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Long-distance axonal regeneration induced by CNTF gene transfer is impaired by axonal misguidance in the injured adult optic nerve

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ABSTRACT

The optic nerve crush injury is a well-accepted model to study the mechanisms of axonal regeneration after trauma in the CNS. The infection of retinal ganglion cells (RGCs) with an adeno-associated virus serotype 2 — ciliary neurotrophic factor (AAV2.CNTF) was previously shown to stimulate axonal regeneration. However, the transfection of axotomized neurons themselves may not be optimal to promote full axonal regeneration in the visual system. Here, we show that the release of CNTF by glial cells is a very powerful stimulus for optic fiber regeneration and RGC survival after optic nerve crush. After 8 weeks, long-distance regeneration of severed optic axons was induced by CNTF until and beyond the optic chiasm. Regenerated axons stayed for at least 6 months in the damaged optic nerve. Strikingly, however, many regenerated axons showed one or several sharp U-turns along their course, suggesting that guidance cues are missing and that long-distance axonal regeneration is limited by the return of the growing axons toward the retina. Even more surprisingly, massive axonal sprouting was observed within the eye, forming a dense plexus of neurites at the inner surface of the retina. These results indicate that massive stimulation of the neuronal growth program can lead to aberrant growth; the absence of local regulatory and guidance factors in the adult, injured optic nerve may therefore represent a major, so far underestimated obstacle to successful axon regeneration.

Introduction

The optic nerve crush model was extensively used to study the mechanisms of axonal growth inhibition and to design new repair strategies for the injured CNS (Benowitz and Yin, 2007; Harvey et al., 2006). In the intraorbital optic nerve crush paradigm, severed axons cannot spontaneously regenerate into the distal part of the optic nerve and most of the retinal ganglion cell (RGC) bodies die by apoptosis after 2 weeks (Berkelaar et al., 1994).

Contrary to BDNF (Mansour-Robaey et al., 1994) or FGF2 (Sapieha et al., 2003), CNTF was shown to stimulate both axonal regeneration and neuronal survival after optic nerve lesion (Lingor et al., 2008; Muller et al., 2007, 2009). Repeated intraocular injections of the recombinant CNTF peptide were efficient at activating axonal growth and neuronal survival but only to a limited extent (Muller et al., 2007). The effects of CNTF are likely restricted in time by the short half life of the recombinant peptide (Dittrich et al., 1994) and by the negative feedback control mediated by the up-regulation of the *suppressor of cytokine signaling 3* (SOCS3) (Smith et al., 2009). To sustain the CNTF delivery in the retina, an adeno-associated virus serotype 2 (AAV2) containing the *Cntf* cDNA was intravitreally injected to selectively infect the RGCs. AAV2.CNTF treatment resulted in significant neuroprotection and regeneration of some optic axons over longer distances (Leaver et al., 2006a,b). However, transducing neurons may not be optimal to deliver survival factors to the retina as only a small number of cells was infected (Leaver et al., 2006b) and protein synthesis is altered in axotomized neurons (Park et al., 2008).

Here, we hypothesized that the Müller glia-mediated release of CNTF may improve neuroprotection and stimulate long-distance axonal regeneration. In the healthy retina, Müller cells fulfill similar homeostatic functions as astrocytes in the rest of the CNS (Bringmann et al., 2006). Müller cell bodies occupy a central position in the retina from where they extend radial processes contacting all types of retinal neurons. In the degenerating retina, Müller cells are resistant to cell death and therefore are ideal intermediates to release neurotrophic factors. After optic nerve lesion, the Müller cell response is characterized by strong reactive gliosis and by a small number of proliferating cells (Wohl et al., 2009). AAVs allow stable, safe and efficient gene transfer and are thus suitable for human gene therapy (Bainbridge et al., 2008;

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Maguire et al., 2008, 2009). An engineered AAV called ShH10 was selected based on its ability to preferentially transduce Müller glia (Klimczak et al., 2009). Here we present the effects of the infection of Müller cells by the ShH10 vector carrying the cDNA of DH-CNTF, a mutant peptide exhibiting a higher affinity for CNTFRα and therefore acting as a super-agonist for this receptor subunit (Saggio et al., 1995). Our results show that glia-targeting AAV.DH-CNTF can promote long-range axonal regeneration. However, the distance covered by the regrowing axons was severely limited by the frequent formation of U-turns in the optic nerve. In addition, we observed massive aberrant axonal sprouting at the inner surface of the retina. Our data suggest that axonal misguidance is a key limiting factor for the long-distance axonal regeneration in the visual system.

Materials and methods

Animals

All surgeries were performed on 2–4 month old male C57BL/6 mice. Animal experiments were performed in agreement with the guidelines of the Veterinary Office of the Canton of Zürich.

ShH10 vector production

The AAV transfer plasmid with a modified form of the ciliary neurotrophic factor gene, DH-CNTF (Fig. 1C) was a generous gift from Dr W. Hauswirth, University of Florida. DH-CNTF recognizes with higher affinity the CNTFRα (Saggio et al., 1995) subunit (Fig. 1C). This construct contained a growth hormone signal peptide to improve the secretion of the CNTFRα superagonist DH-CNTF into the retina. Adeno-associated viral vectors were produced by standard methods. Triple plasmid co-transfection method (Grieger et al., 2006) was followed by ultracentrifugation as previously described (Dalkara et al., 2009). The iodixanol fraction interphase was then extracted and diluted with PBS plus 0.001% Tween 20. This fraction was buffer-exchanged and concentrated using Amicon Ultra-15 Centrifugal Filter Units to a final volume of 100–200 μL. Vector was then titered for DNase-resistant vector genomes by Real-Time qPCR relative to standards. Vector concentrations were calculated in viral genomes/mL with ShH10.GFP and ShH10.DH-CNTF at ~10^{13} vg/mL and AAV2.GFP at 1.8×10^{13} vg/mL. Four weeks after virus delivery, the infectivity of ShH10.GFP was estimated on retinal crossections in 3 different mice (3 tissue sections/mouse). On average, 84 ± 3% (mean ± S.E.M.) of glutamine synthase-positive Müller cells expressed the GFP protein.

