

ORIGINAL RESEARCH ARTICLE | OPEN ACCESS

Mitochondrial dynamics and preconditioning in white matter

Chinthasagar Bastian¹, Stephen Politano¹, Jerica Day¹, Andrew McCray¹, Sylvain Brunet¹, Selva Baltan¹

[Received: 1 December 2017; accepted 16 January 2018; published online 15 February 2018]

Mechanisms of ischemic preconditioning have been extensively studied in gray matter. However, an ischemic episode affects both the gray matter (GM) and white matter (WM) portions of the brain. Inhibition of mitochondrial fission is one of the mechanisms of preconditioning neuronal cell bodies against ischemia. Although axons are anatomical extensions of neuronal cell bodies, injury mechanisms differ between GM and WM. Indeed, axonal dysfunction is responsible for much of the disability associated with clinical deficits observed after stroke; however, the signaling process underlying preconditioning remains unexplored in axons. Using mouse optic nerve, which is a pure isolated WM tract, we show that mitochondria in myelinated axons undergo rapid and profuse fission during oxygen glucose deprivation (OGD) that is mediated by translocation of cytoplasmic Dynamin Related Protein-1 (Drp-1) to mitochondria. OGD-induced mitochondrial fission correlates with reduced mitochondrial motility and loss of axon function. Mitochondrial fragmentation and loss of motility become permanent during the recovery period. Inhibiting mitochondrial fission by administering mitochondrial division inhibitor-1 (Mdivi-1) during OGD preserves mitochondrial shape and motility and promotes axon function recovery. In contrast, preconditioning WM by applying Mdivi-1 only before OGD fails to conserve mitochondrial shape or motility and fails to benefit axon function. Our findings suggest that inhibition of mitochondrial fission during ischemia promotes axon function recovery, but is not sufficient to precondition WM against ischemia. These results raise caution in that approaches to preconditioning neuronal cell bodies may not successfully translate into functional improvement following ischemia.

Keywords: electrophysiology, mitochondrial motility, ATP, myelinated axon, white matter

¹Department of Neurosciences, Cleveland Clinic Foundation, Cleveland, Ohio, 44195.

Correspondence should be addressed to Dr. Selva Baltan (baltans@ccf.org).

Ischemic preconditioning is an endogenous neuroprotective mechanism in which the brain protects itself against future injury by adapting to low doses of an insult (Cadet et al., 2009; Dirnagl et al., 2009). Clinical studies indicate that patients who suffer from a transient ischemic attack show improved clinical recovery after a stroke (Moncayo et al., 2000; Wegener et al., 2004). Therefore, elucidating the complex molecular mechanisms underlying preconditioning is a critical challenge in stroke research (Gidday, 2006). Recent work has shown that mitochondria actively participate in the preconditioning signaling pathway by generating reactive oxygen species (ROS) (Kowaltowski et al., 2000; McLeod et al., 2005; Hirata et al., 2011; Gundimeda et al., 2012) or by inhibiting mitochondrial ATP channels (Auchampach et al., 1992; Yao and Gross, 1993; Jaburek et al., 1998; Garlid et al., 2003). Mitochondria are dynamic organelles that undergo fission, fusion, and intracellular transport while conducting multiple physiological functions including the generation of ATP through oxidative phosphorylation, the buffering of cytosolic Ca^{2+} , and the generation of ROS (van der Bliek et al., 2017). In normal healthy cells, fission and fusion are balanced to maintain mitochondria within a certain range of length appropriate for the maintenance of cellular physiology (Flippo and Strack, 2017). In response to injury, mitochondria undergo fragmentation into small dysfunctional units that in turn generate excessive amounts of ROS, causing cellular damage (Reddy et al., 2012; Balog et al., 2016; Golpich et al., 2017; Wu et al., 2017). Fission of mitochondria is mediated by Dynamin Reactive Protein-1 (Drp-1). Drp-1 is recruited to the mitochondrial outer membrane by a variety of adaptor proteins and then aggregates and forms oligomers in the shape of a ring at the site of future fission along mitochondria (Hatch et al., 2014; Flippo and Strack, 2017). Upon contraction of this ring formation, Drp-1 divides the mitochondrion into two separate mitochondria. Therefore, inhibition of Drp-1 activity has been of interest to attenuate or to prevent mitochondrial injury. In addition to genetic modifications, which produce serious effects on development and longevity (Ishihara et al., 2009; Wakabayashi et al., 2009), pharmacological blockade of Drp-1 with Mdivi-1, which is a blood-brain barrier-permeable inhibitor, provides a powerful tool with greater spatiotemporal control that can be acutely monitored and visualized.

A stroke affects both the gray matter (GM) and white matter (WM) portions of the brain (Mohr 2011). Faithful axon conduction is crucial for signaling among neuronal cell bodies to connect GM and WM in order to achieve and maintain proper function. Despite the anatomical perception that myelinated axons are a natural extension of neurons, axons are independent of their cell bodies in their supply of energy, metabolism, and injury mechanisms. It is important to note that approaches for neuronal protection fail to improve, or even impede, axon function recovery after ischemia (Tekkök et al., 2007; Baltan, 2009, 2012, 2014a). Inhibition of mitochondrial fission has been shown to induce preconditioning tolerance to neurons *in vivo* (Park et al., 2011b; Xie et al., 2013; Zhang et al., 2013a; Zuo et al., 2014, 2016; Jin et al., 2016; Kim et al., 2016; Deryagin et al., 2017) and *in vitro* (Correia et al., 2010; Wang et al., 2014), which raises the question as to whether or not preservation of mitochondria can precondition WM against ischemic injury.

Rodent brains contain only 10% WM by volume, as opposed to the human brain, which contains a significantly higher percentage (50% by volume) (Zhang and Sejnowski, 2000). Thus, the response to ischemia is dominated by neuronal injury in rodent brain. However, the use of optic nerve, which is a pure WM tract, avoids this impediment and provides a platform to test WM injury mechanisms in isolation. We hypothesized that blockade of mitochondrial fission during oxygen glucose deprivation (OGD) with Mdivi-1 will prevent mitochondrial

fragmentation and preserve mitochondrial motility, thereby promoting axon function recovery and mediating ischemic tolerance in WM. We developed a novel technique for live imaging of axonal mitochondria to monitor mitochondrial fission and motility using optic nerves obtained from Thy-1 mito-CFP (+) mice. Together with direct quantification of axon function, we aimed to reveal the interaction between mitochondrial dynamics and preconditioning in WM.

MATERIALS AND METHODS

Animals and Chemicals

All experimental procedures were approved by The Institutional Animal Care and Use Committee of the Cleveland Clinic. Mouse optic nerves (MONs) were obtained from C57BL/6J mice and from mice expressing mitochondrially-targeted cyan fluorescent protein (CFP) on a C57BL/6 background (Thy-1 mito-CFP (+); (Misgeld et al., 2007)). Thy-1 mito-CFP (+) mice were originally obtained at the University of Washington and were later relocated to and bred at the Cleveland Clinic Foundation. Mitochondrial division inhibitor-1 (Mdivi-1) was purchased from Selleck Chemicals (#S7162, Houston, TX). The sources for other chemicals used have been described in detail previously (Baltan et al., 2008; Stahon et al., 2016).

