Loss of the m-AAA protease subunit AFG3L2 causes mitochondrial transport defects and tau hyperphosphorylation

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Abstract

The m-AAA protease subunit AFG3L2 is involved in degradation and processing of substrates in the inner mitochondrial membrane. Mutations in AFG3L2 are associated with spinocerebellar ataxia SCA28 in humans and impair axonal development and neuronal survival in mice. The loss of AFG3L2 causes fragmentation of the mitochondrial network. However, the pathogenic mechanism of neurodegeneration in the absence of AFG3L2 is still unclear. Here, we show that depletion of AFG3L2 leads to a specific defect of anterograde transport of mitochondria in murine cortical neurons. We observe similar transport deficiencies upon loss of AFG3L2 in OMA1-deficient neurons, indicating that they are not caused by OMA1-mediated degradation of the dynamin-like GTPase OPA1 and inhibition of mitochondrial fusion. Treatment of neurons with antioxidants, such as N-acetylcysteine or vitamin E, or decreasing tau levels in axons restored mitochondrial transport in AFG3L2-depleted neurons. Consistently, tau hyperphosphorylation and activation of ERK kinases are detected in mouse neurons postnatally deleted for Afg3l2. We propose that reactive oxygen species signaling leads to cytoskeletal modifications that impair mitochondrial transport in neurons lacking AFG3L2.

Keywords m-AAA protease; mitochondrial transport; N-acetylcysteine; Neurodegeneration; tau

Introduction

Mitochondria are essential organelles, whose functional integrity is ensured by efficient quality control mechanisms, including maintenance of mitochondrial proteostasis by chaperones and proteases, ongoing mitochondrial fusion and fission, and removal of dysfunctional mitochondria by mitophagy (Rugarli & Langer, 2012). Neurons are particularly susceptible to defects of any of these systems, which fail in several neurodegenerative conditions (Schon & Przedborski, 2011). Since neurons are highly polarized and need to traffic mitochondria to sites very distant from where mitochondrial biogenesis takes place, mechanisms are likely to exist to couple transport of mitochondria to their functionality. For instance, PINK1 and parkin target the mitochondrial motor adaptor miro for degradation, thus preventing transport of dysfunctional mitochondria (Wang et al, 2011).

The mitochondrial protease AFG3L2 is a central component of the intra-mitochondrial quality control system. AFG3L2 forms homo-oligomeric complexes or hetero-oligomeric hexamers with the homologous subunit paraplegin to constitute the m-AAA (matrix-ATPase associated with various cellular activities) protease in the inner mitochondrial membrane (Atorino et al, 2003; Koppen et al, 2007). This complex exerts ATP-dependent proteolytic activity, leading either to degradation or processing of specific substrates (Gerdes et al, 2012). Quality control substrates of the m-AAA protease include misfolded polypeptides in the inner membrane, such as subunits of the respiratory chain (Hornig-Do et al, 2012), while the best characterized processing substrate is the mitoribosome subunit MRPL32 (Nolden et al, 2005; Bonn et al, 2011). Mitochondria lacking AFG3L2 show defective assembly of the mitoribosome and reduced levels of mitochondrial protein synthesis (Almajan et al, 2012).

Mutations in AFG3L2 have been linked to two neurodegenerative conditions in humans, a dominant form of spinocerebellar ataxia (SCA28), and a severe recessive form of spastic-ataxia with early-onset, and rapid progression (SPAX5; Di Bella et al, 2010; Pierson et al, 2011). In contrast, mutations in SPG7, encoding paraplegin, are found in patients with a recessive form of hereditary spastic paraplegia (HSP; Casari et al, 1998). Given the pleiotropic roles of
m-AAC proteases in mitochondria, the pathogenic cascade in these diseases is still unclear.

Constitutive Afg3l2 knockout mice are affected by a severe form of spasticity and muscle weakness, and die before 3 weeks of age (Maltecca et al., 2008). Strikingly, these mice display a characteristic neurodevelopmental phenotype, characterized by maintenance of neuronal cell number, but failure to develop spinal and peripheral large caliber axons (Maltecca et al., 2008). Neurons from Afg3l2-deficient mice contain abnormal mitochondria in the cell body and a paucity of neurofilaments in their processes (Maltecca et al., 2008). This phenotype led to the proposal that impaired trafficking of mitochondria and other cargos underlies the developmental failure of the axons. AFG3L2 is also required cell-autonomously for survival of adult neurons. Deletion of the gene in Purkinje cells following development provokes dramatic cell loss associated with secondary inflammation (Almajan et al., 2012). Notably, the first abnormalities observed in neurons lacking AFG3L2 are mitochondrial fragmentation and clustering, further hinting to impaired mitochondrial dynamics and transport (Almajan et al., 2012).

