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Mitochondrial Dynamics in Retinal Ganglion Cell Axon Regeneration and Growth Cone Guidance

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Abstract

Failed axon regeneration and retinal ganglion cell (RGC) death after trauma or disease, including glaucomatous and mitochondrial optic neuropathies, are increasingly linked to mitochondrial dysfunction. Mitochondria are highly dynamic organelles whose size, organization, and function are regulated by a balance between mitochondrial fission and fusion. Mitochondria are ubiquitous in axonal growth cones both in vitro and in vivo and during development and regeneration. However, the roles that mitochondrial fission and fusion dynamics play in the growth cone during axon regeneration are largely unstudied. Here we discuss recent data suggesting mitochondria in the distal axon and growth cone play a central role in axon growth by integrating intrinsic axon growth states with signaling from extrinsic cues. Mitochondrial fission and fusion are intrinsically regulated in the distal axon in the growth cones of acutely purified embryonic and postnatal RGCs with differing intrinsic axon growth potentials. These differences in fission and fusion correlate with differences in mitochondrial bioenergetics; embryonic RGCs with high intrinsic axon growth potential rely more on glycolysis whereas RGCs with low intrinsic axon growth potential rely more on oxidative phosphorylation. Mitochondrial fission and fusion are also differentially modulated by KLFs that either promote or suppress intrinsic axon growth, and altering the balance between mitochondrial fission and fusion can differentially regulate axon growth rate and growth cone guidance responses to both inhibitory and permissive guidance cues.

Keywords

Mitochondria; Growth cone; Regeneration; Retinal ganglion cell; KLF

Introduction

Vision loss after optic nerve injury remains a persistent clinical problem due in part to the failure of injured or diseased retinal ganglion cells (RGCs) to regenerate their axons, which often degenerate after injury, leading to RGC cell death and irreversible vision loss [1]. In RGCs, like other central nervous system (CNS) neurons, the failure to regenerate after injury

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or disease is multifactorial, including reduced intrinsic axon growth ability in adult neurons [2-5], tissue destructive inflammatory responses [6], extrinsic glial-associated inhibitory molecules [7-9], and insufficient neurotrophic factor support [10-12]. Most efforts to improve CNS axon survival and regeneration have focused on overcoming extrinsic glial-associated inhibitors in the injured CNS like Nogo, oligodendrocyte myelin glycoprotein (Omgp), semaphorin 3A, myelin associated glycoprotein, and chondroitin sulfate proteoglycans (CSPGs) [13-19], increasing deficient neurotrophic factor signaling [10,12,20-23], modulating inflammation [24], and manipulating intrinsic signaling molecules like phosphatase and tensin homolog (PTEN) [25,26], SOCS3 [27], and the Krüppel-like family (KLF) family of transcription factors [28,29] among others well reviewed [30]. Addressing extrinsic factors individually or in combination with other extrinsic or intrinsic factors can slow RGC death, promote partial axon regeneration, and in some cases, restore limited visual function in animal models [26]. However, the overall number of axons that regenerate successfully is low, many regenerating axons fail to pathfind correctly [25,31], and many of the molecular manipulations are not clinically translatable, arguing for new strategic and therapeutic approaches to overcoming barriers to successful axon regeneration.

Mitochondria

Mitochondria integrate and modulate numerous cellular signaling pathways [32] and are increasingly recognized as central players in modulating signaling by both intrinsic and extrinsic factors that regulate neuronal survival and axon growth (Figure 1). Generally, mitochondria regulate neuronal growth, maintenance, and function by regulating basic metabolic functions, including ATP production through oxidative phosphorylation, calcium homeostasis, cellular apoptosis, lipid metabolism, and reactive oxygen species (ROS), nitric oxide (NO) and cyclic AMP (cAMP) signaling [33]. Moreover, evidence suggests mitochondria play key modulatory roles in the signaling pathways of many intrinsic and extrinsic factors that regulate axon regeneration [34]. Specifically, mitochondrial fission, fusion, and transport dynamics, appear to regulate numerous signal transduction pathways that control axon growth, suggesting mitochondria in axonal growth cones (GC) are optimally positioned to integrate and modulate signaling from both intrinsic and extrinsic factors that regulate axon growth rate and guidance.

