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The Role of Axon Transport in Neuroprotection and Regeneration

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Abstract

Retinal ganglion cells and other central nervous system neurons fail to regenerate after injury. Understanding the obstacles to survival and regeneration, and overcoming them, is key to preserving and restoring function. While comparisons in the cellular changes seen in these non-regenerative cells with those that do have intrinsic regenerative ability has yielded many candidate genes for regenerative therapies, complete visual recovery has not yet been achieved. Insights gained from neurodegenerative diseases, like glaucoma, underscore the importance of axonal transport of organelles, mRNA, and effector proteins in injury and disease. Targeting molecular motor networks, and their cargoes, may be necessary for realizing complete axonal regeneration and vision restoration.

Keywords

CNS regeneration; axon transport; optic nerve; neuroprotection; glaucoma

INTRODUCTION

Like most other mature neurons in the central nervous system (CNS), retinal ganglion cells (RGCs) cannot regenerate their axons in disease and after injury. In the visual system, RGCs carry visual information along their axons down the optic nerve to the superior colliculus (SC) and the lateral geniculate nucleus (LGN) of the thalamus, among other important brain targets. Therapies to protect or restore vision after axon insult must address RGC survival and axon regeneration, and re-integration of the RGC axons into the appropriate visual circuitry. To combat regenerative failure, many strategies have been devised both alone and in combination to allow partial regeneration of injured RGCs to their visual targets. Over the last 30 years, to understand why adult, mammalian RGCs and other CNS neurons do not regenerate after injury, and convert them into neurons that do regenerate, many groups have asked what are the molecular differences between adult, mammalian CNS neurons and 1) “immature” mammalian CNS neurons, which have a higher intrinsic growth capacity; 2) adult peripheral nervous system (PNS) neurons, which do regenerate their axons after injury;

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and 3) CNS neurons from other species that do show regenerative capacity? Here, we review advances in these parallel strategies, and then discuss a hypothesis that links prior work into a unified model for the role of axon transport in regenerative failure, as well as a new way to target approaches to promote regeneration.

IMMATURE CNS NEURONS HAVE A HIGH REGENERATIVE POTENTIAL

Developing mammalian CNS neurons have a high growth potential that is lost by adulthood. In fact, isolated RGCs from embryonic mice have a far greater *in vitro* growth potential than RGCs isolated from early postnatal mice (Goldberg *et al.*, 2002a). Manipulating growth regulation pathways in mature neurons may promote regeneration, by increasing intrinsic growth factor-driven signaling pathways such as mTOR, cAMP, suppressors of cytokine signaling (e.g., SOCS3), and mitogen-activated protein kinases (Cai *et al.*, 1999; Zhou *et al.*, 2005; Leaver *et al.*, 2006; Smith *et al.*, 2009; Kurimoto *et al.*, 2010; Park *et al.*, 2010), manipulating responsiveness to extrinsic inhibitory factors such as by blocking Nogo receptor expression or activation (Chen *et al.*, 2000; GrandPré *et al.*, 2000; Fischer *et al.*, 2004), and decreasing transcriptional inhibitors of axon growth such as Krüppel-like family transcription factors (KLFs) (Moore *et al.*, 2011, 2009a). Identifying genes or pathways whose pattern of expression in immature, highly regenerative CNS neurons are drastically altered after maturation may lead to candidate regulators of intrinsic growth potential. In the case of KLFs, where developmental upregulation of KLF9 and KLF4 and downregulation of KLF6 and KLF7 are coincident with the reduction of intrinsic regenerative capacity of RGCs, reversing these expression patterns in adult RGCs or in other CNS neuron pathways after injury allows sprouting or long-distance regeneration (Moore *et al.*, 2009a; Blackmore *et al.*, 2012; Apara *et al.*, 2017; Wang *et al.*, 2017). Exploring the molecular mechanisms of the KLFs further, identification of co-factors such as JNK3 and STAT3, and downstream targets such as serotonin receptors and dual-specificity phosphatase 14 (DUSP14) (Qin *et al.*, 2013; Apara *et al.*, 2017; Trakhtenberg *et al.*, 2017; Galvao *et al.*, 2018) have led to broader understanding of the biology of intrinsic capacity for axon growth. What other molecular targets do KLF family transcription factors and other intrinsic regulators of axon growth affect? Advances in sequencing and mass spectrometry technologies can uncover a more complete understanding of the cellular and molecular changes underlying the developmental loss of regenerative capacity.

