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ORIGINAL PAPER

Preserving Mitochondrial Structure and Motility Promotes Recovery of White Matter After Ischemia

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Abstract
Stroke significantly affects white matter in the brain by impairing axon function, which results in clinical deficits. Axonal mitochondria are highly dynamic and are transported via microtubules in the anterograde or retrograde direction, depending upon axonal energy demands. Recently, we reported that mitochondrial division inhibitor 1 (Mdivi-1) promotes axon function recovery by preventing mitochondrial fission only when applied during ischemia. Application of Mdivi-1 after injury failed to protect axon function. Interestingly, L-NIO, which is a NOS3 inhibitor, confers post-ischemic protection to axon function by attenuating mitochondrial fission and preserving mitochondrial motility via conserving levels of the microtubular adaptor protein Miro-2. We propose that preventing mitochondrial fission protects axon function during injury, but that restoration of mitochondrial motility is more important to promote axon function recovery after injury. Thus, Miro-2 may be a therapeutic molecular target for recovery following a stroke.

Keywords Mitochondria · Miro-2 · NOS3 · Mitochondrial dynamics · Ischemia · Stroke

Stroke

Stroke is the 5th leading cause of death in United States and is a major cause of disability (Benjamin et al. 2017). Stroke occurs when part of the brain is blocked from receiving adequate blood supply (glucose and oxygen), which could either be due to a blood clot occluding blood vessels (ischemic stroke) or rupture of a blood vessel (hemorrhagic stroke). The gold standard for ischemic stroke treatment, and the only one currently approved by the Food and Drug Administration (FDA), is tissue plasminogen activator (tPA, Alteplase), which has to be administered within a short time window of 3 h for most patients following confirmation of the diagnosis of ischemic stroke (Marler 2003). Mechanical thrombectomy although highly effective is only recommended for a selected group of patients who have minimal pre-stroke disability (Mistry et al. 2017; Venker et al. 2010).

The human brain consists of roughly equal parts of gray matter (GM) and white matter (WM) (Zhang and Sejnowski 2000). It has been observed that brain WM is almost always involved in ischemic stroke (Wang et al. 2016). Over the past two decades, multiple studies have helped to define how stroke injury mechanisms differ between WM and GM (Baltan et al. 2008, 2018; Baltan 2009, 2014a, 2014b, 2016, Baltan et al. 2011, 2013, 2014; Bastian et al. 2018a, b, c; Dewar et al. 2003; Fern et al. 2014; Malek et al. 2003; Matute 2010, 2011; Murphy et al. 2014; 1998, Stys 2005; Tekkök et al. 2007; Wang et al. 2016). Moreover, many promising pre-clinical studies for ischemic stroke that advanced to clinical trials ultimately failed because the pre-clinical studies focused only on preserving GM and did not validate targets against both GM and WM (Gladstone et al. 2002). In addition, pre-clinical studies were performed predominantly on rodent brain, which is comprised of only ~ 10% WM by volume (Zhang and Sejnowski 2000). Hence, it is of the utmost importance to identify and validate alternate targets for stroke therapy that can preserve both GM and WM to improve post-stroke recovery.

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Stroke Injury Mechanisms in WM

WM is made up of myelinated and unmyelinated axons that transmit electrical impulses as well as supporting glial cells such as oligodendrocytes, astrocytes, microglia, and others. Though strokes were traditionally believed to predominantly affect GM, it has been well-established that WM is also readily susceptible to ischemia. WM injury mechanisms during stroke are incredibly complex (Agrawal and Fehlings 1996; Baltan 2016; Baltan et al. 2011, 2013, 2018; Bastian et al. 2018c; Fern and Ransom 1997; Follett et al. 2000; Matute et al. 1999; Matute 2011; McDonald et al. 1998; Stys 1998, 2004; Tekkök and Goldberg 2001; Tekkök et al. 2007; Wrathall et al. 2018) and change significantly with age (Baltan et al. 2008; Baltan 2009, 2014a, b; Bastian et al. 2018b, c; Stahon et al. 2016).