Mitochondria, Intrinsic and Extrinsic Signaling, and Second Messengers

In previous studies, both intrinsic and extrinsic regulators of axon growth have been shown to regulate mitochondrial activities (Figure 1). For example, intrinsic regulators, like KLFs and PTEN, influence mitochondrial dynamics and function. PTEN is a negative regulator of the mammalian target of rapamycin (mTOR) pathway [35] and both PTEN and mTOR integrate signals regulating mitochondrial transport, oxygen consumption, and oxidative capacity [36-39]. PTEN may also mediate mitochondrial trafficking via PTEN induced Kinase 1 (PINK1). PINK1 forms a multi-protein complex with the calcium-dependent transport protein Miro and the adaptor protein Milton [40], a complex that links kinesin to mitochondria for anterograde transport along microtubules [41,42]. The KLF transcription factors can also differentially alter intrinsic axon growth ability in RGCs. The downstream KLF axon growth effectors in CNS neurons are not yet defined, but potential links to mitochondria exist. Overexpressing KLFs that differentially suppress, KLF4, or enhance, KLF6, axon growth potential [28] differentially regulates mitochondrial organization and mitochondrial DNA replication in the distal axons and growth cones of cultured RGCs [43]. In non-neuronal cells KLF2 is regulated by the mTOR pathway [44], KLF4 can up-regulate Mfn2 expression [45], and KLF6 can up-regulate p21 [46-48] which can enhance axon

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regeneration in the spinal cord [49]. Moreover, p21 can activate p21-activated kinase 5 (PAK5), which localizes to mitochondria [50,51].

Similarly, extrinsic axon growth regulators also act on mitochondrial dynamics and function. BDNF can increase mitochondrial respiration in neurons, via a MAP kinase dependent pathway linked to increased complex I activity [52], whereas decreased BDNF levels are linked to reduced mitochondrial respiration in neurons [53]. Interestingly, the full-length TrkB receptor is present in brain mitochondria [54] and BDNF protein has been localized to mitochondria in axon terminals in the visual system [55], suggesting that neurotrophins may act as signaling molecules directly on mitochondria. Nerve growth factor (NGF) can also regulate mitochondrial activities. In culture, applying NGF locally to either axons or to pre-synaptic terminals can locally direct mitochondrial transport and docking [56-58] by a mechanism that locally increases mitochondrial membrane potential [59] and is directed by the actin cytoskeleton [57,58]. Similarly, locally applying semaphorin 3A, an inhibitory guidance cue [60,61], also directs mitochondrial transport and increases mitochondrial membrane potential ($\Delta\psi_m$) [59]. Both NGF and semaphorin 3A, altered $\Delta\psi_m$ via PI3 kinase and, like BDNF, MAP kinase dependent pathways [59], suggesting that these pathways can regulate $\Delta\psi_m$ and that mitochondrial functions requiring increased $\Delta\psi_m$ may regulate both positive and negative regulators of axon growth. Finally, Nogo, a potent inhibitor of axon growth, has been suggested to regulate electron transport chain (ETC) function by binding to a complex III protein or to a putative Nogo-interacting mitochondrial protein [62]. Surprisingly, despite the number of cues that regulate both signaling pathways that regulate axon growth, little is known about how these intrinsic and extrinsic signaling pathways modulate or are modulated by mitochondrial dynamics in the growth cone.

Mitochondria also share a reciprocal relationship with second messenger systems that regulate axon growth (Figure 1). Mitochondria are prominent in axonal growth cones *in vitro* [63] and *in vivo* [64] and regulate numerous second messenger pathways, including Ca^{2+}, cAMP, and NOS, that can regulate both axon growth rate and growth cone motility and guidance [65-67]. Moreover, mitochondrial organization, in turn, is influenced by second messenger systems [68,69] that alter axon growth, including Ca^{2+} signaling [70-72], ROS signaling [73], NOS signaling [74], and mitophagy [75-77], suggesting mitochondrial dynamics and second messenger signaling share a reciprocal relationship. Though progress has been made in the temporal and spatial control of second messenger transients in the growth cone [78,79], studies on how various second messenger pathways alter mitochondrial dynamics in the growth cone and, in turn, how such changes in mitochondrial dynamics regulate second messenger signaling to regulate axonal growth and guidance are limited [43]. Moreover, little is known about how mitochondrial dynamics function in the axonal growth cone.

