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WldS Prevents Axon Degeneration through Increased Mitochondrial Flux and Enhanced Mitochondrial Ca\textsuperscript{2+} Buffering

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Summary

WldS (slow Wallerian degeneration) is a remarkable protein that can suppress Wallerian degeneration of axons and synapses [1], but how it exerts this effect remains unclear [2]. Here, using Drosophila and mouse models, we identify mitochondria as a key site of action for WldS neuroprotective function. Targeting the NAD\textsuperscript{+} biosynthetic enzyme Nmnat to mitochondria was sufficient to fully phenocopy WldS, and WldS was specifically localized to mitochondria in synaptic preparations from mouse brain. Axiotomy of live wild-type axons induced a dramatic spike in axoplasmic Ca\textsuperscript{2+} and termination of mitochondrial movement—WldS potently suppressed both of these events. Surprisingly, WldS also promoted increased basal mitochondrial motility in axons before injury, and genetically suppressing mitochondrial motility in vivo dramatically reduced the protective effect of WldS. Intriguingly, purified mitochondria from WldS mice exhibited enhanced Ca\textsuperscript{2+} buffering capacity. We propose that the enhanced Ca\textsuperscript{2+} buffering capacity of WldS mitochondria leads to increased mitochondrial motility, suppression of axotomy-induced Ca\textsuperscript{2+} elevation in axons, and thereby suppression of Wallerian degeneration.

Results and Discussion

Mitochondria as a Key Site of WldS Neuroprotective Function

Remarkably, the distal fragments of severed axons survive for weeks after axotomy in the WldS (slow Wallerian degeneration) mouse [3–6]. The WldS mutation resulted from the fusion of two neighboring genes and led to the production of a novel hybrid protein (WldS) composed of the 70 NH-terminal amino acids of the E4 ubiquitin ligase Ube4b, a novel 18 amino acid linker domain, and the NAD\textsuperscript{+} biosynthetic enzyme Nmnat1 [7]. We previously found that expression of mouse Nmnat3 in Drosophila olfactory receptor neuron (ORN) axons provided protection equivalent to WldS 5 days after axotomy [8]. Recent work has shown that Nmnat3 expression in mouse neurons also robustly protects axons [9]. We coexpressed mouse UAS-Nmnat3::Myc and the mitochondrial marker UAS-mito::GFP in Drosophila ORNs. We found Nmnat3::Myc localized in a punctate pattern in ORN axons that precisely overlapped with mito::GFP (Figure 1A), indicating that Nmnat3 localized predominantly, if not exclusively, to mitochondria. We next assayed ORN axon preservation at 10, 20, and 50 days after axotomy. We found that Nmnat3 protected axons at levels indistinguishable from WldS at all time points tested (Figure 1B). Thus the N70 and W18 domains of WldS are dispensable for axon protection if Nmnat activity is targeted to mitochondria. By contrast, expression of Nmnat2 in ORN axons failed to suppress Wallerian degeneration, despite the fact that Nmnat2::Myc was localized throughout the axonal compartment (see Figure S1A available online).

Recently Yahata et al. [9] reported that in mouse neurons, WldS protein is located in mitochondria, cytosol, peroxisome/lysosome, and endoplasmic reticulum (ER) and/or Golgi-enriched cell fractions. We revisited WldS localization in isolated mouse striatum from control and WldS mice by separating the tissues into three fractions: nonsynaptic striatal tissue, striatal synaptosomes without mitochondria, and synaptic mitochondria. We found that WldS was detectable in the nonsynaptic fraction, as would be expected from its predominantly nuclear localization. In addition, we detected WldS in synaptic mitochondria, but not in mitochondria-free synaptic preparations (Figure S1B). These data are consistent with a primarily mitochondrial localization of WldS in axons and synapses in vivo in mouse brain.