Studies in cell lines have shown that mitochondria lacking AFG3L2 are fragmented and fusion-incompetent owing to activation of OMA1, a mitochondrial protease that cleaves the GTPase fusion molecule OPA1 (Ehses et al., 2009; Head et al., 2009). OPA1 is essential for inner membrane fusion (Song et al., 2007). Various stress conditions activate the peptidase OMA1 in the inner membrane that cleaves OPA1, converting long OPA1 forms into short forms and inhibiting mitochondrial fusion (Duvezin-Caubet et al., 2006; Ishihara et al., 2006; Griparic et al., 2007; Ehses et al., 2009; Head et al., 2009; Baker et al., 2014). It is therefore conceivable that mitochondrial fragmentation impairs the axonal transport of mitochondria in Afg3l2-deficient neurons leading to neurodegeneration.

Here, we use primary murine cortical neurons to examine this possibility. We show that depletion of AFG3L2 leads to a specific defect of anterograde transport of mitochondria. Surprisingly, these transport defects are independent of OMA1 activation and mitochondrial fragmentation, but can be rescued by treatment of neurons with antioxidants and by modulation of tau levels. In vivo, deletion of Afg3l2 in cortical and hippocampal neurons leads to tau hyperphosphorylation and activation of ERK kinases. Our data suggest a cross talk between dysfunctional mitochondria and the cytoskeleton, which may have implications for other neurodegenerative conditions characterized by tau aggregations.

Results

Depletion of AFG3L2 in primary neurons leads to fragmentation and defective anterograde transport of mitochondria

To investigate whether mitochondrial transport is affected in neurodegenerative diseases caused by mutations in AFG3L2, we established primary cultures of mouse cortical neurons and downregulated Afg3l2 using RNA interference while plating the neurons. A construct expressing mCherry targeted to mitochondria (mito-mCherry) was co-transfected together with different Afg3l2-specific or control siRNA oligonucleotides (Ehses et al., 2009). We imaged morphology and motility of mitochondria in axons with a length of at least 200 μm. Downregulation of Afg3l2 resulted in mitochondrial fragmentation in comparison with control neurons (Fig 1A, Supplementary Fig S1A). A significantly different distribution of mitochondrial length was observed in neurons with depleted levels of AFG3L2 (Fig 1B, Supplementary Fig S1B). Furthermore, the percentage of axonal length occupied by mitochondrial mass, hereafter referred to as mitochondrial occupancy, was reduced upon depletion of AFG3L2 when compared to control (Fig 1C, Supplementary Fig S1C). Mitochondrial fragmentation often precedes mitophagy that in turn could account for the reduced occupancy (Twig et al., 2008). The best characterized mitophagic pathway involves recruitment of parkin to depolarized mitochondria (Narendra et al., 2008). However, we found no recruitment of parkin to the mitochondrial surface in neurons downregulated for Afg3l2 (Supplementary Fig S2A). Moreover, mitochondrial membrane potential was not significantly affected in most neurons by AFG3L2 deletion (Supplementary Fig S2B and C).

To analyze mitochondrial transport, we scored the percentage of mitochondria moving in the anterograde direction, in the retrograde direction, changing their directionality (defined as oscillatory), or remaining stationary during the time of recording. Strikingly, depletion of AFG3L2 resulted in a selective defect of anterograde movement of mitochondria into the axons (Fig 1D, Supplementary Fig S1D). The number of stationary mitochondria slightly increased, in some cases to statistically significant values (Supplementary Table S1). Finally, we determined whether downregulation of Afg3l2 affects the velocity of mitochondrial movement. We found that the average speed of both anterogradely and retrogradely moving mitochondria was not significantly reduced in Afg3l2-downregulated neurons (Fig 1E).

Mitochondrial transport defects in Afg3l2 knockdown axons are independent of OMA1 activation

It is conceivable that mitochondrial fragmentation causes transport defects. To test this possibility, we downregulated Opa1 in cortical neurons using two different siRNA oligonucleotides and performed live-imaging experiments. As expected, Opa1 downregulation led to mitochondrial fragmentation (Supplementary Fig S3A and B). It is noteworthy that mitochondrial occupancy was also significantly decreased with siRNA-B (Supplementary Fig S3C). However, mitochondria were normally transported into the axons, as previously observed (Misko et al., 2010). This experiment suggests that mitochondrial fragmentation by itself does not impair mitochondrial movement (Supplementary Fig S3D).

To substantiate these findings, we investigated whether Oma1 deletion could alleviate defects in mitochondrial morphology, occupancy, and transport in primary cortical neurons depleted for AFG3L2. In Afg3l2-deficient mouse embryonic fibroblasts (MEFs), long OPA1 forms are processed by the protease OMA1, inhibiting fusion (Ehses et al., 2009). In Oma1 knockout neurons, mitochondrial length was not significantly affected by downregulation of Afg3l2, and mitochondrial occupancy was only slightly reduced (Fig 2A-C). Strikingly, anterograde mitochondrial transport was still impaired upon depletion of AFG3L2 (Fig 2D), indicating that the mitochondrial transport defect is independent of OMA1 activation and mitochondrial fragmentation.
Figure 1. Depletion of AFG3L2 causes mitochondrial defects.

