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# **Age-dependent modifications of axons, mitochondrial dynamics, and Ca<sup>2+</sup> homeostasis underlie the vulnerability of aging white matter to ischemia**

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## **Abstract**

Aging white matter (WM) is increasingly susceptible to neurodegenerative diseases and stroke. Among others, changes in ATP production, mitochondrial dynamics, and Ca<sup>2+</sup> homeostasis contribute to increased susceptibility of aging WM to stroke. We utilized a pure white matter tract, mouse optic nerve (MON), obtained from 2- and 12-month old mice to quantify axon structure and function using electrophysiology, 3D-EM, immunoblots, and ATP assays. Functionally, aging axons did not recover as well after ischemia compared to young axons. Structurally, aging axons became thicker with lower G ratios, indicating increased myelin thickness, as well as increased internodal distances and nodal lengths. Aging axonal mitochondria were larger and thicker with lower incidence of smooth endoplasmic reticulum (SER) association. An increase of mitochondrial fusion proteins with aging resulted in aggregation of mitochondria and together with lower ATP levels suggested mitochondrial dysfunction. Our results suggest that aging alters axonal and mitochondrial structure and function resulting from reduced ATP production that may disrupt Ca<sup>2+</sup> homeostasis to underlie the vulnerability of aging WM to ischemia and neurodegenerative diseases.

## **Introduction**

Stroke is the fourth leading cause of death in the United States, on average killing one person every four minutes (Go et al., 2014). About 87% of all strokes are ischemic strokes, in which blood flow to the brain is blocked, depriving the brain of oxygen and glucose (Go et al., 2014). Aging is a significant risk factor for stroke. It has been previously thought that age-related cardiovascular changes, such as atherosclerosis, would account for the increased risk of stroke with age. [However, intrinsic](#) changes to the central nervous system itself contribute to this increased risk (Baltan et al., 2008).

White matter is injured in most strokes (Baltan et al., 2008). The white matter contains no neural soma and is composed of myelinated axons, oligodendrocytes, and

astrocytes (Baltan, 2009). Scientists have widely used rodents to study ischemic injury, but this method underestimates deficits due to injury of the white matter. Half of the human brain is composed of white matter, compared to about 15% of the rodent brain (Zhang and Sejnowski, 2000). Failure to protect the white matter may be one of the primary reasons that clinical drug trials specifically aimed at protecting neurons, have failed (Baltan et al., 2008). Mouse optic nerve (MON), a pure white matter tract, has been used extensively to study ischemic injury in white matter (Agrawal and Fehlings, 1996; Tekkök and Goldberg, 2001), and has been shown to be sensitive to the aging process making it a useful model for this study (Cavallotti et al., 2002).

Previous studies have shown that aging white matter is increasingly vulnerable to ischemia (Baltan et al., 2008). When aging MONs were stimulated under baseline conditions, they exhibited a larger compound action potential (CAP) area, indicating a higher response to stimulation compared to young MONs (Baltan et al., 2008). When exposed to oxygen glucose deprivation (OGD) for 30, 45, and 60 minutes, aging MONs recovered less of their compound action potential compared to young (Baltan et al., 2008). These findings suggest that there are age-dependent changes in the white matter structure and function that would allow for a higher response to stimulation, but a limited ability to recover after a stress, such as ischemia.

The MON is a dense bundle of myelinated axons that transmit electrical impulses. Oligodendrocytes myelinate the internodal distances of axons by spirally wrapping the oligodendrocytic plasma membrane around the axon. As the turns of myelin ends, the sheath becomes thinner, and eventually stops at the node of Ranvier. The nodes of Ranvier separate successive internodal lengths of axons. In general, the large diameter of the axon

correlates with thicker myelin sheaths and longer internodes (Peters, 2009). The thickness of myelin sheaths is measured by G-ratios, which is a ratio of the axon diameter to the myelinated axon diameter (Peters et. al., 2001). Increased myelination provides insulation for axons to conduct faster action potentials.