Beyond transcriptional regulation, immature and mature CNS neurons have differential axonal transport. In embryonic cortical neurons *in vitro*, axonal transport included integrins important for axonal growth and elongation; in mature cortical neurons, this transport is lost. While most molecular transport is dependent on motor trafficking, axonal integrin transport has some specificity to a specific kinesin KIF4A (Heintz *et al.*, 2014). It is particularly noteworthy that axonal transport of integrins in mature neurons switches from an anterograde to mainly retrograde transport (Franssen *et al.*, 2015).

These findings present a clear opportunity for future research into the axonal transport changes after optic nerve injury. Does integrin transport change more or less than other proteins after injury? Are all kinesin-transported proteins affected the same by injury, or are there kinesin isoform responses specific to different injuries or insults? While not a direct

link, these examples underline how developmental changes in gene expression and axonal transport parallel developmental changes in intrinsic axon growth ability – and a ripe avenue for future regeneration research.

ELECTRICAL STIMULATION AFTER RGC INJURY

An additional element to consider beyond molecular signaling pathways is the functionality of RGCs: electrical activity is critical for transmission of action potentials and visual information, but it turns out it is also beneficial for RGC and other CNS neurons' responsiveness to survival and growth signals. It has previously been shown that electrical activity regulates mitochondrial localization and motility from the cell body in myelinated axons (Ohno *et al.*, 2011). In addition, electrical modulation through eye-opening, brain-derived neurotrophic factor (BDNF), or tetro-dotoxin administration *in vivo* during development greatly impact mitochondrial function and trafficking in RGC axons (our unpublished data), demonstrating a direct connection between activity and transport. RGCs extend longer neurites with concurrent electrical stimulation and growth factor administration *in vitro* (Goldberg *et al.*, 2002b) and *in vivo* (Lim *et al.*, 2016). Increasing conduction in regenerating axons with a voltage-gated potassium channel blocker enhances visual recovery measured at the level of behavior (Bei *et al.*, 2016). This electrical stimulation pathway acts at least in part through activation of adenylate cyclases, and specifically the calcium-sensitive, soluble adenylate cyclase (sAC) *in vitro* and *in vivo* (Corredor *et al.*, 2012; Martinez *et al.*, 2014). Complicating the story, however, is that excessive calcium influx into the axon after injury is a primary step in acute axon degeneration (Knoferle *et al.*, 2010). Pre-loading RGCs with calcium channel blockers before optic nerve crush results in improved survival and regeneration of RGCs (Ribas *et al.*, 2017). How is calcium or downstream cAMP signaling compartmentalized to regulate complex cellular responses? Is there anterograde or retrograde effector transport responsible for long-distance communication? Further work dissecting timing, compartmentalization, and localization of these pathways will be needed to reconcile these data.

PNS NEURONAL REGENERATION INFORMS CNS REGENERATION

Early experiments showing that PNS neuronal grafts can induce CNS axon elongation started a field of comparative work of PNS and CNS neurons, and the glial environments they must regenerate through (Richardson *et al.*, 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982). Exploring differences in the molecular characteristics of the regenerative response after injury between PNS and CNS has uncovered several networks that all could contribute toward inducing RGC regeneration (Smith *et al.*, 2011; Chandran *et al.*, 2016).

For example, cytokines, such as gp130 family members like interleukin-6, are differentially activated after PNS injury as compared to CNS injury (Cafferty *et al.*, 2001). Further studies expanded on the subsequent activation of the JAK/STAT pathway, showing a correlation with enhanced PNS regeneration (Miao *et al.*, 2006). Removing SOCS3, an inhibitor of the JAK/STAT pathway present in high levels following injury in CNS neurons, promotes RGC regeneration (Smith *et al.*, 2009). Surprisingly, STAT3-dependent gene expression is directly

inhibited by KLF family member KLF4 after cytokine activation, potentially explaining one mechanism by which KLF4 deletion promotes RGC regeneration (Qin *et al.*, 2013).

Through differential proteomics and bioinformatic network analysis, c-Myc was identified as a hub protein that was downregulated in RGCs but not in dorsal root ganglion PNS neurons after injury (Belin *et al.*, 2015). Furthermore, overexpression of this protein increased survival and regeneration. As another example, the transcription factor SOX11 was first identified as a modulator of regeneration in the PNS (Jankowski *et al.*, 2009). This was extended to the CNS, showing that SOX11 underlies DLK/LZK-mediated cell death, and that overexpression of Sox11 can induce regeneration of some subtypes of RGCs, although also leading to cell death of other RGC subtypes (Norsworthy *et al.*, 2017; Welsbie *et al.*, 2017). This unveiling of factors that differentially promote survival or regeneration depending on the subtype of neuron was also seen with osteopontin and IGF1, which improved survival and regeneration of the alpha-RGCs that preferentially express the relevant receptors (Duan *et al.*, 2015). As more details emerge about not only CNS–PNS differences but also about the heterogeneity of CNS neuron subtypes and their responses to injury, more work will be needed to fine-tune individualized therapies for regeneration.