The Axonal Growth Cone

Growth cones, the motile tips of axons, determine the rate and the direction axons grow by altering three distinguishable processes: protrusion, engorgement, and consolidation (Figure 2) [80] in response to signaling from cellular [81] and extracellular matrix guidance cues [82] integrated with intrinsic axon growth states [83,84]. During protrusion, actin meshworks in the growth cone's peripheral domain protrude sheet-like, lamellipodia, whereas filamentous actin (F-actin) bundles protrude finger-like, filopodia. These protrusions advance the growth cone's membrane [85], distributing receptors, cell adhesion molecules, and lipids peripherally [86] to detect and modulate extrinsic cue signal transduction [87]. In the central domain, at the base of the growth cone, bundled microtubules extending from the neurite shaft fan out providing structural support and conduits for transporting vesicular cargos, including mitochondria, essential to growth cone

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function and remodeling. Mitochondria are ubiquitous residents of the axonal growth cone's central domain. In actively growing axons, mitochondria are highly dynamic often undergoing rapid fission and fusion events and bi-directional transport along dynamic microtubules extending from the central domain and into the peripheral domain. The persistent presence of mitochondria in the growth cone's central domain is hypothesized to provide the ATP required to support growth cone motility. However, recent observations suggest the role of mitochondria in the growth cone is not so straightforward and that mitochondrial dynamics themselves may play a critical signaling role.

Mitochondrial Dynamics

Mitochondria are heterogeneous organelles, differing in subcellular distribution, membrane potential ($\Delta\psi_m$), and metabolic activity that act individually or in dynamic networks regulated by fission, fusion, transport, docking, biogenesis, and mitophagy (Figure 3) [88-90]. These dynamics are critical to regulating mitochondrial bioenergetics, network organization, and distribution in different neuronal compartments [91-93]. Specifically, mitochondrial fission, fusion, and transport proteins play critical roles in maintaining mitochondrial fidelity and cellular health. Among other adaptor and regulatory proteins, the mitofusins, Mfn-1 and -2 [94], and optic atrophy 1 (OAP-1) [95] cooperate to regulate outer and inner mitochondrial membrane fusion respectively, dynamin related protein (DRP-1) mediates fission [72], and Miro [70] and syntaphilin [96] regulate mitochondrial transport and docking respectively (Figure 2C-2D). Mutations in these proteins can lead to mitochondrial network dysfunction and eventually poor cellular and organism health [97]. In the CNS, dysfunctional mitochondrial fission, fusion, and transport dynamics are broadly linked to impaired neuroplasticity and neuronal degeneration in Alzheimer's disease [98], Huntington's disease [99], Parkinson's disease [100], ALS [101,102], psychiatric disorders [103], and stroke [104] among others well reviewed [97].

In the visual system, mitochondrial dysfunction is also implicated in both traumatic and disease related disorders [105], including RGC axon degeneration after trauma [106,107], glaucoma [102], Charcot-Marie tooth disease (CMT) [108,109], and autosomal dominant optic atrophy (ADOA) [110,111]. Interestingly, these disorders are linked either primarily or secondarily to proteins regulating mitochondrial fission and fusion. For example, mutations in the fusion proteins Mfn-2 or OPA-1 are considered causal to axon loss in CMT type 2A [112] and progressive RGC axon loss in ADOA [111] respectively. Mitochondrial fission abnormalities are also implicated in eye disorders related to Parkinson's and Alzheimer's disease [97], and abnormal brain development [109,113-117]. Moreover, dominant negative mutations in Drp-1 cause lethality in addition to the rapid loss of RGC axons [118]. In glaucomatous eyes, ischemia due to increased ocular pressure can increase OPA-1 release from the inner mitochondrial membranes, leading to increased mitochondrial fission and eventual RGC axon degeneration [119]. Neurodegeneration in these diseases suggests RGC survival and function depend on mitochondrial fission/fusion dynamics, not only to meet the high energy demands in highly polarized cells like neurons [120], but also (as discussed below) to regulate mitochondrial fission/fusion-dependent signaling necessary to regulate RGC axon regeneration.

