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## Review

## Astrocytes as gate-keepers in optic nerve regeneration – A mini-review

Dana M. García, Joseph R. Koke\*

Department of Biology, Texas State University-San Marcos, TX 78666, USA

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## ABSTRACT

Animals that develop without extra-embryonic membranes (anamniotes – fish, amphibians) have impressive regenerative capacity, even to the extent of replacing entire limbs. In contrast, animals that develop within extra-embryonic membranes (amniotes – reptiles, birds, mammals) have limited capacity for regeneration as adults, particularly in the central nervous system (CNS). Much is known about the process of nerve development in fish and mammals and about regeneration after lesions in the CNS in fish and mammals. Because the retina of the eye and optic nerve are functionally part of the brain and are accessible in fish, frogs, and mice, optic nerve lesion and regeneration (ONR) has been extensively used as a model system for study of CNS nerve regeneration. When the optic nerve of a mouse is severed, the axons leading into the brain degenerate. Initially, the cut end of the axons on the proximal, eye-side of the injury sprout neurites which begin to grow into the lesion. Simultaneously, astrocytes of the optic nerve become activated to initiate wound repair as a first step in reestablishing the structural integrity of the optic nerve. This activation appears to initiate a cascade of molecular signals resulting in apoptotic cell death of the retinal ganglion cells axons of which make up the neural component of the optic nerve; regeneration fails and the injury is permanent. Evidence specifically implicating astrocytes comes from studies showing selective poisoning of astrocytes at the optic nerve lesion, along with activation of a gene whose product blocks apoptosis in retinal ganglion cells, creates conditions favorable to neurites sprouting from the cut proximal stump, growing through the lesion and into the distal portion of the injured nerve, eventually reaching appropriate targets in the brain. In anamniotes, astrocytes ostensibly present no such obstacle since optic nerve regeneration occurs without intervention; however, no systematic study of glial involvement has been done. In fish, vigorously growing neurites sprout from the cut axons and within a few days begin to re-nerve the brain. This review offers a new perspective on the role of glia, particularly astrocytes, as “gate-keepers;” i.e., as being permissive or inhibitory, by comparison between fish and mammals of glial function during ONR.

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## Contents

1. Introduction . . . . .	0
2. ONR in mammals . . . . .	0
3. ONR in fish . . . . .	0
4. Astrocytes and markers for astrogliosis . . . . .	0
5. Conclusions . . . . .	0
References . . . . .	0

## 1. Introduction

Significant differences exist among vertebrate animals in developmental and regenerative capacities. It has been known for many

years that animals that develop without extra-embryonic membranes (anamniotes – fish, amphibians) have impressive regenerative capacity, even to the extent of replacing entire limbs. Many fish appear capable of growing indefinitely to fit the available resources – e.g., a goldfish will get bigger in direct proportion to the volume of the tank in which it resides, depending on how many other fish are present. In contrast, animals that develop within extra-embryonic membranes (amniotes – reptiles, birds, mammals) have limited

\* Corresponding author. Tel.: +1 512 395 7497; fax: +1 512 245 8713.  
 E-mail address: [jrkoke@txstate.edu](mailto:jrkoke@txstate.edu) (J.R. Koke).

capacity for regeneration as adults, particularly in the central nervous system (CNS). The size and life span of amniotes are genetically determined, and some theorize that the same mechanisms which regulate growth also prevent regeneration (Cameron et al., 2005). Much is known about the process of nerve development in fish and mammals and about regeneration after lesions in the CNS in fish and mammals. Because the retina of the eye and optic nerve are developmentally and functionally part of the brain and are reasonably accessible in fish, frogs, and mice, optic nerve lesion and regeneration has been extensively used as an experimental system for study of CNS nerve regeneration — particularly in zebrafish and the mouse.

In mice, astrocytes are an obstacle to optic nerve regeneration (ONR); selectively poisoning them is permissive to the tracking of newly formed neurites through the nerve casing en-route to targets in the brain (Cho and Chen, 2008; Cho et al., 2005). In lower vertebrates (anamniotes) there is no evidence that astrocytes present any barrier to regeneration, and few investigators have looked carefully at the role of glia, particularly astrocytes, comparatively in mammals and fish.

