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Review

Optic nerve regeneration in mammals: Regenerated or spared axons?

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ABSTRACT

Intraorbital optic nerve crush in rodents is widely used as a model to study axon regeneration in the adult mammalian central nervous system. Recent studies using appropriate genetic manipulations have revealed remarkable abilities of mature retinal ganglion cell (RGC) axons to regenerate after optic nerve injury, with some studies demonstrating that axons can then go on to re-innervate a number of central visual targets with partial functional restoration. However, one confounding factor inherent to optic nerve crush injury is the potential incompleteness of the initial lesion, leaving spared axons that later on could erroneously be interpreted as regenerating distal to the injury site. Careful examination of axonal projection pattern and morphology may facilitate separating spared from regenerating RGC axons. Here we discuss morphological criteria and strategies that may be used to differentiate spared versus regenerated axons in the injured mammalian optic nerve.

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1. Introduction

Neurons in the mammalian central nervous system (CNS) have only a limited ability to spontaneously regenerate axons after injury. Due to the relative simplicity of the surgical procedure, intraorbital optic nerve crush is widely used as a model to study axon regeneration (as of year 2017, there are 965 PubMed results using the keywords "optic nerve crush"). Indeed, studies that have used the optic nerve crush paradigm have helped identify several key factors that affect axon regeneration. Early studies in rodent models have demonstrated that lens injury transforms RGCs into an active regenerative state, thereby enabling moderate axon growth into the injured nerve (Fischer et al., 2001, 2000; Leon et al., 2000). Since then, many groups have developed various strategies to promote optic nerve regeneration, including increasing the intraretinal supply (either using recombinant protein or viral vectors) of various neurotrophic factors such as BDNF, GDNF and cytokines of the IL6 family, such as CNTF and hyper-IL6 (Hellstrom and Harvey, 2011; Leaver et al., 2006; Leibinger et al., 2016; Pernet et al., 2013), or manipulating various genetic factors within the projecting neurons.

Some of the genetic factors and downstream pathways that appear to regulate aspects of RGC axon regrowth capability include c-Myc, mTOR, Klfs, Pcaf, Pten, Socs3, and Stat3 (Belin et al., 2015; Benowitz et al., 2017; de Lima et al., 2012; Leibinger et al., 2013; Lim et al., 2016; Luo and Park, 2012; Luo et al., 2016; Mehta et al., 2016; Moore et al., 2009; Park et al., 2008; Pernet et al., 2013; Puttagunta et al., 2014;

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Smith et al., 2009; Vigneswara et al., 2014). Moreover, some of the factors identified in the optic nerve model have been shown to affect axon regeneration in CNS neurons other than RGCs. For example, modulating Pten and Klf7 promotes cortical axon regeneration in the injured spinal cord (Blackmore et al., 2012; Liu et al., 2010). Thus, discoveries arising from studies using the optic nerve crush paradigm not only have implications in developing strategies to repair the injured visual system, but can also help us understand the basis for axon regeneration failure in the adult CNS in general.

In both the peripheral and central nervous systems, ensuring a reproducible and complete crush injury, severing all axons, is an essential prerequisite for any research focusing on axon regeneration, because an incomplete lesion will otherwise spare axons that could be misinterpreted later as regenerating profiles (Bauder and Ferguson, 2012; Lee and Lee, 2013; Steward et al., 2003; Tuszynski and Steward, 2012). Such potential false identification is not unique to optic nerve repair work, and this issue has been a subject of intense discussion particularly within the spinal cord injury (SCI) research community. Accordingly, several review and research articles have proposed useful morphological criteria and strategies for distinguishing spared versus regenerating axons in the spinal cord (Steward et al., 2003; Tuszynski and Steward, 2012). Here we discuss and highlight morphological criteria and strategies that may help experimenters to differentiate spared versus regenerated RGC axons in the injured optic nerve.

2. Commonly used strategies are not sufficient

Currently, two strategies are typically used to validate that, after intraorbital crush injury, RGC axons are regenerating, not spared axons.

1. Absence of labeled axons in control animals: For testing potential treatment strategies, control animals (i.e. receiving mock or sham treatment) that show lack of axon regeneration are viewed as evidence that the lesions produced by a particular surgeon or laboratory are complete.
2. Time course experiments: Optic nerves are examined at different survival time points after injury, and the progression of axon regeneration is assessed in histological sections (de Lima et al., 2012; Lim et al., 2016; Park et al., 2008; Sun et al., 2011). During development in mouse and rat, de novo RGC axons growing from the retina can reach the brain within 2–3 days, although in the rat at least, axons from RGCs born later in development may take more than a week to reach their central targets (Dallimore et al., 2002). With further increases in brain size, it is reasonable to think that it would take longer for RGC axons to regenerate and re-innervate the brain in adult animals. Indeed, in adult animals subjected to different growth promoting treatments, it is common to see that it takes at least 3–5 weeks for regenerating RGC axons to reach the optic chiasm (de Lima et al., 2012; Leibinger et al., 2016; Luo et al., 2016; Park et al., 2008; Pernet et al., 2013). As such, if regenerating axons are detected only within the optic nerve and not in the chiasm shortly after injury (i.e. 1 to 14 days post crush), this is generally regarded as evidence that the lesions produced by a particular surgeon or laboratory are complete.

