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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.

Keywords

Axon Regeneration; Axon injury; Retinal ganglion cells; Optic nerve injury; Axon growth

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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; Fischer et al, 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 & Harvey, 2011; Leaver et al, 2006; Leibinger et al, 2016; Pernet et al, 2013a), 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 & Park, 2012; Luo et al, 2016; Mehta et al, 2016; Moore et al, 2009; Park et al, 2008; Pernet et al, 2013b; Puttagunta et al, 2014; 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 & Ferguson, 2012; Lee & Lee, 2013; Steward et al, 2003; Tuszynski & 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 & 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.

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, 2013a). 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. Consider a scenario where incompletely severed axons degenerate in control animals, while some intervention or treatment protects RGCs and their axons. These axons will be mistakenly interpreted as regenerating axons when in fact they are spared, protected axons that would have otherwise degenerated in control animals. 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 experiment 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 & 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 & 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 & Fehlings, 2001; Shi & Sun, 2011).

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 glial scar. These reactive cells can physically and chemically change the course of axons (Blaugrund et al, 1992; Qu & 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, 2013a; Pernet & 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 can 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, 2013a).

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. GFAP) or extracellular matrix (e.g. laminin) to show the location of physical damage. On the other hand, optic nerve regeneration studies do not usually use GFAP staining to outline the injury site. This might be partly because the

investigators think that optic nerve crush produces a clean and consistent injury (i.e. severity and location wise), and presume that injury will leave no axons spared. Moreover, the astrocytic gap is partly closed between 10 and 21 days after optic nerve crush (Hilla et al, 2017), so that looking at optic nerve sections later than that time point (which is usually done) would not prove complete crush. Also, the lesion site in optic nerve may be viewed less “critical” or “interesting” than one would view in SCI studies (in which different parameters of the lesion including the lesion size and the extent of immune cell infiltration are important factors to consider in spinal cord regeneration studies). Even for optic nerve studies however, careful examination of the lesion site should be routine for each operated animal. For instance, after optic nerve crush, the presence of residual GFAP⁺ processes that appear to bridge the optic nerve lesion site (e.g. on the peripheral margin of the nerve; Fig. 3) and long linear axons that project along this injury boundary suggest that the crush injury is incomplete.

It should be noted that, although bundles of long linear RGC axons projecting to the brain are strongly suggestive of axon sparing, one cannot exclude the possibility that certain conditions and specific experimental manipulations render axons to regrow linearly over long distances. However, if this was to be the case, how could RGC axons potentially achieve long linear regrowth? During development, *de novo* RGC axons physically adhere to neuroepithelial cells or immature glial cells (Raper & Mason, 2010) and can grow linearly along the developing tract. During regeneration in the peripheral nervous system, axons often travel linearly as they regrow within tunnels generated by reactive Schwann cells, known as the bands of Büngner (Namgung, 2014). Thus, it is viewed that regrowing axons do not simply “swim” through spaces, but rather they attach and elongate along physical substrates such as the glial cells (Raper & Mason, 2010). In vivo studies using SCI injuries have shown that regenerating CNS axons physically associate with astrocyte processes, and thus the paths of regenerating axons through the lesion site are shaped by astrocyte morphology and orientation (Zukor et al, 2013). In this regard, the extent to which the regenerating RGC axons use astrocytes or other cells (e.g. oligodendrocytes, or infiltrating monocytes) as physical substrates, and the extent to which axon-glial interaction ultimately shapes the path of regenerating RGC axons (e.g. aberrant paths or more direct growth towards the brain) remains to be determined. Nonetheless, post crush injury, the presence of bundles of long linear RGC axons in distal optic nerve should be a warning sign, suggestive of axon sparing. In our view, to conclude otherwise and consider that long linear axons are in fact regenerating axons, requires more rigorous validation than currently is undertaken.

More Stringent Injuries?

As mentioned above, the validation experiments (e.g. time course experiment) provide circumstantial evidence, at best. Perhaps new gold standards are needed in the field of optic nerve regeneration. Many years ago, most scientists in the SCI field concluded that manual injury approaches were insufficiently consistent to be used as a routine and reliable procedure. Indeed, many SCI investigators today believe that the only way to demonstrate convincingly that axons are regenerating, but not spared is to use a complete transection of the cord (Steward et al, 2003; Tuszynski & Steward, 2012). Perhaps investigators could perform similar complete transection to validate optic nerve regeneration, at least for the

most promising strategies. In this regard, it is worth noting that studies that use complete optic nerve transection and peripheral nerve autograft showed long-distance regeneration of severed adult rat RGC axons resulting in extensive reinnervation of central visual targets (Thanos et al, 1997; You et al, 2016). Moreover, lens injury has been shown to promote RGC axon regeneration even after complete optic nerve transection in adult rats (Fischer et al, 2001). Performing this type of surgery (cut and re-adaptation of nerve) is however, already very challenging in the rat optic nerve (Fischer et al, 2001) and it may be more difficult in the even thinner mouse optic nerve (which is often the preferred model due to the availability of genetically modified strains). There are only a handful of reported studies in mouse that have successfully analyzed RGC axon regeneration following optic nerve transection (i.e. peripheral nerve graft model) (Cui & Harvey, 2000; Inoue et al, 2002; Inoue et al, 2000). Having said that, it remains to be seen whether any of the genetic strategies previously shown to promote long distance RGC axon regeneration after optic nerve crush can also promote similar regeneration following complete optic nerve transection.

