of the earlier views.

Targeting of regenerating axons

Widely reproduced in numerous textbooks are pictures from Attardi and Sperry's paper²¹ conveying the view that regenerating fish axons follow their normal pathways right back to their predesignated target sites in the tectum. This view is incorrect since sensitive anatomical fiber tracing techniques have revealed that regenerating axons lose their normal spatiotemporal order and cross in aberrant routes through the tectum^{10,28}. In early phases of their growth through the tectum, the axons ramify widely, covering large tectal areas by numerous side-branches studded with growth cones and filopodia²⁵. With time, however, the axons undergo course corrections. They develop target-directed routes, lose their ectopic branches and form terminal arbors of normal size and morphology over retinotopic territories^{10,24} (Fig. 1).

The same sequence of events – exploratory branching, branch elimination, directed target approach and terminal arbor formation at appropriate sites – is observed when the regenerating axons are deprived of their normal impulse activity by blocking the Na-channel-mediated activity with repeated intraocular injections of tetrodotoxin (TTX). The routes to the targets established by activity-deprived axons and the morphology of the axons resemble closely those of active axons¹².

Successful targeting despite abnormal paths and despite impulse blockade suggests that neither axonal preordering nor activity is necessary for axonal home-finding. The paths through which the axons are connected to their targets are directed and not the result of a random walk, which would have been expected if the axons had no information about the location of the target during their growth through the tectum¹¹. Course corrections and formation of target-directed routes by normal and misrouted axons are consistent with the concept that axons are guided in a field specified by gradients of positional markers¹¹.

Targeting of embryonic axons

In the fish embryo the tectum is quite small (200 μm across). In analogy to the situation in mammalian embryos and during regeneration in fish some earlier speculations were that the embryonic axons might produce large exploratory arborizations and

Fig. 1. Camera lucida tracings demonstrating the paths of a few HRP-labeled axons, which originate from a dorsotemporal sector of the retina (sketch in the upper left). Normally (tectum in the upper row) these axons travel through the ventral half of the tectum and end in small terminal arborizations in a region topographically related to the label sites in the retina (between the two arrows). Regenerating axons (lower row) course in abnormal routes, and branch initially over non-retinotopic regions (left tectum), but then they form target-directed routes and end in small terminal arborizations at their appropriate target sites (right tectum). D = dorsal; V = ventral; N = nasal; T = temporal; R = rostral; C = caudal.

use activity-driven interactions to confine their branches to successively smaller and appropriate locations¹⁸.

Recent data²⁶, however, demonstrated that embryonic axons in fish are surprisingly precise in targeting. They grow directed to their destination where they form small terminal arborizations. Size disparity experiments in which either the temporal or nasal retina was ablated prior to axonal outgrowth supported the selectivity of the embryonic axons. In the absence of temporal axons the nasal axons passed the uninervated rostral tectum to arborize exclusively at their appropriate sites in the caudal tectum. Temporal axons in the absence of nasal axons stayed confined to their appropriate domains in the rostral tectum without invading the vacated caudal tectum (Fig. 2). Neural activity appears not to be of importance for the development of the embryonic map. The retinotopic order developing in TTX-paralyzed embryos is as precise as in non-deprived fish²⁷.

Fig. 2. Dorsal view onto tecta of zebra fish embryo showing a defined group of retinal axons labeled with a fluorescent dye (diI). (a) Nasal axons pass over the rostral tectal half to arborize in retinotopically appropriate caudal tectal regions. (b) Temporal axons innervate exclusively the rostral tectal half, for further explanation, see legend to Fig. 1. Scale bar = 100 μ m.

These data lead to the conclusion that map formation of either developing or regenerating axons does not depend on a special preordering of axons and that neural activity is dispensable for axonal path- and homefinding. Axonal guidance by gradients of positional markers, however, is consistent with these data.

Discrimination of position-specific differences by growing axons in vitro

Provided positional markers play a decisive role in axonal navigation, such markers should preferably be expressed on the surfaces of the tectal cells to be accessible to the axons. In vitro experiments support this notion. An assay developed by Bonhoeffer³⁴ for the chick allows arrangement of cell surface membranes of the rostral and caudal tectum in alternating stripes. On this carpet, axons from temporal retinal explants (of fish and chick) show a specific behavior: they accumulate on rostral, retinotopically related membranes. Thus, the axons recognize and respond to position-specific differences associated with tectal cell surfaces^{31,34}. Embryonic chick axons exhibit this behavior only when the membranes are derived from tecta of defined embryonic ages. Fish axons, in contrast, still show their specific response on membranes of adult tecta, suggesting that the relevant cell surface components are continuously expressed in fish. This correlates well with the continuous growth and the capacity of map (re)formation in these animals³¹.