Axons require a vast supply of energy in the form of ATP, provided by mitochondria, for proper functioning. ATP is also necessary for axons to recover after being exposed to stress, such as ischemia. Mitochondria are dynamic organelles constantly undergoing the processes of fusion and fission. Fusion and fission have been shown to be essential for an extensive exchange of proteins between mitochondria (Liu et al., 2009). Closely interacting with the mitochondria is the smooth endoplasmic reticulum (SER). SER carries out many important cellular functions, such as intracellular storage of  $Ca^{2+}$  and lipid synthesis (Rowland and Voeltz, 2012). The importance of the physical contacts between the SER and mitochondria or the mitochondria-associated membrane has recently gained attention for a potential role in the proper functioning of axons (Rowland and Voeltz, 2012).  $Ca^{2+}$  released from the SER to the mitochondria has been shown to be important for proper mitochondrial function and dynamics, as well as regulation of apoptosis (Rizzuto et al., 1998).

The synergistic activity of mitochondria and SER plays an important role in axon functioning by regulating energy production and  $Ca^{2+}$  homeostasis. We propose that changes to axon size, myelin thickness, internodal distances, nodal lengths, mitochondrial size, fusion and fission proteins, ATP levels, and mitochondrial interaction with SER in aging WM results in vulnerability to damage from stress, such as ischemia, as well as inability to recover following stress.

## **Materials and Methods**

### *Animals*

All experiments were conducted using mouse optic nerves isolated from 2- and 12-month old C57BL6/J mice (Jackson Laboratory) representing young and aging groups, respectively. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic Foundation.

### *3-D Scanning Electron Microscopy*

All chemicals used in the preparation of optic nerves for electron microscopy were purchased from Electron Microscopy Sciences (Hatfield, PA).

Young and aging animals (n=3 for each group) were anesthetized using isoflurane and perfused intracardially with 0.1 M sodium cacodylate buffer containing 4% paraformaldehyde and 2.5% glutaraldehyde. MONs were isolated and washed in cold buffer 3 times. The samples were processed in the following sequence: 2% osmium tetroxide/potassium ferrocyanide for 1 hour, 1% thiocarbonylhydrazide for 20 minutes, and 2% osmium tetroxide solution for 30 minutes, washing with ddH<sub>2</sub>O every 10 minutes in between. Following overnight incubation in saturated uranyl acetate solution, the MONs were washed in three successive portions of ddH<sub>2</sub>O and incubated in lead aspartate solution (0.066g lead nitrate in 10 mL of 0.03 M aspartic acid, pH 5.5) at 60°C for 30 minutes. The samples were again rinsed three times with ddH<sub>2</sub>O. The samples were then dehydrated by an ethanol gradient of freshly prepared ice cold solutions at the following concentrations: 20%, 60%, 80%, 90%, 95% each once, and three times with anhydrous 100% ethanol. Dehydrated MONs were incubated in anhydrous ice-cold acetone for 10 minutes on ice and another 10 minutes at room temperature. Finally, the MONs were

embedded in Epon resin formulated as [10mL EMBed-812, 8 mL DDSA (Dodeceny Succinic Anhydride), 4 mL NMA (Methyl-5-Norbornene-2,3-Dicarboxylic Anhydride) and 0.4 mL DMP-30 (2,4,6-Tri(Dimethylaminomethyl)-Phenol)], first in a 50% solution, then a 100% solution in a mold.

From the resin embedded tissue, a sample block of 0.5 mm x 0.5 mm x 0.5 mm was trimmed using a razor blade and placed on a pin. The MON pin was viewed on a Zeiss serial-blockface scanning electron microscope (3D-SSEM), and 100 nm sections were cut with the diamond knife. The sections were imaged at 4000X magnification with aperture set at 30  $\mu$ m to create an image stack of approximately 300-400 images. The acquired images were in 16 bit .dm3 form, which were converted to 8 bit images for analysis using Fiji (ImageJ) software. After converting to 8 bit, the image stack underwent linear alignment with SIFT (Schindelin et al., 2012). Simple macros, written by Graham Kidd, Ph.D. (Cleveland Clinic Foundation, Dept. of Neuroscience), were used to randomly select axons from the stack for blinded 3D analysis.

### *3-D Reconstruction*

Each image stack was analyzed using Reconstruct and ImageJ software. The image sequence (8 bit .tiff images) was imported into a new series in Reconstruct. The parameters for pixel size and thickness were set to match the imaging parameters. The palette was used to set the specific name and colors for axons and mitochondria. The axons were traced using the *wildfire tool*, but manually corrected. The mitochondria, as well as myelin, were manually traced using the *pencil*. Each trace with the same name was considered an object, and therefore a 3D model could be generated for axons and mitochondria. The *Z trace tool* was used to measure the length of the axons and

mitochondria, as well as internodal and nodal distance, by manually drawing a line through the center of the object. Reconstruct calculated the volume of the objects based on the image parameters. The *object* and *z trace* lists (containing volume and length information) were exported from Reconstruct and imported into Microsoft excel. Corrected volume, axon diameter, g-ratio, and mitochondria per unit volume were calculated. Twenty axons and their myelin, mitochondria, internodal distances, and nodal lengths were traced for each animal.