Optic Nerve Preparation, Electrophysiological Recordings, and Oxygen Glucose Deprivation

Following CO_2 asphyxiation, MONs were obtained from C57BL/6J or Thy-1 mito-CFP (+) mice at 2-3 months of age. MONs were gently cleared off from their dural sheaths and transferred to a Haas-Top perfusion chamber (Harvard Apparatus) and superfused with artificial cerebrospinal fluid (ACSF) containing the following (in mmol/L): 124 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4 , 23 $NaHCO_3$, and 10 glucose. MONs were allowed to equilibrate for at least 15 min in the recording chamber in normal ACSF bubbled with a 95% O_2 /5% CO_2 mixture. All electrophysiological recordings were performed at 37°C. Suction electrodes back-filled with glucose-free ACSF were used for stimulation and for recording compound action potentials (CAPs). The stimulation electrode was connected to a stimulus isolation unit (A365R Stimulus isolator; WPI, Sarasota, FL) and elicited CAPs at 30 s intervals. Stimulus pulse (30 μs duration) strength was adjusted to evoke the maximum CAP possible and then increased another 25% for supramaximal stimulation. The recording electrode was connected to an Axoclamp 900A amplifier (Molecular Devices, San Jose, CA) and the signal was amplified 20 or 50 times, filtered at 3 kHz (SR560, Stanford Research Systems, Sunnyvale, CA). OGD was induced as previously described (Baltan et al., 2008, 2010, 2011; Murphy et al., 2014; Stahon et al., 2016) by switching to glucose-free ACSF (replaced with equimolar sucrose to maintain osmolarity) and a gas mixture containing 95% N_2 /5% CO_2 . OGD was applied for 60 min, glucose-containing ACSF and O_2 were restored, and CAPs were recorded for up to 5 h after the end of OGD. MONs from Thy-1 mito-CFP (+) mice were collected and fixed for subsequent CFP (+) mitochondrial analysis (see below).

Time-Lapse Live Imaging of Mitochondrial Fission and Motility

These experiments were performed exclusively on MONs obtained from Thy-1 mito-CFP (+) mice. After dissection, MONs were transferred to a C-Stim CMC Microscope Chamber System (IonOptix, Westwood, MA) attached to an in-line heater (Cell MicroControls MTCII temperature controller and heater; IonOptix, Westwood, MA) to keep the chamber at 37°C. The MONs were superfused with ACSF bubbled with a 95% O_2 /5% CO_2 mixture at a flow rate of 3 ml/min. OGD was induced by switching to glucose-free ACSF saturated with 95% N_2 /5% CO_2 .

To determine mitochondrial motility in both the anterograde and retrograde directions, Thy-1 mito-CFP (+) MONs were imaged every 1.5 s at 1024 x 1024 resolution from a single microscopic field using an inverted confocal microscope (Leica DMI6000, Buffalo Grove, IL, 40x water immersion objective, numerical aperture, 0.80). The imaging area was set to contain at least three axons (confirmed during analysis) in which there would be at least ten mitochondria moving in either direction. Time lapse images were captured during the last 5 min of baseline (20 min), during OGD (60 min), and during the recovery period (20 min). Imaging parameters were chosen and set to minimize photobleaching. Control experiments consisting of imaging MONs using the same parameters without OGD showed less than 10% CFP fluorescent loss over the same duration (data not shown). Mitochondrial motility, direction, and changes in mitochondrial size were analyzed using ImageJ software combined with the KymoTool Box plugin (Zala et al., 2013). Stationary and motile mitochondria, as well as the direction of motility, were identified and kymographs were generated in order to measure the anterograde and retrograde movements during baseline, OGD (60 min), and recovery.

Confocal Imaging and Pixel Intensity Analysis of Thy-1 mito-CFP (+) Mice

As previously described (Baltan et al., 2011; Baltan, 2012, 2014a; Murphy et al., 2014; Stahon et al., 2016), expression of CFP was imaged using a Leica DMI6000 inverted confocal laser-scanning microscope using Thy-1 mito-CFP (+) mice. Two to three adjacent sections for each MON were imaged with the wavelength set at 456 nm. A total of 10 optical sections of 1 μ m thickness at 512 x 512 pixel size were collected in the z-axis from a single microscopic field using the 40x objective lens (HCX APO, water immersion; numerical aperture, 0.80) under fixed gain, laser power, pinhole, and photomultiplier tube settings. To compare pixel intensity, all sections were processed concurrently with Leica imaging software (LAS-AF version 2.7) using a single channel. The z-stacks were projected into a single plane image before analysis and assessment of pixel intensity.

Western blots

Mitochondria-rich lysate fractions and cytosol fractions were prepared from MON samples (two or three pairs; modified from (Disatnik et al., 2013)). MON samples were homogenized in 75 μ L of isolation buffer (300 mM sucrose, 10 mM HEPES, 2 mM EGTA, pH 7.2, at 4°C) with protease inhibitor and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). After centrifugation at 950 g for 10 min, the supernatant was collected and further spun at 10,000 g for 20 minutes at 4°C. The final pellets suspended in lysis buffer were used as the mitochondrial-rich lysate fractions and the supernatants were used as cytosol fractions. Protein concentration was estimated using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Protein lysates were prepared in 4x Laemmli sample buffer (Bio-Rad, Hercules, CA) and 2-mercaptoethanol at a ratio of 10:1 and then incubated at 95°C for 10 minutes. Equal amounts of protein were loaded into each well (10-22 μ g) of stain-free 4-20% Mini-Protean TGX gels (Bio-Rad, Hercules, CA). After gel electrophoresis, protein samples were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Blots were blocked in 5% dry milk in TBS-Tween (TBS-T; 0.1%) for one hour at room temperature. Both primary (mouse Drp-1 #611112, BD Biosciences) and secondary antibodies were made in blocking buffer. Primary antibody was incubated overnight at 4°C at 1:500. After three washes in TBS-T for 5 min, blots were incubated in secondary antibodies (goat anti-mouse HRP, Jackson ImmunoResearch) at room temperature for 2 hours. Chemiluminescence was detected using Clarity Western ECL substrate (Bio-Rad, Hercules, CA), imaged using a Bio-Rad

Chemidoc MP, and analyzed using Imagemlab software version 4.1 (Bio-Rad, Hercules, CA) for volumetric analysis of protein expression. Drp-1 levels were normalized to voltage-dependent anion channel (VDAC, # 10866-1-AP, Protein Tech Group Inc.) antibody levels in mitochondria-rich fractions and to β -actin (A5441, Sigma-Aldrich) in cytosol fractions, respectively.

Data Acquisition and Statistical Analysis

Optic nerve function was monitored quantitatively as the area under the supramaximal CAP using Clampfit (version 10.2, Molecular Devices, CA). CAP area is a complex spatiotemporal summation of action potentials from individual axons (Stys et al., 1991; Baltan et al., 2008, 2011; Stahon et al., 2016). For comparing CAP area among experimental groups, data from each group of experiments were pooled and CAP areas were normalized to the baseline conditions. Data were normalized by setting the mean of initial baseline values (measured over 15 min) to a value of 1 as previously described (Baltan et al., 2008, 2011; Stahon et al., 2016). All data are presented as mean \pm SEM. Graphpad Prism (ver. 4.0c, La Jolla, CA) was used for statistical analysis. For sample sets containing more than two groups, one-way ANOVA followed by *post hoc* Bonferroni's test for between-group comparisons was used. The p values and significance values are indicated individually for each figure in the text. The n values indicate the number of optic nerves and the numbers in brackets denote mitochondria followed by experiment numbers in histograms quantifying mitochondrial motility.