INTRINSICALLY REGENERATIVE SPECIES

Regenerative failure of the CNS is not a universally conserved phenomenon: in fact, diverse phyla and classes like nematodes (Yanik *et al.*, 2004), fruit-flies (Soares *et al.*, 2014), zebrafish (Cameron, 2000; Sherpa *et al.*, 2008), and reptiles (Lang *et al.*, 1998), demonstrate at least partial neural regeneration after injury. In *C. elegans*, DLK-1 was first shown to promote and regulate adult axon regeneration, regulating the cells to respond to injury, partially through mRNA stabilization, discussed further below (Hammarlund *et al.*, 2009; Yan *et al.*, 2009). This finding was also seen in peripheral nerve regeneration in mice, with DLK required for retrograde transport of phosphorylated STAT3 to the cell body from the damaged axon (Shin *et al.*, 2012). In one study of Drosophila wing regeneration after injury, transgenic screening highlighted JNK pathway inhibition as pro-growth, a finding conserved in mammalian RGCs after injury (Welsbie *et al.*, 2013; Soares *et al.*, 2014; Apara *et al.*, 2017). In fact, it seems the DLK/JNK pathway underlies cellular responsiveness to injury in intrinsically regenerative species, regenerative PNS neurons, and non-regenerative neurons even in humans, either pro-growth or pro-apoptotic depending on the neuronal context (Le Pichon *et al.*, 2017).

In zebrafish, KLF6a and KLF7a together are necessary for RGC regeneration after optic nerve crush, and similarly promote axon growth and regeneration in rodent RGCs and corticospinal neurons (Veldman *et al.*, 2007; Moore *et al.*, 2009b; Blackmore *et al.*, 2012). However, a downstream target in zebrafish was identified as *tuba1a*, a key protein for regeneration in fish that has not been found to be relevant for regeneration in mammals (Veldman *et al.*, 2010). Thus exploring conserved and divergent molecular pathways and functions has helped to understand differences in regenerative capacity between mammals and other species and has led to candidate approaches for promoting regeneration.

COMBINING THERAPIES TO ENHANCE REGENERATION

As many of these regenerative therapies target different pathways, the combination of cell intrinsic and cell extrinsic approaches has led to novel insights and improvements in survival and regeneration of ganglion cells. For example, while the deletion of *PTEN* or *SOCS3* independently lead to extensive RGC regeneration, the co-deletion of *PTEN* and *SOCS3* had a synergistic effect for robust, sustained axon regeneration (Sun *et al.*, 2011). Recently, the combination of KLF9 knockdown with zinc chelation by TPEN was shown to lead to more enhanced regeneration and cell survival than either therapy alone (Trakhtenberg *et al.*, 2018). Visual or electrical stimulation also elicits more profound effects in combination with neurotrophic factors or with manipulation of pro-growth signaling pathways in neurons such as RGCs (Goldberg *et al.*, 2002b; Lim *et al.*, 2016). However, despite the best combinations of transcription factors and growth pathways, relatively few RGC axon reach their target regions. It is likely that a cocktail approach manipulating several factors together may enhance regeneration and indeed be necessary for full visual recovery.

Indeed, we must now ask how regenerated RGC synapses compare to those established during development. Do they have adequate transport of pre-synaptic machinery to maintain synaptic connections? Is exogenous expression of guidance molecules necessary for axon targeting? The answers to these questions and more form the next frontier of visual regeneration research. Despite these advances, limited visual recovery has been seen, underscoring the need for a deeper understanding of cellular changes in injury and disease. Many of the regenerative factors discussed above were hypothesized as candidate therapies due to differential expression in regenerative and non-regenerative neurons. Similarly, differential expression of factors in degenerative and non-degenerative neurons can highlight candidates for survival and maintenance of axons, which when combined with regenerative therapy, will lead to enhanced therapeutic response. Indeed, the link between degenerative molecular pathways and those failing to promote regenerative response may be one fertile area to focus on. With that in mind, insights from degenerative changes seen in conditions like glaucoma may suggest new avenues for vision restoration research.

