SARM1 participates in axonal degeneration and mitochondrial dysfunction in prion disease

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Abstract

Prion disease represents a group of fatal neurodegenerative diseases in humans and animals that are associated with energy loss, axonal degeneration, and mitochondrial dysfunction. Axonal degeneration is an early hallmark of neurodegeneration and is triggered by SARM1. We found that depletion or dysfunctional mutation of SARM1 protected against NAD⁺ loss, axonal degeneration, and mitochondrial functional disorder induced by the neurotoxic peptide PrP<sub>106-126</sub>. NAD⁺ supplementation rescued prion-triggered axonal degeneration and mitochondrial dysfunction and SARM1 overexpression suppressed this protective effect. NAD⁺ supplementation in PrP<sub>106-126</sub>-incubated N2a cells, SARM1 depletion, and SARM1 dysfunctional mutation each blocked neuronal apoptosis and increased cell survival. Our results indicate that the axonal degeneration and mitochondrial dysfunction triggered by PrP<sub>106-126</sub> are partially dependent on SARM1 NADase activity. This pathway has potential as a therapeutic target in the early stages of prion disease.

Key Words: axonal degeneration; mitochondrial dysfunction; NAD⁺ metabolism; NADase; neurodegenerative disease; prion disease; SARM1; sterile alpha and TIR motif-containing 1

Introduction

Prion disease represents a group of transmissible and fatal neurodegenerative diseases in both animals and humans that includes scrapie, bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease (Collinge, 2001). Accumulation of the misfolded form of the cellular prion protein in the central nervous system induces neuronal loss, spongiform vacuolation, and inflammation and is the main cause of neurodegeneration (Prusiner, 1991; Hur et al., 2002; Soto and Satani, 2011; Batlle and Ventura, 2020). The misfolded cellular prion protein is termed PrP<sup>+</sup>. Prion protein peptide 106–126 (PrP<sub>106-126</sub>), a neurotoxic prion protein fragment, has been used by researchers to mimic amyloid-like fibril accumulation and trigger axonal degeneration and neuronal apoptosis in vitro (Prusiner, 1991; Soto and Satani, 2011; Forloni et al., 2019; Groveman et al., 2020; Ishikawa et al., 2021).

In studies of various neurodegenerative diseases, the loss of synapses and axons that precedes neuronal loss is adequate to cause clinical symptoms. (Li et al., 2001; Gunawardena and Goldstein, 2005; Luo and O’Leary, 2005; Stokin and Goldstein, 2006; Gerds et al., 2015). Therefore, axonal degeneration is considered a hallmark of neurodegenerative disorders (Soto and Satani, 2011). Mitochondria are the most important cellular hub for adenosine triphosphate (ATP) production, the key source of cellular energy. Neurons are especially sensitive to mitochondrial dysfunction due to their extreme dependence on mitochondria for energy (Galluzzi et al., 2012; Morán et al., 2012). During ATP biosynthesis, nicotinamide adenine dinucleotide (NAD⁺) functions as an irreplaceable coenzyme. In previous studies, researchers found that the fifth member of the myeloid differentiation primary response 88 family, sterile alpha and Toll-interleukin receptor 1 (TIR) motif-containing 1 (SARM1), was required for axonal degeneration induced by axotomy. In addition, SARM1 knockout rescued symptoms in both in vivo and in vitro models (Osterloh et al., 2012; Henninger et al., 2016). Because injury-induced axonal degeneration has been associated with reduced NAD⁺ concentration, SARM1 may regulate this degeneration via a gain of NAD⁺ cleavage activity following release of SARM1 autoinhibition and dimerization of its TIF domains (Wang et al., 2005; Gerds et al., 2015).