RESULTS

Drp-1 regulates ischemia-induced mitochondrial fission

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 Duchon, 2008). Because ischemia caused extensive fission and fragmentation, leading to smaller-sized mitochondria that correlated with irreversible axon function loss (Baltan et al., 2011), we reasoned that preventing mitochondrial fission could maintain mitochondrial size, shape, and morphology, and also precondition WM as characterized by improved axon function recovery. Drp-1, which is a mitochondrial fission protein, is critical for mitochondrial division, size, and shape (Reddy et al., 2011) and is regulated with age in WM (Stahon et al., 2016). Mdivi-1 is a selective inhibitor of Drp-1 and has been shown to confer protective effects in heart, kidney, retinal ganglion cells, spinal cord, and cerebral ischemia reperfusion models (Brooks et al., 2009; Ong et al., 2010; Park et al., 2011a; Grohm et al., 2012; Liu et al., 2015). Mdivi-1 is reported to suppress the translocation of cytosolic Drp-1 onto mitochondria to prevent fission (Kim et al., 2017; Valenti et al., 2017). To ensure that Mdivi-1 acted on Drp-1 and conserved mitochondrial size in myelinated axons of optic nerve, we quantified the protein levels of Drp-1 in the cytosolic (Figure 1A and B, left panels) and mitochondrial (Figure 1A and B, right panels) fractions. OGD suppressed Drp-1 in the cytosolic fraction ($0.7 \pm 0.04\%$, n = 4) because it is translocated to the mitochondrial fraction ($2.0 \pm 0.4\%$, n = 6; control 1.0 ± 0.0 , n = 7, p < 0.05, one-way ANOVA, Bonferroni's *post hoc* test), while Mdivi-1 (50 μ M) application reversed these effects of OGD. Cytosolic Drp-1 levels increased to $1.55 \pm 0.3\%$, (n = 3, p < 0.05, one-way ANOVA, Bonferroni's *post hoc* test) and mitochondrial Drp-1 levels dropped to $0.94 \pm 0.2\%$, (n = 6, p < 0.05, one-way ANOVA, Bonferroni's *post hoc* test) in MONs pre-treated with Mdivi-1. Consequently, Mdivi-1 application preserved mitochondrial size and CFP (+) fluorescence (Figure 1C, Mdivi-1 throughout). In control optic nerves obtained from Thy-1 mito-CFP (+) mice, axonal mitochondria displayed

elongated tubular CFP (+) structures (Figure 1C, Control). After OGD, as reported previously (Baltan et al., 2011), there was a dramatic reduction in CFP fluorescence and remaining mitochondria presented a punctuate morphology (Figure 1C, OGD) consistent with ischemia-induced fission. Pretreatment of MONs with Mdivi-1 preserved CFP pixel intensity and mitochondrial morphology (Figure 1C, Mdivi-1 throughout), confirming the principal mode of action of Mdivi-1 inhibition of mitochondrial fission in myelinated axons of optic nerve and that diffuse mitochondrial fission observed during OGD irreversibly hampers axon function recovery.

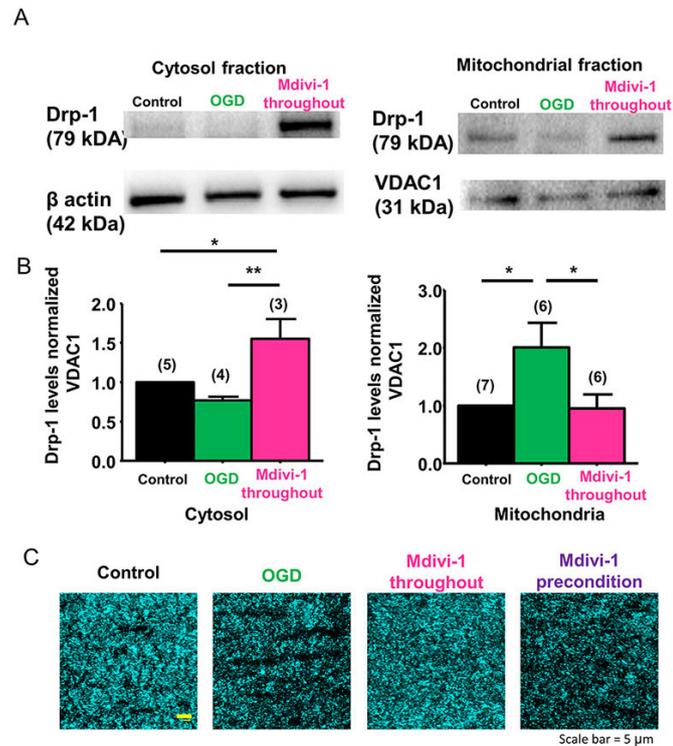


Figure 1: Drp-1 regulates ischemia-induced mitochondrial fission in WM. (A, B) OGD suppressed Drp-1 levels in MONs in the cytosolic fraction ($0.7 \pm 0.04\%$, $n = 4$) and revealed a corresponding increase in the mitochondrial fraction ($2.0 \pm 0.4\%$, $n = 6$; control 1.0 ± 0.0 , $n = 7$, $p < 0.05$). Following application of the Drp-1 inhibitor Mdivi-1 ($50 \mu\text{M}$), cytosolic Drp-1 levels increased to $1.55 \pm 0.3\%$, ($n = 3$, $p < 0.05$) and mitochondrial Drp-1 levels dropped to $0.94 \pm 0.2\%$ ($n = 6$, $p < 0.05$). (C) Confocal microscopy imaging showed that OGD drastically reduced CFP fluorescence in MONs from Thy-1 mito CFP (+) mice. Note that mitochondrial numbers declined and mitochondria became smaller and more round with OGD. Mdivi-1 application before, during, and after OGD preserved CFP pixel intensity and mitochondrial morphology; however, Mdivi-1 preconditioning application failed to preserve CFP fluorescence. (Scale bar, $5 \mu\text{m}$). $n =$ number of MONs; $*p < 0.05$, $**p < 0.01$, one-way ANOVA. Error bars indicate SEM.

Inhibition of mitochondrial fission promotes myelinated axon function recovery following ischemia

To assess whether inhibition of mitochondrial fission promoted axon function recovery, the effects of OGD on CAP area recovery with or without Mdivi-1 was tested using different concentrations of Mdivi-1 (Figure 2A). The functional integrity of axons was quantified by the area under the evoked CAPs. After a 30-min baseline recording, Mdivi-1 was introduced at 1, 10, 20, or $50 \mu\text{M}$ concentrations (pink) for 30 min and the superfusion conditions were maintained for 60 min of OGD and the initial 30 min of reperfusion time (Figure 2A and C). After OGD, CAP area recovered to $21.6 \pm 1.8\%$ ($n = 14$, green) of the maximum-recorded CAP area, whereas a lower dose of Mdivi-1 ($1 \mu\text{M}$) improved CAP area recovery to $25.9 \pm 6.4\%$ ($n = 2$, $p < 0.4$) and higher doses (10, 20, and $50 \mu\text{M}$) improved CAP area recovery to $41.8 \pm 4.1\%$ ($n = 2$, $p < 0.02$), $37.4 \pm 23.5\%$

($n = 2$, $p < 0.4$), and $46.4 \pm 10.3\%$ ($n = 4$, $p < 0.003$, one-way ANOVA, Bonferonni's *post hoc* test), respectively (Figure 2A). Application of $50 \mu\text{M}$ Mdivi-1 provided the greatest amount of protection (Figure 2A-C, pink) when applied throughout the procedure (30 min before, 60 min during, and 30 min after the end of OGD; Figure 2C, pink time course and inset histograms). As expected, this functional recovery correlated with inhibition of mitochondrial fission and consequent preservation of mitochondrial integrity (Figure 1C, Mdivi-1 throughout).