Stroke injury mechanisms follow a sequential order of injury pathways that converge to cause irreversible injury. The first in the temporal order is ionic dysfunction, which occurs as a result of intracellular accumulation of Na^+ and Ca^{2+} (Fern et al. 1995; Ouardouz et al. 2003; Stys et al. 1990; Underhill and Goldberg 2007; Wolf et al. 2001) resulting from ATP depletion and the reversal of Na^+-dependent glutamate transporters on astrocytes (Li et al. 1999; Tekkök et al. 2007). These events initiate the next injury mechanism, the excitotoxic pathway. In this pathway, excessive release of extracellular glutamate leads to myelin damage and death of oligodendrocytes by acting through AMPA/kainate receptors (Tekkök and Goldberg 2001; Tekkök et al. 2007). The third injury mechanism, the oxidative stress pathway, initiates concomitantly with glutamate-induced excitotoxicity (Baltan 2014b; Bastian et al. 2018c) and free radicals are generated due to substrate competition at glutamate-cysteine pumps (Oka et al. 1993) and glutamate-disrupting mitochondrial function (Chang et al. 2006).

Interestingly, age-related changes to axons and axonal mitochondria in WM result in the generation of oxidative stress markers such as increased generation of nitric oxide (NO), protein nitration, and lipid peroxidation (Stahon et al. 2016). Furthermore, these changes correlated with morphological changes to aging axonal mitochondria, which were thicker and longer with lower ATP production levels (Stahon et al. 2016). Consequently, aging WM was highly susceptible to ischemia due to increased oxidative stress (Baltan 2009, 2014b) and a lower energy state. Further studies provided evidence that preserving mitochondria is a universal target in WM for achieving post-stroke recovery independent of age (Baltan et al. 2011; Brunet et al. 2016). More recently, we have reported that inhibition of seemingly independent pathways such as NOS3 (Bastian et al. 2018), Class 1 histone deacetylase (HDAC) (Baltan et al. 2011), and CK2 inhibition (Baltan et al. 2018; Bastian et al. 2018b) promotes axon function recovery by preserving mitochondria.

Mitochondria as a Target for Stroke Therapy

Mitochondria play roles as both initiators and targets of oxidative damage during injury. Stroke results in depletion of oxygen and a rapid loss of ATP following impaired mitochondrial oxidative phosphorylation. Mitochondrial length is determined by the balance between the rates of mitochondrial fission and fusion and is important for controlling the spatiotemporal properties of mitochondrial responses during physiological and pathophysiological processes (Szabadkai and Duchen 2008). Mitochondria and the changes that they undergo during aging and stroke have been important areas of study to obtain potential therapeutic targets for stroke (Bastian et al. 2018; Bastian et al. 2018; Bastian et al. 2018; Bhatti et al. 2017; Ham and Raju 2017; Russo et al. 2018; Springo et al. 2015).

Mitochondria in Aging WM

The processes of fusion and fission contribute to mitochondrial quality control and the response of mammalian cells to stress such that fusion is stimulated by energy demand and stress, while fission generates new organelles and facilitates quality control (Frank et al. 2001; Skulachev 2001; Tondera et al. 2009). In WM, aging axons have fewer mitochondria than young axons, but they are thicker and longer (Baltan 2014b; Stahon et al. 2016). This age-dependent expansion of mitochondrial morphology correlates with a mismatch among mitochondrial shaping proteins such that increases in levels of fusion proteins such as mitofusin-1 (mfn-1) and mitofusin-2 (mfn-2) and decreases in fission protein Drp-1 levels result in conditions that favor mitochondrial fusion in aging axons (Baltan 2014b; Stahon et al. 2016).