Each image stack was then opened in Fiji and inverted for better contrast in order to analyze the SER network. After enhancing the contrast, randomly selected 10  $\mu\text{m}$  sections of axons were isolated from the image stack. Three separated stacks were created for the same area of interest. One was used to trace the axons, mitochondria, and SER; one contained the traced mitochondria and SER; and one contained the traced axon. The first stack was left in 8-bit format, but the other two were converted to RGB format, so that they could be traced. A macro, written by Graham Kidd, Ph.D., was used to trace the SER using the *paint tool* of Fiji. The SER proximity macro was used to generate a color-coded image, as well as generate information about the proximity of SER to mitochondria and the plasma membrane. In the proximity stack, the blue SER represents SER near the plasma membrane; the yellow is near the mitochondria; the white is near the plasma membrane and mitochondria; and the green SER is cytosolic.

#### *Statistical Methods*

Statistical analyses were completed using Graphpad Prism (version 4.0c). Young and aging data were compiled and compared using Student's unpaired t-tests for normally distributed data. For non-normally distributed data, non-parametric tests such as Mann-

Whitney's test and Kruskal–Wallis test were used to compare medians. P-values are reported with 95% confidence.

## Results

Aging white matter is increasingly susceptible to damage from stress, such as ischemia (Baltan et al., 2008). In electrophysiology studies, aging MONs exhibit a higher CAP area ( $2.79 \pm 0.22$  mV.ms;  $n=8$ ) when normalized to young MONs ( $1.00 \pm 0.17$  mV.ms;  $n=10$ ) (student's unpaired t-test,  $p < 0.0001$ ), indicating a larger response of axons from stimulation (Figure 1A-B). However, when the MONs are exposed to OGD for one hour, aging MONs recover only  $10\% \pm 1.5\%$  ( $n=7$ ) of their CAP area compared to a  $22.9\% \pm 3.4\%$  ( $n=7$ ) recovery of young MONs (student's unpaired t-test,  $p = 0.0047$ ) (Figure 1C-D). In order to determine the mechanisms behind this discrepancy, the ultrastructural structures of axons, mitochondria and SER, were examined using 3D-SSEM.

Aging axons (median  $1.1 \mu\text{m}^3/\mu\text{m}$ ; mean  $1.5 \pm 0.1 \mu\text{m}^3/\mu\text{m}$ ;  $n=60$ ) are 50% larger in volume compared to young (median  $0.8 \mu\text{m}^3/\mu\text{m}$ ; mean  $1.0 \pm 0.1 \mu\text{m}^3/\mu\text{m}$ ;  $n=60$ ) (Mann-Whitney test,  $p = 0.0021$ ) (Figure 2A-C). This increase in volume is attributed to an increase in the thickness, quantified as axon diameter, of aging axons (median  $0.9 \mu\text{m}$ ; mean  $1.0 \pm 0.0 \mu\text{m}$ ,  $n=1041$ ) compared to young axons (median  $0.8 \mu\text{m}$ ; mean  $0.9 \pm 0.0 \mu\text{m}$ ;  $n=1260$ ) (Mann-Whitney test,  $p < 0.0001$ ) (Figure 3A-C). Accompanying the larger diameter of aging axons is a decrease in their G-ratios (Figure 3E), which is indirectly proportional to myelin thickness. When splitting the axons into three groups based upon axon cross-sectional area (small, medium, and large), there was a significant increase in myelin thickness of aging axons in the small (median  $0.67$ ; mean  $0.67 \pm 0.00$ ;  $n=215$ ) and medium (median  $0.69$ ; mean  $0.69 \pm 0.00$ ;  $n=469$ ) groups compared to young small (median  $0.70$ ; mean  $0.70 \pm 0.00$ ;



n=315) and medium (median 0.72; mean  $0.72\pm 0.00$ ; n=630) axons (Mann-Whitney test,  $p<0.0001$ ). Due to an increase in volume and thickness, as well as myelin thickness, the aging axons (median 14.0; mean  $13.8\pm 0.7$ ; n=12) are 25% less dense within the optic nerve than young axons (median 18.5; mean  $19.7\pm 1.3$ ; n=12)(Mann-Whitney test,  $p=0.0002$ )(Figure 3D-E), which was calculated by axons per unit area within a cross section.