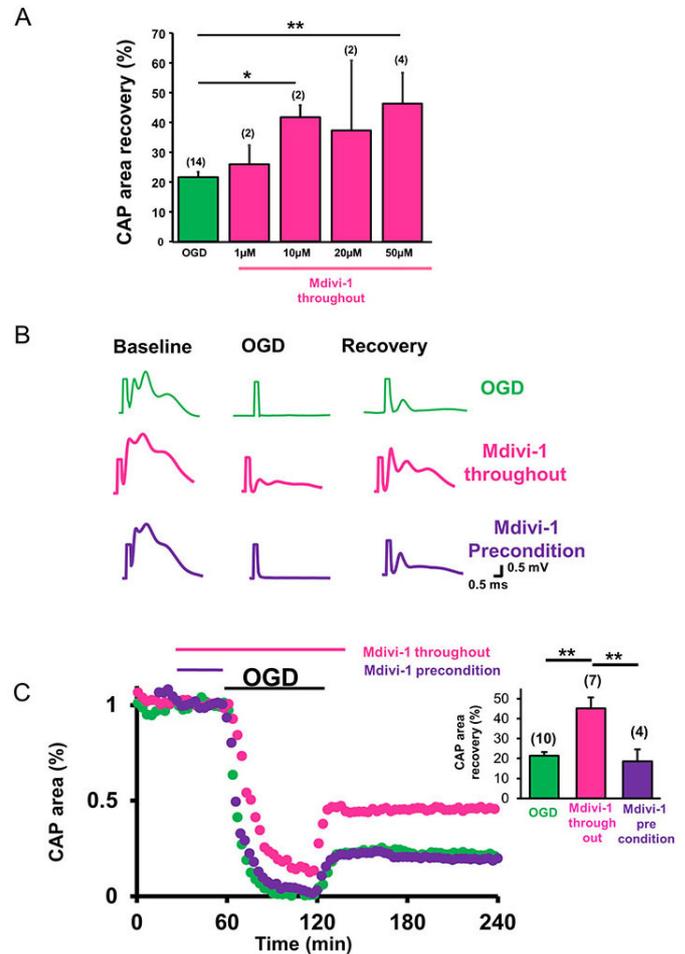


Figure 2: Inhibition of mitochondrial fission promotes WM function after ischemia. (A) Mdivi-1 exerts dose-dependent protection of axon function recovery in MONs following OGD. (B) Representative CAP traces at baseline in control ACSF (a), 60 min OGD (b), and recovery (c) conditions are shown for control (green), Mdivi-1 applied as an all-throughout treatment (pink), and Mdivi-1 applied as a preconditioning treatment (purple). (C) CAP area recovered minimally after OGD (green, $21.6 \pm 1.8\%$, $n = 10$). Mdivi-1 all-throughout application (pink) preserved CAP area during OGD and improved CAP area recovery during reperfusion ($41 \pm 5.0\%$, $n = 7$). Mdivi-1 preconditioning treatment (purple) failed to promote axon function recovery ($18.6 \pm 6.0\%$, $n = 4$). Pink and purple horizontal lines represent Mdivi-1 all-throughout and preconditioning treatment applications, respectively. The inset histograms show quantification of CAP area recovery 2 h after OGD. MONs pretreated with Mdivi-1 (pink) showed improved axon function recovery, whereas the CAP area recovery failed to improve when MONs were preconditioned with Mdivi-1 (purple). $n =$ number of MONs; $*p < 0.05$, $**p < 0.01$, one-way ANOVA. Error bars indicate SEM.

Inhibition of mitochondrial fission fails to precondition myelinated axons against ischemia

Mitochondria have been implicated in neuroprotective signaling underlying preconditioning and represent a promising target in various neurodegenerative diseases (Correia et al., 2010). It was proposed that low levels of mitochondrial ROS

generation induces fission and fusion of mitochondria and alters mitochondrial motility and dynamics to form a mitochondria-free gap, thereby preventing propagation of ROS during oxidative injury to precondition the tissue (Jou, 2008; Correia et al., 2010). We reasoned that by inhibiting mitochondrial fission, Mdivi-1 can regulate mitochondrial motility and subsequently precondition WM to promote axon function recovery as previously reported in GM (Ravati et al., 2000, 2001; Dirnagl and Meisel, 2008; Jou, 2008; Dirnagl et al., 2009). To determine whether inhibition of mitochondrial fission was effective in promoting axon function recovery when applied before the onset of OGD, Mdivi-1 was applied for 30 min after obtaining a stable baseline and then OGD was induced in normal ACSF (Figure 2B and C, purple traces and time course). OGD predictably suppressed the CAP area completely (Figure 2B, purple traces), and preconditioning with Mdivi-1 (50 μ M) failed to promote axon function recovery (Figure 2B and C, purple time course and inset). Consistent with these results, Mdivi-1 application before OGD failed to prevent mitochondrial fission or to preserve mitochondrial size and shape (Figure 1C, right panel).

Mitochondrial fission alters mitochondrial motility

Mitochondria underwent extensive fission during ischemia, but whether mitochondrial fragmentation correlates with loss of motility and whether preservation of mitochondrial motility correlates with axon function recovery were still unanswered questions. Time-lapse live imaging of mitochondria showed that mitochondria move bi-directionally, change direction, or become stationary in response to OGD (Figure 3). Optic nerves were placed and stabilized for 15 min in a perfusion chamber and continuously superfused with oxygenated ACSF (95% O₂/5% CO₂) at a rate of 3 ml/min at 37°C. Once an area was chosen where mitochondria in myelinated axons were clearly visible, mitochondria were imaged every 1.5 s. These mitochondria were observed to be very dynamic organelles that underwent fission and fusion constantly. Coordinated motility of mitochondria ensures that metabolically active areas are adequately supplied with ATP such that injured mitochondria are replaced with healthy ones during injury. Motile and stationary mitochondria were identified using kymographs (Figure 3A), which represent 2-dimensional images of stationary and motile mitochondria during the entire time-lapse sequence. Vertical lines represent the stationary mitochondria (Figure 3A), while oblique lines represent motile mitochondria (Figure 3A). Specifically, blue lines denote mitochondria moving in the anterograde direction and tan lines indicate mitochondria moving in the retrograde direction. Note that a small but similar number of mitochondria moved in either direction (Figure 3A, tan and blue lines), while the majority remained stationary (Figure 3A, vertical lines). The size of mitochondria in young myelinated axons varies between 1-8 μ m and the average mitochondrial velocity varies between 0.6 - 1.7 μ m/s (Stahon et al., 2016). Mitochondria maintained a stable speed under control conditions. Onset of OGD caused a 50% drop in mitochondrial motility both in the anterograde (Figure 3B and 3C, blue histograms, OGD 0.57 ± 0.03 , n = 157 vs. Baseline 1.0 ± 0.02 , n = 318, p < 0.001, One-way ANOVA, Bonferonni's *post hoc* test) and retrograde directions (Figure 3B and 3C, tan histograms, OGD 0.48 ± 0.03 , n = 95 vs. Baseline 1.0 ± 0.03 , n = 243, p < 0.001, one-way ANOVA, Bonferonni's *post hoc* test). Recovery in the anterograde direction was not different (Figure 3B and 3C, blue histograms, Recovery 0.71 ± 0.04 , n = 139, p > 0.05, one-way ANOVA, Bonferonni's *post hoc* test). Interestingly recovery in the retrograde direction was different (Figure 3B and 3C, tan histograms, Recovery 0.73 ± 0.03 , n = 162, p < 0.001, one-way ANOVA, Bonferonni's *post hoc* test). Mdivi-1 caused a prominent increase in

mitochondrial motility in both directions within 10 min of application (Figure 3B, pink histograms, one-way ANOVA, p < 0.001), suggesting that inhibition of mitochondrial fission enhanced mitochondrial transport within myelinated axons. Note that the baseline effect of Mdivi-1 was more prominent in the anterograde direction (Figure 3B **Anterograde**, pink histograms with blue border, Mdivi-1 throughout Baseline 1.60 ± 0.09 , n = 68, p < 0.001) when compared to retrograde direction (Figure 3B **Retrograde**, pink histograms with tan border, Mdivi-1 throughout Baseline 1.39 ± 0.08 , n = 63, p < 0.001, one-way ANOVA, Bonferonni's *Post-test*). This increase in mitochondrial motility persisted during OGD (60 min) (Figure 3B **Anterograde**, pink histograms with blue border, Mdivi-1 throughout OGD 0.89 ± 0.06 , n = 53, vs. OGD 0.57 ± 0.03 , n = 157, p < 0.001; **Retrograde**, pink histograms with tan border, Mdivi-1 throughout OGD 0.87 ± 0.04 , n = 69, vs. OGD 0.48 ± 0.03 , n = 95, p < 0.001, one-way ANOVA, Bonferonni's *post hoc* test) and recovery (20 min) (Figure 3B **Anterograde**, pink histograms with blue border, Mdivi-1 throughout Recovery 0.99 ± 0.03 , n = 72, vs. Recovery 0.71 ± 0.04 , n = 139, p < 0.001; **Retrograde**, pink histograms with tan border, Mdivi-1 throughout Recovery 0.95 ± 0.05 , n = 55, vs. Recovery 0.73 ± 0.03 , n = 162, p < 0.05, one-way ANOVA, Bonferonni's *post hoc* test) in both directions. Together with the electrophysiology results, these findings suggest that application of Mdivi-1 promotes axon function recovery and preserves mitochondrial motility by preventing mitochondrial fission during ischemia.