WldS Suppresses Termination of Mitochondrial Motility after Injury and Axotomy-Induced Increases in Axonal Ca\textsuperscript{2+}

We assayed mitochondrial dynamics in live Drosophila axons using the Tdc2-Ga4 driver, which is expressed in only three axons per segment of larval peripheral nerve, by driving UAS-mCD8::mCherry (to label axonal membranes) and UAS-mito::GFP (to label mitochondria; Figure 3A). In uninjured axons, we found no differences in the total number of mitochondria, mitochondrial morphology, or mitochondrial size when we compared control and WldS-expressing axons (Figures S1A and S1B). In control animals we found that ~35% of mitochondria were motile before injury, but all motility terminated after laser axotomy (Movie S1). In striking contrast, we found that laser axotomy of WldS-expressing axons had no effect on mitochondrial movement (Movie S2; Figure 1C).

Axon injury in mammals leads to extracellular Ca\textsuperscript{2+} entry, which is necessary and sufficient for Wallerian degeneration [10]. Mitochondrial motility is known to be potently modulated by Ca\textsuperscript{2+} [11, 12]. We therefore sought to determine whether Drosophila axons showed axotomy-induced changes in axonal Ca\textsuperscript{2+}, and whether axonal Ca\textsuperscript{2+} signaling was modulated by WldS. We drove the expression of the genetically encoded Ca\textsuperscript{2+} indicator GCaMP3 in axons and measured changes in GCaMP3 signals in distal axon segments after laser-induced axotomy. In control animals, we found a rapid increase in Ca\textsuperscript{2+} levels within seconds after axotomy, with Ca\textsuperscript{2+} levels peaking within 1 min after cut, and then returning toward baseline levels over the next hour (Figures 2A–2C;
Immediately after axotomy for 5 min. Axotomy was induced by severing axons with a Micropoint laser ablation system and confirmed by a breakage of mCD8::mCherry-labeled axons. n ≥ 10 live samples for each genotype and time point. ***p < 0.001.

Movie S3). However, >1 hr after axotomy, axonal Ca²⁺ levels remained significantly elevated above baseline (~20% increase). Strikingly, whereas baseline Ca²⁺ levels in WldS+ axons were indistinguishable from those in controls (Figure S3C), in WldS− axons, injury-induced Ca²⁺ bursts were almost completely eliminated: Ca²⁺ levels only rose to ~15% control levels and returned to baseline within 1.5 min (Figure 2C; Movie S4).

In previous work, we generated a collection of UAS-regulated WldS-derived molecules that suppress Wallerian degeneration to varying degrees [8] (Figure 1A). We assayed axotomy-induced changes in GCaMP3 fluorescence in axons expressing each of these molecules in live larval preparations. As with WldS, we found no evidence for changes in axonal mitochondrial number, morphology, or size in these backgrounds (Figures S2A and S2B). However, we found a striking correlation between axon protective function and suppression of axotomy-induced increases in axonal Ca²⁺: WldS and Nmnat3 strongly suppressed Wallerian degeneration and postinjury axonal Ca²⁺ increases; Nmnat1ΔN16::WldS, and WldS-dead partially suppressed Wallerian degeneration and postinjury axonal Ca²⁺ increases; Nmnat1dead, which lacks NAD⁺ biosynthetic activity and provided no protection from Wallerian degeneration [8], did not affect postinjury axonal Ca²⁺ increases. These changes were evident in both the distal and proximal axon segment and affected both peak axonal Ca²⁺ intensities and recovery times to baseline Ca²⁺ levels (Figures 2C–2E; Figures S3A and S3B).

WldS Enhances Mitochondrial Movement, which Is Essential for Maximal Axonal Protection after Injury Because mitochondrial motility is Ca²⁺-modulated, we reasoned that changes in axonal Ca²⁺ buffering might affect axonal mitochondrial motility. We therefore assayed mitochondrial flux in axons expressing WldS-derived neuroprotective molecules. Surprisingly, we found a significant change in basal mitochondrial motility: in control animals, ~35% of total axonal mitochondria were motile; however, ~65% of mitochondria were motile in WldS+ axons. Moreover, we found that molecules that provide partial suppression of Wallerian degeneration:

**Figure 1. Mitochondria as a Focal Point for WldS-Mediated Axon Protection**

(A) Mouse Nmnat3::Myc localizes to mitochondria in Drosophila axons. 22a-Gal4 was used to drive UAS-Nmnat3::Myc and UAS-mito::GFP. Insets show boxed region.

