Mitofusin 2 protects cerebellar granule neurones against injury-induced cell death

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Mitofusin 2 (Mfn2) plays a central role in the regulation of mitochondrial fusion. Most importantly, mitofusin 2 loss of function significantly prevents cell death in the absence of any apoptosis. More importantly, whereas wild-type Mfn2 and the hydrolysis-deficient mutant of Mfn2 (Mfn2RasG12V) function equally to promote fusion and lengthening of mitochondria, the activated Mfn2RasG12V mutant shows a significant increase in the protection of neurons against cell death and release of proapoptotic factor cytochrome c. These findings highlight a signaling role for Mfn2 in the regulation of apoptosis that extends beyond its role in mitochondrial fusion.

It has been recently demonstrated that the apoptotic program includes the regulated induction of mitochondrial fragmentation (1, 2). In addition, it has been shown that the rate of mitochondrial fusion is reduced early in the apoptotic process (3, 4), which together with the activation of the fission machinery leads to a morphological shift of the mitochondria to the fragmented state. The regulatory mechanisms and functional importance of these events during death remain unclear. For example, it is not clear whether the inhibition of mitochondrial fusion is an essential step in apoptosis or if fragmentation is promoted mainly because of the increase in fission. In addition, it has been shown that the loss of either Drp1 or hFis1 delayed, but did not block cell death, questioning the importance of the mitochondria morphological shift in the apoptotic cascade (5, 6). In this context, there is an emerging emphasis on the examination of mitochondrial fusion in the context of cell death.

Mitochondrial fusion is regulated by at least three essential GTPases, the outer membrane-anchored proteins mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2) along with the intermembrane space GTPase, Opa1 (7, 8). Although the functions of Mfn2 overlap with Mfn1 in the process of mitochondrial fusion (9), there are clear distinctions between these two GTPases. Perhaps most informative of their distinct function is their biochemical difference in nucleotide binding and hydrolysis properties, where Mfn1 has a faster GTPase hydrolysis rate and higher affinity for nucleotide relative to Mfn2 (10, 11). In addition, Mfn1, but not Mfn2, has been shown to genetically interact with Opa1 (12), a member of the dynamin family of mechanoenzymes. This relationship with Opa1 would suggest that Mfn1 plays a central role with Opa1 in the fusion process. In an in vitro mitochondrial docking assay, expression of Mfn1 significantly enhanced the tethering reaction, whereas Mfn2 resulted in tethering with low efficiency, suggesting a secondary role for Mfn2 in docking (10). Recently, compelling evidence has emerged supporting additional roles for Mfn2 that goes beyond the regulation of the mitochondrial fusion. First, Mfn2 is colocalized in punctate with Bax and Drp1 at sites of future fission (13). This suggests that Mfn2 activity may affect mitochondrial recruitment of Bax and Drp1 during cell death. Second, Mfn2, but not Mfn1, can interact directly with Ced9 or BclXL in HEK293 cells, suggesting a mechanism for cross-talk with anti-apoptotic Bcl family proteins (3). Third, Mfn2, not Mfn1, has been shown to modulate metabolism through function of complex 1, IV, and V (14, 15). Fourth, it was also shown that cytotoxic Bax plays a specific role in the steady state activity of Mfn2 as a regulator of mitochondrial fusion (16). Most importantly, Mfn2 seems to be critical for the function of the nervous system.

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The abbreviations used are: Mfn, mitofusin; CGN, cerebellar granule neurones; MOI, multiplicity of infection; ROS, reactive oxygen species; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GFP, green fluorescent protein; OCT, ornithine carbamyl transferase; YFP, yellow fluorescent protein; DIV, days in vitro.
as point mutations in this molecule have been associated with Charcot Marie Tooth neuropathy type 2A (17). Understanding the role of mitofusin 2 in response to acute neuronal injury is therefore crucial for development of novel therapeutic strategies. Because of the importance of Mfn2 in fusion as well as its importance in the nervous system, we therefore asked whether Mfn2 is involved in the regulation of acute injury using primary cerebellar granule neurons. To examine the role of this GTPase in apoptosis signaling, we have constructed adenoviral vectors carrying both wild type and a hydrolysis-deficient mutant of Mfn2, Mfn2RasG12V (18), and Mfn2RasG12V lentivirus for long term transduction of these neurons. We asked: (a) whether Mfn2 could protect neurons against different mechanisms of injury and (b) whether this protection is by promoting mitochondrial fusion and shifting the morphological equilibrium or through an additional role for Mfn2 that is distinct from the stimulation of mitochondrial fusion.