Comprehensive Analysis and Improved Imaging

Visualization of growing axons in living animals unequivocally proves that certain axons are damaged (and regenerated). Indeed, several SCI and PNS studies have utilized in vivo optical imaging for examining axon regeneration (Bareyre et al, 2011; Lorenzana et al, 2015). Although there is a precedent for in vivo imaging of optic nerve in adult rats (Knoferle et al, 2010), this technique is considered to be very challenging (and undoubtedly even more so in mouse). The anatomical limits imposed by the surrounding tissues and skull make it difficult to expose the optic nerve for real time imaging. As such, validation of optic nerve regeneration currently relies on examining tissues that are dissected out from animals at given time points.

Any strategy for studying axon regeneration that depends on sectioning pieces of tissues, such as the optic nerve, often sacrifices the ability to follow individual axons for more than a few 10's of microns. It is not uncommon for only subsets of sections to be studied. This makes it impossible to follow individual axons as they pass through various way points. If axon bundles (as illustrated in Fig. 1) are identified close to peripheral margin of the optic nerve, this should be viewed as a likely indicator for spared axons and an incomplete crush. However, under conditions of strong initial regeneration (for instance by lens injury or PTEN knockout), such axon bundles may be hidden by regenerating axons over moderate distances (i.e. near the lesion site) while the longer, spared axons would be visible at more distal locations. For this reason, careful examination of sections covering the nasal/medial and temporal/lateral margins (see Fig. 1) of an injured nerve and regions closely beyond the lesion site as well as chiasm (or brain) is essential. Especially in cases where labeled RGC axons are found in central brain targets, many optic nerve sections close to the lesion site should be carefully surveyed. There have been important advances in the 3D visualization of tissues in the last 5 years, including tissue clearing combined with fluorescent light sheet microscopy and confocal microscopy (Erturk & Bradke, 2013; Erturk et al, 2012; Luo et al, 2013; Luo et al, 2014; Pernet et al, 2013a; Soderblom et al, 2015). These techniques allow imaging and visualization of the entire nerve tissues without histological sectioning, and thus permit comprehensive examination of the axon trajectory and growth (i.e. visualization

of the origin of the regenerating axon and where it ends, including the degree to which an axon turns or projects linearly). Incorporating the use of these kinds of methods to study regeneration of RGC axons could provide more compelling evidence that a given treatment or combination of treatments does indeed induce significant (e.g. full length) axon regeneration in the mature visual system.

Concluding Remarks

In any study focused on analyzing the regeneration of axons, it is important that a result is reproducible and that most animals of one specific experimental group show similar results. Documentation of outliers is preferable. Control animals that were subjected to the same experimental procedure in the same surgical session (so that all conditions and surgical equipment are as identical as possible) should consistently show no lengthy regeneration. In addition of course, experimenters should be well-trained by experienced personnel, carry out sufficient numbers of controls to check for technique, and should be blinded to the identity (and phenotype) of the experimental groups to avoid any additional bias when carrying out crush surgeries.

Optic nerve crush has been widely used to study axon regeneration, and will continue to be a useful source of discovery. Given the impact of findings arising from optic nerve for blind and SCI patients worldwide, there needs to be rigorous validation that distinguishes spared RGC axons from regenerating axons. As mentioned above, there is currently no method that directly proves an axon is a regenerated profile. Emergence of new staining and imaging methods is sought after in this regard (e.g. identification of specific markers of growth cone/newly generated axon in vivo, or establishment of labeling method that monitors the addition of lipids or proteins in growing axons). At present, vigilant examination of axon morphology and pattern remains perhaps as the best method. Continuous and linear axons extending over long distances are most likely signs of axon sparing. Careful monitoring of these distinguishing features must be a key component of all optic nerve regeneration studies.

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Figure 1. Example of optic nerve section with incomplete crush showing linear and continuous axons

A, A mouse optic nerve was incompletely crushed and isolated 3 weeks after surgery. To stimulate axon regeneration lens injury was performed simultaneously to nerve injury. Microphotograph shows a section of this nerve with spared axons on the edge that project through and beyond the optic chiasm. To label axons cholera toxin β subunit (CTB) was unilaterally injected into the injured eye 2 days prior to euthanasia. (A') and (A'') higher magnification of the boxed area in A. Spared axons project as a narrow bundle near the lesion (arrows in A' and A''). **B**, bundle of spared axons with variable width along the optic nerve. Depending on the orientation and angle at which the optic nerve is sectioned (i.e. cryosectioned), spared axons in some tissue sections appear narrow near the lesion area, but are often wider and more obvious in the distal optic nerve regions (red rectangle). Asterisk indicates the lesion site. Scale bars, 200 μ m.

Figure 2. Examining signs of regenerating axons in whole tissue or in tissue sections

A, Assessment of regenerated RGC axons in a cleared, whole optic nerve. PTEN/SOCS3 knockout mice at 17 days post-injury (Luo et al, 2013). In this animal, the brain contained no CTB-labelled RGC axons. **B** and **C**, 3D neurite tracing of single fiber showing different growth patterns. Some axons loop back towards the eye as shown in **B**, and others generate branches as they extend within the optic nerve as shown in **C** (Luo et al, 2013). **D** and **D'**, Regenerating axons are often scattered with a degree of fragmentation and turning. (**D'**) higher magnification of the boxed area in **D**. (pointed with yellow arrows). Asterisk, lesion site. Scale bars, 150 μ m in **A** and **D**; 50 μ m in **C**.

Figure 3. Linear axons along GFAP⁺ astrocytic processes in the optic nerve lesion site strongly suggest axon sparing

AAV-shPTEN was injected intravitreally to induce regeneration and followed by an incomplete crush a week later. Antibody against GFAP is used to examine the location of lesion area. Yellow boxed area in the upper image shows a region containing an astrocytic bridge (i.e. likely to be a region with incomplete crush). In the sections of this particular optic nerve, long linear axons projecting along the periphery margin were seen similar to those seen in Figure 1. Scale bar, 50 μ m.