Fused mitochondria is an adaptation in response to the lower ATP levels observed in aging axons and results in shared components, thereby helping to maintain energy output during stress (Westermann 2010). However, this also results in reduced mitochondrial numbers, which when combined with increased axonal volume results in parts of the axon being without mitochondria. The number of mitochondria directly correlates with the level of cellular metabolic activity. An interruption in mitochondrial dynamics due to a mismatch in

mitochondrial shaping proteins results in reduced ATP production in aging axons. In addition, aging axons also show increased levels of oxidative stress markers (4-HNE, 3-NT, and NO), which can lead to mitochondrial impairment and resulting stress (Stahon et al. 2016). Morphological alterations compromise mitochondrial function and the resultant reduced energy production and increased oxidative stress underlie the increased vulnerability of WM to an ischemic attack.

Inhibition of Mitochondrial Fission Promotes Axon Function Recovery During Stroke

During stroke, increased intracellular Ca^{2+} causes mitochondrial Ca^{2+} overload that results in inhibition of ATP production and breakdown of phospholipids, proteins, and nucleic acids. Stroke also causes extensive fission and fragmentation, leading to smaller-sized mitochondria that correlate with irreversible axon function loss (Baltan et al. 2011). Drp-1, which is a mitochondrial fission protein, is critical for mitochondrial division, size, and shape (Bastian et al. 2018a; Reddy et al. 2011) and is regulated with age in WM (Stahon et al. 2016). Inhibition of Drp-1 by Mdivi-1 is protective in several tissues such as heart, kidney, retinal ganglion cells, spinal cord, and cerebral ischemia reperfusion models (Brooks et al. 2009; Grohm et al. 2012; Liu et al. 2015; Ong et al. 2010; Park et al. 2011). Mdivi-1 prevents translocation of cytosolic Drp-1 onto mitochondria and thus inhibits fission (Kim et al. 2017; Valenti et al. 2017). We reported that Mdivi-1 acts on Drp-1 and preserves mitochondrial size in myelinated axons of mouse optic nerve (MON). Oxygen–glucose–deprivation (OGD) that mimics ischemia suppresses Drp-1 protein levels in the cytosolic fraction (Fig. 1a, left panels) because it translocates onto the mitochondrial fraction (Fig. 1a, right panels), while Mdivi-1 application reverses these effects of OGD (Fig. 1a). In Thy-1 mito-CFP (+) transgenic mice that express endogenous CFP (+) fluorescent mitochondria, Mdivi-1 application preserves mitochondrial size. In control MONs obtained from Thy-1 mito-CFP (+) mice, axonal mitochondria displayed elongated tubular CFP (+) structures (Fig. 1b, Control). OGD causes a dramatic reduction in CFP fluorescence and remaining mitochondria displays a punctuate morphology (Fig. 1b, OGD) consistent with ischemia-induced fission. Pretreatment of MONs with Mdivi-1 preserves CFP pixel intensity and mitochondrial morphology (Fig. 1b, Mdivi-1 pre-OGD); however, post-OGD application is not protective (Fig. 1b, Mdivi-1 post-OGD). Thus, the principal mode of action of Mdivi-1 is inhibition of mitochondrial fission in myelinated axons, with protection only being observed when applied during injury.

