Mechanisms Underlying Optic Nerve Regeneration and Navigation: Roles of Glial Cells and Cell Adhesion Molecules

Kevin K. Park and Marcio Ribeiro

Department of Neurosurgery, Miami Project To Cure Paralysis,
University of Miami Miller School of Medicine

Glaucoma Research Foundation and National Eye Institute

INTRODUCTION

Optic neuropathies including glaucoma are characterized by damage to the optic nerve which results in axonal injury and to varying degrees of immediate or protracted retinal ganglion cell (RGC) death. In addition to the cell death, a major limitation to achieving functional recovery after optic nerve damage is RGCs' inability to regenerate axons. Recently, significant progress has been made in promoting regeneration of injured RGC axons in adult mice. Correct pathfinding of axons to their appropriate target areas is a critical step towards wiring up the nervous system. Recently, studies have identified major problems in axon pathfinding in adult visual system; many regenerating RGC axons grow aberrantly in the adult mouse optic nerve and brain. Thus, in addition to promoting elongation of injured RGC axons, understanding axon guidance mechanisms in adult visual system is of a paramount importance. During development, astroglial cells guide pioneering axons. In adult nervous system, regenerating axons in spinal cord grow on astrocyte processes. Various surface and extracellular matrix molecules including cadherins and fibronectin expressed in astrocytes have been shown to mediate axon-glial cell interaction and promote axon regeneration and navigation. However, the extent to which astrocytes and these proteins play a role in RGC axon regeneration and navigation in adult animals remains largely undetermined.

DESIGN & METHODS

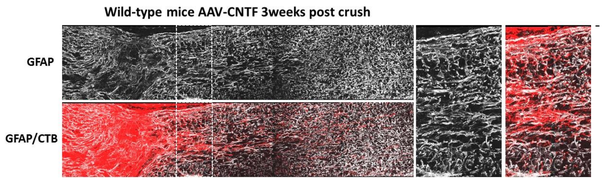
In Vivo Characterization of Astrocytic Gene Effects: To determine the role of astroglial-associated cell adhesion molecules and extracellular matrix proteins in RGC axon regeneration/navigation, we examine regeneration in mutant mice in which gene of interest is deleted specifically in astrocytes. To this end, we use the inducible, GFAPcreERT2 mouse in which tamoxifen administration results in expression of Cre recombinase in GFAP-expressing cells (i.e. astrocytes). We generate GFAPcreER; Ncadf/f; Rosa26-YFP (and GFAPcreER; fibronectinf/f; Rosa26-YFP mice). Since the floxed mice are bred to Rosa26 reporter mice, Ncad-deleted cells will also express a reporter protein (i.e. YFP) and allow comprehensive monitoring of knockout cells. Adult GFAPcre;Ncad;YFP mice received daily i.p. injection of tamoxifen (125 mg/kg) for five consecutive days. Two weeks after the last tamoxifen injection, animals were euthanized and optic nerve dissected out for immunohistochemistry. GFAPcre;GFP mice and GFAPcre;Ncad;YFP mice without tamoxifen injection serve as control animals. These mice received intravitreal AAV-ciliary neurotrophic factor (CNTF) injection (at 3 weeks after the last tamoxifen injection) and intraorbital optic nerve crush for 10 seconds using micro-forceps (at 1 week after AAV-CNTF injection) and euthanized at 2-3 weeks after optic nerve crush. Optic nerve were cryosectioned at 10 µm thickness.

Astrocyte Isolation: GLT-1-eGFP mice were perfused in PBS and the optic nerves were dissected in DMEM without serum. The optic nerves were then minced and dissociated in 21 Units/mL of Papain and 0.25% Trypsin/EDTA during 25 min with gentle shaking.

Quantitative RT-PCR: The total RNA from the GLT1-eGFP optic nerve cells was extracted using Absolutely RNA Nanoprep Kit from Agilent Technologies (USA), accordingly to the manufacturer instructions. The cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR from Invitrogen (USA).

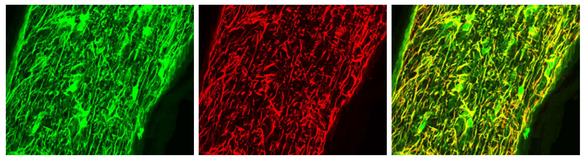
RESULTS

Regenerating retinal ganglion cell axons associate closely with astrocytes in the injured optic nerve



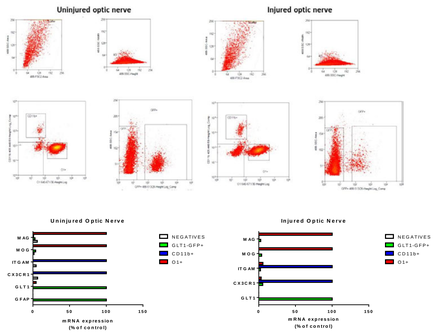
Glial fibrillary acidic protein (GFAP) (astrocyte marker) staining in optic nerve obtained 3 weeks after injury. The mice received AAV2-CNTF injection one week before crush. Cholera toxin subunit B (CTB) alexa fluor 555 conjugate was injected intravitreally three days before perfusion to label regenerating axons.

