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Research Paper

Zinc chelation and Klf9 knockdown cooperatively promote axon regeneration after optic nerve injury

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ABSTRACT

The inability of axons to regenerate over long-distances in the central nervous system (CNS) limits the recovery of sensory, motor, and cognitive functions after various CNS injuries and diseases. Although pre-clinical studies have identified a number of manipulations that stimulate some degree of axon growth after CNS damage, the extent of recovery remains quite limited, emphasizing the need for improved therapies. Here, we used traumatic injury to the mouse optic nerve as a model system to test the effects of combining several treatments that have recently been found to promote axon regeneration without the risks associated with manipulating known tumor suppressors or oncogenes. The treatments tested here include TPEN, a chelator of mobile (free) zinc (Zn^{2+}); shRNA against the axon growth-suppressing transcription factor Klf9; and the atypical growth factor oncomodulin combined with a cAMP analog. Whereas some combinatorial treatments produced only marginally stronger effects than the individual treatments alone, co-treatment with TPEN and Klf9 knockdown had a substantially stronger effect on axon regeneration than either one alone. This combination also promoted a high level of cell survival at longer time points. Thus, Zn^{2+} chelation in combination with Klf9 suppression holds therapeutic potential for promoting axon regeneration after optic nerve injury, and may also be effective for treating other CNS injuries and diseases.

1. Introduction

The inability of neurons in the central nervous system (CNS) to survive or to regenerate damaged axons over long distances limits recovery after multiple types of damage, including spinal cord injury (Hug and Weidner, 2012), stroke (Benowitz and Carmichael, 2010; Sozmen et al., 2012), and traumatic or ischemic optic neuropathy (Benowitz et al., 2015; Ghaffarieh and Levin, 2012; Wang et al., 2013; You et al., 2013). In the case of the optic nerve, manipulation of various factors can promote long-distance regeneration and some reinnervation of central target areas, but these treatments have generally involved intraocular inflammation and/or deletion or knock-down of genes that can act as tumor suppressors, raising questions about their clinical utility (Bei et al., 2016; Belin et al., 2015; de Lima et al., 2012a; de Lima et al., 2012b; Duan et al., 2015; Kurimoto et al., 2010; Lee et al., 2010; Lim et al., 2016; Liu et al., 2010; Park et al., 2008; Smith et al., 2009; Sun et al., 2011).

Here, we used the optic nerve crush (ONC) model in mice to evaluate combinatorial effects of three recently established treatments that stimulate retinal ganglion cells (RGCs), the projection neurons of the eye, to re-grow injured axons over long-distances. We focused on treatments that do not involve manipulation of factors such as Pten, Socs3, Sox11, Bcl2, and Myc (Belin et al., 2015; Chen et al., 1997; de Lima et al., 2012b; Jayaprakash et al., 2016; Kurimoto et al., 2010; Park et al., 2008; Smith et al., 2009; Sun et al., 2011), which are established tumor suppressors or oncogenes (Cory et al., 2003; He et al., 2003; Keniry and Parsons, 2008; Kuo et al., 2015; Nilsson and Cleveland, 2003; Rigby et al., 2007). We tested combinatorial effects of the following treatments: the Zn^{2+} chelator TPEN (Li et al., 2017), shRNA gene therapy-mediated knockdown (KD) of the axon-growth suppressing transcription factor Klf9 (Apara et al., 2017), and the atypical growth factor oncomodulin (Ocm) combined with a cAMP analog (CPT-cAMP) (Kurimoto et al., 2010; Yin et al., 2009; Yin et al., 2006).

We used a Zn^{2+} chelator based on our recent finding that injury to

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the optic nerve induces a rapid and profound elevation of mobile Zn^{2+} in the retina, and that intraocular injection of Zn^{2+}-selective chelators such as TPEN [*N*,*N*,*N*′,*N*′-tetrakis(2-pyridyl methyl) ethylenediamine] lead to long-lasting RGC protection and considerable axon regeneration (Li et al., 2017). Other studies have shown that intraocular inflammation also induces substantial levels of optic nerve regeneration and that this effect is mediated in large part by the atypical growth factor oncomodulin (Ocm), which binds to its cognate receptor on RGCs in a cAMP-dependent manner (Kurimoto et al., 2010; Yin et al., 2009; Yin et al., 2006). The combination of Ocm and a cAMP analog (e.g., CPT-cAMP) captures most of the effects of intraocular inflammation on axon growth without the potentially harmful effects of intraocular inflammation, although Ocm/cAMP has only a small effect on cell survival (Andereggen et al., 2015; Yin et al., 2006). Our other recent work has shown that several members of the Kruppel-like transcription factor (KLF) family contribute to the developmental changes in RGCs' intrinsic capacity to extend axons, and that knock-down of KLF-4 or KLF-9, two developmentally-upregulated suppressors of axon growth, promotes axon regeneration in mature mice (Apara et al., 2017; Moore et al., 2009).

Here, we found that out of these approaches, a co-treatment with TPEN and Klf9 KD had a substantially stronger effect than either one alone, while the other combinatorial treatments produced only marginally stronger effects than the individual treatments. Thus, Zn^{2+} chelation in combination with Klf9 suppression holds therapeutic potential for promoting RGC survival and axon regeneration after optic nerve injury and potentially in other parts of the CNS as well.