To confirm that preservation of mitochondrial motility predicts the extent of axon function recovery, we applied Mdivi-1 just before OGD for 30 min. Live-imaging of mitochondria showed a similar increase in mitochondrial motility in both directions upon application of Mdivi-1 (Figure 3C **Anterograde**, purple histograms with blue border, Mdivi-1 throughout Baseline 1.60 ± 0.09 , n = 68, vs. Baseline 1.0 ± 0.02 , n = 318, p < 0.001; Figure 3B **Retrograde**, purple histograms with tan border, Mdivi-1 throughout Baseline 1.39 ± 0.08 , n = 63, vs. Baseline 1.0 ± 0.03 , n = 243, p < 0.001, one-way ANOVA, Bonferonni's *post hoc* test). However, mitochondrial motility was suppressed during OGD (Figure 3C **Anterograde**, purple histograms with blue border, Mdivi-1 throughout OGD 0.74 ± 0.03 , n = 62, vs. OGD 0.57 ± 0.03 , n = 157, p > 0.05; **Retrograde**, purple histograms with tan border, Mdivi-1 throughout OGD 0.66 ± 0.04 , n = 53, vs. OGD 0.48 ± 0.03 , n = 95, p > 0.05, one-way ANOVA, Bonferonni's *post hoc* test) and did not improve during the recovery period (Figure 3C **Anterograde**, purple histograms with blue border, Mdivi-1 throughout Recovery 0.76 ± 0.05 , n = 42, vs. Recovery 0.71 ± 0.04 , n = 139, p > 0.05; **Retrograde**, purple histograms with tan border, Mdivi-1 throughout Recovery 0.72 ± 0.05 , n = 38, vs. Recovery 0.73 ± 0.03 , n = 162, p > 0.05, one-way ANOVA, Bonferonni's *post hoc* test), which is consistent with failure of axon function recovery. Therefore, we propose that inhibition of mitochondrial fission directs mitochondrial motility, which consequently determines axon function recovery, and that preservation of mitochondrial motility is an indication of improved axon function recovery.

DISCUSSION

Our results showed that inhibition of mitochondrial fission, specifically inhibition of Drp-1 by Mdivi-1, during an ischemic episode modifies mitochondrial motility and determines the extent of axon function recovery in a completely myelinated WM tract, the optic nerve. This protective effect of Mdivi-1 was dose-dependent and correlated with preservation of mitochondrial integrity. While these results propose mitochondrial dynamics as a plausible target to reduce ischemic injury and to restore function in WM, Drp-1 inhibition failed to offer ischemic preconditioning tolerance to myelinated axons,

in contrast to its effect on neuronal cell bodies (Grohm et al., 2012; Zhang et al., 2013b; Wang et al., 2014; Cui et al., 2016). We propose that regulating mitochondrial dynamics is necessary to promote functional recovery, but is not sufficient to provide ischemic tolerance to WM function.

One of the main findings of our study is that inhibition of Drp-1 during ischemia prevents mitochondrial fission, preserves CFP fluorescence, sustains axon conduction, and confers a dose-dependent promotion of axon function recovery. In myelinated axons, an ischemic episode causes widespread mitochondrial fission mediated by translocation of cytoplasmic Drp-1 onto mitochondria. This in turn reduces the ability of mitochondria to produce the ATP that is essential for sustaining the excitability and function of axons (Baltan et al., 2011; Baltan, 2012, 2014b). Consequently, axon function is completely lost during injury (Tekkök et al., 2007; Baltan et al., 2008, 2013; Baltan, 2014a). During recovery, mitochondrial fission became permanent, as characterized by the loss of CFP (+) fluorescence in Thy-1 mito-CFP (+) mice and subsequently a small portion of axons (~20%) regained function (Tekkök et al., 2007; Baltan et al., 2008, 2013; Baltan, 2014a). The reported beneficial effects on neurons and improved functional recovery in our study make Mdivi-1 an ideal candidate for therapy to restore function when applied during ischemia.

A novel finding in our study was that Mdivi-1 not only prevents mitochondrial fission, but also enhances mitochondrial motility without affecting normal axon function. Mdivi-1 application preserved mitochondrial motility in both the anterograde and retrograde directions during OGD and during recovery, which was associated with improved axon function recovery. This suggests that mechanisms of fission and mitochondrial motility are interconnected. Previous studies have showed that Miro, 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+} , 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 (Russo et al., 2009; Morlino et al., 2014; Melkov et al., 2016) along microtubules. Mdivi-1 preserves ATP levels during ischemia reperfusion injury (Li et al., 2016), thus effectively abolishing impairments to mitochondrial movement in both directions and blocking mitochondrial fission to improve axon function recovery.

An unexpected aspect of our study was the lack of preconditioning-induced tolerance when Mdivi-1 was applied