AXON TRANSPORT IN GLAUCOMA AND OTHER NEURODEGENERATIVE DISEASES

Glaucoma is the leading cause of irreversible blindness worldwide and is predicted to affect 80 million people by 2020 (Quigley and Broman, 2006). The biggest risk factor is age; increased intraocular pressure (IOP) is currently the only modifiable risk factor. Vision loss occurs due to dysfunction and death of RGCs and their axons. Furthermore, widespread damage can be seen throughout the visual system, with degenerative changes in the LGN and the visual cortex (Yücel *et al.*, 2000). The molecular pathophysiology of glaucoma is still poorly understood, but increasing evidence implicates interference with axonal transport mechanisms.

Decreased axoplasmic flow between the RGC cell bodies and their axon terminals in the SC or LGN in the face of increased IOP remains one of longest standing hypotheses for pathophysiologic mechanism in this disease. Since the 1970s, studies into axoplasmic

transport in glaucomatous degeneration have shown an association between increased IOP and decreased anterograde and retrograde protein transport (Anderson and Hendrickson, 1974, 1977; Minckler *et al.*, 1977; Quigley and Anderson, 1977; Quigley *et al.*, 1979; Crish *et al.*, 2010). Indeed there is a strong link between many neurodegenerative diseases and dysfunctional axon transport (Appel, 1981). Causative mutations in genes that directly or indirectly lead to axon transport deficits may underlie at least a portion of the neuronal death seen in Huntington's disease (Trushina *et al.*, 2004), amyotrophic lateral sclerosis (ALS) (Pasinelli and Brown, 2006; Nicolas *et al.*, 2018), Parkinson's disease (Saha *et al.*, 2004), and Alzheimer's disease (Zhang *et al.*, 2004; Wu *et al.*, 2009). Specifically, decreases in axonal transport precede and possibly contribute to axonal and microtubule, and then somatic, degeneration (Stokin *et al.*, 2005; Morfini *et al.*, 2009).

What mechanistic insights can be derived from such consistent, distal-to-proximal cellular neurodegeneration? Identifying the molecular cargoes of bidirectional cellular transport mechanisms and ensuring adequate transport of these molecules to their targets may be a key component to achieving long-distance regeneration and re-innervation of RGCs to the brain.

DENDRITIC AND SYNAPTIC DEGENERATION AND TRANSPORT IN GLAUCOMA

RGCs require functional connections with pre-synaptic neurons in the retina and post-synaptic neurons in the brain to maintain transmission of visual information, and re-establishing and maintaining synaptic communication is vital to survival of RGCs (Della Santina *et al.*, 2013). In different glaucoma models, the DBA/2J mouse and a microbead injection-induced IOP model of glaucoma, axon transport fails early, with synaptic transmission and axon and dendritic dysfunction preceding the eventual RGC death, implicating axon transport in disease pathology (Buckingham *et al.*, 2008; Sappington *et al.*, 2010; Ou *et al.*, 2016; Ward *et al.*, 2014). Additional work has also highlighted early dendritic field reorganization in different RGC subtypes, before measurable axonal degeneration, and well before cell death (Della Santina *et al.*, 2013; El-Danaf and Huberman, 2015). Thus failure of long-distance transport down axonal or dendritic neurites may underlie early phases of degeneration.

MOLECULAR MOTORS UNDERLYING TRANSPORT ARE LINKED TO NEURODEGENERATIVE DISEASE

In neurons, microtubule motor proteins, dyneins, and kinesins, drive organelle and molecular axonal transport (Vale *et al.*, 1985; Hirokawa, 1998; Teng *et al.*, 2005; Hirokawa *et al.*, 2009). Given the importance of axon transport in the homeostatic maintenance of neuronal survival, disruptions to these motor proteins underly a variety of neurological diseases. For example, Charcot–Marie–Tooth disease type 2A can be caused by mutations in KIF1B1, and congenital fibrosis of the extraocular muscles (which is a neuropathy, not a myopathy) can be caused by mutations in KIF21A (Zhao *et al.*, 2001; Yamada *et al.*, 2003). Kinesins can form axonal aggregates in some neurodegenerative conditions like Alzheimer's disease, especially in cases with certain amyloid precursor protein mutants, with blockages occurring

before the characteristic amyloid plaque accumulations (Stokin *et al.*, 2005). A loss-of-function mutation in KIF5A results in hereditary spastic paraplegia, characterized by a progressive loss of function and degeneration of upper motor neurons, starting with synaptic degradation (Reid, 2003; Xia *et al.*, 2003; Morfini *et al.*, 2009). Different loss-of-function mutations in KIF5A, all affecting the cargo binding domain, are causative in some cases of ALS (Nicolas *et al.*, 2018). Charcot–Marie–Tooth syndromes can include optic atrophy among the peripheral neuropathies that define the disease, supporting the premise of shared axon transport-related pathophysiologies among axonopathies. The vital role of molecular transport in injury and disease, and their corresponding variety of mRNA, protein, and mitochondrial cargoes, highlight the necessity of addressing axonal transport when attempting to regenerate and re-innervate CNS neurons.