GLT1-eGFP mouse labels optic nerve astrocytes



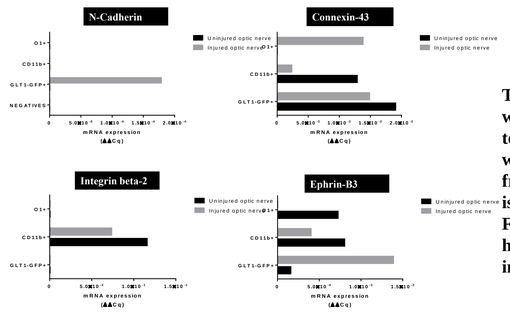
GLT1-eGFP mouse, in which eGFP was inserted downstream of the Slc1a2 (GLT-1) promoter, display a high eGFP expression and colocalization with GFAP (astrocytic marker) positive cells in adult optic nerve.

Astrocyte isolation from adult GLT1-eGFP mouse optic nerves

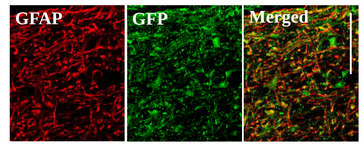


GLT1-eGFP mice received optic nerve crush and 7 day later the optic nerve cells were isolated from the injured and uninjured optic nerves. The eGFP positive cells (astrocytes), the O1 positive cells (mature oligodendrocytes), the CD11b positive cells (microglia/macrophages) were separated by fluorescence-activated cell sorting (FACS). The mRNA levels of cell-marker genes (GLT1 and GFAP for astrocytes; MOG and MAG for mature oligodendrocyte; CX3CR1 and ITGAM/CD11b for microglial cells) indicate that the FACS-isolated cells are predominantly astrocytes.

Cell adhesion and guidance molecule mRNA levels in injured optic nerves astrocytes



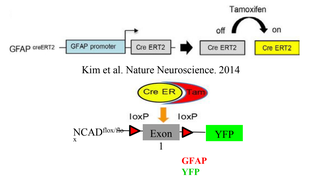
The mRNA levels of different genes whose protein products are known to mediate axon-glial interaction were analyzed. mRNA was obtained from different cell types (i.e. cells isolated using a combination of FACS and flow cytometry). *Ncad* is highly expressed in astrocytes of injured optic nerve.

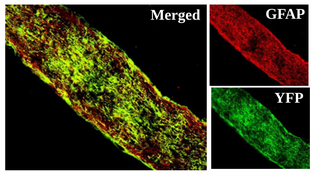


NCAD is expressed in astrocytes in the injured optic nerve

Immunohistochemistry performed on optic nerve sections. Animals were perfused 14 days after crush injury.

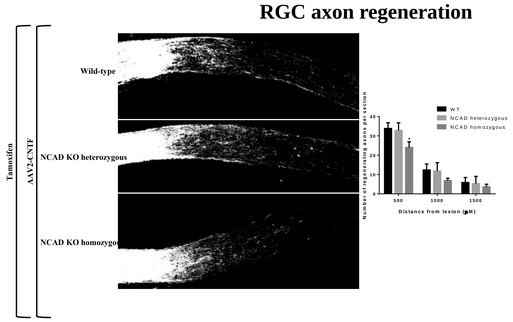
A genetic mouse model to induce astrocyte specific gene deletion





5 days of tamoxifen injection resulted in a high level of Cre recombination in astrocytes

NCAD deletion in optic nerve astrocytes has only a modest effect on RGC axon regeneration



Representative optic nerve sections 3 weeks after optic nerve crush. AAV-CNTF was injected to induced axon regeneration. Three days before perfusion the mice received intravitreal injection of CTB to trace the regenerating axons. NCAD deletion in astrocytes slightly decreases the number of regenerating axons near the lesion site but no changes were observed more distal to injury site.

CONCLUSIONS

- Regenerating RGC axons use astrocytes as physical substrates.
- NCAD deletion in astrocytes does not change significantly the axon regeneration rate.
- NCAD is known to be critical as an adhesion molecule to promote axon-glia interactions during development and in in vitro. However, it does not seem to have the same important role in adult mice, suggesting that the molecular mechanisms underlying axon-glial interaction and growth during development and in adult mice might be distinct.

NEXT STEPS

These results form an important basis for future studies to further examine the roles of glial cells in guiding regenerating RGC axons after injury, and to define the molecular mechanisms that regulate axon navigation and pathfinding in the injured adult visual system. In future studies, we plan to investigate how RGC axons might be able to grow properly towards the brain by using the glial cells in the optic nerve, and further examine different cell adhesion molecules that mediate this process.

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