Neuronal death and axonal degeneration in prion disease are linked with NAD⁺ depletion (Wang et al., 2015) and can be relieved by NAD⁺ replenishment. In a previous study, we investigated prion disease-induced mitochondrial dysfunction in both cell line and animal models (Wu et al., 2019). Because mitochondria process NAD⁺ to synthesize ATP and SARM1 can link to the outer mitochondrial membrane via its mitochondrial targeting sequence domain (Panneerselvam et al., 2012), we hypothesized that axonal degeneration associated with prion disease is partially regulated by SARM1 protein activation and its subsequent destruction of NAD⁺. To verify this hypothesis, we examined the effect of SARM1 knockdown/null mutation on the NAD⁺ depletion, mitochondrial dysfunction, and axonal degeneration induced by treatment with PrP<sub>106-126</sub>. In addition, we determined the effect of NAD⁺ replenishment on mitochondrial dysfunction and mitochondrial and axonal morphology in cells incubated with PrP<sub>106-126</sub>.

Materials and Methods

Ethics statement

All animals in this study were treated in accordance with the Guidelines for the Care and Use of Animals of the National Animal Transmissible Spongiform Encephalopathy Laboratory, College of Veterinary Medicine, China Agricultural University, Beijing, China.

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of the China Agricultural University on the Review of Welfare and Ethics of Laboratory Animals. The study protocol was approved by the China Agricultural University Administration Office of Laboratory Animals (approval No. AW20211202-2-1) on September 1, 2020.

**Cell culture and treatment**

Primary neurons were prepared from the cerebral cortex of 1-day-old neonatal Sprague-Dawley rats (Beijing Bio-Long, China) and served as control group. The neuronal cultures were grown on poly-L-lysine and laminin-precoated coverslips in Neurobasal medium (HyClone, Logan, UT, USA) with 1% penicillin-streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO2 incubator. After 1 week of culture, the neuronal cultures were treated with 10 μM PrP peptide or with siRNA for 12 hours in serum-free medium comprising Neurobasal-A Medium (Invitrogen) containing 1% penicillin-streptomycin and 1% fetal bovine serum. The cell cultures were used for western blotting, apoptosis assay, and immunocytochemistry when they were ~70–80% confluency. Secondary neuronal cultures were treated with siSARM1 in the same way, while the primary neuronal cultures were treated with siRNA. Both primary neurons and N2a cells were harvested after the experimental processes, collected into a tube after washing softly by PBS, and then incubated with the JC-1 probe for 30 minutes at 37°C. Following the washing step, the tubes were analyzed using flow cytometry. The ATP determination kit (Beyotime) was used to measure cellular ATP level, which was performed at low temperature. Cells in the culture plate were lysed with the lysis buffer from the kit and incubated in black 96-well plates with 100 μl detection reagent; 50 μl samples were added to each well. Luminescence of each well was measured using a luminometer (TECAN, Männedorf, Austria).

**Measurement of NAD level**

The NAD/NADH-Glo assay kit (Promega, Madison, WI, USA) was used to determine cellular NAD. After experimental processes, N2a cells were re-seeded at a concentration of 30,000 cells/well in flat white 96-well plates and incubated with detection reagent for 30 minutes at 37°C according to manufacturer instructions for 30 minutes. Luminescence was recorded using a plate reader (TECAN). Data are expressed as means ± standard error of the mean (SEM) and were analyzed using one-way analysis of variance followed by Bonferroni’s post hoc tests. All experiments were performed in triplicate using three independent cultures from different passages. The significance level was 0.05.

**Statistical analysis**

Data were expressed as means ± standard error of the mean (SEM). Data were analyzed using a one-way analysis of variance followed by Bonferroni’s post hoc test. All experiments were performed in triplicate using three independent cultures from different passages. The significance level was 0.05.