The results of our studies demonstrate that mitochondrial fragmentation in response to neuronal injury is dramatic and occurs as an early event. Furthermore, we show that Mfn2 signals mitochondrial fusion in neurons and protects against neuronal death. Our results indicate that the increased activation of mitochondrial fusion by expression of Mfn2 or Mfn2RasG12V cause an equally dramatic increase in the mitochondrial lengths to greater than 30 μm extending throughout the processes. Interestingly, while both wild type Mfn2 and Mfn2RasG12V result in the equal lengthening of mitochondria, the Mfn2RasG12V is most protective against neuronal cell death and release of pro-apoptotic factors. These findings emphasize a signaling role for Mfn2 that goes beyond its role in the regulation of mitochondrial fusion.

**EXPERIMENTAL PROCEDURES**

**Primary Neuronal Cultures and Adenoviral Construction—**Cerebellar granule neurons (CGNs) were cultured from CD1 mice at postnatal day 7 or 8 as described previously (19). Recombinant adenoviral vectors carrying ornithine carbamyl transferase (OCT), human mitofusin 2 (Mfn2), or its active mutant, Mfn2RasG12V, expression cassettes were prepared using AdEasy system, as described previously (20). Mfn2 antisense adenoviral vectors carrying ornithine carbamyl transferase (OCT), human mitofusin 2 (Mfn2), or its active mutant,
graphs of the same fields were taken with light microscopy, and the number of cells infected was compared with the total number of cells in the field. To measure toxicity the number of infected dead cells was compared with the total number of infected cells in the field.

Cell Viability Assays—Cell death was measured by condensed nuclear morphology revealed by Hoechst staining. MTT or Live/Dead assay (supplemental Fig. S1) was used to confirm the results of Hoechst staining, as described previously (21). In each replicate, three to five different fields were randomly chosen per treatment group. Representative samples were photographed using a Zeiss Axiosvert 100 (Oberkochen, Germany) fluorescence microscope equipped with a QICam Digital camera (QImaging Corporation, Burnaby, Canada) and Northern Eclipse software (Empix Imaging Inc. Mississauga, ON, Canada). The total number of cells in each field was counted. Cell death was expressed as a percentage of total cells.

DNA Damage, K⁺ Deprivation, and Reactive Oxygen Species (ROS)-induced Cell Death—To model in vitro DNA damage-induced cell death, CGNs were treated with 10 μM camptothecin (Sigma-Aldrich) following 2 days in vitro (2 DIV). Hydrogen peroxide (H₂O₂) was used to model ROS-induced cell death. CGNs were treated with H₂O₂ following 2 DIV for 5 min after which the medium was replaced with conditioned medium taken from the parallel cultures with no treatment. Because of unstable nature of H₂O₂, the concentration used in each replicate was optimized prior to each treatment and 75–100 μM was used to induce 50–70% cell death following 24 h of treatment. To model K⁺ deprivation-induced cell death, neurons were transduced at the time of plating with purified lentiviruses 1.5 MOI and after 7 days in vitro the media containing 25 mM K⁺ was changed to a low potassium media of 5 mM.

Cytochrome c Release—Neurons were fixed and stained with cytochrome c and/or Tom20 and Hoechst following treatment with hydrogen peroxide or camptothecin. For each replicate, a total of 100 neurons were counted from 3–5 different fields. In each case, a Z stack of the field was taken for analysis using a Zeiss 510 meta confocal microscope. A diffuse cytochrome c staining or complete lack of staining was identified as release.