The increased axon diameter correlated with a 25% increase of internodal distances in aging axons (median median 153.4  $\mu\text{m}$ ; mean  $155.7\pm 6.7$   $\mu\text{m}$ ; n=61) compared to young (median 122.9  $\mu\text{m}$ ; mean  $126.4\pm 5.5$   $\mu\text{m}$ ; n=62)(Mann-Whitney test,  $p=0.0027$ )(Figure 4A-C). Nodal length was also increased in aging axons (median 2.4  $\mu\text{m}$ ; mean  $2.6\pm 0.1$   $\mu\text{m}$ ; n=121) compared to young (median 2.2  $\mu\text{m}$ ; mean  $2.2\pm 0.1$   $\mu\text{m}$ ; n=121)(Mann-Whitney test,  $p=0.0015$ )(Figure 4A-B, D), which also showed correlation with axon diameter (n=121,  $R=0.44169$ ,  $p<0.0001$ )(figure 4E). A correlation was observed between increased internodal distance and nodal length for aging axons (n=121,  $R=0.19764$ ,  $p=0.03$ )(Figure 3F). This trend was not observed in young axons. These axonal changes are consistent with the increased CAP area of aging MONs observed in electrophysiology studies.

Although the aging axons increased in diameter, the number of mitochondria did not compensate for the larger volume (Figure 5A-C). Aging axons (median 0.2; mean  $0.2\pm 0.0$ ; n=60) have 30% fewer mitochondria per unit volume than young axons (median 0.3; mean  $0.3\pm 0.0$ ; n=60)(Mann-Whitney test,  $p<0.0001$ ). The decreased number of mitochondria may be due to the increased size and amount of large (median 0.4  $\mu\text{m}^3$ ; mean  $0.5\pm 0.0$   $\mu\text{m}^3$ ; n=397) and very large (median 1.0  $\mu\text{m}^3$ ; mean  $1.1\pm 0.0$   $\mu\text{m}^3$ ; n=48) aging mitochondria compared to large (median 0.4  $\mu\text{m}^3$ ; mean  $0.4\pm 0.0$   $\mu\text{m}^3$ ; n=194) and very

large (median  $0.8 \mu\text{m}^3$ ; mean  $0.9 \pm 0.0 \mu\text{m}^3$ ;  $n=8$ ) young mitochondria (Mann-Whitney test,  $p < 0.0001$ ) (Figure 5D). Large mitochondria were determined from the 75<sup>th</sup> percentile of young mitochondria, while the 99<sup>th</sup> percentile represented the very large mitochondria. Along with very large mitochondria, aging axons also have very long mitochondria (median  $11.0 \mu\text{m}$ ; mean  $13.3 \pm 0.8 \mu\text{m}$ ;  $n=12$ ) that are not as prevalent in the young axons (median  $9.9 \mu\text{m}$ ; mean  $9.9 \pm 0.2$ ;  $n=8$ ) (Mann-Whitney test,  $p=0.05$ ) (Figure 5E). All three of these findings were confirmed with fluorescent imaging of optic nerves obtained from Thy-1 CFP+/+ C57BL6/J mice, which express cyan fluorescent protein in their neuronal mitochondria (Figure 5F-G).