**Results**

**Axonal degeneration induced by PrP<sup>106–126</sup> incubation**

In prion disease, the most characteristic pathological change is neuronal degeneration, which causes dramatic clinical symptoms (Prusiner, 1999; Hur et al., 2002; Soto and Satani, 2011). Neuronal cell damage in prion disease models has been investigated in our previous studies (Yang et al., 2015; Song et al., 2016), which focused on morphological changes in the ultrastructure of primary cultured cortical neurons and axonal loss by spinal cord neurons after PrP<sup>106–126</sup> incubation. We found that the axons of primary cultured cortical neurons were severely shortened after 12 hours of incubation with 200 μM PrP<sup>106–126</sup> and PrP<sup>106–126</sup> conjugated to DUTP nick-end labeling (TUNEL) Apoptosis Assay Kit (Beyotime) was used. This procedure was performed away from light. The samples were prepared as described for immunofluorescence microscopy until antibody incubation. Then, the samples were incubated with TUNEL stain reagent for 1 hour at 37°C. The nuclei were visualized using a Nikon confocal imaging system. Images were analyzed using ImageJ software version 1.52a.

**SARM1 is required for PrP<sup>106–126</sup>-induced axonal degeneration**

In recent studies of neurodegenerative diseases, the role of SARM1 in axonal degeneration has gradually become clearer (Osterloh et al., 2012; Gerds et al., 2013; Henninger et al., 2016; Essuman et al., 2017). To determine whether SARM1 is involved in axonal degeneration induced by PrP<sup>106–126</sup> incubation, we first examined the level of SARM1 expression in N2a cells. According to our western blot results, SARM1 expression was increased significantly in N2a cells treated with PrP<sup>106–126</sup> for 12 or 24 hours (Figure 2A and B). Since SARM1 knockout (Beyotime, Cat# A0453, RRID: AB_2890132, 1:400) was conducted as treatment after treatment with PrP<sup>106–126</sup>, we explored the role of SARM1 in this effect with SARM1 knockdown using siRNA (Figure 2C). Western blotting showed...
that SARM1 knockdown N2a cells maintained their level of beta-tubulin III following treatment with PrP\(^{106–126}\) (Figure 2D and E). Next, we overexpressed full-length SARM1 using plasmid transfection (500 ng for 48 hours) in SARM1 knockdown N2a cells (Figure 2F) and showed that treatment with PrP\(^{106–126}\) reduced the beta-tubulin III expression level of the transfected cells (Figure 2G and H). In addition, SARM1 knockdown (Figure 2I) protected the axonal structure of primary cultured cortical neurons during PrP\(^{106–126}\) incubation (Figure 2J and K). Our findings indicate that SARM1 is involved in axonal degeneration caused by PrP\(^{106–126}\) and that knockdown of SARM1 protects neurons from this pathological change.

PrP\(^{106–126}\)-induced mitochondrial morphological damage and dysfunction are partly mediated by SARM1

Considering that neurons consume a large amount of energy, mitochondria are critical organelles involved in many neurodegenerative diseases (Pacelli et al., 2015; Swerdlow, 2018; Wang et al., 2021). In our previous studies, mitochondrial fragmentation and dysfunction were observed in neuron disease models (Li et al., 2018; Wu et al., 2019; Zhang et al., 2020). In other studies, SARM1 function was also associated with mitochondrial respiration and mitochondrial dysfunction-induced cell death (Hammars et al., 2014; Murata et al., 2018). Therefore, we hypothesized that SARM1 plays a role in PrP\(^{106–126}\)-induced axonal degeneration via regulation of mitochondrial function. After incubation with 200 μM PrP\(^{106–126}\) for 12 hours, mitochondria in primary cultured cortical neurons were imaged using fluorescence microscopy. TOMM40 staining showed that PrP\(^{106–126}\)-treated neurons had a remarkably fragmented mitochondrial network, whereas untreated control neurons had filamentous, continuous mitochondria, which suggested mitochondrial changes. The mitochondria of primary neurons that were treated with SARM1 siRNA and then incubated with PrP\(^{106–126}\) maintained their tubular morphology (Figure 3A). Mitochondrial length decreased significantly in PrP\(^{106–126}\)-treated neurons; however, in PrP\(^{106–126}\)-treated SARM1 knockdown neurons, the length significantly decreased and fusion was observed (Figure 3B). To ascertain mitochondrial function, we measured the ATP level of PrP\(^{106–126}\)-treated N2a cells. After PrP\(^{106–126}\) incubation, the ATP level of N2a cells was reduced to approximately 50% of that in the untreated control ATP loss was prevented by SARM1 knockdown and accelerated by overexpression of SARM1 (Figure 3C).