Time Lapse Microscopy—CGNs were seeded on 4-well plates (Nalgene Nunc International, Rochester, NY) with attached glass coverslips coated with poly-D-lysine (VWR International), and infected with the YFP-tagged ornithine carbamyl transferase (OCT-YFP), a mitochondrial matrix protein, at the time of seeding. Following each treatment, neurons were imaged to track individual mitochondria in real time. The coverslips were mounted in a temperature-controlled chamber (37 °C) in regular growth media supplemented with 20 mM HEPES (pH 7.4), and visualized with an Olympus ×100 oil immersion objective, numerical aperture 1.4, on an Olympus IX80 Laser scanning confocal microscope operated by FV1000 software v1.4a. The YFP was excited with a 515-nm line of a multiple line Ar laser, the Mitofluor Red was excited with the 543-nm line of He/Ne green laser, and the Alexa 647 was excited with the 633-nm line of He/Ne red laser. All images shown demonstrate cells that are representative of
moderate infection efficiencies and that have been obtained from at least three independent experiments.

**Immunofluorescence**—At each time point, neurons were fixed for 30 min with ice-cold 4% paraformaldehyde in 1× phosphate-buffered saline (1× PBS) and then rinsed twice with 1× PBS. Cells were permeabilized with 300 µl of ice-cold 0.4% Triton-X in 1× PBS for 10 min. Cells were stained with the primary antibodies in 10% normal goat serum-0.4% Triton X/PBS for 1 h. The cells were washed for 3× 5 min with ice-cold 1× PBS. Cells were incubated with the secondary antibodies to either TOM 20 (1:250, a kind gift from Dr. Gordon Shore) (22) or cytochrome c (1:250; BD Biosciences, Franklin Lake, NJ) in 10% normal goat serum-0.4% Triton X/PBS for 1 h. The cells were washed for 5 min and stained with Hoechst for 5 min. Following Hoechst staining, neurons were washed with 1× PBS for 3× 5 min and mounted. Representative samples were photographed using a Zeiss Axiovert 100 (Oberkochen, Germany) fluorescence microscope equipped with a QICam Digital camera (Q Imaging Corporation) and Northern Eclipse software (Empix Imaging Inc.).

**Mitochondrial Length Measurement**—Whole cell images were acquired by exciting at 549 nm with the CY3 filter (Chroma Technology Corp., Rockingham, VT). Mitochondrial length was measured by tracing the mitochondria using Northern Eclipse software. Mitochondrial length varied remarkably even in control neurons. For comparison purposes mitochondria were classified into different categories with a length ranging from less than 0.5 µm, 0.5–1 µm, 1–2 µm, 2–3 µm, and greater than 3 µm.

**Quantification and Statistical Analysis**—For cell death studies, a minimum of 500 cells per field (three fields per replicate) was scored for each treatment at the indicated time points. For mitochondrial length measurements, a minimum of 500 mitochondria for each treatment (per replicate) was scored. The data represent the mean and S.D. from three independent experiments (n = 3), where n stands for each independent experiment. p values were obtained using two-way analysis of variance and Tukey post-hoc tests or Student’s t test. A p value <0.05 was considered significant and was indicated on the graphs by an asterisk.

**RESULTS**

**Mitochondrial Fragmentation Occurs Following DNA Damage-induced Neuronal Death**—It has been suggested that mitochondria remodel following acute neuronal injury (23). We therefore asked whether mitochondria undergo fragmentation or remodeling following DNA damage-induced neuronal death. The DNA damage model induced by topoisomerase inhibitor, camptothecin, occurs physiologically following stroke or trauma and is believed to contribute to the extensive neuronal loss after acute injury (24). To model in vitro DNA damage-induced cell death, primary cerebellar granule neurons (CGNs) were treated with 10 µM camptothecin. This concentration of camptothecin was shown to induce a slow cell death resulting in about 40% neuronal loss by 24 h (Fig. 1D). To track individual mitochondria in real time, we created adenovirus vectors containing the 32 amino acid targeting signal of ornithine carbamyl transferase fused to YFP (OCT-YFP) (25), and infected the CGN cultures at the time of seeding. Mitochondrial dynamics were documented within the first 16 h using fluorescence time lapse microscopy. We quantified the percentage and timing of mitochondrial fragmentation following treatment with camptothecin. CGN cultures were fixed and stained with anti-
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TOM20, a mitochondrial protein import receptor (22) and Hoechst to identify cell nuclei at different time points following treatment (Fig. 1B). Mitochondria in neurons exhibited variable length. The data were therefore binned into different length categories from less than 0.5 to greater than 3 μm. Quantification of mitochondrial lengths showed that immediately following exposure to camptothecin, 96% of mitochondria had a length of greater than 0.5 μm, as in the control neurons (Fig. 1C). Of these 41 ± 1% ranged within 0.5–1 μm; 47 ± 4% had a length of between 1 and 2 μm, and 9.3 ± 0.9% had a length of 2–3 μm. At 6 h following treatment with camptothecin, 20 ± 2.5% of the mitochondria were fragmented with a length of less than 0.5 μm. Following 12 h of treatment, however, there was a dramatic change in morphology where 56 ± 3% of mitochondria exhibited a length of less than 0.5 μm. The fragmentation was maximal by 24 h where 70.2 ± 0.4% of mitochondria exhibited a length of less than 0.5 μm. Only 3.4 ± 1.7% of mitochondria had a length of 2–3 μm at 24 h. These results demonstrate that mitochondrial fragmentation is initiated 3–6 h following exposure of neurons to camptothecin and there is a remarkable difference in the mitochondria pool by 12 h.

