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Review

Assessment of retinal ganglion cell damage in glaucomatous optic neuropathy: Axon transport, injury and soma loss

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ABSTRACT

Glaucoma is a disease characterized by progressive axonal pathology and death of retinal ganglion cells (RGCs), which causes structural changes in the optic nerve head and irreversible vision loss. Several experimental models of glaucomatous optic neuropathy (GON) have been developed, primarily in non-human primates and, more recently and commonly, in rodents. These models provide important research tools to study the mechanisms underlying glaucomatous damage. Moreover, experimental GON provides the ability to quantify and monitor risk factors leading to RGC loss such as the level of intraocular pressure, axonal health and the RGC population. Using these experimental models we are able to gain a better understanding of GON, which allows for the development of potential neuroprotective strategies. Here we review the advantages and disadvantages of the relevant and most often utilized methods for evaluating axonal degeneration and RGC loss in GON. Axonal pathology in GON includes functional disruption of axonal transport (AT) and structural degeneration. Horseradish peroxidase (HRP), rhodamine-B-isothiocyanate (RITC) and cholera toxin-B (CTB) fluorescent conjugates have proven to be effective reporters of AT. Also, immunohistochemistry (IHC) for endogenous AT-associated proteins is often used as an indicator of AT function. Similarly, structural degeneration of axons in GON can be investigated via changes in the activity and expression of key axonal enzymes and structural proteins. Assessment of axonal degeneration can be measured by direct quantification of axons, qualitative grading, or a combination of both methods. RGC loss is the most frequently quantified variable in studies of experimental GON. Retrograde tracers can be used to quantify RGC populations in rodents via application to the superior colliculus (SC). In addition, *in situ* IHC for RGC-specific proteins is a common method of RGC quantification used in many studies. Recently, transgenic mouse models that express fluorescent proteins under the Thy-1 promoter have been examined for their potential to provide specific and selective labeling of RGCs for the study of GON. While these methods represent important advances in assessing the structural and functional integrity of RGCs, each has its advantages and disadvantages; together they provide an extensive toolbox for the study of GON.

Abbreviations: APP, amyloid precursor protein; AT, axonal transport; BDNF, brain derived neurotropic factor; Brn3a/3b, Brain-specific homeobox/POU domain protein 3a/3b; CDK5, Cyclin-dependent kinase 5; CFP, cyan fluorescent protein; CFP+, CFP positive; ChAT, choline acetyltransferase; ChAT+, CHAT positive; CSLO, confocal scanning laser ophthalmoscopy; CTB, cholera toxin B; DAB, diaminobenzidine; FG, fluorogold; FG+, fluorogold positive; GCL, ganglion cell layer; GON, glaucomatous optic neuropathy; HRP, horseradish peroxidase; IHC, immunohistochemical/immunohistochemistry; IOP, intraocular pressure; LGN, lateral geniculate nucleus; MAP, microtubule associated protein; NeuN, neuronal nuclei; NF, neurofilament; ON, optic nerve; ONH, optic nerve head; RBPMS, RNA binding protein with multiple splicing; RGC, retinal ganglion cell; RITC, rhodamine-B isothiocyanate; RITC+, RITC positive; SC, superior colliculus; Sncg, γ-synuclein.

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1. Introduction

The retina converts light stimuli from the visual environment into electrical signals that converge on retinal ganglion cells (RGCs). RGC subtypes are morphologically distinct and have specialized functions that further process the information sent to the brain (reviewed by Sanes and Masland, 2015). This signal is conducted via long RGC axons that extend through diverse extracellular terrain. From the RGC soma, axons form the retinal nerve fiber layer, pass through the optic nerve head (ONH), and continue centrally as the optic nerve (ON) and optic tracts. These projections terminate primarily in either the lateral geniculate nucleus (LGN) in higher primates (Jeffries et al., 2014) or the superior colliculus (SC) in rodents (Wang et al., 2010a). RGC axons are conduits for transporting important cellular components from cell body to terminal and vice versa (Morgan, 2004). Consequently, injury to the axon often results in RGC death and loss of visual function (Dieterich et al., 2002; Gellrich et al., 2002; Watanabe et al., 2001). Glaucomatous optic neuropathy (GON) is a leading cause of vision loss due to RGC death (Quigley, 1999). Assessing axonal transport and degeneration along with assessing morphologic and functional changes to RGCs, and ultimately their loss, in experimental models is essential for improving our understanding of GON pathophysiology. The aim of this review is to highlight the most commonly used techniques to assess and quantify RGC damage *ex vivo* in experimental models of GON (for an in depth discussion on *in vivo* techniques, refer to Fortune, 2015; in this special issue). While these techniques are extensively described in the literature for various applications and combinations, here we present an overview and objective discussion of each method to allow researchers to determine the most appropriate tools for their studies. As such, we cannot give a blanket endorsement of a specific technique because it is dependent on many experimental details, and investigators will have to consider the relative merits of each approach in the context of their particular studies.