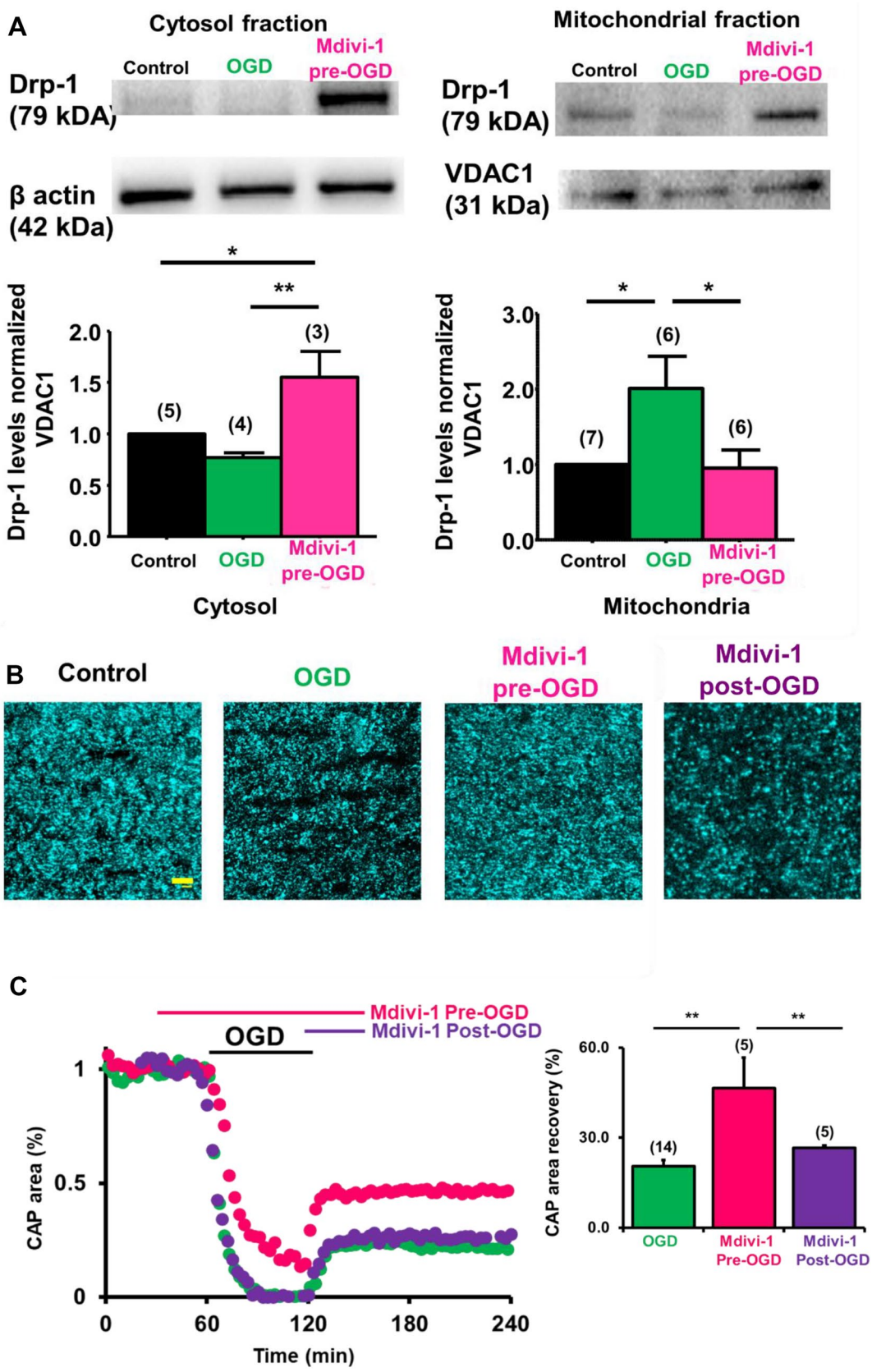
Preserving mitochondrial motility promotes axon function recovery after stroke

Electrophysiological studies to assess the functional integrity of axons exposed to OGD with or without Mdivi-1, quantified by the area under evoked compound action potentials (CAPs), show the effects of Mdivi-1 on axon function when applied 30 min before OGD, 60 min during OGD, and 30 min following reperfusion time (Fig. 1c). Without any intervention, CAP area typically recovers to ~ 20% of baseline following OGD. Mdivi-1 pre-OGD (Fig. 1c, pink) application improves CAP area recovery to 45%. Expectedly, this improvement in functional recovery correlates with inhibition of mitochondrial fission and consequent preservation of mitochondrial integrity (Fig. 1b, Mdivi-1 pre-OGD). In contrast, Mdivi-1 post-OGD application neither preserve mitochondrial fragmentation (Fig. 1b, Mdivi-1 post-OGD) nor show a significant improvement in axon recovery (Fig. 1c, purple). In conclusion, inhibition of mitochondrial fission alone post-OGD is not sufficient to preserve axon function.