Does neurodegeneration follow a general decline in transport, or is the transport of specific, key cargoes causative in these different diseases. In the last few decades, increasing evidence has shown that kinesin subtypes and adaptor proteins have at least partial cargo specificity (Chevalier-Larsen and Holzbaur, 2006). For example, KIF1A and KIF1B of the Kinesin 3 family transport synaptic vesicle precursors synaptophysin and synaptotagmin, but do not transport syntaxin 1A or SNAP25 (Okada *et al.*, 1995), whereas KIF5 motors do transport syntaxin 1A and SNAP25, and also transport synaptotagmin (Toda *et al.*, 2008). The physiologic relevance of this partial specificity and partial redundancy is not fully elucidated but could reflect compensatory mechanisms for vital cargo to maintain cellular function and survival.

Regulation of these motors' active state and specific cargoes also depends on cell signaling cascades and post-translational modifications such as phosphorylation and changes in adaptor proteins. For KIF5 motors, protein kinase A phosphorylation inhibits the binding of synaptic vesicles and glycogen synthase kinase-3 phosphorylation inhibits the binding of membrane organelles (Sato-Yoshitake *et al.*, 1992; Morfini *et al.*, 2002). In mitochondrial trafficking, the adaptor proteins Milton and Miro bridge KIF5 motors to the mitochondria in a calcium dependent manner (Glater *et al.*, 2006; MacAskill *et al.*, 2009). The wide heterogeneity of these identified cargoes leads directly to the question of how specificity is effected, if they form functional groups, and how they change after injury or during regeneration. What are the key molecules and organelles transported in axons? And, which are affected in RGC axons in optic neuropathies like glaucoma with associated axon transport loss?

AXONAL TRANSPORT OF MITOCHONDRIA

Mitochondria are perhaps the most studied organelle being shuttled up and down the axon by motor proteins. Mitochondria are responsible for ATP generation by oxidative phosphorylation, generation of reactive oxygen species, calcium buffering, among many other functions (Werth and Thayer, 1994). Dysregulation of mitochondria and mitochondrial distribution can lead to apoptotic cell death. Mitochondrial trafficking is essential for neurite outgrowth *in vitro* (Morris and Hollenbeck, 1993), and *in vivo* transport of mitochondria after injury has recently been more appreciated. *In vivo* imaging of mitochondria in the retina has shown a general decrease in motility and transport in aged mice compared to adult

mice, as well as a reduced number of transported mitochondria in a glaucoma model (Takihara *et al.*, 2015). Interestingly, aged mice are more susceptible to the mitochondrial transport disruption of glaucoma compared to younger adult mice, correlating with the increased incidence of glaucoma as human age. Mitochondria traffic to injured axons in *C. elegans* is required for normal regeneration (Han *et al.*, 2016). Similarly, after optic nerve crush in mice, the mitochondrial protein Armcx1 is upregulated during injury in a regenerative condition, and further overexpression enhances both survival and regeneration of RGCs. This effect is hypothesized to be due to an increased in mobilization of mitochondria after injury, consistent with the results seen in *C. elegans* and in the mammalian sciatic nerve (Cartoni *et al.*, 2016; Han *et al.*, 2016; Zhou *et al.*, 2016). Thus promoting increased mitochondrial transport promotes regenerative responses in the mammalian optic nerve, although it is not yet understood how regulation of this mitochondrial redistribution and energetics modulation contributes to survival and regeneration.