MMP loss is also a signal of mitochondrial dysfunction; therefore, we assessed the MPP using the JC1 assay. The monomer form of JC1 in damaged mitochondria appears green, while the aggregate form in healthy mitochondria appears red. The red/green ratio reflects the MMP and would decrease with MMP loss. In concert with the ATP measurements mentioned above, PrP\(^{106–126}\) incubation lowered the MMP compared with the untreated control. The change in MMP induced by PrP\(^{106–126}\) incubation was prevented by SARM1 knockdown and aggravated by SARM1 overexpression (Figure 3D).

These results indicate that SARM1 takes part in the mitochondrial morphological changes and dysfunction induced by PrP\(^{106–126}\) incubation.

PrP\(^{106–126}\)-induced mitochondrial dysfunction and axon degeneration via the NAD\(^+\)-hydrolase activity of SARM1

In a previous study, Wang et al. (2015) proposed NAD\(^+\) replenishment for neuroprotection in patients with prion disease. Because SARM1 performs cleavage of NAD\(^+\), which has been associated with axonal degeneration (Gerds et al., 2015; Essuman et al., 2017), we conjectured that SARM1 mediates axonal degeneration and mitochondrial dysfunction via its NAD\(^+\)-hydrolase activity. We found that PrP\(^{106–126}\) incubation decreased the percentage of cellular NAD\(^+\). In cells incubated with PrP\(^{106–126}\) and treated with SARM1 siRNA, the NAD\(^+\) level was similar to that in control cells; however, cells overexpressing SARM1 showed enhanced loss of NAD\(^+\) (Figure 4A). Next, we investigated the effects of NAD\(^+\) supplementation on axonal and mitochondrial morphology in an in vitro prion model by observing β-NAD-treated primary neurons using fluorescence microscopy. In primary cultured cortical neurons incubated with PrP\(^{106–126}\), we observed their filamentos and tubular shape and longer average length compared with the fragmented mitochondria of the PrP\(^{106–126}\)-treated group (Figure 4B and C). ATP level and MMP were measured to assess mitochondrial function. The mitochondria in PrP\(^{106–126}\)-treated N2a cells showed a decrease in length after treatment with β-NAD. Therefore, NAD treatment protected cells from PrP\(^{106–126}\)-induced ATP loss, even in cells overexpressing SARM1. However, SARM1 overexpression impaired recovery of the ATP level (Figure 4D).

In concert with the findings described above, after PrP\(^{106–126}\) incubation, the MMP of NAD-treated cells was maintained with compared with that of cells treated with PrP\(^{106–126}\) alone. Moreover, we observed the protective effect of NAD supplementation on MMP during PrP\(^{106–126}\) incubation (Figure 4E). In cells incubated with PrP\(^{106–126}\), αs in the β-NAD-treated group were significantly longer (Figure 4B and F). NAD\(^+\) treatment also restored the higher expression of the tubulin III protein. Not surprisingly, overexpression of SARM1 limited the recovery of beta-tubulin III expression (Figure 4G and H).

Our results suggest that the NAD\(^+\) hydrolase activity of SARM1 plays a role in PrP\(^{106–126}\)-induced mitochondrial dysfunction and axonal degeneration, and that NAD\(^+\) supplementation can prevent these deleterious effects of PrP\(^{106–126}\) on mitochondria and axons.