Intraocular injections

ShH10 vectors or the anterograde tracer cholera toxin β subunit conjugated to alexa-594 (CTb, Molecular Probes) were injected as previously described (Pernet et al., 2005). To infect the Müller cells, 1 μL of ShH10.DH-CNTF or ShH10.GFP was intravitreously injected 4 weeks before optic nerve crush or tissue analysis, a time that allowed optimal transgene expression in vivo (Cheng et al., 2002; Klimczak et al., 2009).

Neuronal survival and soma diameter measurement

RGC survival was examined after intraorbital optic nerve crush injury at ~0.5 mm from the back of the eye. Two weeks after injury the animals were intracardially perfused with 4% paraformaldehyde (PFA). The RGCs were observed by immunofluorescent staining for β3Tubulin on retinal flat-mounts. β3Tubulin has previously been shown to be a specific and reliable marker to label all RGCs (Cui et al., 2003). To do so, the primary antibody was diluted in a solution of PBS containing 0.3% of Triton-X-100, 5% of normal serum and 0.05% sodium azide to prevent bacterial contamination. Then, after washings the retinae were incubated for 3 days with a goat anti-mouse secondary antibody coupled to alexa 594 or Cy3 at 4 °C. The β3Tubulin-positive RGCs were imaged in the 4 quadrants of the retina using a Leica SPE-II confocal microscope equipped with a 40× oil immersion objective (NA 1.25). Image stacks were acquired in the ganglion cell layer with a step size of 0.5 μm and a resolution of 1,024×1,024 pixels (0.27 μm/pixel). The number of RGC cell bodies was quantified in grids of 62,500 μm^2 at 1 mm and 1.5 mm from the optic disk. The density of surviving RGCs was calculated in individual quadrants or in the whole retina per mm^2.

Axonal regeneration analysis

To study axonal regeneration, a knot was tied with a 9-0 suture to fully constrict and crush the optic nerve intraorbitally. The suture was then carefully removed and a fundus examination allowed us to control the retinal blood supply from the ophthalmic artery. One day before fixation with paraformaldehyde (4%), the optic axons were anterogradely traced by injecting 1.5 μl of 0.5% CTb into the vitreous body. Axons labeled with CTb-594 were visualized on longitudinal sections of optic nerve (14 μm) with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss) and images were taken with a CCD video camera at 20×. The number of growing axons per optic nerve was estimated at 500 μm, 750 μm, 1,000 μm, 1,250 μm, 1,500 μm, 2,000 μm, 3,000 μm and 4,000 μm after the crush site (Pernet et al., 2005). Optic nerve slices were examined in 3–6 animals per condition. An estimation of the number of axons per optic nerve (Σ) was calculated with the following formula: $\Sigma_d = \Pi \times R^2 \times (\text{average number of axons/mm}) / T$. The sum (Σ) of axons at a given distance (d) was obtained using the average optic nerve radius (R) of all optic nerves, and a thickness (T) of the tissue slices of 14 μm (Leon et al., 2000). For statistical analysis, an ANOVA followed by a Bonferroni's or Dunnett's *post hoc* test was applied for multiple comparisons. Animals presenting ischemia or retinal hemorrhages were excluded from the analysis.

For the study of ShH10.DH-CNTF-induced axonal regeneration at 6 months post-lesion, RGCs were infected by injecting 1 μL of AAV2.GFP. GFP-filled axons were examined in whole-mounted optic nerves with a Leica SPE-II confocal microscope equipped with a 40× oil immersion objective (NA 1.25) or a 10× objective (NA 0.3). Three-dimensional (3D) image stacks were reconstructed with the Imaris software (Bitplane AG, Zürich, Switzerland).

Retina, optic nerve processing and immunofluorescence

Adult mice were sacrificed by injecting an overdose of anesthetic intraperitoneally. After intracardiac perfusion with PBS (0.1 M) and 4% PFA, the eyes were rapidly dissected by removing the cornea and the lens. For retinal crossections, the eye cups were postfixed in 4% PFA overnight at 4 °C. The tissues were then cryoprotected in 30% sucrose overnight and frozen in OCT compound (optimal cutting temperature, Tissue-TEK, Sakura) with a liquid nitrogen-cooled bath of 2-methylbutane. Optic nerves and retinal sections were cut (14 μm) with a cryostat and collected on Superfrost Plus slides (Menzel-Glaser). For immunohistochemistry procedure, tissue slices were blocked with 5% BSA or normal serum, 0.3% Triton X-100 in PBS for 1 h at room temperature to avoid unspecific cross-reactivity. Then, primary antibodies were applied in 5% BSA or normal serum, 0.3% Triton X-100 in PBS overnight at 4 °C. After PBS washes, sections were incubated with the appropriate secondary antibody for 1 h at room temperature, and mounted with MOWIOL anti-fading medium (10% Mowiol 4–88 (w/v) (Calbiochem), in 100 mM Tris, pH 8.5. 25% glycerol (w/v) and 0.1% 1,4-diazabicyclo[2.2.2]octane (DABCO)). Primary antibodies were: rabbit anti-Phospho-Stat3 (1:100, Cell Signaling, #9131), rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, Dako, #20334), rabbit anti-β3Tubulin (1:1,000, abcam, #ab18207), mouse anti-glutamine synthetase (GS, 1:200-1:400, Chemicon, #MAB302), mouse anti-β3Tubulin