For chick axons it has been shown that they are not attracted by rostral membranes, but rather avoid a repulsive component associated with the caudal membranes³⁵. Fish axons are also repulsed by caudal membranes³⁰. Moreover, when adult fish axons are exposed to embryonic chick membranes, they elongate on rostral but not on caudal membranes³². This is suggestive evidence that embryonic chick and adult fish axons are sensitive to and respond to a similar molecular component on fish and chick tectal membranes.

Time lapse videomicroscopy has allowed us to monitor the encounter of fish axons with lanes of these tectal membranes (Bastmeyer and Stuermer, unpublished data). Temporal axons grow readily into and elongate in territories containing rostral membranes. However, when they meet caudal membranes, the growth cones retract. If they have no way to escape, such as in a dead-end lane, the axons cease to grow (Fig. 3). This

Fig. 3. Drawings of single frames from a continuous time-lapse video sequence. The growth cone elongates on a laminin lane and is bounded on two sides and in the last two frames on 3 sides by cell surface membranes from the caudal tectum (shaded areas). This growth cones derives from the temporal retina and is sensitive to repulsive components associated with the caudal membranes. On the left above each frame the duration of this sequence is given in hours and minutes.

axonal response supports the notion that the growth of fish axons is inhibited by a repulsive component on caudal membranes.

Such *in vitro* assays have provided insight into the dynamic interactions of growing axons with elements of their natural targets. Although somewhat artificial, such assays give reasons to hope that cell–cell interactions involved in axonal guidance and target recognition may eventually be understood on a molecular level.

Arbor plasticity in vivo

In very recent experiments we have extended our time-lapse video microscopy studies *in vitro* to observations *in vivo* to investigate how retinal axons in the living zebra fish embryo navigate towards and form arborizations at their retinotopic target sites in the tectum (Käthner and Stuermer, unpublished data). Consistent with our earlier analysis on fixed brains²⁶ is our present observation that the growth cones do not deviate much from the path that takes them directly to their retinotopic target sites. Here, then, the growth cones alter their shape and bifurcate. The primary branches elongate and give, over hours, rise to numerous side-branches and branchlets so that the arbor increases in size. These secondary and even the primary branches of the arbor are, however, not stable. In a rapid sequence, the branches are withdrawn and new ones extended (Fig. 4), so that the arbor after 12 h differs significantly in shape from the arbor formed first. The arbor stays more or less over the territory of tectum where branching was initiated but appears to ‘optimize’ its position by branch extension and retraction. These rapid changes in arbor morphology were not only observed in embryos at 70–80 h when arbors are known to form, but also in embryos at 120 h, where the retinotopic map has long been fully established.

Fig. 4. An axon arbor stained with dil in the tectum of a developing zebra fish embryo. *Left panel:* Photomicrographs from 2 single frames 15 min apart of a continuous time-lapse sequence. During this time interval the arbor has changed its appearance as seen more clearly in the drawings in the *right panel* (kindly provided by R. Käthner).

That arbors once formed over retinotopic sites are variable rather than stable structures was unexpected in that this probably involves a rapid making and breaking of synapses. On the other hand, it is known that axon arbors in fish cannot remain stable and 'locked in place'. As the fish grows, its retinae and tecta enlarge in size by adding new neurons. The growth pattern of retina and tectum, however, is geometrically incongruent. The retina grows in rings and the tectum by adding new cells only at its caudal pole¹⁷. This implies that the retinal axon arbors 'shift' to maintain a geometrically simple well-organized retinotopic map^{8,22,28}. In other words the arbors move gradually from previous to new retinotopically appropriate loci in the enlarging tectum.

Arbor plasticity, the extension and retraction of branches, observed in the living zebra fish could potentially be the basis for shifting. Our time-lapse studies will have to be extended to older embryos to substantiate this speculation.

AXONAL REGENERATION

Barely understood is another phenomenon unique to the retinotectal system of fish and amphibians. The retinal ganglion cell in fish and, to some extent, in amphibians are able to readily regenerate their axons after optic nerve transection. Our current working hypothesis views the capacity of axonal regeneration to depend on (1), the ability of the injured nerve cell to re-extend an axon; (2) the cellular and acellular environment that provides growth-permissive and -inhibiting molecules relevant for the elongating axon, and (3) the expression of specific cell surface molecules on the growing axons that enables

them to interact with each other and with their environment during their growth. To this point our experiments have addressed the second and third points.