The reduced NADase activity of the SARM1(S548A) mutant limits mitochondrial dysfunction and axonal degeneration triggered by PrP\(^{106–126}\)

To confirm that the NAD\(^+\) hydrolase activity of SARM1 is a critical factor in mitochondrial dysfunction and axonal degeneration, we obtained a plasmid containing the S548A mutant SARM1 sequence, as S548A SARM1 has reduced NAD\(^+\) cleavage activity (Murata et al., 2018). After transfecting the SARM1(S548A) plasmid into SARM1-silenced N2a cells (Figure 5A), we measured the intracellular NAD\(^+\) level in cells treated with PrP\(^{106–126}\) and cells that were not treated. NAD\(^+\) depletion induced by PrP\(^{106–126}\) incubation in the SARM1-silenced N2a cells was limited and similar to that in the SARM1-silenced group but dramatically higher than that in the SARM1-overexpressed group (Figure 5B). Next, we measured the mitochondrial function of SARM1(S548A) N2a cells (Figure 5C and D). In contrast to cells overexpressing SARM1, SARM1(S548A) N2a cells did not show a loss of ATP or reduced MMP following PrP\(^{106–126}\) incubation. The intracellular ATP concentration and MMP of SARM1(S548A) N2a cells were maintained at levels similar to those in the SARM1-silenced group. Analysis of beta-tubulin III expression via western blotting yielded similar results (Figure 5E). After transfection with PrP\(^{106–126}\), N2a cells following PrP\(^{106–126}\) incubation. These results suggest that the NAD-hydrolase activity of SARM1 is required for PrP\(^{106–126}\)-induced mitochondrial dysfunction and axonal degeneration in cells incubated with PrP\(^{106–126}\).

SARM1 is involved in PrP\(^{106–126}\)-induced neuronal death

Because neuronal apoptosis is a characteristic of prion disease (Forlioni et al., 2019; Wu et al., 2019), and cell survival is a key clinicopathologic factor, we investigated the effect of SARM1 NAD\(^+\) hydrolase activity in cells incubated with PrP\(^{106–126}\). Apoptosis was assessed using the TUNEL assay in control, SARM1-silenced, SARM1-overexpressed, and SARM1(S548A)-expressed N2a cells (Figure 6A and B). The apoptotic cell percentage was increased in N2a cells following PrP\(^{106–126}\) incubation. This increase was prevented in SARM1-silenced cells and enhanced in SARM1-overexpressed cells. In the SARM1(S548A)-expressed group, PrP\(^{106–126}\) incubation failed to trigger apoptosis. Next, we assayed cell viability using the CCK8 assay (Figure 6C). SARM1 silencing protected PrP\(^{106–126}\)-incubated cells from dying, while SARM1 overexpression increased the percentage of apoptotic cells. In addition, cells that were maintained by NAD\(^+\) supplementation were significantly increased in PrP\(^{106–126}\)-treated cells, confirming the results of the apoptosis assays. These results indicate that the NAD\(^+\) hydrolase activity of SARM1 is critical in PrP\(^{106–126}\)-induced cell death and that SARM1 silencing or loss of function protects against this pathologic event.

Pharmacological supplementation with NAD\(^+\) promotes N2a survival in prion disease

As shown in Figure 4, NAD\(^+\) supplementation protected mitochondrial morphology and function from the deleterious effects of PrP\(^{106–126}\); therefore, we assessed the effect of NAD\(^+\) supplementation on cell survival. The results of apoptosis and viability assays showed that NAD\(^+\) supplementation inhibited apoptosis induced by PrP\(^{106–126}\), and protected cells in the SARM1-overexpressed group, NAD\(^+\) supplementation inhibited apoptosis and protected cells from cell death induced by PrP\(^{106–126}\); however, the protective effect was diminished by SARM1 overexpression (Figure 7A–C). These results confirm that NAD\(^+\) supplementation rescued PrP\(^{106–126}\)-induced apoptosis but that this effect was suppressed by SARM1 overexpression.
Figure 2 | SARM1 contributes to axon degeneration in PrP<sub>106–126</sub>-treated cells. (A) Western blots of SARM1 in PrP<sub>106–126</sub>-treated N2a cells. (B) Quantitative results for SARM1 expression. (C) Representative western blot results for SARM1 protein in N2a cells after transfection with SARM1 siRNA for 48 hours. (D) Western blots of beta-tubulin III in SARM1 knockdown N2a cells treated with PrP<sub>106–126</sub> compared with control. (E) Quantitative results for the data shown in D. (F) Representative western blot results for SARM1 protein in N2a cells transfected with SARM1 plasmid for 48 hours after 12 hours SARM1 siRNA transfection. (G) Western blots of beta-tubulin III in SARM1 overexpressed N2a cells treated with PrP<sub>106–126</sub> compared with control. (H) Quantitative results for the data shown in G. (I) Representative western blot results for SARM1 protein in primary neurons after transfection with SARM1 siRNA for 48 hours. (J) Primary neurons were transfected with SARM1 siRNA for 48 hours before incubation with PrP<sub>106–126</sub>. Neuronal structure is shown in fluorescent green (stained by Alexa Fluor 488). After incubation with PrP<sub>106–126</sub>, axonal length was shortened significantly. SARM1 knockdown protected axons from PrP<sub>106–126</sub>-induced degeneration. Scale bars: 10 μm. (K) Analysis of axon length. Data are expressed as the mean ± SEM. All assays were repeated three times. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance followed by Bonferroni’s post hoc test). GADPH: Glyceraldehyde-3-phosphate dehydrogenase; PrP<sub>106–126</sub>: PrP peptide 106–126; SARM1: sterile alpha and Toll-like receptor motif-containing 1; siRNA: small interfering RNA.