AXONAL TRANSPORT OF MRNA

Effectors from the cell body arrive at pre-synaptic terminals, growth cones, dendrites, and sites of injury by two methods: local axonal translation after transport of mRNA, and direct long-distance transport of proteins. Before the detection of mRNA transport into mammalian axons, local translation was predicted based on an efficiency hypothesis: over long distances, transport of few mRNA molecules that could be translated many times over at the desired location conserves energy over synthesizing these proteins at the cell body and transporting them (Spaulding and Burgess, 2017). Evidence for local translation in PNS neurons has first been shown through radioactive protein synthesis labeling *in vitro* and *in vivo*, followed by microscopic evidence of ribosomes *in vivo* (Koenig, 1991; Eng *et al.*, 1999; Bleher and Martin, 2001). The presence of ribosomes and mRNA in the axons of mature CNS neurons is a pre-requisite for local translation. PolyA and rRNA are seen in developing hippocampal neurons *in vitro*, and axonal protein synthesis contributes to growth cone stabilization in isolated, regenerating DRG neurons *in vivo* (Kleiman *et al.*, 1994; Zheng *et al.*, 2001). Furthermore, specific mRNA molecules whose transport is increased after injury have been seen in both PNS and CNS axons *in vivo*. (Hanz *et al.*, 2003; Willis *et al.*, 2011). RNA-binding proteins and ribosomes for local translation have been found in peripheral neurons (Zheng *et al.*, 2001; Spillane *et al.*, 2013), and bound to mitochondria in RGC axons *in vitro* (our unpublished data). Isolated mRNA from purified axons of cortical neurons using a specialized microfluidic chamber also revealed many transcripts related to RNA translation machinery and transport (Taylor *et al.*, 2009). Indeed even with these data in CNS neurons, having less translation machinery in CNS axons than PNS axons (Verma *et al.*, 2005) may contribute to the differential regenerative capacities between these two populations. Is a relative lack of mRNA transport and/or locally translated effector proteins a fundamental reason for regenerative failure? While further work in this field is necessary to determine if increased translation after injury in RGC axons can improve regeneration, progress has been made in identifying and targeting specific mRNA transport pathways.

Are there links between mRNA transport and molecular pathways implicated in neuroprotection or regeneration? One strong example involves the dual leucine zipper kinase

(DLK-1) pathway, which was first shown to lead to *cebp-1* mRNA stabilization and local translation in *C. elegans* (Yan *et al.*, 2009), and later tied to RGC survival in mice (Watkins *et al.*, 2013; Welsbie *et al.*, 2013). In a high-throughput siRNA assay, DLK inhibition was seen to be pro-survival in primary RGCs given an axonal injury. Similarly, inhibition of leucine zipper kinase (LZK), whose *C. elegans* homolog is DLK-1, in conjunction with DLK knockdown, more completely prevents RGC death both *in vitro* and *in vivo* (Welsbie *et al.*, 2017). Downstream effectors of this pathway have been identified, including SOX11, MEF2A, JUN, and ATF2, and a number of these also affect RGC survival and optic nerve regeneration. It is not known, however, whether DLK or these DLK/LZK pathway effectors lead to mRNA stabilization and local translation or transport changes in mammalian axons, similar to DLK's mechanism of action in *C. elegans*.

Even more broadly, how can we discover the identities of these pools of mRNA that are actively being translated in RGC axons? Ribotrap techniques to isolate actively translating RNA in the visual system, and identifying them with RNA-Seq, takes this exploration of *in vivo* axonal translation a step further (Shigeoka *et al.*, 2016). Briefly, affinity-tagged ribosomes are expressed in a cell-specific manner, cross-linked, and isolated. The bound mRNA to these ribosomes, the "translatome," gives insight into actively translating mRNA in a specific cell type. In neurons with spatial separation of compartments, such as RGCs, optic nerve, and synaptic terminals can be isolated to identify locally translating axonal proteins. Quantifying changes of intra-axonal protein synthesis in the normal, injured, and regenerating optic nerves as compared to intrinsically regenerating axons will identify aspects of the translatome most relevant to neuro-regeneration.

AXONAL TRANSPORT OF PROTEINS

Directly transported proteins have been identified and studied in glaucoma and acute optic nerve injury, with an experimental focus on strong candidates for involvement in neurodegeneration, such as the transport of BDNF (Pease *et al.*, 2000). To truly appreciate the complement of proteins transported normally or disrupted in glaucoma or other insults, unbiased methods are needed for broad identification. Mass spectrometry for proteins and lipids continue to show the most promise for tackling such questions. As these technologies continue to advance, methods for subdividing these pools, including time resolution for synthesis and degradation of these molecules and compartmentalized sequencing, will provide a clearer picture of molecular interactions.