A Representative kymographs of axons (shown above respective kymographs) from neurons co-transfected with mito-mCherry and control or Afg3l2 siRNAs. Schematic of different color-coded transport types is shown below. Scale bars, 10 μm.

B The percentage of mitochondria belonging to a given length bin was averaged from three independent experiments (120–150 mitochondria were measured in each experiment). Bars represent SEM. Chi-square test: $P = 0.002$.

C Quantification of mitochondrial occupancy per axon ($n = 3$ experiments; 7–8 axons per experiment).

D Quantification of mitochondrial transport types in the axon. Data represent mean ± SEM of three independent experiments. 7–8 axons from each experiment were analyzed. $P$-value was determined with Student’s $t$-test.

E Average mitochondrial velocity in the anterograde and retrograde direction. Data represent mean ± SEM of three independent experiments. The velocity of mitochondria from at least 5 axons per experiment was analyzed.
Figure 2. OMA1 ablation does not rescue mitochondrial transport defect of AFG3L2-depleted neurons.

A Representative kymographs of axons (shown above respective kymographs) from neurons isolated from Oma1 knockout mice co-transfected with mito-mCherry and control or Afg3L2 siRNAs. Schematic of different color-coded transport types is shown below. Scale bars, 10 μm.

B The percentage of mitochondria belonging to a given length bin was averaged from three independent experiments (105–200 mitochondria were measured in each experiment). Bars represent SEM.

C Quantification of mitochondrial occupancy per axon (n = 3 experiments; at least 8 axons per experiment).

D Quantification of mitochondrial transport types in the axon. Data represent mean ± SEM of three independent experiments. At least 8 axons from each experiment were analyzed. P-value was determined with Student’s t-test.
Afg3l2 deficiency leads to disruption of the microtubule network and tau hyperphosphorylation in vivo

The previous data suggest that dysfunctional mitochondria in Afg3l2 knockout neurons might be sensed in the cell body leading to inhibition of the anterograde transport. To elucidate the underlying mechanism, we examined the cerebral cortex of mice constitutively lacking Afg3l2 (Afg3l2<sup>Emvo6/Emvo6</sup> mice, from now on referred to as Afg3l2 KO; Maltecca et al., 2008). Nissl staining of serial coronal sections of the brain at postnatal day 14, shortly before Afg3l2 KO mice died, showed a reduction in the thickness of the cerebral cortex, while the organized six-layered structure was preserved (Fig 3A). Cortical neurons in layer V appeared shrunken and lacked processes, a hallmark of degeneration (Fig 3A).

We performed in situ hybridization experiments to evaluate the expression of laminar-specific genes, such as Cux2 for layers II–IV, Rorβ for layer IV, and Er81 for layer V (Molyneaux et al., 2007). These genes were expressed in the respective layer, indicating normal migration and specification of cortical neurons during development (Fig 3B). However, Er81-positive neurons were decreased, pointing to pronounced secondary degeneration of neurons in layer V.

We then examined neuronal projections by staining brain sections with antibodies against phosphorylated neurofilaments (SMI31) to detect axons, microtubule-associated protein 2 (SMI52) to label dendrites, and myelin basic protein (MBP) to decorate myelin sheaths. A drastic reduction of myelinated axonal projections containing neurofilaments was found in Afg3l2 KO mice, while dendrites were affected to a lesser degree (Fig 3C). These findings are consistent with previous observations in the spinal cord of these mice (Maltecca et al., 2008). To further characterize neuronal alterations in the absence of AFG3L2, we performed ultrastructural studies of the cerebral cortex. Neurons demonstrated pronounced mitochondrial abnormalities. Mitochondria appeared swollen, lost their tubular morphology, and had disrupted cristae pushed to the organelle periphery (Fig 3D). Microtubules in control dendrites and axons were aligned along the major axis, while they were fragmented and disorganized in Afg3l2-deficient neuronal processes (Fig 3D).

The microtubule binding protein tau plays an important role in microtubule stabilization (Morris et al., 2011). Tau hyperphosphorylation detaches tau from the microtubules, ultimately leading to their instability and fragmentation, similar to our observations in Afg3l2-deficient neuronal projections. We therefore assessed the levels of phosphorylated tau species in the brain of Afg3l2 KO mice. Hyperphosphorylation of tau can be detected with the AT8 antibody, which recognizes epitopes (serine 202 and threonine 205) that are pathologically hyperphosphorylated in Alzheimer’s disease and other tauopathies. Immunoreactivity for AT8 antibodies was greatly enhanced in neurons of Alzheimer’s disease and other tauopathies. Immunoreactivity for with the AT8 antibody, which recognizes epitopes (serine 202 and threonine 205) that are pathologically hyperphosphorylated in Alzheimer’s disease and other tauopathies. Immunoreactivity for AT8 antibodies was greatly enhanced in neurons of Alzheimer’s disease and other tauopathies.