The increased size of aging mitochondria is attributed to an imbalance of mitochondria shaping proteins: Mfn-1, Mfn-2, Opa-1, and Dlp-1. Mfn-1 and Mfn-2, fusion proteins of the outer mitochondrial membrane, are increased in aging MONs (Mfn-1:  $1.69 \pm 0.19$ ,  $n=13$ ; Mfn-2:  $1.79 \pm 0.33$ ,  $n=19$ ) when normalized to young MONs (Mfn-1:  $1.00 \pm 0.11$ ,  $n=13$ ; Mfn-2:  $1.00 \pm 0.04$ ,  $n=16$ ) (Student's unpaired t-test, Mfn-1:  $p=0.003$ , Mfn-2:  $p=0.024$ ) (Figure 6A-B, E). Opa-1, fusion protein of the inner mitochondrial membrane, is decreased in aging MONs ( $0.64 \pm 0.09$ ,  $n=12$ ) when normalized to young MONs ( $1.00 \pm 0.07$ ,  $n=11$ ) indicating improper fusion of aging mitochondria (Student's unpaired t-test,  $p=0.003$ ) (Figure 6C, E). Dlp-1, mitochondrial fission protein, is also decreased in aging MONs ( $0.70 \pm 0.07$ ,  $n=14$ ) when compared to young MONs ( $1.00 \pm 0.07$ ,  $n=13$ ) (Student's unpaired t-test,  $p=0.005$ ) (Figure 6D-E). The imbalance of mitochondrial shaping proteins disrupts mitochondrial dynamics, which are essential to the proper functioning of mitochondria. Along with this disruption in dynamics, aging MONs ( $66 \pm 9\%$ ,  $n=6$ ) produce 34% less ATP than young ( $100 \pm 6\%$ ,  $n=6$ ) (Student's unpaired t-test,  $p=0.01$ ) (Figure 6F).

Proper mitochondrial dynamics and functioning are dependent on mitochondrial association with the SER network. The SER network of aging axons is greatly reduced, inhabiting 17% less of the axon volume ( $4.7 \pm 0.2\%$ ,  $n=30$ ) than in young axons ( $5.6 \pm 0.2\%$ ,  $n=30$ ) (Student's unpaired t-test,  $p=0.0076$ ) (Figure 7A-C). 50% less of the SER network is associated with mitochondria in aging axons (median 4.6%; mean  $5.0 \pm 0.7\%$ ;  $n=24$ ) than young axons (median 7.2%; mean  $9.1 \pm 1.1\%$ ;  $n=29$ ) (Mann-Whitney test,  $p=0.01$ ) (Figure 7A-B, D-F). This decrease in mitochondrial-associated SER may disrupt the  $Ca^{2+}$  homeostasis of aging axons. Age-dependent changes to the mitochondria and its associated SER network may contribute to aging WM's decreased recovery following ischemic stress.

## **Discussion**

Previous studies have shown that aging white matter is increasingly vulnerable to ischemic stress due to changes in the central nervous system (Baltan et al., 2008). Here we show that ultrastructural changes to the axons, mitochondria and its associated SER network may underline this increased vulnerability.

Previous electrophysiology studies (Baltan et al., 2008) have shown that aging MONs exhibit an increased CAP area compared to young MONs. The axon diameter and thickness of the myelin sheath have been shown to determine the velocity of action potential propagation, which can be used as criteria for determining the electrical function of a neuron (Colello et al. 1994). Aging results in thicker myelin sheaths because oligodendrocytes remain active during aging, adding layers to the myelin sheath increasing its thickness. Oligodendrocytes also play a role in regulating the axon diameter, so as the axons are being myelinated, they are also growing in volume (Colello et al. 1994). Not only are aging axons larger in volume and thickness, but they are also insulated by a thicker

myelin sheath resulting in a faster saltatory conduction in aging MONs than young. A longer distance between nodes is also advantageous, because the action potential quickly jumps between nodes. If nodes are farther spread out along the axon, the action potential will travel faster. Nodal regions require much of the energy produced in axons for the proper functioning of ion pumps, such as  $\text{Na}^+/\text{K}^+$ -ATPase. Increased internodal distances may be an adaptation resulting from decreased ATP production. The faster saltatory conduction of aging axons may result in more efficient electrical function under normal conditions.

Although aging axons have a larger response under control conditions, they recover only  $10 \pm 1.5\%$  of their CAP area after 60 minutes of OGD, compared to young axons exhibiting a  $22.9 \pm 3.4\%$  recovery. Due to lower ATP levels, aging axons adapt their structure and function for more efficient conduction. Although these adaptations are advantageous under normal conditions, age dependent ultrastructural changes of mitochondria and its associated SER network may contribute to the decreased ability of aging WM to recover after ischemia.

Although aging axons have grown in volume, they have not subsequently increased their number of mitochondria. Aging mitochondria are very large and long, which may decrease movement compared to shorter mitochondria (Misgeld et. al., 2007). Due to axons' extensive length, movement of mitochondria is critical for their distribution in order to sufficiently supply local ATP, as well as buffer  $\text{Ca}^{2+}$ , along with other mitochondrial functions.