## 2. ONR in mammals

A large body of literature describes research (with results sometimes conflicting) on ONR as a model of CNS regeneration or for study of retinal pathologies and stem cells. When the optic nerve of a mouse is severed, the distal portion of axons leading to the brain degenerate. Initially, the cut end of the axons on the proximal, eye-side of the injury may sprout neurites which begin to grow into the lesion. However, most often extension of neurites is brief if it happens at all, as the retinal ganglion cells (RGCs) whose axons make up the optic nerve quickly die apoptotically. Several lines of investigation have developed to explore why this is true in mammals but not in fish, amphibians, and to a lesser extent, birds; animals which maintain retinal growth throughout life and respond with robust regeneration to retinal or optic nerve injury (Hitchcock and Raymond, 2004).

A recent study of ONR in mice examined the role of ciliary neurotrophic factor (CNTF), thought to be important for survival and neurite extension by RGCs after ON injury. The authors showed that the CNTF receptor was lost rapidly from RGC axons near the crush site and that this loss spread proximally and distally from the lesion over a period of 2 days. On the other hand, 24 h later antibody to CNTF receptor intensely labeled previously unlabeled glia, both astrocytes and oligodendrocytes, near the lesion. These findings were interpreted to suggest that loss of the CNTF receptor from axons rendered RGCs unresponsive to CNTF, leading to cell death, and that the appearance of the receptor on glia promoted scarring (Miotke et al., 2007). A related finding is that CNTF activates astrocytes both *in vivo* and *in vitro*, causing them to release fibroblast growth factor 2 (Wang et al., 2008a,b).

In somewhat contradictory findings, Muller et al. (2007) reported that induction of intraocular inflammation by lens injury or intravitreal injection of zymosan (yeast cell wall extract) results in survival of RGCs after ON lesion and promotion of axon growth via CNTF release from retinal Müller glia and astrocytes. Whereas others had proposed that the protective effect of lens injury on RGCs might be due to macrophage-derived oncomodulin (Yin et al., 2006), Muller et al. (2007) present convincing evidence that intraocular inflammation induces retinal astrocytes to release CNTF which binds to RGCs via receptor tyrosine kinases and acts through the JAK/STAT pathway to switch RGCs to the regenerative state.

Chondroitin sulfate has also been reported as an inhibitor of axonal regeneration in mammals (Becker and Becker, 2002). In a recent study of ONR in rats, chick neural tube stem cells were transplanted into the cut rat optic nerve where they survived and produced a variety of growth factors (oncomodulin, CNTF, BDNF, and  $\beta$ - and  $\gamma$ -crystallins). In addition, the chick stem cells activated matrix metalloprotease MMP-2 in themselves and MMP-14 in GFAP-positive optic nerve astrocytes which resulted in degradation and disappearance of matrix chondroitin

sulfate proteoglycans. Under these conditions, the cut axons intermingled with the chick neural tube stem cells, and then passed through the injury site, entered the distal cut optic nerve and migrated long distances to the thalamus and mid-brain (Charalambous et al., 2008).

Other groups have concentrated on the expression of Nogo and the Nogo receptor, showing that knocking out expression of Nogo-A, -B and -C or inhibiting their receptors encourages neurite extension into an optic nerve lesion (Su et al., 2008). In embryonic mice, Nogo expression has been shown in vimentin positive glia, and Nogo receptor on neurites (Wang et al., 2008a,b). In adult rats, Nogo is produced by oligodendrocytes in the CNS, presumably including in the optic nerve (Chen et al., 2000).

The chemorepellant axon guidance protein semaphorin has also been implicated in failure of ONR in rats. The semaphorin receptor NP-1 has been shown to be expressed abundantly in *Xenopus* RGCs, and semaphorin secreted from the tectum and optic tract from an as yet unidentified cell type controls guidance of growing axons by stage-dependent collapse, turning, or branching (Campbell et al., 2001). Shirvan et al. (2002) reported “marked” induction of semaphorin in retinas of axotomized rats and that injection of antibodies against semaphorin 3A at the same time as axotomy was effective in preventing RGC apoptosis. A more recent immunohistochemical and biochemical study of the rat optic nerve by Nitzan et al. (2006) reported that axotomy which spares the vascular supply and neural scaffold causes loss of the normal optic nerve organization and invasion of the injury site by microglia, oligodendrocytes, and astrocytes. A concomitant increase in semaphorin 3A at the injury site is sustained for at least 28 days, along with a lesser, transient increase in the retina of the injured eye, although it was not clear what the source of semaphorin was in either location.