(B) Mitochondrial Nmnat3 fully mimics WldS in axon protective function. 22a-Gal4 was used to drive UAS-Nmnat3 or UAS-WldS in a background where axons were labeled with membrane-tethered GFP (UAS-mCD8::GFP), n ≥ 20 antennal lobes for each. ***p < 0.001. Error bars represent ± SEM.

(C) WldS suppresses axotomy-induced termination of mitochondrial motility. Mitochondrial movement was assessed in live open-filet preparations of third-instar Drosophila larvae immediately after axotomy for 5 min.
degeneration (Nmnat1, ΔN16::WldS, and WldS-dead) led to a modest, but significant, increase in the number of motile mitochondria, whereas molecules that maximally suppress Wallerian degeneration (WldS, N16::Nmnat1, and Nmnat3) led to a robust increase in the number of motile mitochondria (Figure 3B). This change in mitochondrial flux in WldS axons appears to represent a decrease in docked mitochondria, an increase in motile mitochondria, but no significant change in pause and/or release rates for individual mitochondria (Figure 3C).

Is increased mitochondrial flux critical for WldS-mediated axon protection? The adaptor protein Miro functions to tether mitochondria to cytoskeletal motor proteins and modulate mitochondrial movement in a Ca^{2+}-dependent fashion [12]. Impressively, mutations in miro dominantly decrease mitochondrial motility [13]. We therefore crossed strong alleles of miro (miro^{SD32} and miro^{SD26}) and a UAS-regulated version of Miro (UAS-myc::Miro), which when overexpressed acts as a dominant-negative [13], into the WldS background and assayed mitochondrial flux. We found that miro mutants or expression of Myc::Miro dominantly suppressed mitochondrial movement in controls. In addition, we found that loss of Miro function also decreased mitochondrial movement in the presence of WldS to levels found in control animals (Figures S2C and S2D). Remarkably, reduced miro function also dominantly suppressed the neuroprotective effects of WldS in ORN axotomy assays. In animals with reduced miro function, axon loss was evident by 5 days after axotomy, with synaptic regions showing significant degeneration and, by 30 days after axotomy, the protection afforded by WldS is almost completely blocked (Figures 3D and 3E).

Mitochondria from WldS-Expressing Axons Show Enhanced Ca^{2+} Buffering Capacity and Resistance to Formation of the Permeability Transition Pore

Mitochondria are major sinks for cellular Ca^{2+} in both axons and synapses [14]. A powerful mechanism by which WldS could exert all of these effects would be by altering mitochondrial Ca^{2+} buffering capacity. We therefore assessed mitochondrial Ca^{2+} cycling and/or buffering capacity in cortical mitochondria isolated from young (~p25) wild-type (WT) and WldS mice. Mitochondrial isolations [15, 16] from both control and WldS animals yielded healthy, well-coupled mitochondria (Figures 4A and 4B). No apparent difference was observed in the Ca^{2+} uptake rates in mitochondria isolated from WldS versus control mice (Figures 4A and 4B). In contrast, the threshold for mitochondrial permeability, indicated by the loss of membrane potential (Figure 4A) and mitochondrial release of Ca^{2+} (Figure 4B), was significantly greater in mitochondria from WldS mice (Figure 4C). Thus, following increases in cytoplasmic Ca^{2+}, WldS mitochondria isolated from mouse brain buffer higher loads of Ca^{2+} before releasing it back into the cytoplasmic compartment via the mitochondria permeability transition pore (PTP).