Deletion of Afg3l2 in adult cortical neurons causes tau hyperphosphorylation and activation of PKA and ERK1/2 kinases

Tau is highly phosphorylated during embryonic development and in the early postnatal periods at several residues. However, after the majority of axons have reached their target, tau phosphorylation is downregulated (Wang & Liu, 2008). Tau hyperphosphorylation in Afg3l2 null mice may therefore reflect the axonal developmental failure. To test whether lack of AFG3L2 can also lead to tau hyperphosphorylation in adult neurons, we crossed Afg3l2<sup>Emvo6/Emvo6</sup> mice (Almajan et al., 2012) with a transgenic line expressing the Cre recombinase under the control of the CamKIIα promoter, leading to efficient depletion of Afg3l2 in postnatal neurons of the cortex, and the hippocampus (Minichiello et al., 1999; Supplementary Fig S4A). Deletion of Afg3l2 in neurons (Afg3l2<sup>Emvo6</sup>KO) leads to prominent neurodegeneration. At 8 weeks of age, Afg3l2<sup>Emvo6</sup>KO mice display evident loss of neurons in both the cortex and the hippocampus, as shown by Nissl staining (Fig 4A). Remarkably, tau hyperphosphorylation was prominent in degenerating neurons and involved similar residues as in the constitutive Afg3l2 KO mice (Fig 4B and C). Thus, tau phosphorylation is activated during the neurodegenerative process that is triggered by the loss of AFG3L2.

To address the mechanism underlying tau phosphorylation, we examined the activation of potential tau kinases in the Afg3l2<sup>Emvo6</sup>KO model. The main tau kinase GSK3β was found to be inhibited by phosphorylation at serine 9, and activation of MARK, CDK5, or its activator p35 was not observed (Supplementary Fig 5B). In contrast, deletion of Afg3l2 in adult neurons increased the levels of the phosphorylated active forms of mitogen-activated protein kinase (MAPK) ERK1/2 and of cAMP–dependent protein kinase A (PKA; Fig 4D). Both kinases have been previously implicated in tau phosphorylation in pathological conditions (Martin et al., 2013). MAP kinases are activated by oxidative stress, which in turn can lead to tau hyperphosphorylation (Chu et al., 2004; Petersen et al., 2007). We therefore examined the brains of Afg3l2 null and NKO mice for signs of oxidative damage, but could not find accumulation of carboxylated proteins or increased levels of the antioxidant SOD2 (Supplementary Fig S5).

We conclude from these experiments that the MAP kinases ERK1/2 and PKA are activated upon loss of AFG3L2 in adult neurons of the cortex and hippocampus leading to tau hyperphosphorylation in vivo.

Tau downregulation affects mitochondrial transport in AFG3L2-depleted neurons

Tau binds selectively to anterograde-directed motors and can therefore regulate anterograde transport of mitochondria (Dixit et al., 2008). The finding of tau hyperphosphorylation in the absence of AFG3L2 in vivo prompted us to examine whether tau levels in primary cortical neurons can affect the mitochondrial transport and morphology defects, associated with acute Afg3l2 downregulation. Mapt-specific siRNA oligonucleotides were tested for their ability to downregulate overexpressed tau in cultured MEFs (Supplementary Fig S6). Afg3l2 siRNA oligonucleotides were transfected together with a control siRNA or Mapt siRNA in cortical neurons, and mitochondrial morphology and transport were evaluated. Notably, tau depletion did not affect mitochondrial length, occupancy, transport,
and velocity in wild-type neurons (Fig 5A–E, Table S1). However, mitochondria were less fragmented, and a partial but significant rescue of the anterograde transport defect was detected upon concomitant downregulation of Afg3l2 and Mapt when compared to AFG3L2 depletion alone (Fig 5). Thus, modulation of tau levels affects mitochondrial transport in pathological conditions.

Antioxidants suppress mitochondrial transport defects in AFG3L2-depleted neurons

As oxidative stress triggers tau hyperphosphorylation, we explored the possibility that increased reactive oxygen species (ROS) production affects mitochondrial motility. Firstly, we
tested whether primary neurons downregulated for Afg3l2 show signs of increased ROS by staining them with CellROX green. This dye becomes highly fluorescent upon oxidation and labels the DNA of both nucleus and mitochondria. However, we did not observe consistent, statistically significant differences in the fluorescence between control and AFG3L2-depleted neurons (Supplementary Fig S7A and B). This result could be due to limited sensitivity of the dye that is suitable to detect massive changes in ROS levels, such as those induced by treating neurons with menadione (Supplementary Fig S7C and D). We therefore asked whether interfering with basal ROS levels by culturing neurons in the presence of antioxidants, such as N-acetylcysteine (NAC) or vitamin E, has an impact on mitochondrial motility. NAC is a drug that affects the redox state of cells and indirectly acts as antioxidant by increasing the pool of reduced glutathione (Kelly, 1998), while vitamin E scavenges the peroxyl radicals and prevents the propagation of free radicals (Niki & Traber, 2012). Both drugs efficiently reduce the levels of ROS induced in primary neurons by menadione (Supplementary Fig S7C and D). Moreover, both NAC and vitamin E reduce the levels of phospho-tau in cultured neurons in vitro (Supplementary Fig S7E and F). Strikingly, NAC treatment of primary neurons leads to a global change in cytoskeletal components, by reducing the levels of β-tubulin, tyrosinated and acetylated tubulin, actin, and total tau (Supplementary Fig S7E).