Mitochondrial motility along microtubules is regulated by protein complexes, of which ATP and the calcium-dependent protein mitochondrial Rho GTPase-2 (Miro-2) play a major role (Guo et al. 2005; Melkov et al. 2016; Russo et al. 2009; Saotome et al. 2008). Miro-2 has been shown to be increasingly associated with neurodegenerative diseases that are characterized by mitochondrial dysfunction (Tang 2015). Dysregulation of Miro-2 leads to mitochondrial arrest in movement and clearance (Wang et al. 2011). Miro-2 also affects both anterograde (Macaskill et al. 2009; Wang and Schwarz 2009) and retrograde motility (Morlino et al. 2014) and the fusion-fission dynamics of mitochondria (Misko et al. 2010; Tang 2015).

Time-lapse live imaging studies of mitochondria in MONs show that axonal mitochondria move bi-directionally, change direction, or become stationary in response to OGD (Bastian et al. 2018a). Mitochondria are dynamic organelles whose coordinated motility ensures that metabolically active areas of axons are adequately supplied with ATP. Moreover, injured mitochondria are replaced with healthy ones following injury. Kymograph analysis of mitochondrial motility shows that preservation of mitochondrial motility against OGD is only achieved with pre-OGD Mdivi-1 application (Bastian et al. 2018a), parallel to which axon function recovery also shows improvement (Fig. 1c, Mdivi-1 Pre-OGD).

Interestingly, L-NIO, which is a NOS3 blocker, preserves axon function recovery in MONs when administered either pre-OGD (Fig. 2a, cyan) or post-OGD (Fig. 2a, pink). This improvement in axon function recovery is also associated with preservation of mitochondrial motility (Fig. 2d, e).


A
Cytosol fraction
Control
OGD
Mdivi-1 pre-OGD
Drp-1 (79 kDA)
β actin (42 kDa)
Mitochondrial fraction
Drp-1 (79 kDA)
VDAC1 (31 kDa)
Drp-1 levels normalized VDAC1
Cytosol
Mitochondria
B
Control
OGD
Mdivi-1 pre-OGD
Mdivi-1 post-OGD
C
Mdivi-1 Pre-OGD
Mdivi-1 Post-OGD
OGD
CAP area (%)
Time (min)
CAP area recovery (%)

◂**Fig. 1** Drp-1 regulates ischemia-induced mitochondrial fission in WM. **a** OGD suppresses Drp-1 levels in MONs in the cytosolic fraction and shows a corresponding increase in Drp-1 in the mitochondrial fraction. Following application of the Drp-1 inhibitor Mdivi-1, cytosolic Drp-1 levels increase and mitochondrial Drp-1 levels decrease. **b** CFP fluorescence studies on MONs from Thy-1 mito-CFP (+) mice show loss of CFP fluorescence and a reduction in mitochondrial numbers when exposed to OGD. Mitochondria become smaller and rounder with OGD. Mdivi-1 applied pre-OGD, but not post-OGD, preserves mitochondria. **c** Time course of electrophysiological axon function studies from MONs shows a loss of axon function with 60 min of OGD, followed by ~ 20% recovery. Mdivi-1 applied pre-OGD (pink) preserves CAP area during OGD and improves CAP area recovery during reperfusion. Mdivi-1 post-OGD treatment (purple) fails to promote axon function. (Scale bar, 5 µm). n = number of MONs; $^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA. Error bars indicate SEM. Modified figure from Bastian et al. (2018a)