2. Results

We tested three pro-regenerative approaches individually and then in 2-way combinations for possible additive or synergistic effects in promoting RGC survival and axon regeneration after optic nerve crush (ONC) injury in mice, a widely used model of CNS injury, and more specifically, traumatic optic neuropathy. Where information was available, we used the doses and timing of treatments as previously described (Kurimoto et al., 2010; Li et al., 2017; Yin et al., 2009; Yin et al., 2006) (experimental time-line is shown in Fig. 1): For localized gene therapy, we injected intravitreally 3 μl of a high titer (1×10^{12}) adeno-associated virus serotype 2 (AAV2) expressing anti-KLF9 shRNAs with a GFP marker, or GFP control, two weeks prior to ONC injury. For targeted drug delivery, we injected intravitreally immediately after ONC 3 μl of either Ocm (15 ng/μl) combined with CPT-cAMP (50 μM; as described previously for this treatment, Yin et al., 2006), or TPEN (100 μM; also injected 4 days later as described previously for this treatment, Li et al., 2017), or phosphate-buffered saline (PBS) as a control. Across studies, the investigator performing the surgeries and quantification was masked to the treatment (i.e., the tube with virus or drug was prepared and coded by another researcher) until the end of the experiment. Animals that received viral injections were randomly selected for subsequent injections of different drugs or PBS alone. The animals were euthanized two weeks after injury and analyzed for axon regeneration in the optic nerve and RGC survival in the retina as described in Methods (Fig. 1). A few animals that developed cataract in the injured eye were excluded.

As expected, the negative control group (AAV2-GFP injected prior to nerve injury, PBS injected shortly after injury) showed very few axons growing beyond the injury site, whereas each of the individual experimental treatments promoted considerable regeneration (Figs. 2–3; Table 1). Of these, pre-injury KLF9 KD resulted in greater regeneration than post-injury administration of Ocm + CPT-cAMP or TPEN (Figs. 2–3; Table 1). The extent of axon regeneration induced by Ocm + CPT-cAMP or TPEN was comparable to previous reports from our lab (Li et al., 2017; Yin et al., 2009; Yin et al., 2006).

Combining KLF9 KD with TPEN resulted in substantially stronger regeneration than either one alone, enabling ~200 axons to regenerate more than half-way down the optic nerve in just two weeks (total length of optic nerve ~5 mm) (Figs. 2–3; Table 1). A few axons even entered the optic chiasm in these mice (Fig. 4). In contrast, the effect of combining Ocm + CPT-cAMP with either TPEN or KLF9 KD was only marginally stronger than the individual treatments (Figs. 2–3; Table 1). Some of the treatments improved RGC survival compared to mice receiving the control virus + PBS injections, although the treated groups did not differ significantly from one another even though the averages differed slightly (Fig. 5).

Next, we investigated the long-term effect of the lead combinatorial treatment of TPEN and KLF9 KD. The experiment was performed as above except that animals were sacrificed at 6 weeks after injury. Regenerating axons in these studies were labeled with intravitreal injection of the anterograde tracer cholera toxin subunit B (CTB), as our prior work has shown that levels of GAP-43 immunostaining begin to decline several weeks after the initiation of axon regeneration (Kurimoto et al., 2010). Mice were also tested at 4 weeks after injury and the day before sacrifice for possible recovery of simple visual functions using the optomotor response (OMR) (de Lima et al., 2012b). The number of regenerated axons at 6 weeks after injury was unchanged from what we observed at 2 weeks (Fig. 6A–B), although a few axons regenerated through the optic chiasm and into the ipsilateral optic tract, with some stalling at the midline (Fig. 6C–F). Mice receiving the combinatorial treatment showed ~7-fold higher levels of RGC survival than controls (Fig. 6G–H). These studies did not continue past 6 weeks after injury, and no evidence of visual recovery was found on the OMR tests by the latest time-point we tested, although the experiments were focused primarily on anatomic and not functional endpoints.

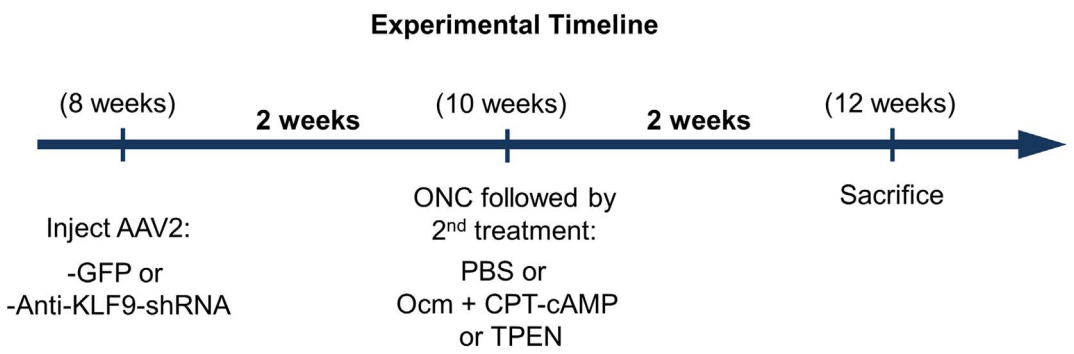

Fig. 1. Experimental timeline. Adult mice were pre-treated with anti-Klf9 shRNA gene therapy or GFP control virus 2 weeks prior to optic nerve crush (ONC) injury. The indicated treatments were injected intravitreally immediately after the injury and animals were sacrificed for histological analysis 2 weeks later. For the long-term experiment shown in Fig. 6, mice were sacrificed 6 weeks after injury.