2. Axonal injury and transport

This section examines current methods for evaluating RGC axonal health including: (a) assessment of axonal transport (AT) function with transport tracers and immunohistochemical (IHC) labeling of endogenous AT-associated proteins; (b) techniques to assess axon structural integrity; and (c) ON grading systems to quantify axon survival.

2.1. Evaluation of AT

In AT, kinesin motors carry molecular cargo such as synaptic vesicles and other active zone proteins anterogradely along microtubules towards the cell terminal (Goldstein et al., 2008), while dynein motors transport signaling endosomes carrying neurotrophic molecules retrogradely towards the cell body (Cosker et al., 2008). Disruption of AT may represent one of the first signs that RGCs are under stress in GON (Chidlow et al., 2011). Changes to AT are evaluated with actively transported tracer molecules or IHC for endogenous AT-associated proteins.

2.1.1. Application of actively transported molecules

Tracers intended to evaluate AT function must travel along the axon via active transport, rather than passive diffusion, and, given that AT is an ATP-driven process, must be applied to live tissue. For anterograde transport, subretinal or intravitreal injection can be used. For retrograde transport, methods include injection or application of a tracer-soaked gelpad to RGC target structures such as the SC (Thanos et al., 1987; Wang et al., 2008) or LGN (Minckler et al., 1977; Naito, 1989). Tracers enter the cell either by receptor-mediated uptake, or are internalized during vesicle endocytosis (Lanciego and Wouterlood, 2011). While there is an wide range of tracers available (reviewed by Köbbert et al., 2000; Lanciego and Wouterlood, 2011 and Vercelli et al., 2000), only a few, namely horseradish peroxidase (HRP), rhodamine-B-isothiocyanate (RITC) and cholera toxin B (CTB) fluorescent conjugates, have been consistently used in the last decade.

2.1.1.1. Horseradish peroxidase (HRP). HRP becomes incorporated into neurons via pinocytosis and remains within vesicles that are transported both anterogradely and retrogradely (Lanciego and Wouterlood, 2011). Following fixation and sectioning, HRP within tissue is reacted with diaminobenzidine (DAB) or other electron donors to form an electron dense black/brown precipitate visualized by light- or electron microscopy (Köbbert et al., 2000; Lanciego and Wouterlood, 2011). Fig. 1A and B show HRP staining of RGC axons and somas. The rate of HRP transport in RGC axons depends on the direction of transport and species examined (Table 1).

HRP has been used as a reporter of AT in the visual system for many years. Minckler et al. (1977) observed accumulation of retrogradely transported HRP posterior to Bruch's membrane and in the lamina cribrosa of monkeys following acute intraocular pressure (IOP) elevation to 25–150 mmHg for 12–28 h. Shorter durations (2–4 h) of IOP elevation in rats also decrease retrograde HRP transport from the SC to retina (Johansson, 1986, 1988). More recently, Wang et al. (2008) demonstrated reversible HRP transport blockade with acute application of endothelin-1, a regulator of neurovascular tone implicated in GON (Chauhan, 2008; Yorio et al., 2002), to the retrobulbar nerve. HRP filled the entire ON within 2 h of application to the SC, however, after 48 h no HRP was visible, suggesting that it was fully transported and metabolized (Wang et al., 2008). HRP has also been used to evaluate anterograde AT in experimental GON by episcleral vein cauterization (Diaz et al., 2005). While HRP is a reliable marker of both anterograde and retrograde AT, the enzymatic reaction necessary to visualize it precludes further IHC investigation.

2.1.1.2. Rhodamine-B-isothiocyanate (RITC). RITC, a red-fluorescing probe (emission 573 nm), was first used as a tracer by Thanos and Bonhoeffer (1983) for labeling developing neurons in the chick retinotectal pathway, and subsequently in other species (Table 1).

Like HRP, RITC is endocytosed and transported in vesicles throughout the axon, soma and dendrites (Fig. 1C, E and G), appearing granular at high magnification (Fig. 1F). While it is transported bi-directionally, RITC provides an apparently superior signal within the axon for anterograde transport (Thanos et al., 1987, Fig. 1C and E). RITC remains in RGCs for at least 30 days without extracellular leakage (Thanos et al., 1987) and is resilient to further IHC processing (Köbbert et al., 2000; Thanos and Bonhoeffer, 1983). Balaratnasingam et al. (2007, 2008) used RITC in conjunction with IHC for structural proteins to examine disruption of anterograde AT and changes in the axonal cytoskeleton simultaneously after acute IOP elevation in pig. RITC transport was disrupted in the ONH coupled with decreased staining for neurofilament, a major structural protein in the axon, between 3 and 6 h of IOP elevation (Balaratnasingam et al., 2007, 2008).

2.1.1.3. Cholera toxin B (CTB) fluorescent conjugates. CTB is the beta subunit of the toxin secreted by *Vibrio cholerae* bacterium, and facilitates the internalization of the toxin upon binding to GM1 ganglioside receptors on lipid rafts in the cell membrane (Wernick et al., 2010). Conjugating CTB to a fluorophore thus allows visualization of endocytotic vesicles for tracking of AT. The CTB molecule