It has been convincingly shown in several studies that prevention of reactive astrogliosis in the optic nerve along with induction of expression of Bcl-2, a gene encoding the Bcl2 IAP (inhibitor of apoptosis) protein, results in structural regeneration of the mouse optic nerve, including re-ervation of appropriate targets in the brain although it is not clear that vision is restored (Cho and Chen, 2008; Cho et al., 2005). In mice, Bcl2 expression in retinal neurons turns off prenatally, while in fish and frogs expression continues throughout life and is upregulated during ONR (Chierzi et al., 1999; Cristino et al., 2000).

While the above-mentioned studies all indicate the importance of glial/RGC interactions in ONR, they differ in the location of the glia being studied — in the retina or optic nerve itself. Thus, even though it seems clear that mammalian ONR may be prevented by glial–RGC interactions and developmental silencing of Bcl-2, an overall theoretical framework that accommodates the large body of experimental results reported for mammalian ONR is lacking. Thus some investigators have turned to fish (zebrafish in particular because of the known genome and mutants available) models of ONR, in which ONR is robust, to do comparative analyses with the goal of identifying key differences that would allow formulation of such a theoretical framework.

## 3. ONR in fish

Few parallels can be drawn between studies of optic nerve regeneration made in fish and mammals, mainly because the complement of genes whose expression has been studied in one taxon differs from what has been studied in the other. One exception is Bcl-2, which may play a permissive role in ONR in both mice and fish, as just mentioned (Cho et al., 2005). A related phenomenological similarity may be the occurrence of apoptosis in RGCs consequent to optic nerve lesion. Although this phenomenon remains mostly unexplored in zebrafish *in situ*, Zhou and Wang (2002) reported changes in the number and distribution of RGCs after optic nerve crush injury. They found approximately 40,000 to 56,000 RGCs in the adult zebrafish retina, the densities of which depended on location. After ON crush, approximately 20% of the RGCs were lost during ONR in a wave of apoptosis proceeding from the central to peripheral retina.

In a recent study of *in vitro* organotypic culture of adult zebrafish retina, Kustermann et al. (2008) report a low rate of less than 10% RGC apoptosis as determined by TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) in the retinal explants after 3 days in culture. However, after 10 days *in vitro* TUNEL staining indicated about 50% of the RGC population were apoptotic, apparently as a result of the axotomy required to prepare the retinal explant and because of detachment of the retinal pigment epithelium that took place *in vitro* during culture. The loss of RGCs in the explants did not appear to be compensated by cell proliferation as determined by BrdU uptake and labeling, although extensive proliferation was observed among other cell types. Interestingly, Kustermann et al., did not observe the extensive hypertrophy of RGCs in their axotomized explants that others have reported in retinas of axotomized gold fish during ONR (Devadas et al., 2000). These differences between *in vivo* and *in vitro* findings suggest that signals that are present in the intact organism, and missing in the explanted retina, may be responsible for RGC survival and activation of axonal regrowth. Thus more work needs to be done to determine whether axonal sprouting from RGCs reflects survival of a subpopulation or *de novo* differentiation of RGCs from stem cell precursors.