Conclusions

The mechanistic action of WldS has remained controversial, but recent work has established a nonnuclear role for WldS [2] after injury [17]. In this study, we show that WldS is localized to mitochondria in vivo. It is important to note that protein localization studies with WldS must be interpreted cautiously—the primarily nuclear localization of WldS suggested a nuclear role for WldS and initially misled the field [2]. However, we also find that WldS increases mitochondrial Ca^{2+} buffering capacity and results in maintained mitochondrial motility after axotomy. Taken together, these data argue strongly that the mitochondrial compartment is a key site of action for WldS in vivo.

We have shown that axonal injury in live Drosophila preparations leads to a dramatic and transient rise in axonal Ca^{2+}. Increased axonal Ca^{2+} has been observed in mammals after acute nerve crush [18] and entry of extracellular Ca^{2+} is necessary and sufficient for Wallerian degeneration [10]. Impressively, WldS expression resulted in a striking suppression of
Mitochondria Mediate WldS Axonal Protection

(A) and (B) TMRE (tetramethylrhodamine, ethyl ester; membrane potential indicator) and CaG5N (extramitochondrial Ca2+ indicator) fluorescence were monitored over time simultaneously for each sample of nonsynaptic mitochondria. As illustrated in TMRE traces for the first 3 min, the addition of pyruvate and malate (PM) an oxidative substrate, causes a marked downward deflection at 1 min due to increased mitochondrial membrane potential ($\Delta$ψm). Following ADP (A) addition, the loss of $\Delta$ψm is indicated by upward deflection at 2 min as $\Delta$ψm is utilized to phosphorylate ADP to ATP via proton flow thru the ATP synthase. The ATP synthase inhibitor, oligomycin (O) addition at 3 min results in maximum $\Delta$ψm as proton flow is inhibited. The Ca2+ infusion began at 5 min (infusion rate 160 nmol of Ca2+/mg protein/min) and was monitored by CaG5N fluorescence and is illustrated by the initial upward deflection followed by constant signal due to mitochondrial Ca2+ uptake into the matrix. The subsequent rise in CaG5N fluorescence accompanied by a loss of membrane potential signifies mitochondrial permeability transition and subsequent release of mitochondrial Ca2+. (C) Quantification of mitochondrial Ca2+ buffering capacity (nmol/mg protein) indicates that WldS nonsynaptic mitochondria sequestered significantly higher amounts of Ca2+ compared to the control group (n = 6/group, * p < 0.05, unpaired t test).

Figure 4. WldS Brain Mitochondria Display Higher Ca2+ Load Capacity than Age-Matched Wild-Type (NJ) Controls

(A and B) TMRE (tetramethylrhodamine, ethyl ester; membrane potential indicator) and CaG5N (extramitochondrial Ca2+ indicator) fluorescence were monitored over time simultaneously for each sample of nonsynaptic mitochondria. As illustrated in TMRE traces for the first 3 min, the addition of pyruvate and malate (PM) an oxidative substrate, causes a marked downward deflection at 1 min due to increased mitochondrial membrane potential ($\Delta$ψm). Following ADP (A) addition, the loss of $\Delta$ψm is indicated by upward deflection at 2 min as $\Delta$ψm is utilized to phosphorylate ADP to ATP via proton flow thru the ATP synthase. The ATP synthase inhibitor, oligomycin (O) addition at 3 min results in maximum $\Delta$ψm as proton flow is inhibited. The Ca2+ infusion began at 5 min (infusion rate 160 nmol of Ca2+/mg protein/min) and was monitored by CaG5N fluorescence and is illustrated by the initial upward deflection followed by constant signal due to mitochondrial Ca2+ uptake into the matrix. The subsequent rise in CaG5N fluorescence accompanied by a loss of membrane potential signifies mitochondrial permeability transition and subsequent release of mitochondrial Ca2+. (C) Quantification of mitochondrial Ca2+ buffering capacity (nmol/mg protein) indicates that WldS nonsynaptic mitochondria sequestered significantly higher amounts of Ca2+ compared to the control group (n = 6/group, * p < 0.05, unpaired t test).