Mitochondrial Dynamics are Intrinsically Regulated in RGCs

In non-neuronal cells, mitochondrial morphology, biogenesis, distribution, and signaling change with the state of the cell [121-123]. However, little is known about how mitochondrial fission/fusion dynamics function in the neuronal growth cone. RGCs' intrinsic ability to rapidly regenerate axons declines progressively in postnatal RGCs shortly after birth compared to embryonic RGCs [2]. In culture, this decline in axon growth ability

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correlates with changes in mitochondrial fission/fusion dynamics; fewer, smaller mitochondria in the distal axon and growth cone support rapid axon regeneration [43], whereas longer more fused mitochondria do not (Figure 3B). For example, RGCs with enhanced axon growth potential, such as embryonic RGCs or RGCs overexpressing the axon growth enhancer KLF6, have smaller, more dynamic mitochondria. Moreover, chronically inhibiting the fission protein DRP-1 prior to axon initiation, which increases mitochondrial fusion (Figure 4), significantly reduces mitochondrial number and size in RGCs axons and growth cones without altering initial axon growth rates. In contrast, slow growing postnatal RGC axons harbor longer mitochondria in their distal axons and growth cones [43], which are less mobile, consistent with studies in cultured peripheral neurons [124] and the localization of immobile mitochondria to non-motile regions like branch points [125]. Whether similar differences in mitochondrial fission/fusion dynamics are specific to RGCs or applicable to regenerating axons in general remains to be determined.

Mitochondrial Bioenergetics and Axon Growth

Mitochondrial fission/fusion dynamics regulate mitochondrial bioenergetics [126,127], which appears to play an important role in pro-growth signaling. Consistent with smaller, less dense mitochondria in fast growing axons, embryonic RGCs appear to rely less on mitochondrial oxidative phosphorylation and more on cytoplasmic glycolysis compared to postnatal RGCs, which rely primarily on oxidative phosphorylation (Figure 3C) [43]. This is consistent with the low oxygen environment during prenatal development and the onset of respiration in postnatal development [128]. Thus, in contrast to the conventional roles prescribed to growth cone mitochondria [63], these data support the hypothesis that increased oxidative phosphorylation may suppress axon regeneration and suggest a switch from primarily oxidative phosphorylation to glycolysis, known as the Warburg effect (Figure 3D) [129], may be necessary to enhance postnatal axon regeneration. A switch from oxidative phosphorylation to glycolysis, or at least an increase in glycolytic metabolites from neighboring cells [130], is necessary for cancer metastasis [131] and proper retinal progenitor [132] and stem cell [133] proliferation and biosynthesis, consistent with the hypothesis that pro-growth signaling pathways are supported by glycolysis [134]. Thus, postnatal RGCs and other mature CNS neurons may be unable to meet the unique metabolic demands required to support axon regeneration [100,135]. In addition, mitochondrial bioenergetics also regulate the molecular relationships between mitochondria and both cytoskeletal [136] and cytoplasmic proteins [137]. Thus, changes in mitochondrial fission/fusion dynamics and mitochondrial bioenergetics, and the immobilization of mature actively respiring mitochondria may help explain the decline in postnatal RGC axon growth ability and the distinct differences in embryonic and postnatal RGC axon growth ability. Unquestionably, the evolutionary development of highly polarized neurons that make up the nervous system depended upon mitochondrial activities. However, perhaps mature mitochondria are more suited to support neuronal maintenance than active axon growth.