The presence of NAC in the culture medium did not affect the distribution of mitochondrial length or the occupancy of mitochondria in control neurons. Surprisingly, the percentage of mitochondria moving in the anterograde direction and their velocity was significantly increased in control neurons when cultured in medium supplemented with NAC (Fig 6A–F, Supplementary Table S1). Moreover, NAC treatment significantly suppressed the defects observed upon Afg3l2 downregulation: mitochondrial length, mitochondrial occupancy, and the percentage of mitochondria moving in anterograde direction increased to levels comparable to control (Fig 6A–D).

We then depleted AFG3L2 in cortical neurons grown in medium containing vitamin E. Vitamin E induced slight fragmentation of mitochondria in control neurons, which is reflected in a reduced occupancy (Fig 7A and B). Consistently, vitamin E did not rescue mitochondrial morphology and occupancy of AFG3L2-depleted neurons when compared to controls (Fig 7A and B). However, a significant improvement in the anterograde transport of mitochondria was observed (Fig 7C, Supplementary Table S1). Mitochondrial velocities of anterogradely and retrogradely moving mitochondria were not significantly affected by vitamin E (Fig 7D and E). We conclude that ROS, present at physiological levels, play a pivotal role in coupling quality control of mitochondria to their transport.

Discussion

Neurodegenerative diseases linked to mutations in components of the m-AAA protease, paraplegin and AFG3L2, are characterized by late-onset axonal degeneration in cortical motor or cerebellar neurons. Our study shows for the first time that the frequency of anterograde transport of mitochondria, but not the speed of movement, is affected in neurons with reduced levels of AFG3L2, suggesting that defective mitochondrial trafficking contributes to the pathogenic mechanism in the human diseases. Mitochondrial transport defects in Afg3l2-deficient neurons are restricted to mitochondria moving from the cell body toward the growth cone. A similar phenotype has been previously described in cortical neurons isolated from mice lacking spastin, a microtubule-severing protein involved in HSP (Kasher et al., 2009), in motor neurons derived from SOD1G93A transgenic mice, a model of amyotrophic lateral sclerosis (De Vos et al., 2007), and in amyloid β-treated neurons (Reddy et al., 2012). Even mild impairment of anterograde transport in patients carrying heterozygous mutations in AFG3L2 could lead to progressive depletion of mitochondria from axons causing late-onset neurodegeneration. It is tempting to speculate that impaired anterograde transport of mitochondria underlies the developmental failure of large myelinated axons in absence of AFG3L2 in the mouse.

Surprisingly, our data uncouple mitochondrial trafficking defects from OMA1 activation and mitochondrial fragmentation. Stress-activated OMA1-mediated processing of OPA1 is not required for mitochondrial transport defects in the absence of AFG3L2. Furthermore, reduced OPA1 levels do not affect mitochondrial transport, despite leading to fragmentation, in agreement with previous studies (Misko et al., 2010). Our data therefore suggest that alteration of mitochondrial transport and increased processing of OPA1 in absence of AFG3L2 are independent events. Notably, we observed a strict correlation between the degree of mitochondrial fragmentation and the reduction in mitochondrial occupancy, suggesting increased mitochondrial turnover likely by a parkin-independent mechanism.

How are Afg3l2-deficient mitochondria hindered from being transported toward the growth cone? We identify tau as a regulator...
of transport of dysfunctional Afg3l2-deficient mitochondria. The primary function of the microtubule-associated protein tau is to bind and stabilize microtubules (Wang & Liu, 2008). Tau was found to largely affect access of the anterograde motor kinesins to microtubules (Dixit et al., 2008). Consistently, tau overexpression inhibits kinesin-dependent transport of cargos, including mitochondria (Ebneth et al., 1998). Loss of tau function in the mouse still allows normal mitochondrial transport (Yuan et al., 2008), whereas tau depletion is beneficial in certain pathological conditions; for example, it improves axonal transport defects of mitochondria in neurons treated with Aβ peptides (Vossel et al., 2010). Similarly, we show that reducing tau levels can partially rescue the mitochondrial transport defects associated with Afg3l2 downregulation.