Time course experiments that visualize the axons at early time points after injury are an important inclusion to address an issue that may not be resolved by just having the control animals (i.e. receiving mock or sham treatment) in the study design. Considering a scenario where incompletely severed axons degenerate in control animals, while some intervention or treatment protects RGCs and their axons, these axons would be mistakenly interpreted as regenerating axons. For instance, interventions designed to enhance regrowth that are given before the crush (e.g. adeno associated virus-mediated transgene expression) may also enhance the durability of injured but not completely severed axons in a way that differs from control animals.

Although inclusion of appropriate control animals and time course experiments are valuable steps, they do not, ipso facto, prove that any labeled fibers seen distal to the injury site are regenerated axons. It is worth noting that stretched or damaged (but not severed) axons that survive an initial injury have disrupted microtubule organization that could potentially result in altered axon transport for several weeks (Dengler-Crish et al., 2014; Echevarria et al., 2017; Hanke and Sabel, 2002; Prilloff et al., 2012; Tang-Schomer et al., 2012; Wang et al., 2011). Indeed, there are reports suggesting that anterograde transport of neuronal tracers (e. g MiniRuby) in damaged (but not severed) RGC axons could be disrupted for up to at least 3 weeks after incomplete crush injury and then recover via intrinsic axon repair mechanisms (Hanke and Sabel, 2002). These studies have raised a possibility that neuronal tracers may fail to fully illuminate those damaged (but structurally connected) axons, at least transiently and during the early phase of injury, potentially confounding the use of serial labeling approaches to argue for regeneration. Others have reported that surviving but compressed axons may also be physiologically abnormal for a certain period of time, probably due to altered myelin structure and potassium channel activity, leading to conduction failure and loss of function (Nashmi and Fehlings, 2001; Shi and Sun, 2011).

3. Characteristic of spared axons in the injured optic nerve that should be monitored

The degenerating optic nerve consists of interstitial arrays of cells and debris, many of which will physically obstruct and alter the trajectory of axons. Moreover, in and near the lesion site, blood-born macrophages accumulate and astrocytes form a glial scar. These reactive cells can physically and chemically change the course of axons (Blaugrund et al., 1992; Qu and Jakobs, 2013; Selles-Navarro et al., 2001), making it unlikely for axons to travel linearly through the lesion site (Luo et al., 2013; Pernet et al., 2013; Pernet and Schwab, 2014). As such, bundles of linear and continuous axons that project seemingly unimpeded through the lesion site and then along the length of the distal optic nerve are, at the very least, strongly suggestive of axon sparing (Fig. 1). Many regeneration studies produce optic nerve lesions using micro-forceps. Even when carried out with care, micro-forceps may sometimes fail to injure the entire width of the nerve, thus leaving a small region of intact tissue usually located towards the periphery of the nerve. Characteristics of spared RGC axons after optic nerve crush include:

- Bundles of axons found at the edge of the optic nerve near the crush site that project linearly over long distances (Fig. 1A). However, depending on the orientation and angle at which the optic nerve is sectioned under a microtome (e.g. cryosection), linear axons in some tissue sections are narrow near the lesion site but can be more visible with variable widths in the distal regions of the nerve (Fig. 1B). In this regard, it is likely that even the sparing of 0.5–1.0% of axons (e.g. ~500 axons in adult mouse) could give the impression of extensive axon regeneration within the optic nerve (Fig. 1B). Furthermore, since many RGC axons form arbors within appropriate brain targets, sparing of even a few axons give the impression of extensive re-innervation in the brain. For example, 20–40% of the superior colliculus can contain anterogradely labeled RGC axons when all but the most medial and lateral margins of the rat optic tract are cut and the extent of the innervation increased by appropriate trophic stimulation (Harvey et al., 2012).
- Spared axons appear continuous with only limited fragmentation (Fig. 1) which is typical for the normal RGC projection in uninjured animals. In contrast, regenerating axons are often scattered and may show some degree of axon turning, and even branching, near the injury and in the distal optic nerve (Fig. 2) (Leaver et al., 2006; Luo et al., 2013; Pernet et al., 2013).

Regeneration studies particularly those involving SCI often include immunohistochemical examination of the lesion site. Typically, these studies use antibodies against phenotypic markers of reactive glia (e.g.