To confirm whether this change in morphology was because of mitochondrial membrane scission rather than organelle swelling, we performed a video analysis of mitochondria within cells treated with camptothecin. In many videos, mitochondria were observed to clearly divide into much smaller fragments upon 6 h of treatment with camptothecin. Therefore, although we did observe some mitochondrial swelling (Fig. 1A, box), we also observed mitochondrial fission (Fig. 1A and supplemental movie 1).

To ask whether mitochondrial fragmentation correlates with the cell death, the rate of cell death was evaluated by counting the percentage of cells exhibiting pyknotic nuclei. Pyknotic nuclei indicative of apoptosis were observed following 12 h of treatment (Fig. 1D). Cell death increased to 20 ± 3% at 12 h and was maximal at 24 h (39 ± 2.6%) within the given time frame. Based on these results, we conclude that mitochondrial fragmentation is initiated 6–9 h prior to the degradation of the nucleus following DNA damage-induced cell death.

Mitochondrial Fragmentation Is an Early Event Following Oxidative Stress—In many types of acute neuronal injury such as stroke, a primary cause of death is the exposure to ROS, which initiates a complex signaling cascade (26). We next asked whether mitochondrial fragmentation also occurs following oxidative stress. CGN cultures were infected with OCT-YFP at the time of seeding and treated with H2O2 for 5 min after which the medium was replaced by conditioned media. Time lapse microscopy studies revealed that mitochondria undergo fragmentation within 1 h following treatment (Fig. 2A and supplemental movie 2). Mitochondria were also documented to transition from rod-like to spherical following fragmentation. This kind of mitochondrial remodeling following fission was indeed a common event in this mode of cell death (Fig. 2B and supplemental movie 3). Interestingly exposure of neurons to hydrogen peroxide for longer than 5 min resulted directly in mitochondrial remodeling within first 20 min (Fig. 2C and supplemental movie 4). This concentration was toxic to cells as 100% of neurons were dead following first 2 h of treatment. Interestingly, unlike treatment with camptothecin where the fragmented mitochondria remained highly motile, the motility of fragmented mitochondria was substantially attenuated upon treatment with hydrogen peroxide (Fig. 2D and supplemental movies 1 and 5). To quantify the timing and percentage fragmentation of mitochondria and its correlation to cell death, mitochondrial and nuclear morphology were evaluated (Fig. 3A). Immediately following exposure to ROS, greater than 94% of mitochondria had a length of greater than 0.5 μm (Fig. 3B). Neurons treated with hydrogen peroxide exhibited signs of mitochondrial fragmentation as early as 3 h where 31 ± 6% of mitochondria had a length of less than 0.5 μm. Mitochondrial fragmentation continued to increase to 62.3 ± 1.4% and 88.5 ± 1.2% at 12 and 24 h, respectively. To ask whether the onset of mitochondrial fragmentation correlates with cell death, apoptosis was examined by Hoechst to detect pyknotic nuclei (Fig. 3C). The results of cell death studies were confirmed using colorimetric MTT survival assay (data not shown). At 12 h following treatment, 30 ± 2.5% of neurons exhibited pyknotic nuclei.