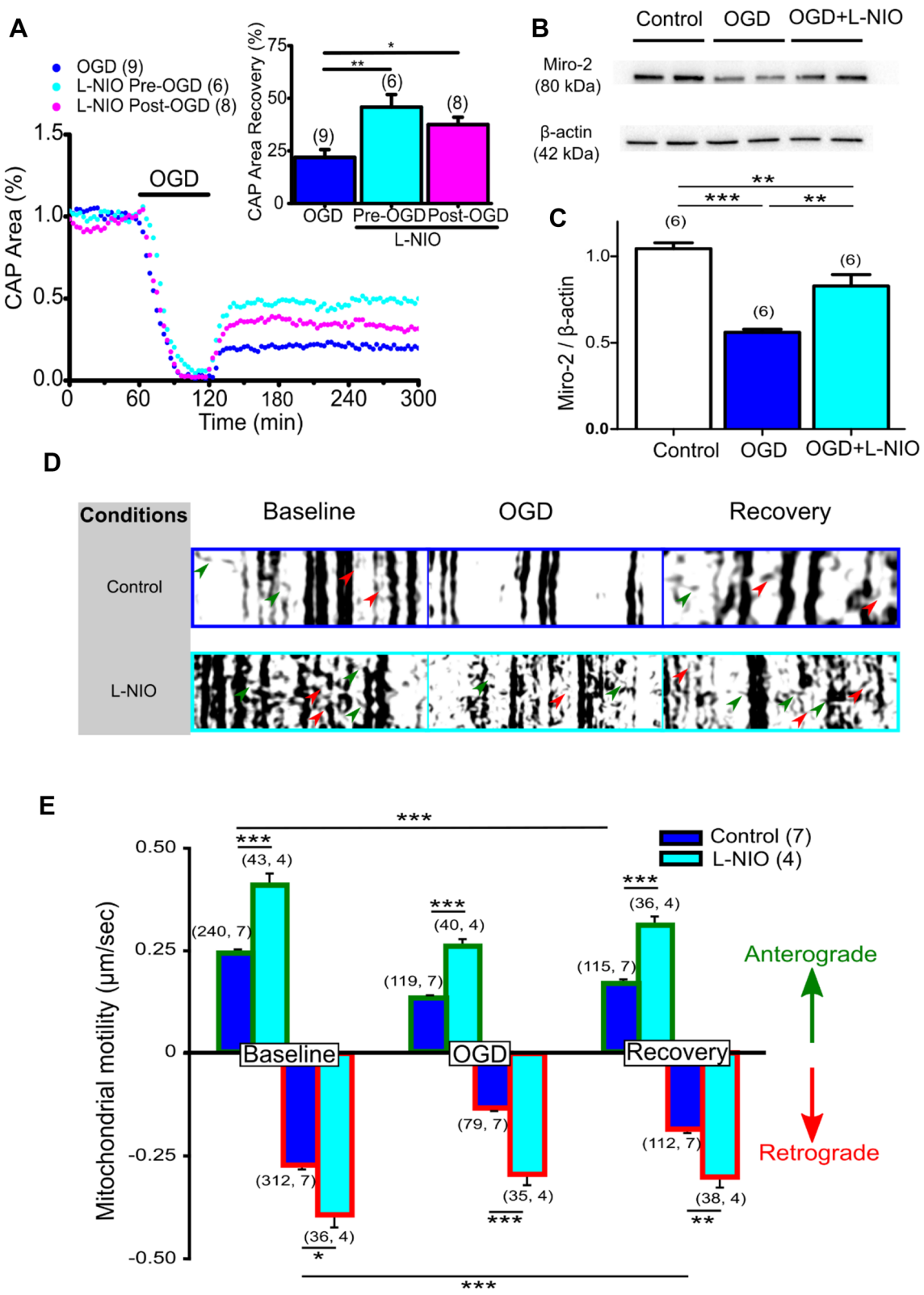
Time-lapse live imaging of mitochondria, using MONs obtained from Thy-1 mito-CFP(+) mice, shows mitochondrial movement in the anterograde (Fig. 2e, Control, green arrows) and retrograde (Control, red arrows) directions. Vertical lines represent the stationary mitochondria, while diagonal lines represent motile mitochondria (Fig. 2d). Under control conditions, the majority of mitochondria are stationary, and those that move maintain a stable speed. Onset of OGD stalls mitochondrial motility to 50% of baseline levels both in the anterograde (Fig. 2e, top blue histograms) and retrograde directions (Fig. 2e, bottom blue histograms). Application of L-NIO for 10 min before the onset of OGD causes a prominent increase in mitochondrial motility under control conditions in the anterograde and retrograde directions (Fig. 2e, top and bottom cyan histograms). This increase in mitochondrial motility persists during OGD and recovery in both directions. Furthermore, OGD results in a decrease in Miro-2 protein levels in optic nerves and L-NIO application preserves Miro-2 levels in MONs exposed to OGD (Fig. 2b). Electrophysiology and CFP imaging results propose that NOS3 inhibition promotes axon function recovery by preventing mitochondrial fission and by preserving mitochondrial structure and motility during ischemia via conserving Miro-2 levels. These results suggest that mechanisms of fission and mitochondrial motility are interconnected.