The temporal and spatial regulation of growth cone motility is fundamental to the patterning of the nervous system [138,139], neuroplasticity in learning and memory [140], and regeneration [141]. Though many of the cytoskeletal mechanisms underlying growth cone motility [85,142] and growth cone protrusive initiations [143,144] have been described, the precise control over the level and the location of lamellar and filopodial protrusion in the growth cone is unclear. Interestingly, increasing mitochondrial fusion by acutely inhibiting DRP-1 pharmacologically (Figure 4A and 4B) reversibly suppresses both axon growth rate and lamellar protrusion but not filopodial protrusion (Figure 5A and 5B), independent of ATP production (Figure 4C), suggesting that mitochondrial fission/fusion dynamics play a central role in controlling growth cone motility. In addition to filopodial signaling [145], lamellae play important sensory roles, mediating neurotrophic factor [87] and second

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messenger signaling feedback loops [146] that can locally amplify lamellar protrusions to regulate the direction and the rate of growth. Thus, these results also suggest that multiple different cues could act on a single control point regulating mitochondrial fission/fusion dynamics to, in turn; regulate axon growth rate and growth cone motility.

Several lines of evidence suggest mitochondrial fission/fusion dynamics play an important role in neuronal architecture. Mitochondrial dynamics can regulate axon guidance responses; increasing mitochondrial fusion either genetically or pharmacologically alters growth cone turning responses both to inhibitory, CSPGs, and to permissive netrin and fibronectin guidance cues (Figure 6) [43]. Mitochondrial dynamics also appear to play a role in axogenesis [147], synapse formation [148], dendritic arbor morphology [149], and dendritic spine and filopodial protrusion [150]. Though the underlying molecular mechanisms of how mitochondrial fission/fusion dynamics regulate such diverse axonal and dendritic processes are unknown, some evidence points to the differential regulation of filamentous actin dynamics through the WASP-ARP2/3 complex. Inhibiting protrusion locally at the growth cone by extrinsic cues can redirect protrusion proximally to promote branching [151,152] at sites where mitochondria are localized [152,153]. At these sites, mitochondria appear to regulate the formation of F-actin patches [154] that can promote lamellar and filopodial nucleation by ARP2/3 [155,156], which also localizes to pre-branch sites [144] and can be regulated by mitochondrial associated WAVE1 [157]. Interestingly, inhibiting ARP2/3 in cultured cerebellar granule neurons can induce a similar phenotype as increasing mitochondrial fusion in cultured RGCs axons [43], reducing lamellar but not filopodial protrusion [158]. WAVE1 also regulates ARP2/3 in growth cones [157]. However, whether WAVE1 also associates with or is activated by mitochondria in growth cones is unknown. Finally, mitochondrial dynamics also appear to play a role in regulating microtubule dynamics [159] which can regulate neurite initiation [160] and extension in PC12 cells [161] and primary cultured neurons [162,163] possibly by locally effecting actin dynamics [164]. How mitochondrial dynamics differentially regulate lamellar and filopodial protrusions, which are regulated by distinct sets of proteins [165], within the neuronal soma, neurites, and growth cones remains an interesting unanswered question.

Conclusion

The development of therapeutics aimed to enhance RGC axon regeneration as well as other CNS neuronal population is a persistent challenge due to the number of intrinsic and extrinsic factors known to suppress axon regeneration in the injured CNS. Numerous lines of evidence suggest many of these factors share a reciprocal relationship with mitochondrial dynamics and functions. Thus, understanding how mitochondrial dynamics integrate and modulate multiple intrinsic and extrinsic signaling pathways to regulate axon growth and guidance as well as how mitochondrial dynamics and bioenergetics function in the growth cone may reveal novel therapeutic strategies for treating neurodegenerative diseases and for enhancing regeneration in the injured CNS.

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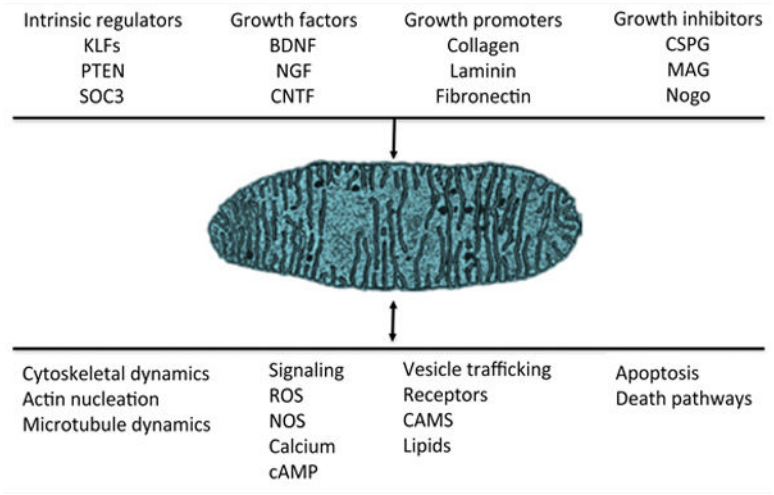

Figure 1.