Aging mitochondria are visibly undergoing increased fusion and decreased fission. Mitochondria are primarily solitary units that periodically undergo fusion followed by

**Comment [BS1]:** Do you think this is due to lower ATP levels so axon function and structure adapted to more efficient conduction.

fission in order to share materials, such as proteins. The fission event can be asymmetrical due to a difference in the daughter membrane potentials, which can be utilized to sort defective mitochondria (Youle and van der Bliek, 2012). Opposing trends in fusion and fission can be used to mediate stress. Increased fusion and decreased fission helps to overcome low levels of stress that occur with aging (van der Bliek et al., 2013). This mechanism may be beneficial under normal aging conditions because mixing with the contents of functional mitochondria can restore the function of partially defective mitochondria.

Normally outer membrane fusion is coordinated with inner membrane fusion, but in some instances outer membrane fusion can still occur without inner membrane fusion. Outer membrane fusion is regulated by inactivation through ubiquitin-mediated degradation, but inner membrane fusion is mediated by proteolytic cleavage at a few sites (van der Bliek et al., 2013). In mammalian cells, there is an S1 site present in all isoforms of Opa-1, which is cleaved when there are low levels of ATP. This inducible cleavage is mediated by Oma1, a small intermembrane protease, which subsequently prevents inner membrane fusion by Opa-1 and may cause changes to the cristae architecture. The decrease in ATP levels observed in aging WM may contribute to the decreased levels of Opa-1. The changes to the cristae architecture may result in further decreased ATP production by the mitochondria, contributing to aging WM's decreased recovery following ischemic stress. Swelling of cristae architecture could also stimulate reactive oxygen species production (Schafer et al. 2006). An increase in reactive oxygen species has been shown as a source of the damage resulting from ischemia.

Decreased fission of aging mitochondria may not only be due to the decreased amount of Dlp-1, but also the decreased association of SER with the mitochondria in aging axons. Dlp-1 has been shown to also play a role in SER distribution (Pitts et. al., 1999). The decrease of Dlp-1 may also contribute to the decrease of mitochondrial associated SER. Recent discoveries have shown that ER tubules often associated with mitochondria at points of fission, wrapping around the mitochondria before fission occurs (Friedman et al., 2011). During times of high levels of stress, mitochondria tend to exhibit increased fission and decreased fusion. With a decreased amount of Dlp-1 and mitochondria-associated SER, aging mitochondria may not be able to undergo fission properly during ischemic stress.

The SER associated with mitochondria also plays a critical role in  $Ca^{2+}$  buffering. SER provides intracellular  $Ca^{2+}$  storage. Mitochondrial membrane proteins require  $Ca^{2+}$  binding for functioning, so the release of  $Ca^{2+}$  from the SER is critical to the function of mitochondria.  $Ca^{2+}$  influences mitochondria's ability to make ATP and is also required for fission by stimulating Dlp-1 (Raturi and Simmen, 2013). Mitochondria must be within a small radius of the SER in order to be responsive to the  $Ca^{2+}$  released from the ER, so the decreased amount of SER near the mitochondria may impair  $Ca^{2+}$  homeostasis in aging axons. Regulation of  $Ca^{2+}$  is very important during and following ischemia because of excitotoxicity resulting from increased levels of intracellular  $Na^+$  and  $Ca^{2+}$  concentrations.

### **Conclusion**

Aging WM has undergone changes in order to compensate for the stress of aging. By increasing the axon diameter, myelin thickness, and internodal distance, the axons are able to increase their electrical efficiency, even with a depleted energy supply. However, even though Changes of the mitochondrial dynamics and the SER network are normally

advantageous to aging WM, under ischemic conditions they result in increased vulnerability to damage. Addressing decreases of Opa-1 and Dlp-1, as well as restoring the disrupted the SER network, may be novel pre-treatments for decreasing the vulnerability of aging WM to ischemia.

No conclusions?

?

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## Figure Legends

**Figure 1. Aging axons show higher CAP area but recover less after ischemia.** (A) The baseline CAP area of 2-month-old (young) compared to 12-month-old (aging) MONs obtained from C57BL6/J mice. (B) Aging MONs ( $2.79 \pm 0.22$  mV.ms;  $n=8$ ) exhibit a two times larger CAP area under baseline conditions compared to young ( $1.00 \pm 0.17$  mV.ms,  $n=10$ ).  $***p < 0.0001$ , Student's unpaired t-test. (C-D) The 60 min OGD reduced CAP area recovery of aging ( $10 \pm 1.5\%$ ;  $n=7$ ) compared with young ( $22.9 \pm 3.4\%$ ;  $n=7$ ) MONs.  $**p = 0.0047$ , Student's unpaired t-test. Error bars indicate SEM.