Historically, studies in goldfish (Benowitz *et al.*, 1981; Perry *et al.*, 1985), tadpoles (Szaro *et al.*, 1984), toads (Skene and Willard, 1981), and mammals (McKerracher *et al.*, 1990) using radiolabeled amino acids have shown that there is a global loss of axonal transport following nerve injury, with selective increases in proteins of certain molecular weights. In non-regenerating mammalian RGCs, there is a preferential loss of slow compared to fast axonal transport, which may underlie some of the differences in regenerative potential between species. Studies that try to dissect the identities of axonal proteins and protein changes are confounded by proteins originating from non-axonal sources, such as glia (Perry *et al.*, 1985). Approaches to separate axonal from glial proteins have included isolating axoplasm, e.g., from ligated sciatic nerves with or without injury; however, biological variability

limited attempts to quantify protein differences even when using clustering methods to correlate and group transport machinery to a set of proteins (Michaelevski *et al.*, 2010). Nonetheless, there is evidence of increased anterograde transport of structural components of translation machinery and mRNAs in PNS neurons that may not occur in CNS neurons after injury or in degenerative disease. Together these findings paint a picture of a coordinated cellular response to injury that relies on transporting both formed proteins and the machinery to synthesize new proteins (See Fig. 1).

Even better than such indirect, bioinformatics-based approaches to identify and separate axonally transported proteins from glial proteins after injury would be direct detection of anterogradely or retrogradely transported proteins *in vivo*. This has been challenging, primarily due to the low proportion of transported axonal proteins compared to those from the surrounding white matter milieu. Are regenerated RGCs able to adequately transport synaptic proteins and mRNA for local translation from the cell body? In fact, what proteins make up the pre-synaptic compartment in RGCs, and do these differ by RGC subtype or target region? While synaptosomal proteomics have improved with novel compartment labeling techniques (Ting *et al.*, 2016), these have for the most part been restricted to cell culture.

Recent advances in mass spectrometry-compatible signal detection *in vivo* have allowed a re-evaluation of this open question in transport biology (Schiapparelli *et al.*, 2014), to the point that we can now directly detect changes in CNS axoplasmic protein transport in injury and disease. Labeling a group of proteins with an affinity tag, such as biotin, is a common method of separating proteins of interest from the background. In such paradigms, these labeled proteins are enriched with streptavidin pull-down, and once isolated, trypsinized, and identified with mass spectrometry. This technique has mostly been limited to situations where labeled proteins are a large portion of total proteins, as can be controlled in cell culture, but is a challenge when labeled proteins are only a small fraction of total proteins. *In vivo*, the percentage of proteins transported from a cell body to the axon is low compared to all the proteins found in the optic nerve, resulting in a high false-positive rate of contaminating unlabeled proteins. To overcome this limitation, reversing the order of the technique, trypsinizing all protein, and then pulling down and searching by mass spec for only the biotinylated peptides can now allow direct detection and high specificity even in rare samples (Schiapparelli *et al.*, 2014). A second technical improvement is the ability to multiplex tags with slightly different molecular weight biotin groups, similar to tandem mass tagging, allowing for quantitative differences in protein abundance between conditions (Thompson *et al.*, 2003). We have been exploring these approaches in optic nerve injury and regeneration, and our early findings suggest feasibility of the technique and identification of promising candidates.

COULD AXON TRANSPORT BE ONE UNIVERSAL EFFECTOR REGULATING REGENERATIVE FAILURE OR SUCCESS?

Re-examining recent advances in regeneration with a renewed and technically improved focus on axonal transport underscores that many of these strategies may depend or be

enhanced by functioning molecular motor networks. For example, KLF4 has been directly linked to mitochondrial biogenesis and autophagy in non-neuronal cells, and proper translocation of mitochondrial to the site of injury is critical for axon growth (Jang and Arany, 2015). While KLF4 deletion promotes partial RGC regeneration, could negative regulation of mitochondrial translocation limit the effectiveness of this therapy? Dissection of the KLF9–Dusp14 link in RGC regeneration may potentially unveil a direct link between nuclear gene expression changes and the transport of signals sent to the RGC axon. As discussed above, the DLK/LZK pathway, which ties in upstream to the role of Sox11 in RGC survival and regeneration, is also known to affect mRNA stabilization and translation in other species. Could transport failure after injury be a cause of incomplete regeneration with visual recovery seen in each of these studies?

As mentioned previously, functional axonal transport depends on multiple kinesins and dynein. How are these motors, and their respective cargoes, differentially expressed and regulated after injury? What is the redundancy and specificity of cargo transport between motors? In uninjured neurons, loss of KIF4a reduces integrin trafficking, but overexpression is not able to increase integrin transport to the axonal compartment (Heintz *et al.*, 2014). Approaching this problem from the opposite side, how do known regenerative or survival therapies affect kinesin expression, and protein translocation? Identifying the upstream regulators of transport through comparisons between regenerative and non-regenerative neurons, and specifically targeting them, may unmask the intrinsic growth capacities of adult CNS neurons.