Tau hyperphosphorylation is an important downstream event to the lack of AFG3L2 in cortical neurons in vivo. During embryonic
and early postnatal periods, tau is hyperphosphorylated at several sites, including Thr181, Ser199, Ser202, and Thr205. Notably, the same residues are also hyperphosphorylated in degenerating neurons of Alzheimer’s disease patients (Wang & Liu, 2008). Upon phosphorylation, tau detaches from microtubules and can potentially improve motility of cargos, such as mitochondria. Consistently, degeneration caused by missorting of tau into neurons can be rescued by increasing its phosphorylation via the tau kinase MARK2 (Thies & Mandelkow, 2007). However, prolonged tau hyperphosphorylation promotes self-aggregation into cytoplasmic inclusions and leads to neurodegeneration (Ballatore et al., 2007).

Hyperphosphorylated tau at residues recognized by the AT8 antibody was recently found to inhibit mitochondrial transport in cortical neuronal axons by affecting spacing of microtubules (Shahpasand et al., 2012). In conclusion, tau hyperphosphorylation might be beneficial at early stages in the pathogenic process, but it is conceivable that sustained tau hyperphosphorylation impairs anterograde transport of mitochondria in Afg3l2-deficient neurons. In agreement with such a scenario, neuronal microtubules appear severely disrupted in absence of AFG3L2. Notably, interfering with axonal transport by mutating kinesin-1, or by downregulating miro or milton in Drosophila, was sufficient to activate kinase pathways responsible for tau hyperphosphorylation (Falzone et al., 2009, 2010; Iijima-Ando et al., 2012), suggesting that loss of axonal mitochondria enhances tau pathology.

Accumulation of fibrillary tau inclusions (neurofibrillary tangles) in the central nervous system has been implicated in onset and progression of aging-associated disorders that manifest clinically with progressive cognitive and/or motor impairment (Ballatore et al., 2007). However, the causative link between tau hyperphosphorylation and upstream events in tauopathies is still unclear. Here, we demonstrate that mitochondrial dysfunction can directly trigger tau hyperphosphorylation upon genetic ablation of a mitochondrial protease. Similarly, we found abnormal tau hyperphosphorylation and deposition of tau filaments in Phb2-deficient degenerating neurons (Merkwirth et al., 2012). Prohibitins form large protein scaffolds in the inner mitochondrial membrane that interact with the m-AAA protease (Merkwirth & Langer, 2009). Together, our data provide genetic evidence for a causal relationship between mitochondrial dysfunction and tau hyperphosphorylation, as

Figure 5. Tau levels affect mitochondrial transport in AFG3L2-depleted neurons.

A The percentage of mitochondria belonging to a given length bin was averaged from three independent experiments (120–210 mitochondria were measured in each experiment). Bars represent SEM. P-values were calculated with the chi-square test. Control vs. Afg3l2/Control: \( P = 7 \times 10^{-3} \), control vs. Afg3l2/Mapt: \( P = 9 \times 10^{-5} \), Afg3l2/Control vs. Afg3l2/Mapt: \( P = 5 \times 10^{-3} \).

B Quantification of mitochondrial occupancy per axon (n = 3 experiments; 7–9 axons per experiment).

C Quantification of mitochondrial transport types in the axon. Data represent mean ± SEM of three independent experiments. 7–9 neurons from each experiment were analyzed.

D, E Average mitochondrial velocity in the anterograde (D) and retrograde (E) direction. Data represent mean ± SEM of three independent experiments. The velocity of mitochondria from 3 axons per experiment was analyzed.

Data information: P-values in (B) and (C) were determined with Student’s t-test.
Figure 6. Mitochondrial defects in AFG3L2-depleted neurons are rescued by NAC.

A Representative kymographs of axons (shown above respective kymographs) from neurons co-transfected with mito-mCherry and Afg3l2 siRNAs in the absence or presence of NAC. Schematic of different color-coded transport types is shown below. Scale bars, 10 µm.

B The percentage of mitochondria belonging to a given length bin was averaged from four independent experiments (78–195 mitochondria were measured in each experiment). Bars represent SEM. P-values were calculated with the chi-square test. Control vs. Afg3l2: \( P = 8 \times 10^{-4} \); Afg3l2 vs. Afg3l2 with NAC: \( P = 1 \times 10^{-9} \).

C Quantification of mitochondrial occupancy per axon (n = 4 experiments; 8–10 axons per experiment).

D Quantification of mitochondrial transport types in the axon. Data represent mean ± SEM of four independent experiments. At least 8 axons from each experiment were analyzed.

E, F Average mitochondrial velocity in the anterograde (E) and retrograde (F) direction. Data represent mean ± SEM of four independent experiments. The velocity of mitochondria from 3 axons per experiment was analyzed.

Data information: P-values in (C–E) were determined with Student’s t-test.
suggested by other studies (Hoglinger et al., 2005; Escobar-Khondiker et al., 2007; Melov et al., 2007; Ohsawa et al., 2008).