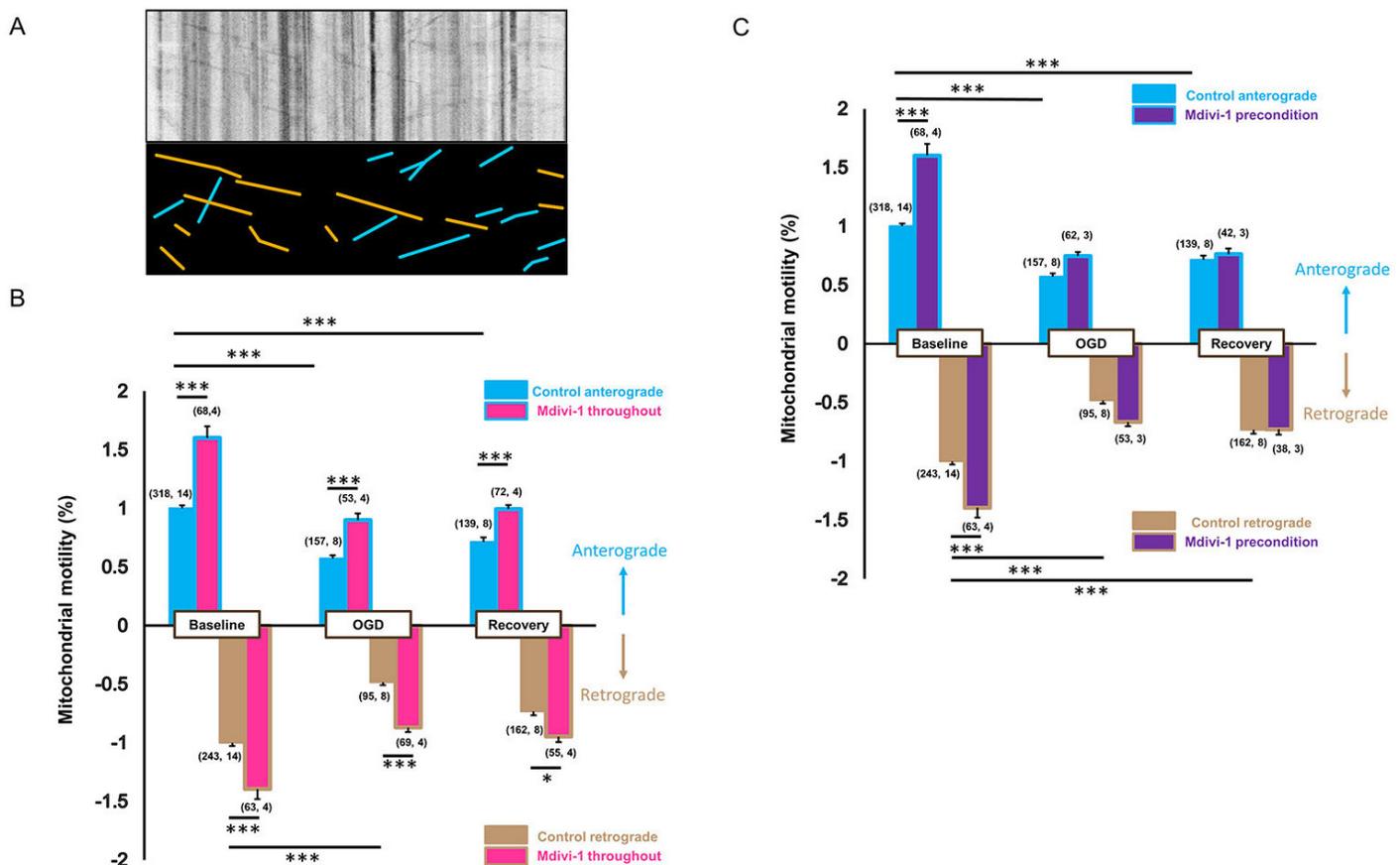


Figure 3: Mdivi-1 preserves mitochondrial motility against ischemia only when applied during OGD. (A) A representative kymograph created from CFP (+) live mitochondrial imaging shows immotile mitochondria as vertical lines. Note the diagonal lines representing mitochondrial movement in axons in the anterograde direction (blue) as well as the retrograde direction (tan). (B) Quantification of mitochondrial motility from kymographs demonstrated a 50% reduction in mitochondrial motility, both in the anterograde (blue histograms) and retrograde directions (tan histograms), which showed minimal recovery in the anterograde direction reperfusion. Mdivi-1 caused a prominent increase in mitochondrial motility in both directions within 10 min of application (pink histograms), suggesting that inhibition of mitochondrial fission enhanced mitochondrial transport within the myelinated axons. Note that the baseline effect of Mdivi-1 was more prominent in the anterograde direction (pink with blue border histograms). This increase in mitochondrial motility persisted during OGD (60 min) and recovery (20 min) in both directions. n = number of MONs; $*p < 0.05$, $***p < 0.001$, One-way ANOVA with Bonferonni's *post hoc* test. (C) Mitochondrial motility analysis showed a similar increase in mitochondrial motility in both directions upon preconditioning application of Mdivi-1 (purple histograms). However, mitochondrial motility was suppressed during OGD and did not improve during the recovery period in either the anterograde or retrograde direction. $***p < 0.001$, One-way ANOVA with Bonferonni's *post hoc* test.

only before injury. Many lines of evidence have posited that mitochondria constitute a convergence point of preconditioning in *in vitro* and *in vivo* models of cerebral ischemia (Perez-Pinzon et al., 2005; Dave et al., 2006). Mdivi-1 is a small molecule that is readily permeant through the blood-brain barrier and it provides ischemic tolerance to neurons by maintaining mitochondrial integrity and function. Previously, we reported that maintaining mitochondrial integrity during or after ischemia provides axon function protection and promotes recovery by conserving ATP production and reducing excitotoxicity (Baltan et al., 2011, 2013; Baltan, 2014b). Among well-established mediators of preconditioning (Barone et al., 1998; Kowaltowski et al., 2000; McLeod et al., 2005; Dave et al., 2006; Cadet et al., 2009; Dirnagl et al., 2009; Marsh et al., 2009; Hirata et al., 2011; Gundimeda et al., 2012; Hibert et al., 2013; Hamner et al., 2015), recent studies link mitochondrial biogenesis, specifically dynamics to preconditioning (Correia et al., 2010). Mdivi-1 application led to a prominent increase in mitochondrial motility during baseline conditions. When Mdivi-1 was applied during OGD, mitochondrial motility was conserved during ischemia and improved further during recovery. This increase in mitochondrial motility, together with preserved mitochondrial integrity, implicated Mdivi-1 application as an ideal candidate to induce ischemic tolerance to WM against injury, comparable to the protection achieved in neurons. However, applying Mdivi-1 only before injury failed to precondition WM against ischemia. This is not due to a general lack of Mdivi-1 effects when applied only before ischemia, since our live imaging results confirm that Mdivi-1 application instantly modifies mitochondrial dynamics, as evidenced by their enhanced motility during the baseline period. This increase in motility is preserved during OGD, albeit to a smaller extent compared to Mdivi-1 application during ischemia. However, the enhanced motility of mitochondria vanishes during the recovery period. Based on the interplay between mitochondrial motility and mitochondrial fission, it is plausible that motile mitochondria are an indicator of better functional recovery. In particular, the extent of mitochondrial motility during recovery directs functional recovery. The downstream molecular mechanisms of this protection are currently under investigation.

In conclusion, this is the first study to investigate whether inhibition of mitochondrial fission induces ischemic preconditioning tolerance to axons in an isolated pure WM tract. Conserving mitochondrial structure sustains mitochondrial motility during ischemia and confers functional protection. However, this approach fails to precondition axon function against ischemia, in contrast to its protective effects on neuronal survival. These findings support the concept that manipulations to precondition the brain should consider interventions to be beneficial for both the gray and white matter.

Acknowledgements

The authors thank the Chris Nelson for his assistance in editing the manuscript. These studies were funded by NIH grants R56AG033720 and R01AG033720 to S.B.

References

Auchampach JA, Grover GJ, Gross GJ (1992) Blockade of ischaemic preconditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc Res* 26:1054–1062.

Balog J, Mehta SL, Vemuganti R (2016) Mitochondrial fission and fusion in secondary brain damage after CNS insults. *J Cereb Blood Flow Metab* 36:2022–2033.

Baltan S (2009) Ischemic injury to white matter: an age-dependent process. *Neuroscientist* 15:126–133.

Baltan S (2012) Histone deacetylase inhibitors preserve

function in aging axons. *J Neurochem* 123:108–115.

Baltan S (2014a) Age-dependent mechanisms of white matter injury after stroke. In: *White matter injury in stroke and CNS disease* (Baltan S, Carmichael ST, Matute C, Xi G, Zhang JH, eds), pp 373–403. New York, NY: Springer US.

Baltan S (2014b) Excitotoxicity and mitochondrial dysfunction underlie age-dependent ischemic white matter injury. *Adv Neurobiol* 11:151–170.

Baltan S, Besancon EF, Mbow B, Ye Z, Hamner MA, Ransom BR (2008) White matter vulnerability to ischemic injury increases with age because of enhanced excitotoxicity. *J Neurosci* 28:1479–1489.

Baltan S, Inman DM, Danilov CA, Morrison RS, Calkins DJ, Horner PJ (2010) Metabolic vulnerability disposes retinal ganglion cell axons to dysfunction in a model of glaucomatous degeneration. *J Neurosci* 30:5644–5652.

Baltan S, Morrison RS, Murphy SP (2013) Novel protective effects of histone deacetylase inhibition on stroke and white matter ischemic injury. *Neurotherapeutics* 10:798–807.

Baltan S, Murphy SP, Danilov CA, Bachleda A, Morrison RS (2011) Histone deacetylase inhibitors preserve white matter structure and function during ischemia by conserving ATP and reducing excitotoxicity. *J Neurosci* 31:3990–3999.

Barone FC, White RF, Spera PA, Ellison J, Currie RW, Wang X, Feuerstein GZ (1998) Ischemic preconditioning and brain tolerance: temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke* 29:1937–50-1.

Brooks C, Wei Q, Cho S-G, Dong Z (2009) Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. *J Clin Invest* 119:1275–1285.

Cadet JL, McCoy MT, Cai NS, Krasnova IN, Ladenheim B, Beauvais G, Wilson N, Wood W, Becker KG, Hodges AB (2009) Methamphetamine preconditioning alters midbrain transcriptional responses to methamphetamine-induced injury in the rat striatum. *PLoS One* 4:e7812.