**Figure 2. Aging axons are larger in volume and thicker compared to young.** 3D-Scanning electron micrograph blocks made of 200 slices of 100 nm thickness from young (A) and aging (B) MONs. Scale bar 5  $\mu\text{m}$ . Longitudinal section electron micrographs, 100 nm thick, along with reconstructed axons from young (A) and aging (B) MONs. Scale bar 2  $\mu\text{m}$ . (C) Axon volume per unit length is increased in aging (median  $1.1 \mu\text{m}^3/\mu\text{m}$ ; mean  $1.5 \pm 0.1 \mu\text{m}^3/\mu\text{m}$ ;  $n=60$ ) compared to young (median  $0.8 \mu\text{m}^3/\mu\text{m}$ ; mean  $1.0 \pm 0.1 \mu\text{m}^3/\mu\text{m}$ ;  $n=60$ ) MONs.  $**p = 0.0021$ , Mann-Whitney test.

**Figure 3. Aging axons have thicker myelin and are less dense than young.** Cross section electron micrographs, 100 nm thick, from young (A) and aging (B) MONs. Scale bar 2  $\mu\text{m}$ . (C) Axon diameter is increased in aging (median  $0.9 \mu\text{m}$ ; mean  $1.0 \pm 0.0 \mu\text{m}$ ,  $n=1041$ ) compared to young (median  $0.8 \mu\text{m}$ ; mean  $0.9 \pm 0.0 \mu\text{m}$ ;  $n=1260$ ) MONs.  $***p < 0.0001$ , Mann-Whitney test. (D) The number of axons per unit area is decreased in aging (median 14.0; mean  $13.8 \pm 0.7$ ;  $n=12$ ) compared to young (median 18.5; mean  $19.7 \pm 1.3$ ;  $n=12$ ) MONs.  $***p = 0.0002$ , Mann-Whitney test. (E) G-ratios are increased in aging small (cross section axon area  $< 0.37 \mu\text{m}^2$ ) (median 0.67; mean  $0.67 \pm 0.00$ ;  $n=215$ ) and medium (cross section axon area  $0.37-0.90 \mu\text{m}^2$ ) (median 0.69; mean  $0.69 \pm 0.00$ ;  $n=469$ ) compared to young small (median 0.70; mean  $0.70 \pm 0.00$ ;  $n=315$ ) and medium (median 0.72; mean  $0.72 \pm 0.00$ ;  $n=630$ ) axons, but are not significantly different for large axons (cross section axon area  $> 0.90 \mu\text{m}^2$ ).  $***p < 0.0001$ , Mann-Whitney test.

**Figure 4. Aging axons have longer internodal distances and nodal lengths.** 3D-reconstruction of axon with internodal distance shown with inset of nodal length from a young (A) and an aging (B) MON. (C) Internodal distances are significantly longer in aging (median median 153.4  $\mu\text{m}$ ; mean  $155.7 \pm 6.7 \mu\text{m}$ ;  $n=61$ ) compared to young (median 122.9  $\mu\text{m}$ ; mean  $126.4 \pm 5.5 \mu\text{m}$ ;  $n=62$ ) MONs.  $**p = 0.0027$ , Mann-Whitney test. (D) Nodal lengths are longer in aging (median 2.4  $\mu\text{m}$ ; mean  $2.6 \pm 0.1 \mu\text{m}$ ;  $n=121$ ) compared to young (median 2.2  $\mu\text{m}$ ; mean  $2.2 \pm 0.1 \mu\text{m}$ ;  $n=121$ ) MONs.  $**p = 0.0015$ , Mann-Whitney test. (E) Nodal length increases with axon diameter for 12-month-old MONs ( $R=0.44169$ ,  $n=121$ ).  $***p < 0.0001$ . (F) Internodal distance correlates with nodal length in 12-month-old axons ( $R=0.19764$ ,  $n=121$ ).  $*p = 0.03$ .