In both Phb2 and Afg3l2 mouse models, we observed activation of MAPKs (Merkwirth et al., 2012). MAPKs have been shown to phosphorylate tau at several residues both in vitro and in vivo (Zhu et al., 2002) and are activated by several cell stresses, including oxidative stress (Son et al., 2013). Consistently, we find that antioxidants efficiently reduce levels of phospho-tau in primary neurons. Increased levels of oxidized proteins were detected in the cerebellum of Afg3l2 heterozygous mice (Maltecca et al., 2009), while it is conceivable that neurons die before oxidative damage occurs in the brain of Afg3l2 knockout mice. Failure to detect a significant increase of ROS levels in Afg3l2-deficient neurons may be due to the limited sensitivity of available fluorescent dyes (Murphy et al., 2011). However, in support of the hypothesis that ROS play a signaling role in regulating mitochondrial transport, we found that treatment with antioxidants, such as NAC and vitamin E, can rescue the anterograde transport defects of Afg3l2-deficient mitochondria. Interestingly, NAC regulates both the frequency of transport and the velocity of mitochondria in wild-type neurons, stressing the hypothesis that modulating the physiological levels of ROS can affect mitochondrial transport. NAC has a more pronounced effect than vitamin E on mitochondrial transport in Afg3l2-deficient neurons. NAC was previously shown to rescue the mitochondrial transport defects observed in dopaminergic neurons treated with MPP+, a toxin that inhibits mitochondrial complex I activity (Kim-Han et al., 2011). Neurons treated with NAC display a reduction of the levels of actin and β-tubulin. Thus, the beneficial effect of NAC may be also exerted by modulation of redox modifications of cytoskeletal components. In fact, actin has been previously shown to sense oxidative load and to be affected by NAC (Dalle-Donne et al., 2001; Farah & Amberg, 2007). An intriguing hypothesis is that ROS-activated signaling cascades can affect mitochondrial transport not only by impinging on tau phosphorylation, but also by targeting motor proteins, or mitochondrial adaptors. Future experiments will be required to investigate this possibility in depth.

In conclusion, we propose a pathogenic model, where deficiency of AFG3L2 leads on one side to mitochondrial fragmentation, via...
OMA1-mediated processing of OPA1, and on the other side to increased production of ROS (Fig 8). Increased ROS may result from respiratory incompetence due to defective mitochondrial translation (Almajan et al., 2012). ROS may trigger activation of stress-induced kinases, which phosphorylate tau in an attempt to reduce its attachment to the microtubules, thus promoting mitochondrial transport. However, with time, insoluble hyperphosphorylated tau aggregates, disrupts microtubules, and affects mitochondrial anterograde transport. The axonal transport defect may further contribute to sustain pathological tau hyperphosphorylation (Fig 8). Mitochondrial transport defects ultimately lead to late-onset mitochondrial depletion from axons and neurodegeneration. Our data may be of relevance for several neurodegenerative conditions characterized by mitochondrial dysfunction and tau accumulation and open up the possibility that treatment with antioxidants may be beneficial in models of 

Material and Methods

Animal experiments

All animal procedures were conducted in accordance with European (EU directive 86/609/EEC), national (TierSchG), and institutional guidelines and were approved by local authorities (Landesamt für Natur, Umwelt, und Verbraucherschutz Nordrhein-Westfalen) under the license 87–51.04.2010.A219. Afg3l2<sup>e<sub>Emv66/Emv66</sub></sup> mice were obtained by breeding heterozygous Afg3l2<sup>+/Emv66</sup> (Maltecca et al., 2008) in a mixed FVB-C57BL/6 background. Conditional Afg3l2 floxed mice (Almajan et al., 2012) were mated with CaMKIIα<sup>cre</sup> (Minichiello et al., 1999) to achieve Afg3l2 forebrain neuron-specific knockouts. Mice carrying the Oma1 gene exon 3 flanked by loxP sites were generated by gene targeting in C57BL/6 background. Oma1 knockout mice were produced by crossing homozygous Oma1 floxed mice with a transgenic line expressing the Cre recombinase under the β-actin promoter.

Tissue preparation

Mice were deeply anesthetized and perfused transcardially with 4% paraformaldehyde (PFA) in PBS. Tissues were dissected and postfixed in 4% PFA for immunohistochemistry and in 2% glutaraldehyde in 0.12 M phosphate buffer for electron microscopy. For biochemical analyses, tissues were immediately frozen in dry ice and stored at −80°C until use.

RNA in situ hybridization

PCR products obtained by amplification of mouse brain cDNA were used as templates to transcribe either sense or antisense digoxigenin-labeled riboprobes using the DIG RNA labeling kit (Roche). Oligonucleotides used to generate the PCR products contained the SP6 promoter. Sequences are available upon request. RNA in situ hybridization was performed on vibratome sections, as previously described (Tiveron et al., 1996).