Correia SC, Carvalho C, Cardoso S, Santos RX, Santos MS, Oliveira CR, Perry G, Zhu X, Smith MA, Moreira PI (2010) Mitochondrial preconditioning: a potential neuroprotective strategy. *Front Aging Neurosci* 2:1–13.

Cui M, Ding H, Chen F, Zhao Y, Yang Q, Dong Q (2016) Mdivi-1 Protects Against Ischemic Brain Injury via Elevating Extracellular Adenosine in a cAMP/CREB-CD39-Dependent Manner. *Mol Neurobiol* 53:240–253.

Dave KR, Saul I, Prado R, Busto R, Perez-Pinzon MA (2006) Remote organ ischemic preconditioning protect brain from ischemic damage following asphyxial cardiac arrest. *Neurosci Lett* 404:170–175.

Deryagin OG, Gavrilova SA, Gainutdinov KL, Golubeva A V., Andrianov V V., Yafarova GG, Buravkov S V., Koshelev VB (2017) Molecular Bases of Brain Preconditioning. *Front Neurosci* 11:427.

Dirnagl U, Becker K, Meisel A (2009) Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use. *Lancet Neurol* 8:398–412.

Dirnagl U, Meisel A (2008) Endogenous neuroprotection: mitochondria as gateways to cerebral preconditioning? *Neuropharmacology* 55:334–344.

Disatnik M-H, Ferreira JCB, Campos JC, Gomes KS, Dourado PMM, Qi X, Mochly-Rosen D (2013) Acute Inhibition of Excessive Mitochondrial Fission After Myocardial Infarction Prevents Long-term Cardiac Dysfunction. *J Am Heart Assoc* 2:e000461–e000461.

Flippo KH, Strack S (2017) Mitochondrial dynamics in

- neuronal injury, development and plasticity. *J Cell Sci* 130:671–681.
- Garlid KD, Dos Santos P, Xie Z-J, Costa ADT, Paucek P (2003) Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K(+) channel in cardiac function and cardioprotection. *Biochim Biophys Acta* 1606:1–21.
- Gidday JM (2006) Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci* 7:437–448.
- Golpich M, Amini E, Mohamed Z, Azman Ali R, Mohamed Ibrahim N, Ahmadiani A (2017) Mitochondrial Dysfunction and Biogenesis in Neurodegenerative diseases: Pathogenesis and Treatment. *CNS Neurosci Ther* 23:5–22.
- Grohm J, Kim S-W, Mamrak U, Tobaben S, Cassidy-Stone A, Nunnari J, Plesnila N, Culmsee C (2012) Inhibition of Drp1 provides neuroprotection in vitro and in vivo. *Cell Death Differ* 19:1446–1458.
- Gundimeda U, McNeill TH, Elhiani AA, Schiffman JE, Hinton DR, Gopalakrishna R (2012) Green tea polyphenols precondition against cell death induced by oxygen-glucose deprivation via stimulation of laminin receptor, generation of reactive oxygen species, and activation of protein kinase C α . *J Biol Chem* 287:34694–34708.
- Guo X, Macleod GT, Wellington A, Hu F, Panchumarthi S, Schoenfield M, Marin L, Charlton MP, Atwood HL, Zinsmaier KE (2005) The GTPase dMiro is required for axonal transport of mitochondria to *Drosophila* synapses. *Neuron* 47:379–393.
- Hamner MA, Ye Z, Lee R V, Colman JR, Le T, Gong DC, Ransom BR, Weinstein JR (2015) Ischemic Preconditioning in White Matter: Magnitude and Mechanism. *J Neurosci* 35:15599–15611.
- Hatch AL, Gurel PS, Higgs HN (2014) Novel roles for actin in mitochondrial fission. *J Cell Sci* 127:4549–4560.
- Hibert P, Prunier-Mirebeau D, Beseme O, Chwastyniak M, Tamareille S, Lamon D, Furber A, Pinet F, Prunier F (2013) Apolipoprotein a-I is a potential mediator of remote ischemic preconditioning. *PLoS One* 8:e77211.
- Hirata N, Shim YH, Pravdic D, Lohr NL, Pratt PF, Weihrach D, Kersten JR, Warltier DC, Bosnjak ZJ, Bienengraeber M (2011) Isoflurane differentially modulates mitochondrial reactive oxygen species production via forward versus reverse electron transport flow: implications for preconditioning. *Anesthesiology* 115:531–540.
- Ishihara N, Nomura M, Jofuku A, Kato H, Suzuki SO, Masuda K, Otera H, Nakanishi Y, Nonaka I, Goto Y-I, Taguchi N, Morinaga H, Maeda M, Takayanagi R, Yokota S, Mihara K (2009) Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. *Nat Cell Biol* 11:958–966.
- Jabůrek M, Yarov-Yarovoy V, Paucek P, Garlid KD (1998) State-dependent inhibition of the mitochondrial KATP channel by glyburide and 5-hydroxydecanoate. *J Biol Chem* 273:13578–13582.
- Jin Z, Wu J, Yan L-J (2016) Chemical Conditioning as an Approach to Ischemic Stroke Tolerance: Mitochondria as the Target. *Int J Mol Sci* 17:351.
- Jou M-J (2008) Pathophysiological and pharmacological implications of mitochondria-targeted reactive oxygen species generation in astrocytes. *Adv Drug Deliv Rev* 60:1512–1526.
- Kim H, Lee JY, Park KJ, Kim W-H, Roh GS (2016) A mitochondrial division inhibitor, Mdivi-1, inhibits mitochondrial fragmentation and attenuates kainic acid-induced hippocampal cell death. *BMC Neurosci* 17:33.
- Kim J-H, Park S, Kim B, Choe Y, Lee D (2017) Insulin-stimulated lipid accumulation is inhibited by ROS-scavenging chemicals, but not by the Drp1 inhibitor Mdivi-1. *PLoS One* 12:e0185764.
- Kowaltowski AJ, Vercesi AE, Fiskum G (2000) Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides. *Cell Death Differ* 7:903–910.
- Li Y, Wang M, Wang S (2016) Effect of inhibiting mitochondrial fission on energy metabolism in rat hippocampal neurons during ischemia/reperfusion injury. *Neurol Res* 6412:1–8.
- Liu J-M, Yi Z, Liu S-Z, Chang J-H, Dang X-B, Li Q-Y, Zhang Y-L (2015) The mitochondrial division inhibitor mdivi-1 attenuates spinal cord ischemia-reperfusion injury both in vitro and in vivo: Involvement of BK channels. *Brain Res* 1619:155–165.
- Marsh BJ, Stevens SL, Hunter B, Stenzel-Poore MP (2009) Inflammation and the emerging role of the toll-like receptor system in acute brain ischemia. *Stroke* 40:S34–7.
- McLeod CJ, Aziz A, Hoyt RF, McCoy JP, Sack MN (2005) Uncoupling proteins 2 and 3 function in concert to augment tolerance to cardiac ischemia. *J Biol Chem* 280:33470–33476.
- Melkov A, Baskar R, Alcalay Y, Abdu U (2016) A new mode of mitochondrial transport and polarized sorting regulated by Dynein, Milton and Miro. *Development* 143:4203–4213.
- Misgeld T, Kerschensteiner M, Bareyre FM, Burgess RW, Lichtman JW (2007) Imaging axonal transport of mitochondria in vivo. *Nat Methods* 4:559–561.
- Mohr, J P, Wolf A Philip, Grotta James, Moskowitz Michael, Mayberg Marc KR (2011) *Stroke: Pathophysiology, Diagnosis, and Management*. Saunders.
- Moncayo J, de Freitas GR, Bogousslavsky J, Altieri M, van Melle G (2000) Do transient ischemic attacks have a neuroprotective effect? *Neurology* 54:2089–2094.
- Morlino G, Barreiro O, Baixauli F, Robles-Valero J, González-Granado JM, Villa-Bellosta R, Cuenca J, Sánchez-Sorzano CO, Veiga E, Martín-Cófreces NB, Sánchez-Madrid F (2014) Miro-1 links mitochondria and microtubule Dynein motors to control lymphocyte migration and polarity. *Mol Cell Biol* 34:1412–1426.
- Murphy SP, Lee RJ, McClean ME, Pemberton HE, Uo T, Morrison RS, Bastian C, Baltan S (2014) MS-275, a class I histone deacetylase inhibitor, protects the p53-deficient mouse against ischemic injury. *J Neurochem* 129:509–515.
- Ong S-B, Subrayan S, Lim SY, Yellon DM, Davidson SM, Hausenloy DJ (2010) Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. *Circulation* 121:2012–2022.
- Park SJ, Park YJ, Shin JH, Kim ES, Hwang JJ, Jin D-H, Kim JC, Cho D-H (2011a) A receptor tyrosine kinase inhibitor, Tyrphostin A9 induces cancer cell death through Drp1 dependent mitochondria fragmentation. *Biochem Biophys Res Commun* 408:465–470.
- Park SW, Kim K-Y, Lindsey JD, Dai Y, Heo H, Nguyen DH, Ellisman MH, Weinreb RN, Ju W-K (2011b) A selective inhibitor of drp1, mdivi-1, increases retinal ganglion cell survival in acute ischemic mouse retina. *Invest Ophthalmol Vis Sci* 52:2837–2843.
- Perez-Pinzon M a, Dave KR, Raval AP (2005) Role of reactive oxygen species and protein kinase C in ischemic tolerance in the brain. *Antioxid Redox Signal* 7:1150–1157.
- Ravati A, Ahlemeyer B, Becker A, Klumpp S, Krieglstein J (2001) Preconditioning-induced neuroprotection is mediated by reactive oxygen species and activation of the transcription factor nuclear factor-kappaB. *J Neurochem* 78:909–919.
- Ravati A, Ahlemeyer B, Becker A, Krieglstein J (2000) Preconditioning-induced neuroprotection is mediated by