Our recent studies show that mitochondria undergo extensive fission during ischemia, and that mitochondrial fragmentation correlates with loss of mitochondrial motility (Bastian et al. 2018a, c). Blockade of mitochondrial inhibition before the onset of OGD protects axon function recovery against ischemia, however, post-OGD protection is achieved only by preserving mitochondrial motility by conserving Miro-2 levels. (Bastian et al. 2018c). Whether Miro-2 and Drp-1 interacts to maintain mitochondrial dynamics remains to be explored.

Conclusion

In this review, we have discussed how inhibition of axonal mitochondrial fission and preservation of axonal mitochondrial motility protects WM only when initiated before ischemia, while inhibition of NOS3 signaling protects WM either before or after an ischemic injury (Fig. 3). NOS3 inhibition preserves Miro-2 levels, which is a Ca^{2+}-sensing member of the microtubular mitochondrial complex that determines the motility of mitochondria, depending upon the availability of ATP and Ca^{2+}. In addition, Miro-2 is intricately linked to the mitochondrial fission protein Drp-1 (Saotome et al. 2008). Miro adaptor protein exhibits GTPase activity and forms complexes with kinesin for anterograde mitochondrial transport (Guo et al. 2005; Russo et al. 2009) and dynein for retrograde mitochondrial transport (Melkov et al. 2016; Morlino et al. 2014; Russo et al. 2009) along with Trafficking kinesin-binding protein (TRAK 1/2) along microtubules (Fig. 3). Mdivi-1 preserves ATP levels during ischemia reperfusion injury (Li et al. 2016), thus effectively abolishing impairments to mitochondrial motility in both directions and blocking mitochondrial fission to improve axon function recovery. NOS3 blockade preserves mitochondrial motility, inhibits fission, and shows post-OGD preservation of axon function recovery.

Further experiments are warranted to understand whether NOS3 inhibition after stroke affects Drp-1 dynamics to effectively protect WM in addition to preserving mitochondrial shape and motility. This protection may be partly achieved by attenuation of NO and related free radicals to preserve mitochondria in axons and thus improve axon function recovery after stroke (Bastian et al. 2018c). Mitochondrial preservation to promote brain function is a universal target against stroke in both GM and WM independent of age. Further exploration of the role of mitochondrial signaling in WM may reveal more effective therapeutic targets for WM that may be useful for neurodegenerative diseases that primarily affect WM such as multiple sclerosis and traumatic brain injury.