Glial cells

In vitro studies have provided evidence that the glial cells of the regenerating goldfish optic nerve differ significantly from glial cells in mammals. They co-express markers that identify them as astrocytes and oligodendrocytes². Although they carry oligodendrocyte-specific markers, they possess highly growth-supporting surfaces. This contrasts with mammalian oligodendrocytes which inhibit regeneration not only of mammalian¹⁹ but also of goldfish retinal axons⁴. They obviously possess highly growth-supporting cell surfaces^{2,5}. On substrates that would not allow axonal growth, axons do regenerate from retinal explants if these non-permissive substrates are covered by goldfish glial cells. The axons then grow rapidly on the surfaces of the cells (Stuermer, unpublished data). Further characterizations of the glial cell surface properties are underway.

Cell surface proteins on growing retinal axons

By raising monoclonal antibodies (Mabs) against cell surface components of the regenerating optic nerve, we have obtained 3 Mabs, ϵ 587, E21, and M-802(Reggie), that specifically bind to growing and regenerating axons. Each Mab recognizes a different protein on Western blots of 200 kd, 84 kd and 50 kd, respectively^{33,36} (Wehner and Stuermer, unpublished data).

In normal fish with uninjured nerves the largest proportion of the retinal axons do not express the molecules recognized by these Mabs. Only the few growing axons which derive from the new ganglion cells constantly produced at the retinal margin and which form a coherent bundle in the nerve and tract are labeled. After optic nerve transection and during axonal regeneration, however, all axons distal to the cut re-express the proteins of the 3 Mabs (compare Figs. 6 and 7). The regenerating axons carry these proteins on the surfaces up into their finest processes in the retinorecipient layers of the tectum and in tissue culture into the growth cones and filopodia (Fig. 5). The expression of these molecules decreases and ceases finally at 6–9 months after optic nerve section.

Fig. 5. Goldfish retinal axons and growth cone in vitro, stained with the monoclonal antibody ϵ 567. Scale bar = 100 μ m (kindly provided by J. Vielmetter).

Fig. 6. Sections through a normal (right) and regenerating (left) optic nerve of goldfish, stained with an antibody against NCAM. In the normal nerve only the young axons from the new ganglion cells at the retinal peripheral margin are NCAM-positive (arrow). During regeneration, however, all axons re-express NCAM. Scale bar = 100 μm .

This time correlates with the restoration and stabilization of the retinotopic map^{24,25}.

A similar pattern of expression was found for the neural cell adhesion molecule NCAM, detected by an antibody (Mab D₃) against chick NCAM 180³ (Figs. 6 and 7).

Whether the re-expression of NCAM and the antigens recognized by the Mabs ϵ 587, E21 and Reggie is essential for and causally related to axonal regeneration in fish is not yet known and awaits functional tests. To this point, the 3 cell surface antigens of ϵ 587, E21 and Reggie have all been enriched by immunoaffinity purification steps and will be tested for sequence homologies with known proteins and can hopefully be cloned (Vielmetter, Wehner, Lottspeich and Stuermer, in progress).

Fig. 7. Sections through the tectum innervated by normal (a) and regenerating (b) axons. Only the regenerating axons in the retinorecipient layers of the tectum (white bars) express NCAM. Scale bar = 100 μm , reproduced with kind permission of The Company of Biologists Limited (1988).

POSSIBLE DETERMINANTS OF AGE-RELATED FIBER ORGANIZATION OF THE NORMAL FISH OPTIC NERVE

The fiber organization in the normal goldfish optic nerve is unique. The axons therein are ordered by age, i.e. their radial retinal origin⁷. This order is a consequence of the continuous addition of new ganglion cells to the retinal margin. The axons of these new cells always travel in apposition to the young unmyelinated axons of the just preceding generation⁷. NCAM is known to mediate the adhesion of neurons and the fasciculation of axons¹³. Only the young axons carry high levels of NCAM on their surfaces, which indicates that NCAM-mediated adhesion is involved in young-axon-to-young-axon association. Since NCAM expression is confirmed to young axons (Fig. 6) and lost with further maturation, NCAM could function as a guidance cue to the growth cones of the most recent axons. In other words, the unique spatiotemporal distribution of NCAM in adult fish could determine the path for the new axons³. In the regenerating optic nerve in which all axons express NCAM (Fig. 6), this age-related fiber order is not restored²³. Whether the antigens of our novel Mabs subserve a similar function as that proposed for NCAM remains to be seen.

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