The overall picture of glial-astrocyte/RGC interactions during ONR in fish is even foggier than the situation in mammals, although certainly interesting work has been done. Using goldfish, Nona et al. (1994) performed optic nerve crush and showed a network of GFAP-positive, reactive astrocytes appearing on either side of the crush site within 7 days of injury. This appearance coincided with the regrowth of the crushed axons. However, the damaged area itself remained GFAP-negative, and astrocytes were excluded from the lesion during regeneration. Nona et al. (2000) went on to report that even though goldfish ON regenerates new nerve fibers quickly, myelination of the new fibers does not occur until they reach the brain. Thus it appears that regenerating axons, not glial cells, determine when myelin formation begins in regenerating optic nerve. In goldfish, the site of ON injury is invaded by Schwann cells of unknown provenance which do not initially attach to or myelinate regenerating axons. These Schwann cells begin to divide after the new axons reach the brain, and only then small numbers exit the cell cycle, attach to axons, and form myelin. After 3 months, remyelination was complete in the injured site. The regenerating axons distal (toward the brain) to the injury site were myelinated by oligodendrocytes in synchrony with the Schwann cells in the restored lesion (Nona et al., 1992). If analogy to mammalian systems is appropriate, the findings in goldfish suggest several possibilities: first, reactive astrocytes recruit Schwann cells to the injured area. In primary cultures, astrocytes obtained from neonatal rats express neuregulins (and their receptors) which *in vivo* attract Schwann cells to axons during development, resulting in myelination in the PNS (Francis et al., 1999). Second, RGC axons inhibit the differentiation of Schwann cells until their axons reach targets in the brain. The mechanism delaying myelination could involve the Notch signaling pathway; in the optic nerve of developing rats oligodendrocytes express Notch1 while RGCs express Jagged1, which acts as a ligand for Notch. This interaction appears to inhibit oligodendrocyte differentiation until proper mapping of RGC axons to the optic tectum is complete (Wang et al., 1998).

Astrocytes may also modulate the microenvironment during ONR in a way that is permissive for axonal tracking. Mack and Wolburg (2006) report that fish astrocytes are interconnected by tight junctions and desmosomes. They investigated regrowth of lesioned axons in optic nerve of Burton's mouthbrooder (*Astatotilapia burtoni*) and found expression of tight junctional proteins occludins protein-1 and claudin-1 in astrocytes located near newly assembled RGC axons. The authors interpreted this finding to indicate different properties of astrocytic membranes modify neuroglial interactions, which may in turn determine a permissive microenvironment for ONR. Astrocytes in mammals are interconnected by gap junctions in the CNS, but tight junctions have not been reported.

Schweitzer et al. (2007) report that in the oligodendrocytes of the developing ON pathway in zebrafish, Contactin1a mRNA is highly

expressed, whereas in the adult fish expression is only found in glial cells in the optic fiber layer of the retina. Contactin proteins are members of the immunoglobulin superfamily and are thought to play a role in cell recognition. After ON lesion, they report that both oligodendrocytes and RGCs upregulate Contactin1a to levels significantly higher than those found during development. They go on to suggest that oligodendrocytes may play important roles in successful CNS regeneration, perhaps exerting an influence most significantly proximal to the injury site (Schweitzer et al., 2007).

Veldman et al. (2007), in an elegant study using laser microdissection to remove RGCs from retinal sections from optic nerve-injured zebrafish, employed DNA microarray analysis, qPCR, and other techniques to identify regeneration-specific genes. They found evidence that ONR is not a simple recapitulation (at least from the RGC perspective) of development, and they identified two genes in particular, KLF6a and KLF7a – a.k.a Krüppel-like factors – that together were necessary for ONR to occur (Veldman et al., 2007).

In a related study, Saul et al. (2008) used DNA microarray analysis to compare changes in retinal expression at different time points after optic nerve injury, using a sham vs. experimental approach. Since this analysis included RNA from whole retina, including retinal glia, differential expression of genes in cells other than retinal ganglion cells may have been detected. The authors selected from the 8000 plus genes analyzed six for verification by quantitative PCR (Table 1). Further work is underway using FISH to determine if the differential expression is cell-type specific.

If one includes injured fish retina as a model of CNS regeneration, one can begin to see a possible similarity between mammals and fish in the role of FGF. Fausett et al. (2008) have shown that retinal injury induces Müller glia (retinal astrocytes) to express alpha1-tubulin and pax6, which in turn induce the conversion of Müller glia into retinal progenitor cells. They further showed that binding of acheate-scute complex-like 1a (ascl1a) to a critical enhancer region in the alpha1-tubulin promoter is necessary for induction of alpha1-tubulin and pax6. In other zebrafish developmental pathways, FGF3 has been shown to be an upstream regulator of ascl1a (Pogoda et al., 2006); and also involved in astrocyte activation (Santos-Silva et al., 2007). FGF is secreted by mammalian astrocytes activated in response to CNTF (Wang et al., 2008a,b). It is possible that mechanical injury to retina causes activation of astrocytes which then initiate transformation of Müller glia to retinal progenitors via an FGF -> ascl1a -> alpha1-tubulin, pax6 pathway.