- reactive oxygen species. *Brain Res* 866:23–32.
- Reddy PH, Reddy TP, Manczak M, Calkins MJ, Shirendeb U, Mao P (2011) Dynamin-related protein 1 and mitochondrial fragmentation in neurodegenerative diseases. *Brain Res Rev* 67:103–118.
- Reddy PH, Tripathi R, Troung Q, Tirumala K, Reddy TP, Anekonda V, Shirendeb UP, Calkins MJ, Reddy AP, Mao P, Manczak M (2012) Abnormal mitochondrial dynamics and synaptic degeneration as early events in Alzheimer's disease: implications to mitochondria-targeted antioxidant therapeutics. *Biochim Biophys Acta* 1822:639–649.
- Russo GJ, Louie K, Wellington A, Macleod GT, Hu F, Panchumarthi S, Zinsmaier KE (2009) Drosophila Miro is required for both anterograde and retrograde axonal mitochondrial transport. *J Neurosci* 29:5443–5455.
- Saotome M, Safiulina D, Szabadkai G, Das S, Fransson A, Aspenstrom P, Rizzuto R, Hajnóczky G (2008) Bidirectional Ca²⁺-dependent control of mitochondrial dynamics by the Miro GTPase. *Proc Natl Acad Sci U S A* 105:20728–20733.
- Stahon KE, Bastian C, Griffith S, Kidd GJ, Brunet S, Baltan S (2016) Age-Related Changes in Axonal and Mitochondrial Ultrastructure and Function in White Matter. *J Neurosci* 36:9990–10001.
- Stys PK, Waxman SG, Ransom BR (1991) Na⁽⁺⁾-Ca²⁺ exchanger mediates Ca²⁺ influx during anoxia in mammalian central nervous system white matter. *Ann Neurol* 30:375–380.
- Szabadkai G, Duchon MR (2008) Mitochondria: the hub of cellular Ca²⁺ signaling. *Physiology (Bethesda)* 23:84–94.
- Tekkök SB, Ye Z, Ransom BR (2007) Excitotoxic mechanisms of ischemic injury in myelinated white matter. *J Cereb blood flow Metab* 27:1540–1552.
- Valenti D, Rossi L, Marzulli D, Bellomo F, De Rasmio D, Signorile A, Vacca RA (2017) Inhibition of Drp1-mediated mitochondrial fission improves mitochondrial dynamics and bioenergetics stimulating neurogenesis in hippocampal progenitor cells from a Down syndrome mouse model. *Biochim Biophys Acta* 1863:3117–3127.
- van der Bliek AM, Sedensky MM, Morgan PG (2017) Cell Biology of the Mitochondrion. *Genetics* 207:843–871.
- Wakabayashi J, Zhang Z, Wakabayashi N, Tamura Y, Fukaya M, Kensler TW, Iijima M, Sesaki H (2009) The dynamin-related GTPase Drp1 is required for embryonic and brain development in mice. *J Cell Biol* 186:805–816.
- Wang J, Wang P, Li S, Wang S, Li Y, Liang N, Wang M (2014) Mdivi-1 prevents apoptosis induced by ischemia-reperfusion injury in primary hippocampal cells via inhibition of reactive oxygen species-activated mitochondrial pathway. *J Stroke Cerebrovasc Dis* 23:1491–1499.
- Wegener S, Gottschalk B, Jovanovic V, Knab R, Fiebach JB, Schellinger PD, Kucinski T, Jungehülsing GJ, Brunecker P, Müller B, Banasik A, Amberger N, Wernecke KD, Siebler M, Röther J, Villringer A, Weih M, MRI in Acute Stroke Study Group of the German Competence Network Stroke (2004) Transient ischemic attacks before ischemic stroke: preconditioning the human brain? A multicenter magnetic resonance imaging study. *Stroke* 35:616–621.
- Wu J, Luo X, Thangthaeng N, Sumien N, Chen Z, Rutledge MA, Jing S, Forster MJ, Yan L-J (2017) Pancreatic mitochondrial complex I exhibits aberrant hyperactivity in diabetes. *Biochem Biophys reports* 11:119–129.
- Xie N, Wang C, Lian Y, Zhang H, Wu C, Zhang Q (2013) A selective inhibitor of Drp1, mdivi-1, protects against cell death of hippocampal neurons in pilocarpine-induced seizures in rats. *Neurosci Lett* 545:64–68.
- Yao Z, Gross GJ (1993) Role of nitric oxide, muscarinic receptors, and the ATP-sensitive K⁺ channel in mediating the effects of acetylcholine to mimic preconditioning in dogs. *Circ Res* 73:1193–1201.
- Zala D, Hinckelmann MV, Saudou F (2013) Huntingtin's Function in Axonal Transport Is Conserved in *Drosophila melanogaster*. *PLoS One* 8:1–10.
- Zhang K, Sejnowski TJ (2000) A universal scaling law between gray matter and white matter of cerebral cortex. *Proc Natl Acad Sci U S A* 97:5621–5626.
- Zhang N, Wang S, Li Y, Che L, Zhao Q (2013a) A selective inhibitor of Drp1, mdivi-1, acts against cerebral ischemia/reperfusion injury via an anti-apoptotic pathway in rats. *Neurosci Lett* 535:104–109.
- Zhang X, Yan H, Yuan Y, Gao J, Shen Z, Cheng Y, Shen Y, Wang R-R, Wang X, Hu W-W, Wang G, Chen Z (2013b) Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. *Autophagy* 9:1321–1333.
- Zuo W, Yang PF, Chen J, Zhang Z, Chen NH (2016) Drp-1, a potential therapeutic target for brain ischaemic stroke. *Br J Pharmacol* 173:1665–1677.
- Zuo W, Zhang S, Xia C-Y, Guo X-F, He W-B, Chen N-H (2014) Mitochondria autophagy is induced after hypoxic/ischemic stress in a Drp1 dependent manner: the role of inhibition of Drp1 in ischemic brain damage. *Neuropharmacology* 86:103–115.