FIGURE 4. Mfn2 expression induces mitochondria fusion in CGNs. CGNs were infected at the time of plating with recombinant adenoviral vectors containing an expression cassette for Mfn2 or Mfn2RasG12V at 50 MOI. Whole cell lysates were analyzed in parallel with the control (no virus) by Western blot using an antibody against FP (A). CGNs were infected at the time of plating with recombinant adenoviral vectors containing an expression cassette for Mfn2 (C) or Mfn2RasG12V (D) or GFP control (B) at 50 MOI. E, neurons expressing the indicated constructs were fixed and stained with TOM 20 to assess changes in mitochondrial morphology following increased expression of Mfn2 and Mfn2RasG12V. Mitochondrial length of the indicated group is classified based on the frequency at different lengths (less than 0.5, 0.5–1, 1–2 and greater than 3 μm). n = 3; *p < 0.05; mag bar, 20 μm; cyan, nuclei; green, mitochondria.
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whereas cell death was maximal by 24 h when 50 ± 2% of neurons exhibited pyknotic nuclei. These results show that mitochondrial fragmentation was detected 3 h after treatment and 9 h before the apoptotic nuclei were considerably detected. These findings suggest that mitochondrial fragmentation may serve as an early apoptotic signaling event in this mode of injury.

Together, our results demonstrate that mitochondrial fragmentation is an early common event following acute injury in CGNs. We therefore asked whether preventing the fragmentation of the mitochondria by activation of the mitochondrial fusion machinery could prevent cell death induced by DNA damage and oxidative stress.

Increased Activation of Mfn2 Blocks Mitochondrial Fragmentation and Protects Neurons against Acute Injury—To examine whether activating mitochondrial fusion could protect neurons against cell death, we created adeno-virus vectors containing the CFP-tagged wild-type Mfn2 and the hydrolysis-deficient, constitutively active mutant Mfn2RasG12V (18). Primary neurons were infected 48 h prior to exposure to oxidative stress or DNA damage. We first examined whether these proteins could affect the mitochondrial morphology in untreated neurons. Cells were infected in parallel with adenovirus vectors carrying Mfn2, Mfn2RasG12V, or GFP control, and the mitochondrial morphology was evaluated 48 h later (Fig. 4). To confirm protein expression a Western blot analysis was performed with neurons infected with Mfn2 or Mfn2RasG12V (Fig. 4A). In unchallenged neurons (Fig. 4, B and E) the majority of mitochondria had an average length between 1 and 2 μm (53.8 ± 1.3%); however, increased expression of Mfn2:CFP (Fig. 4C) or Mfn2RasG12V:CFP (Fig. 4D) resulted in a significant increase in the length of the mitochondria. The majority of mitochondria were greater than 3 μm as a result of increased levels of Mfn2 (33.97 ± 5%) and Mfn2RasG12V:CFP (42.9 ± 4.2%) expression (Fig. 4E). These results demonstrate that enhanced activation of Mfn2 results in increased mitochondrial length.