Histology and Immunohistochemistry

Postfixed brains were embedded in 6% agar, and 30-μm sections were cut using a vibratome (Leica). Free-floating sections were stained with 0.25% thionine solution. Immunohistochemistry with AT8 antibody (Thermo Scientific) was performed with M.O.M. Immunodetection kit (Vector Laboratories), according to the manufacturer’s protocol. Sections were mounted using Eukitt medium (Fluka). For immunofluorescence, sections were permeabilized and blocked in 0.4% Triton X-100 and 10% goat serum in TBS and incubated with SMI 31, SMI 52, and anti-MBP antibodies (Covance) diluted in 5% goat serum in TBS. Following incubation with
anti-mouse Alexa Fluor 488 (Life technologies), sections were mounted using FluorSave Reagent (Calbiochem). Images were acquired using an Axio-Imager M2 microscope equipped with Apotome 2 (Zeiss).

Western blot analysis

Tissues were homogenized in RIPA buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Roche) on ice. After high-speed centrifugation, the supernatant was harvested, and protein concentration was determined using Bradford assay (Bio-Rad). 20–50 μg proteins were separated by 12% SDS–PAGE and blotted onto PVDF membrane (Millipore). The membrane was blocked in 5% milk containing 0.1% Tween-20 in TBS for 1 h at room temperature and probed with primary antibodies overnight at 4°C. We used AT8, AT270, anti-phospho-Tau-Ser396 antibodies from Pierce, anti-Tau1, anti-ERK1/2, anti-GAPDH from Millipore, anti-PKA C– and anti-mouse Alexa Fluor 488 (Life technologies), sections were mounted using FluorSave Reagent (Calbiochem). Images were acquired using an Axio-Imager M2 microscope equipped with Apotome 2 (Zeiss).

Mitochondria in the axons were imaged using a Perkin-Elmer Ultra View spinning disc confocal microscope, equipped with a CCD camera (Hamamatsu, C9100-50), at 60× magnification (oil-immersion objective, N.A = 1.49, illumination wavelength = 561 nm). Video recordings were acquired at 1,000 × 1,000 pixel resolution every 10 seconds for a period of 10 min (61 image stacks/movie). The stage enclosing the dishes was set to 37°C and 5% CO2. Imaging of the axons was performed 72 h after plating the neurons, and they were identified as processes arising from the soma twice or thrice longer than other processes. Axons selected for analyses had a minimum length of 200 μm. Images were acquired at least 50 μm distal to the soma.

Kymograph generation

Mitochondrial movements along an axon were visualized using kymographs, generated with a custom written script (Matlab R2011b incl. image processing toolbox, Mathworks). Initially, an optimal axon centerline between two manually set endpoints was automatically obtained, based on a maximum projection of all image stacks (imaged mitochondria). Then, kymographs were obtained by generating a maximum projection for each image stack (time point) leading to orthogonal projection of maximum intensities in a neighborhood of approximately 1 μm onto the axon centerline.

Assessment of mitochondrial length, occupancy, and axonal transport

Mitochondrial length was quantified from the first image of the video recordings, by drawing a line along the major axis or along the diameter of circular mitochondria. This analysis was performed in Volocity 6.1 (PerkinElmer). Mitochondrial occupancy was calculated as the total length of all mitochondria in an axon divided by axonal length. For quantification of mitochondrial transport, we classified mitochondrial movement over time as anterograde, retrograde, or oscillatory. Mitochondria were defined as oscillatory if they changed direction to move a distance greater than 5 μm, while they were considered stationary if they moved less than 5 μm during the video recordings. In case of a fusion event, daughter mitochondria were considered as separate. In case of a fusion event, mitochondrion after fusion was considered as separate from the parent mitochondria. Classification of mitochondrial movements was manually performed from the video recordings obtained in Volocity 6.1 (PerkinElmer) as the position of soma was noted while image acquisition. Mitochondrial velocity was calculated for anterograde and retrograde movements in all the timeframes where a mitochondrion was moving. After establishing a cut-off value of 0.1 μm/s, the average velocity was calculated for each individual mitochondrion.

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Statistical analysis

Mitochondrial length was measured from at least three independent experiments. Data were binned, averaged across experiments, and statistical evaluation was performed using the chi-square test. Unpaired two-tailed Student’s t-test was used for statistical comparison of mitochondrial occupancy, transport, and average velocity. Data are presented as mean ± standard error (SEM). In all cases, at least three independent experiments were performed, and 8–10 axons analyzed. The P-values were calculated using Graphpad Prism software (version 6.02) and are indicated when lower than 0.05.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

AKK performed imaging experiments, with the help of ACS; SW, SM, AK, PM, and DH performed experiments on the mouse models; MJB produced code required for generating kymographs; SW, SM, AK, PM, and DH performed imaging experiments, with the help of ACS; NK developed the

References

Thies E, Mandelkow EM (2007) Mis-sorting of tau in neurons causes degeneration of synapses that can be rescued by the kinase MARK2/Par-1. J Neurosci 27: 2896 – 2907