Figure 3 | SARM1 is involved in changes in mitochondrial morphology and function caused by PrP<sub>106–126</sub> treatment. (A) Primary neurons were transfected with SARM1 siRNA for 48 hours before PrP<sub>106–126</sub> incubation. After incubation with PrP<sub>106–126</sub>, the mitochondrial network was fragmented; however, in the SARM1 knockdown group, mitochondria kept their tubular morphology after PrP<sub>106–126</sub> incubation. Neuronal structure is shown in fluorescent green (Alexa Fluor 488), while mitochondria appear red (Alexa Fluor 555). Scale bars: 10 μm. (B) Analysis of mitochondrial length. (C) Relative intracellular ATP level. Control, SARM1-silenced, and SARM1-overexpressed N2a cells were incubated with PrP<sub>106–126</sub>. (D) Mitochondrial membrane potential. Control, SARM1-silenced, and SARM1-overexpressed N2a cells were incubated with PrP<sub>106–126</sub>. Data are expressed as means ± SEM. All assays were repeated three times. *P < 0.05, **P < 0.001 (one-way analysis of variance followed by Bonferroni’s post hoc test). ATP: Adenosine triphosphate; PrP<sub>106–126</sub>: PrP peptide 106–126; SARM1: sterile alpha and Toll-like receptor motif-containing 1; siRNA: small interfering RNA; TOMM40: translocase of outer mitochondrial membrane 40.
Figure 4  | NAD-hydrolase activity of SARM1 plays a role in PrP<sub>106–126</sub>-induced axonal degeneration and mitochondrial disorders.

(A) Relative intracellular NAD level. Control, SARM1-silenced, and SARM1-overexpressed N2a cells were incubated with or without PrP<sub>106–126</sub> for 12 hours. After PrP<sub>106–126</sub> incubation, the intracellular NAD level dropped significantly; however, in SARM1-silenced cells, the NAD level was protected. SARM1-overexpression enhanced the decrease in intracellular NAD. Neuronal structure is shown in fluorescent green (Alexa Fluor 488), while mitochondria appear red (Alexa Fluor 555). Scale bars: 10 μm. (C) Analysis of mitochondrial length. (D) Relative intracellular ATP levels in control and SARM1-overexpressed N2a cells incubated with PrP<sub>106–126</sub> and without NAD treatment. (E) Mitochondrial membrane potential in control and SARM1-overexpressed N2a cells incubated with PrP<sub>106–126</sub> with and without NAD treatment. (F) Analysis of axon length. (G) Western blots of beta-tubulin III levels in control and SARM1-overexpressed N2a cells incubated with PrP<sub>106–126</sub> with and without NAD treatment. (H) Quantitative results of the data shown in G. Beta are expressed as means ± SEM. All assays were repeated three times. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance followed by Bonferroni’s post hoc test).