We next asked whether activation of Mfn2 could prevent mitochondrial fragmentation and ultimately protect neurons against death induced by neuronal injury. To test whether activation of Mfn2 could protect neurons against DNA damage, parallel cultures were exposed to 10 μM camptothecin. The mitochondrial morphology and apoptosis were evaluated following 24 h (Fig. 5A). Mitochondrial fragmentation was dramatically inhibited with both Mfn2:CFP or Mfn2RasG12V:CFP expression (Fig. 5A). Following 24 h of treatment, GFP-infected neurons showed only 9.6 ± 1.1% of mitochondria measuring greater than 3 μm, whereas the expression of Mfn2:CFP and Mfn2RasG12V:CFP resulted in 3.4 ± 3.2% and 42.17 ± 7% of mitochondria being greater than 3 μm. Interestingly, the group of mitochondria measuring greater than 3 μm had a widely varied length distribution, with some single mitochondria spanning long projections and measuring greater than 30 μm. These results reveal that expression of Mfn2 or constitutive activation by expression of Mfn2RasG12V could equally prevent the breakdown of the mitochondria typically seen following DNA damage. Most importantly, increasing the levels of Mfn2 resulted in increased protection against cell death induced by DNA damage (Fig. 5C). Following 24 h of treatment, 34 ± 1.6% of neurons exhibited pyknotic nuclei in the GFP control, and this was reduced to 23.9 ± 1.6% in the Mfn2 cultures. Increased activation of Mfn2 through delivery of Mfn2RasG12V:CFP was significantly more protective than the wild type counterpart as it reduced cell death to 12.3 ± 2.1% (Fig. 5C). These results demonstrate that whereas Mfn2 and Mfn2RasG12V result in equal fusion of mitochondria, the GTP bound form of Mfn2RasG12V shows a 2-fold increase in protection of these neurons against DNA damage.
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We then examined whether activation of Mfn2 could protect neurons against injury induced by ROS. Neuronal cultures were infected in parallel with adenovirus-expressing GFP control, Mfn2:CFP, and Mfn2RasG12V:CFP. After 48 h, cells were exposed to H$_2$O$_2$ and mitochondrial morphology and cell death were evaluated as described above. Increased expression of Mfn2:CFP or enhanced activity of Mfn2 by delivery of Mfn2RasG12V:CFP protected neurons against the ROS-induced fragmentation, and resulted in significantly increased mitochondrial lengths (Fig. 6A). At 24 h following treatment with hydrogen peroxide, 3.09 ± 1.81% of mitochondria had a length of greater than 3 μm in cells expressing the GFP control (Fig. 6B). This number increased to 30.65 ± 5.7% and 35.05 ± 7.9% in the parallel cultures expressing Mfn2:CFP and Mfn2RasG12V:CFP, respectively (Fig. 6B). More importantly, whereas expression of wild type Mfn2 resulted in an increased survival with an intermediate 47.8 ± 3.2% of cells remaining alive, Mfn2RasG12V led to a dramatic 2–3-fold increase in survival, with 68 ± 4.77% of cells expressing Mfn2RasG12V:CFP remaining viable relative to only 24.59 ± 1.62% in GFP-expressing controls (Fig. 6C).

These data demonstrate that increased levels of Mfn2 prevents the breakdown of the mitochondria in response to injury and maintains the mitochondrial integrity in neurons. Most importantly, stabilization of the GTP-bound form of Mfn2 provides additional protection against death induced by ROS and DNA damage. These results highlight a novel therapeutic target to maintain neuronal survival after acute injury.

Mfn2 Protects Neurons against Injury by Attenuating Cytochrome c Release—Cytochrome c protein, a critical component of the electron transport chain, is normally localized to the mitochondria intermembrane space where it is sequestered within the cristae. Permeabilization of the outer mitochondria membrane results in partial release of the accessible cytochrome c into the cytosol; however the release of the majority of mitochondrial cytochrome c pool demands structural remodeling of mitochondria cristae (27, 28). We therefore asked whether mitofusin 2 regulates release of cytochrome c following different cell death stimuli. 24 h following treatment of the neurons with camptothecin, the neurons were fixed and stained with an antibody against cytochrome c, Tom 20, and/or Hoechst. 61.85 ± 1.31% of neurons had their cytochrome c released from the mitochondria in the LacZ control group. The cytochrome c release was decreased to 43.29 ± 4.6% in the wild type mitofusin group and to 22.32 ± 1.77% in the Mfn2RasG12V group (Fig. 7F). Similarly, following treatment with hydrogen peroxide, Mfn2 and Mfn2RasG12V attenuated release of cytochrome c to 47.8 ± 2.21% and 26.42 ± 0.98%, respectively when compared with control at 84.6 ± 12.7% (Fig. 7F). Our data not only indicate that activation of mitofusin 2 protects neurons against cell death upstream of cytochrome c release, which positions the function of Mfn2 within the apoptotic cascade in primary neuronal models of cell death.