Mitochondria are positioned opportunistically to modulate numerous signaling pathways. Numerous intrinsic and extrinsic factors that modulate axon growth also modulate mitochondrial dynamics and function. In turn, mitochondria regulate and are regulated by signaling pathways that regulate axon growth.

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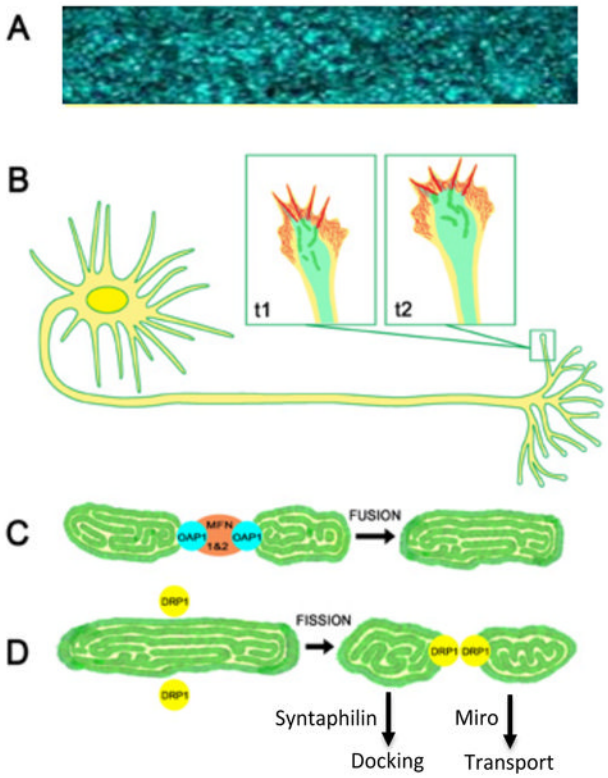

Figure 2.

A. Mitochondria (blue) are highly dynamic organelles localized throughout retinal ganglion cells axons shown in this live whole mount image of the optic nerve [166]. **B.** Growth cones (boxed regions) at the tips of growing axons and dendrites have a protrusive, actin-rich, peripheral domain (red) that pushes the growth cone's membrane peripherally. A transition zone separates the peripheral domain from the microtubule rich central domain (light green). Within the central domain, mitochondria (dark green) are highly dynamic, often undergoing fission and fusion events that constantly change the organization of the mitochondrial network in active growth cones. **C.** Mitochondrial fission and fusion events are regulated by distinct classes of proteins. Mfn1 & 2 and Oap1 regulate mitochondrial outer and inner membrane fusion respectively, whereas **D.** DRP-1 is a major factor in mediating mitochondrial fission. Additional proteins, like Miro and syntaphilin, among others, regulate mitochondrial transport and docking respectively.

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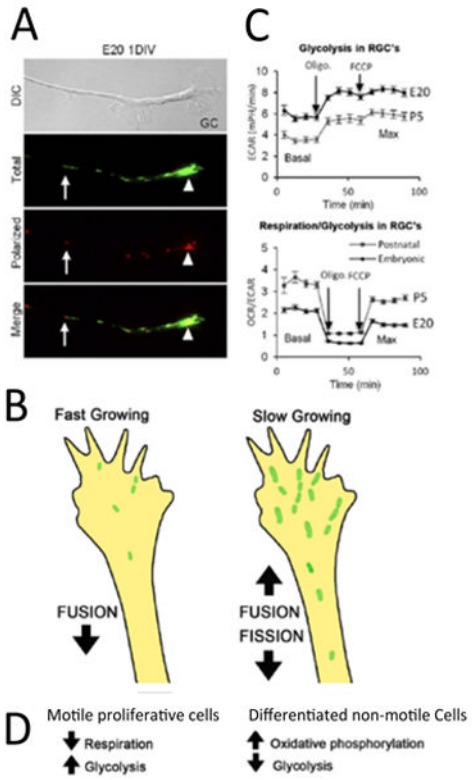

Figure 3.