#### 4. Astrocytes and markers for astrogliosis

Astrogliosis is a characteristic response of astrocytes in the central nervous system (CNS) to trauma and a broad spectrum of disease processes. Morphologically, reactive astrocytes display hypertrophy of the cell body and nucleus, elongation of cytoplasmic processes, hyperplasia and an increase in immuno-detectable glial fibrillary acidic protein (GFAP) (Malhotra et al., 1990). For more recent reviews of gliosis in general, see Pekny (2004) and Pekny et al. (2007). Reactivity of Müller glia in the eye is described by Bringmann et al. (2006); however, significant differences exist in the response to optic nerve or retinal injury between the behavior of retinal and optic nerve glia of fish and mammals.

**Table 1**

Genes differentially expressed in retina following optic nerve lesion in zebrafish (Saul et al., 2008)

Frizzled homolog 2 (U49412)	Up vs. sham at 3 h
Homeobox gene A-11 (NM_131147)	Up vs. sham at 3 h
Activating transcription factor 3 (NM_200964)	Up vs. sham at 24 h
Lunatic fringe homolog (NM_130971)	Up vs. sham at 24 h
Noggin 2 (NM_130992)	Up vs. sham at 24 h
Annexin A2a (AY178796)	Up vs. sham at 168 h
Similar to SLIT and TRK, member 4 (XM_6813090)	Down vs. sham at 168 h

GFAP is an intermediate filament protein specific to mature astrocytes, and its increased expression is considered a marker for reactive astrocytes. Malhotra et al. (1984) reported creation of monoclonal antibody (Mab J1–31) raised against plaque materials taken from brains of patients who had suffered from multiple sclerosis, which appeared to label only reactive astrocytes both *in situ* and *in vitro* (Malhotra et al., 1995). Preliminary characterization of the antigen revealed it to be a protein of MW 68–70 kDa with both a cytoplasmic and nuclear localization. Further work provided strong evidence that Mab J1–31 recognizes a phosphorylated epitope that occurs on GFAP and on lamin B, explaining its appearance in both cytoplasm and nuclei. In addition Mab J1–31 labeling showed a developmental relationship: cells engaged in rapid growth and DNA synthesis exhibit strong Mab J1–31 staining in nuclei, whereas quiescent cells do not (García et al., 2003). Further evidence of J1–31 specificity for reactive astrocytes was shown by activation of adenylyl cyclase in F98 cells with forskolin which resulted in a significant increase in Mab J1–31 labeling of nuclear and cytoplasmic structures, consistent with the known locations of lamins and GFAP. A similar increase in labeling intensity was not observed using antibodies specific for lamin B or GFAP. Downstream targets of cAMP, including PKA and Ca<sup>2+</sup> channels, were inhibited in an attempt to elucidate the kinase responsible for phosphorylating the J1–31 antigen. Inhibitors of PKA had no effect on the forskolin induced increase in Mab J1–31 labeling, but treating F98 cells with verapamil, a known inhibitor of Ca<sup>2+</sup> channels, caused strong attenuation of the forskolin-induced increase in Mab J1–31 labeling. These results support the notion that Mab J1–31 recognizes an epitope on GFAP and lamin B when that epitope contains a phosphorylated amino acid, most likely serine. Furthermore, that residue may be phosphorylated in a Ca<sup>2+</sup>-dependent manner as a result of adenylyl cyclase activation (Ramsey et al., 2005). Therefore, Mab J1–31 is particularly useful in studies of proliferating astrocytes and will likely be informative when applied to studies of the role of astrocytes in ONR.

## 5. Conclusions

The existing literature on optic nerve regeneration in mammals and fish reveals a diversity of questions asked and approaches taken, which lead to interesting, significant remaining questions. These questions call for a more thorough analysis of glial response to optic nerve injury which includes retinal glia as well as optic nerve and brain glia. In addition, understanding neuronal responses and the interactions between neurons and glia will be essential to provide conceptual scaffolding that will support a model to account for differences between fish and mammals in ONR.

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