Transduction of Neurons by a Mfn2RasG12V Lentivirus Protects Neurons against Potassium Deprivation-induced Apoptosis—Cell excitability is a critical determinant of neuronal survival during brain development (29). K$^+$ channels set both the resting membrane potential and the duration of the action potential. Opening of these channels can influence neuronal death or neuronal survival (30). Low K$^+$ exposure of granule neurons initiates a complex set of proapoptotic, metabolic, and signal transduction mechanisms that include up-regulation of c-Jun target genes and inhibition of glycolysis (31). Also, it has been recently demonstrated that upon K$^+$ deprivation, CGN exhibit an immediate reduction in mitochondrial respiration, a decrease in ATP turnover which correlates with decreased calcium concentration (32) and depletion of NFκB(33). Because cerebellar granule neurons yield a classic...
model for depolarization-induced apoptosis, we asked whether mitofusin 2 protects against this mode of cell death. We constructed a lentivirus for the Mfn2RasG12V to transduce CGN. The media containing 25 mM K\(^+\)/H\(_{11001}\) was changed to the media of 5 mM K\(^+\)/H\(_{11001}\) following 7 DIV and following 24 h in the low potassium media, the percentage of cell death was evaluated using the “Live/dead” assay. The percentage cell death declined from 74.06% in the control group to 49.1% in the Mfn2RasG12V group. Following correction for the basal cell death (15% in CTL to 22% in Mfn2\(_{\text{RasG12V}}\) group) Mfn2\(_{\text{RasG12V}}\) counts for a greater than 50% protection against K\(^+\)/H\(_{11001}\) deprivation-mediated apoptosis (Fig. 8).

**DISCUSSION**

The results of our studies support a number of conclusions: first we show that mitochondrial fragmentation occurs as an early event in response to injury in cerebellar granule neurons. The extent of mitochondrial fragmentation, however, is variable and depends on the mode of neuronal injury and the severity of the death stimuli. Second, expression of Mfn2 prevents mitochondrial fragmentation in response to oxidative stress and DNA damage-induced neuronal death. Third, we show that in addition to stimulating the fusion machinery, Mfn2 protects neurons against different modes of neuronal injury including DNA damage, oxidative stress, and K deprivation-induced apoptosis. Most importantly, we demonstrate that while the wild type Mfn2 and the constitutively activated mutant Mfn2\(_{\text{RasG12V}}\) function equally to promote fusion and

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**FIGURE 7.** Mfn2 attenuates cytochrome c release following DNA damage and ROS-mediated injury. CGNs were infected as described and treated with either H\(_2\)O\(_2\) (75 \(\mu\)M) or camptothecin (10 \(\mu\)M). The cells were fixed and stained with cytochrome c and Tom 20 following 24 h of treatment with camptothecin or hydrogen peroxide. Z stack sections of different fields were taken for each replicate. A, colocalization of Tom 20 and cytochrome c in the control neurons. B, representative field demonstrating release of cytochrome c from mitochondria following treatment with camptothecin and C, following treatment with hydrogen peroxide. D, cytochrome c and Tom 20 colocalization in the neurons infected with Mfn2\(_{\text{RasG12V}}\) following treatment with hydrogen peroxide. E, percentage of cytochrome c release following camptothecin treatment in CTL, Mfn2, and Mfn2\(_{\text{RasG12V}}\) group. F, quantification of cytochrome c release following treatment with hydrogen peroxide in CTL, Mfn2, and Mfn2\(_{\text{RasG12V}}\) group. n = 3; *, p < 0.05.
lengthening of mitochondria, neuronal protection against acute injury is much more effective in the GTPase hydrolysis-deficient Mfn2 mutant versus the wild type Mfn2. Furthermore, mitofusin 2 exerts its protective effect at an early stage upstream of cytochrome c release. Finally, down-regulation of mitofusin 2 induces cell death in the absence of any apoptotic stimuli. Taken together, these findings implicate an anti-apoptotic role for Mfn2 during death models representative of acute neuronal injury and neuronal development.

Mfn2 has been proposed to function along with its homologue Mfn1 as a direct tethering/fusion component (8, 10). Our results indicate that the increased activation of mitochondrial fusion by expression of Mfn2 or Mfn2 RasG12V could cause a dramatic increase in the mitochondrial lengths to greater than 30 μm. This neuronal response exhibiting a dramatic lengthening of the mitochondria is unlike that previously found in other cell types where the ectopic expression of Mfn2 resulted in mitochondria fusion within a non-motile perinuclear cluster (18). Neurons may therefore be unique in their ability to respond to these factors and may express a distinct repertoire of proteins that regulate mitochondrial fusion relative to other cell types.