In nascent RGC axons, mitochondrial organization and bioenergetics correlate with axon growth rate. **A.** DIC image of a distal RGC axon and growth cone. JC-1 monomer emission at 530 nm (green) reveals total mitochondria and JC-1 J-aggregate emission at 590 nm (red) reveals the high potential, polarized, regions within mitochondria, demonstrating heterogeneity within the mitochondrial network. Mitochondria are detected as individuals (arrows) and as complexes (arrowheads) that differ in their degree of polarization. **B.** Glycolysis is higher in fast growing embryonic RGCs whereas the basal and maximal OCR (oxygen consumption rate)/ECAR (extracellular acidification rate) ratios are greater in P5 RGCs, indicating a greater reliance on oxidative phosphorylation in slow growing RGC axons [43]. **C.** Schematic showing that fast growing RGCs are associated with small active mitochondria in the growth cone compared to slower growing postnatal RGC axons, which harbor more, longer mitochondria likely due to differences in mitochondrial fission and fusion dynamics. **D.** Data from RGCs agree with data from motile, proliferative cells, which rely more on glycolysis, whereas differentiated, non-motile cells, rely primarily on oxidative phosphorylation.

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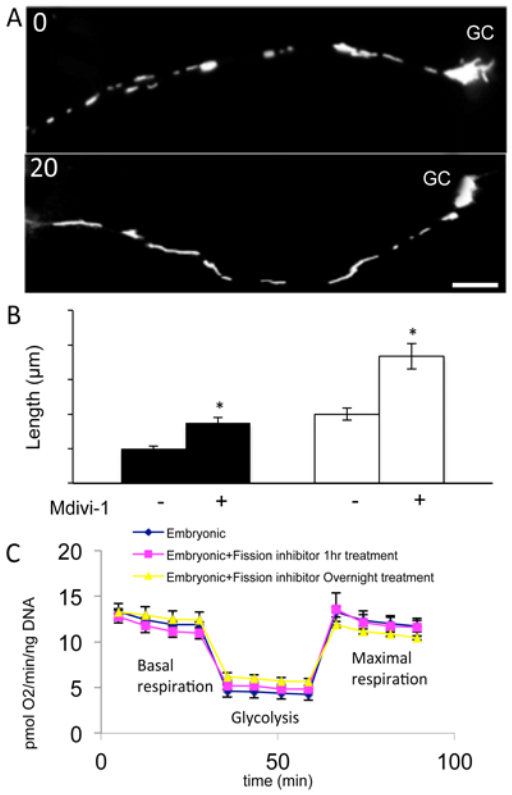

Figure 4.

Inhibiting DRP-1 with Mdivi-1 induces long reticulated mitochondria within minutes without altering mitochondrial ATP production. **A.** Cultured RGCs labeled with mitotracker, before and 20 minutes after Mdivi-1 (20 μm). **B.** After Mdivi-1, mitochondrial length doubles in both the faster growing embryonic and the slow growing RGC axons. **C.** Neither acute nor chronic Mdivi-1 alters basal or maximal respiration or glycolysis [43]. Bar is 10 μm.