In summary, recent large-scale òmics studies, both transcriptomic and proteomic, have opened up the ability to quantitatively probe all changes in the neuronal cell body – which may generate a deluge of differentially expressed candidates – and now changes in transport to other compartments, like the axon, which may narrow such molecular candidates to those most relevant for axonal degeneration and regeneration. Indeed probing these together may suggest a mechanistic link between gene transcription, protein expression, and molecular transport to affected axons. We hypothesize that understanding such mechanistic links will not only impact axon regeneration approaches, but also address myelination in regenerating fibers, electrophysiology and axon conductance of action potentials, and formation of synapses, all key to restoring the diversity of visual responses and behavior.

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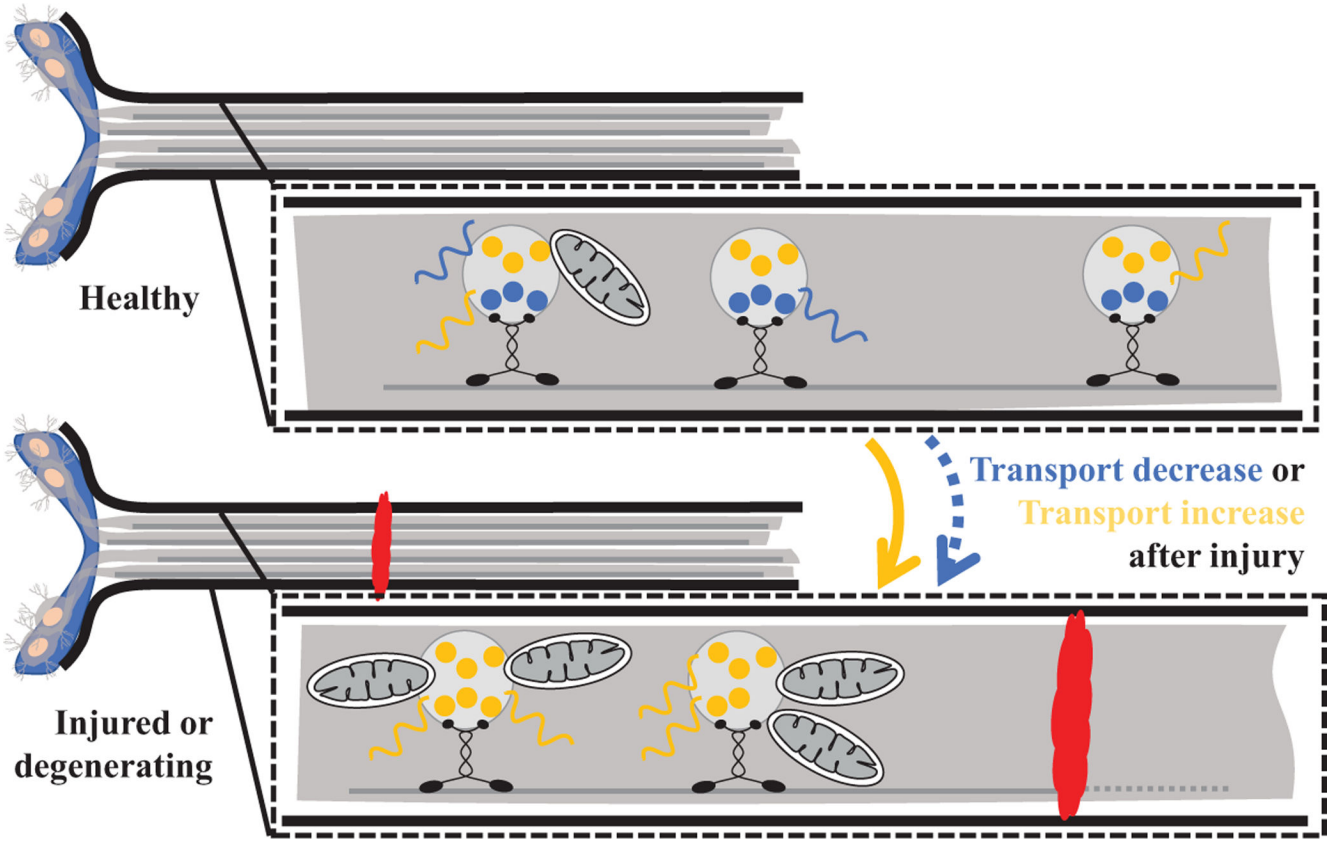

Figure 1.

After axonal injury, anterograde transport of mitochondria, mRNA, and proteins are all affected. Certain proteins and mRNA decrease in transport, while the transport of other specific transcripts, proteins, and mitochondria toward the injury site may increase.