**Figure 5. Aging axonal mitochondria are less abundant, but larger in volume.** 3D-reconstruction of an axon with mitochondria from a young (A) and aging (B) MON. Scale bar 2  $\mu\text{m}$ . (C) Number of mitochondria per unit volume is decreased in aging (median 0.2; mean  $0.2 \pm 0.0$ ;  $n=60$ ) compared to young (median 0.3; mean  $0.3 \pm 0.0$ ;  $n=60$ ) axons.  $***p < 0.0001$ , Mann-Whitney test. (D) Aging axons have an increase in the size and amount of large (median  $0.4 \mu\text{m}^3$ ; mean  $0.5 \pm 0.0 \mu\text{m}^3$ ;  $n=397$ ) and very large (median  $1.0 \mu\text{m}^3$ ; mean  $1.1 \pm 0.0 \mu\text{m}^3$ ;  $n=48$ ) mitochondria compared to large (median  $0.4 \mu\text{m}^3$ ; mean  $0.4 \pm 0.0 \mu\text{m}^3$ ;  $n=194$ ) and very large (median  $0.8 \mu\text{m}^3$ ; mean  $0.9 \pm 0.0 \mu\text{m}^3$ ;  $n=8$ ) mitochondria in young axons.  $***p < 0.0001$ , Mann-Whitney test. (E) Aging axons also have very long mitochondria (median  $11.0 \mu\text{m}$ ; mean  $13.3 \pm 0.8 \mu\text{m}$ ;  $n=12$ ) that are not as prevalent in the young axons (median  $9.9 \mu\text{m}$ ; mean  $9.9 \pm 0.2$ ;  $n=8$ ).  $*p = 0.05$ , Mann-Whitney test. Confocal images of young (F) and aging (G) MONs obtained from Thy-1 CFP+/+ C57BL6/J mice show longer, thicker mitochondria with aging. Scale bar 5  $\mu\text{m}$ .

**Figure 6. Aging mitochondria have reduced ATP levels associated with an imbalance of mitochondrial shaping proteins.** Representative western blots for Mfn-1 (A), Mfn-2 (B), Opa-1 (C), and Dlp-1 (D). All protein levels were normalized to  $\beta$ -actin. (E) Quantification of the fold change in protein expression from young to aging MONs. Mfn-1 and Mfn-2 are increased in aging MONs (Mfn-1:  $1.69 \pm 0.19$ ,  $n=13$ ; Mfn-2:  $1.79 \pm 0.33$ ,  $n=19$ ) when normalized to young MONs (Mfn-1:  $1.00 \pm 0.11$ ,  $n=13$ ; Mfn-2:  $1.00 \pm 0.04$ ,  $n=16$ ) (Mfn-1  $**p = 0.003$ , Mfn-2  $*p = 0.024$ ). Opa-1 is decreased in aging MONs ( $0.64 \pm 0.09$ ,  $n=12$ ) when normalized to young MONs ( $1.00 \pm 0.07$ ,  $n=11$ ) ( $**p = 0.003$ ). Dlp-1 is also decreased in aging MONs ( $0.70 \pm 0.07$ ,  $n=14$ ) when compared to young MONs ( $1.00 \pm 0.07$ ,  $n=13$ ) ( $**p = 0.005$ ). Student's unpaired t-test. (F) Aging MONs ( $66 \pm 9\%$ ,  $n=6$ ) produce 34% less ATP than young ( $100 \pm 6\%$ ,  $n=6$ )  $**p = 0.01$ , Student's t-test.

**Figure 7. Aging axons show disruption to the SER network with less association with mitochondria, as well as disrupted SER stress proteins.** 3D reconstruction of the SER network (green) and mitochondria (red) in young (A) and aging (B) axons. 3D reconstruction of SER network distribution: the cytosolic SER (green), plasma membrane associated SER (blue), and the mitochondrial associated SER (yellow). Scale bar 0.5  $\mu\text{m}$ . (C) Aging axons ( $4.7 \pm 0.2\%$ ,  $n=30$ ) have decreased SER network per axon volume compared to young ( $5.6 \pm 0.2\%$ ,  $n=30$ ).  $**p = 0.0076$ , Student's t-test. (D) Less of the SER network is associated with mitochondria in aging axons (median 4.6%; mean  $5.0 \pm 0.7\%$ ;  $n=24$ ) than young axons (median 7.2%; mean  $9.1 \pm 1.1\%$ ;  $n=29$ )  $*p = 0.0103$ , Mann-Whitney test. (E-F) Quantification of the SER network distribution. Mitochondrial associated SER (yellow) is decreased in aging axons compared to young.