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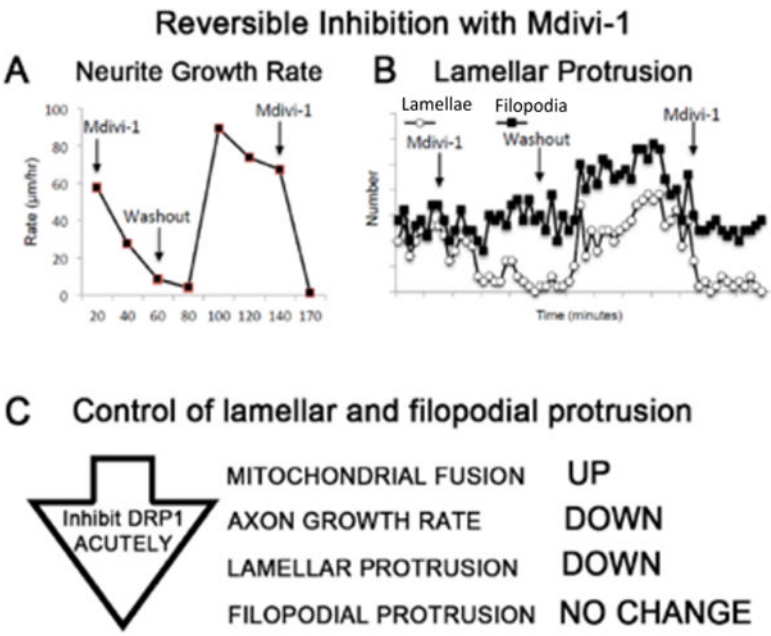

Figure 5.

The DRP-1 fission protein inhibitor, Mdivi-1, reversibly reduces neurite growth rate and lamellar, but not filopodial protrusion. **A.** Neurite growth rate and **B.** lamellar protrusion (white circles) were reversibly and repeatedly inhibited by Mdivi-1. Filopodial protrusion frequency (black squares) was unchanged with the initial Mdivi-1 perfusion, but increased briefly after washout [43]. **C.** Summary of acute effects of inhibiting DRP-1 in already established axons.

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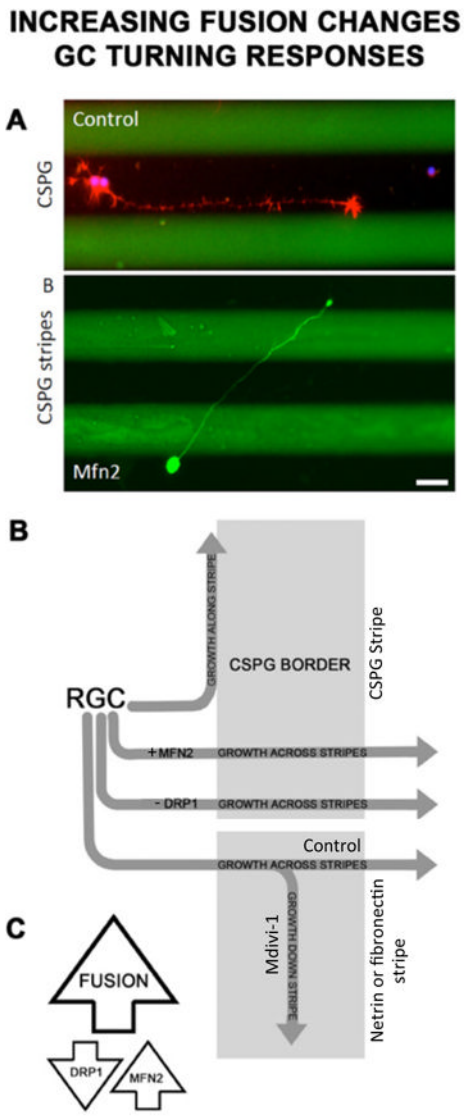

Figure 6.

Increasing fusion chronically, either genetically or pharmacologically, alters RGC growth cone responses to both inhibitory and to permissive guidance cues. **A.** RGCs normally turn and fail to cross chondroitin sulfate proteoglycan (CSPG) borders (green). **B.** Overexpressing Mfn2 or treating RGCs with Mdivi-1 (not shown) prior to axon initiation permits RGC growth cones to cross CSPG stripes without altering neurite growth rate (not shown). **B.** Schematic of increasing fusion on RGC axon decision-making. Untreated RGCs, turn and grow along CSPG stripes whereas Mfn2 overexpressing or DRP-1 inhibited RGC axons cross CSPG stripes. Inhibiting DRP-1 with Mdivi-1 also alters RGC responses to permissive cues netrin-1 and fibronectin by decreasing crossing from netrin-1 and fibronectin onto laminin [43]. Bar is 10 μm. **C.** Summary: Increasing fusion by either inhibiting DRP-1 mediated fission or increasing fusion by overexpressing Mfn2 alters growth cone decision making.