A key question is whether the longer tubular mitochondria are more supportive of survival than the short fragmented mitochondria or alternatively; do molecules involved in the fusion machinery interact with cell death signaling? Previous studies have demonstrated that inducing fusion by overexpression of Mfn1 or a dominant negative mutant of Drp1 protects against nitric oxide-mediated cell death (34). Unlike Mfn1, which interacts with Opa1 to induce fusion, Mfn2 has been associated with apoptotic signaling proteins (16, 18). Interestingly, we show here that expression of both the wild type Mfn2 and the constitutively active mutant, Mfn2 RasG12V, had similar effects on mitochondrial lengthening; however, the hydrolysis-deficient mutant exhibited a more profound protection against cell death. These distinct biological responses suggest that the protection from death may not be due only to the increased fusion because both the wild type and mutant Mfn2 result in similar increases in mitochondrial length. Instead, the data suggest that the nucleotide state of Mfn2 may regulate other interactions on the mitochondrial membrane that are critical for the cell death. Our data supporting an additional role for Mfn2 beyond the activation of fusion is consistent with recent findings that demonstrate interactions with the Bcl family proteins. First Mfn2 is colocalized in punctate with Bax and Drp1 at sites of future fission and affects mitochondrial recruitment of Bax or Drp1 during cell death, indicating a spatial relationship between fusion and fission during cell death (13). Second, it was
shown that cytosolic Bax plays a specific role in the steady state activity of Mfn2 as a regulator of mitochondrial fusion (16). In that study, the mitochondria within the Bax/Bak double knockout (DKO) cells demonstrated a reduced rate of mitochondrial fusion. Mfn2 is normally found in a punctate pattern (18); however, in Bax/Bak DKO cells, the protein circumscribed the outer membrane (16). The introduction of Bax into the DKO cells resulted in a stable shift of Mfn2 within the outer mitochondrial membrane into foci, which rescued the rates of fusion. Interestingly, the GTP-bound form of Mfn2 did not respond to Bax/Bak expression and retained its highly mobile, even distribution along the outer membrane regardless of Bax/Bak expression levels (16). Given that this mutant was not affected by Bax/Bak expression, it is possible that Mfn2 RasG12V is also resistant to Bax/Bak-induced changes on the membrane during apoptotic stimuli. This resistance of Mfn2 RasG12V to assemble into Bax-dependent foci may interfere with the efficient assembly of pro-apoptotic complexes required for the progression of cell death. This would at least partially explain the increased protective activity of the activated mutant to multiple Bax-dependent apoptotic stimuli that we have observed in primary neurons. Finally, Mfn2 can interact directly with Ced9 or BclXl in HEK293 cells further suggesting a mechanism for protective cross-talk with antiapoptotic Bcl family proteins (3). Because Mfn2 protein levels have not yet been shown to be reduced during apoptosis, the nucleotide state of Mfn2 could be required to mediate cross talk with the apoptotic machinery. We envision a model whereby the GTP-bound form of Mfn2 may interact with the Bcl-2 family of proteins, remaining circumscribed along the outer membrane, functioning in a protective manner and protect against cell death. In contrast, the GDP-bound form would be susceptible to modulation by Bax to allow foci formation and assembly of the death machinery on the outer membrane. Future studies will be required to determine how this activity is regulated in the context of the apoptosis signaling cascade.

Finally, there is growing evidence to support the idea that the machineries that govern mitochondrial fusion are linked to the metabolic processes within the organelle. For example, Mfn2 has been shown to modulate metabolism through function of complex I, IV, and V (14, 15). Consistent with this idea, down-regulation of fusion proteins (Mfn1 and Mfn2) led to fragmented mitochondria with reduced oxygen consumption and electrochemical potential (35). This suggests that mitochondrial fusion is likely to be a central player in relating mitochondrial dynamics to mitochondrial metabolism, and could also be a mechanism for modulating cell death during neuronal injury.

In conclusion, we show that mitochondria undergo extensive fragmentation in acute neuronal injury and that activating the mitochondrial fusion machinery can protect neurons against injury induced cell death. These results demonstrate the importance of mitochondrial dynamics in acute injuries such as trauma and stroke. Demonstrating that the control of the nucleotide state of Mfn2 can dramatically affect the outcome of cell death suggests that Mfn2 may serve as an accessible therapeutic target for the treatment of these human diseases. Future work to investigate the factors that control the nucleotide state of Mfn2 and to delineate the interaction with the apoptotic machinery should further elucidate these mechanisms.

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