A
OGD (9)
L-NIO Pre-OGD (6)
L-NIO Post-OGD (8)
CAP Area (%)
OGD
Time (min)
CAP Area Recovery (%)
OGD
Pre-OGD
Post-OGD
L-NIO
B
Control
OGD
OGD+L-NIO
Miro-2 (80 kDa)
β-actin (42 kDa)
C
Miro-2 / β-actin
Control
OGD
OGD+L-NIO
D
Conditions
Baseline
OGD
Recovery
Control
L-NIO
E
Control (7)
L-NIO (4)
Mitochondrial motility (μm/sec)
Baseline
OGD
Recovery
Anterograde
Retrograde

◂**Fig. 2** NOS3 inhibition promotes axon function recovery following OGD by preserving mitochondrial motility and Miro-2 levels against ischemia. **a** Time course of electrophysiological axon function studies shows a loss of axon function with 60 min of OGD, followed by ~ 20% recovery. L-NIO application pre-OGD or post-OGD improves axon function recovery following OGD. **b** and **c** MONs exposed to 60 min of OGD show a decrease in Miro-2 protein levels. Miro-2 plays a crucial role in regulating mitochondrial motility. L-NIO application preserves Miro-2 levels. **d** Kymographs constructed from Thy1-CFP (+) live mitochondrial imaging to study mitochondrial motility show stationary mitochondria as vertical lines and motile mitochondria as diagonal lines. Green arrows represent anterograde direction and red arrows represent retrograde direction. **e** Quantification of mitochondrial motility shows a 50% reduction in mitochondrial motility, both in the anterograde (blue with green border histograms) and retrograde (blue with red border histograms) directions with OGD. L-NIO application enhances mitochondrial transport during OGD (60 min), which improves further during recovery (20 min) in both directions n = number of MONs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; one-way ANOVA followed by Bonferonni's post hoc test. Adapted from Bastian et al. (2018c)

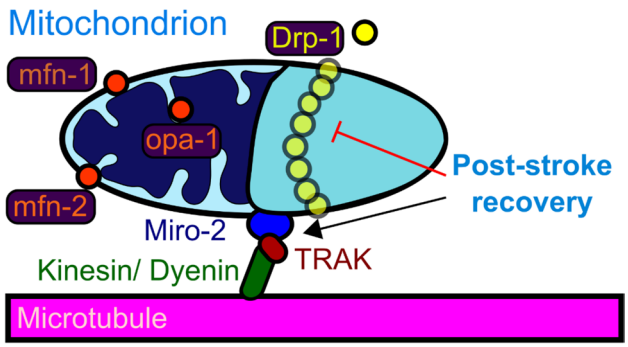


Fig. 3 Preservation of mitochondrial motility and attenuation of mitochondrial fission are both essential to enhance post-stroke recovery in WM. Schematic represents axonal mitochondria attached to the microtubule by the mitochondrial motor complex, which consists of Miro-2 and TRAK proteins. Kinesin is attached to this complex via a microtubule for transport in the anterograde direction and dynein is similarly attached for transport in the retrograde direction. The mitochondrial shaping proteins mfn-1, mfn-2, and opa-1 are associated with the mitochondrial membrane, whereas Drp-1 is predominantly found in the cytosol under physiological conditions. Ischemia activates translocation of Drp-1 to the mitochondria to form a ring that initiates mitochondrial fission. Post-stroke recovery of axons is only achieved by inhibiting translocation of Drp-1 (thus inhibiting fission, red line) and preserving Miro-2 levels (black arrow). *mfn-1* mitochondrial fusion protein 1, *mfn-2* mitochondrial fusion protein 2, *opa-1* optic atrophy protein 1, *Drp-1* dynamin-related protein 1, *Miro-2* Mitochondrial Rho GTPase 2, *TRAK 1/2* Trafficking kinesin-binding protein

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical Approval All experimental procedures were performed according to the principles of the Guide for the Care and Use of Laboratory Animals (National Association for Biomedical Research) and approved by The Institutional Animal Care and Use Committee (IACUC) of the Cleveland Clinic. Experimental procedures were performed and reported in compliance with the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments).

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