this axotomy-induced rise in axonal Ca2+. The most plausible explanation for this enhanced buffering is that increased ATP and energy production observed in WldS+ mitochondria [9]—presumably via increased mitochondrial NAD+ production, though we cannot formally exclude essential roles for other substrates of Nmnat—is linked to increased mitochondrial membrane potential ($\Delta$ψm), and thereby increased Ca2+ entry through the $\Delta$ψm-regulated mitochondrial Ca2+ uniporter [19]. This model is supported by our observation that WldS+-expressing mitochondria isolated from mouse brain exhibit an enhanced ability to maintain their membrane potential and avoid PTP formation in the face of increasing extramitochondrial Ca2+. In the future, it will be important to confirm that such changes are also observed in Drosophila axonal mitochondrial physiology in vivo in WldS+-expressing neurons.

Axonal Ca2+ spikes could result solely from entry of extracellular Ca2+ into the axon after injury. This would be consistent with the observation that blocking Ca2+ channels inhibits Wallerian degeneration [10, 18]. Mitochondria are a well-established sink for Ca2+ in axons [14] and here we show that WldS+ mitochondria exhibit enhanced Ca2+ buffering capacity and resistance to Ca2+-induced formation of the permeability transition pore (PTP). Indeed PTP formation appears to be a key final execution step in Wallerian degeneration [20–22]. We therefore favor a model whereby extracellular Ca2+ enters the axon after axotomy and normally acts as a switch to activate Wallerian degeneration. In WldS axons, this Ca2+ is instead rapidly buffered by mitochondria, thereby blocking induction of axonal destruction. Consistent with this model, uncoupling mitochondria, which suppresses mitochondrial Ca2+ uptake [23, 24], completely abrogates the protective effect of WldS in vitro [25]. WldS+-expressing neurons exhibit a roughly 2-fold increase in the number of motile versus stationary mitochondria compared to WT controls, which could result from changes in mitochondrial Ca2+ buffering. Notably, genetic suppression of enhanced mitochondrial flux using mutations in miro also resulted in a remarkable suppression of WldS-mediated axonal protection in vivo. However, because this suppression was only partial, additional factors beyond increases in mitochondrial motility must also contribute to WldS-mediated axonal protection. For example, axonal energy supplies are likely closely intertwined with mitochondrial transport and bioenergetics. WldS+ mitochondria are known to exhibit an enhanced ability to generate ATP [9]. This change in bioenergetics, coupled with increased mitochondrial motility in WldS+ axons, might enhance distribution of ATP or other mitochondrially-derived metabolites. At the same time, enhanced mitochondrial motility could also speed the removal of metabolic byproducts normally processed by mitochondria. Similarly, increased mitochondrial motility in axons could further enhance mitochondrial Ca2+ buffering in WldS+ axons because motile mitochondria would be predicted to traverse more “axonal space” and perhaps be exposed to more Ca2+ than stationary mitochondria. Together, these could have the combined effect of increasing energy delivery, removing harmful byproducts, and increased buffering of Ca2+, a signal that can potently activate axonal degeneration.

A role for mitochondria in the WldS neuroprotective mechanism is intriguing because defects in mitochondria respiration and dynamics are emerging as critical underlying factors in a number of neurological disorders [26]. For example, in mouse models of ALS (SOD1 transgenics), anterograde [27] and retrograde [28] mitochondrial transport is reduced, altered mitochondrial trafficking has been observed in models of Alzheimer’s disease [29], and mutant, but not WT Huntington, protein blocks mitochondrial movement in cortical neurons [30]. However, in the majority of models, whether these mitochondrial alterations are a cause or consequence of disease remains an open question [26]. Our study shows, reciprocally, that enhanced mitochondrial flux is associated with and is required for maximal axon protection by WldS.
Supplemental Information

Supplemental Information includes four figures, Supplemental Experimental Procedures, and four movies and can be found with this article online at doi:10.1016/j.cub.2012.02.043.

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