Figure 5  | Expression of SARM1(S548A) improves mitochondrial dysfunction and reduces axonal degeneration induced by PrP<sub>106–126</sub>.

(A) Western blot results for SARM1 protein in N2a cells transfected with the SARM1(S548A) plasmid for 48 hours after 12 hours SARM1 siRNA transfection. (B) Relative intracellular NAD level. Control, SARM1-silenced, SARM1-overexpressed, and SARM1(S548A)-expressed N2a cells were incubated with or without PrP<sub>106–126</sub>. (C) Relative intracellular ATP levels in control, SARM1-silenced, SARM1-overexpressed, and SARM1(S548A)-expressed N2a cells incubated with or without PrP<sub>106–126</sub>. (D) Mitochondrial membrane potential in control, SARM1-silenced, SARM1-overexpressed, and SARM1(S548A)-expressed N2a cells incubated with or without PrP<sub>106–126</sub>. (E) Western blots of beta 3-tubulin in control, SARM1-silenced, SARM1-overexpressed, and SARM1(S548A)-expressed N2a cells incubated with or without PrP<sub>106–126</sub>. (F) Quantitative results of the data shown in E. Data are expressed as means ± SEM. All assays were repeated three times. **P < 0.01, ***P < 0.001 (one-way analysis of variance followed by Bonferroni’s post hoc test). ATP: Adenosine triphosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NAD: nicotinamide adenine dinucleotide; ns: not significant; PrP<sub>106–126</sub>: PrP peptide 106–126; SARM1: sterile alpha and Toll-like receptor motif-containing 1; siRNA: small interfering RNA; TOMM40: translocase of outer mitochondrial membrane 40.
Discussion

Prion disease represents a group of fatal central nervous system diseases that exhibit typical features of neurodegeneration, spongiform vacuolation, and inflammation (Soto and Satani, 2011). The pathogenesis of prion disease remains unclear, but axonal degeneration, mitochondrial dysfunction, and neuronal death have been described and neuronal loss is thought to be the cause of clinical symptoms and death (Hür et al., 2002; Song et al., 2016; Forloni et al., 2019). Evidence from the last decade suggests that SARM1 is associated with axonal degeneration in various pathologic conditions, such as injury, mitochondrial dysfunction, and Leber congenital amaurosis (Osterloh et al., 2012; Gerds et al., 2013; Summers et al., 2014; Ko et al., 2020; Sasaki et al., 2020). We demonstrated that SARM1 knockdown N2a cells maintained a higher level of axon formation-associated beta-tubulin III expression following PrP<sub>106–126</sub> incubation. In addition, re-establishing expression of SARM1 led to PrP<sub>106–126</sub>-induced beta-tubulin III loss. SARM1 depletion also protected the axons of primary cultured cortical neurons from the deleterious effects of PrP<sub>106–126</sub>. This finding is in accordance with the studies mentioned above that used other disease models.

Fragmentation of axons and mitochondria after PrP<sub>106–126</sub> incubation is an indicator of neuronal dysfunction. As the main producer of energy in cells, mitochondria are involved in many neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and prion disease (Packelli et al., 2015; Swerdlov, 2018; Wu et al., 2019; Zhang et al., 2020; Wang et al., 2021). SARM1 function has been associated with mitochondrial respiration and mitochondrial dysfunction-induced cell death (Summers et al., 2014; Murata et al., 2018). As we expected, depletion of SARM1 resulted in maintenance of the axonal and mitochondrial morphology following PrP<sub>106–126</sub> incubation. Next, we determined whether SARM1 was associated with PrP<sub>106–126</sub>-induced changes in mitochondrial function. We found that downregulation of SARM1 expression limited the effects of PrP<sub>106–126</sub> on ATP level and MMP. Not surprisingly, overexpression of SARM1 enhanced the PrP<sub>106–126</sub>-induced loss of ATP and reduction of MMP. Overexpression of SARM1 alone does not always lead to axonal damage or mitochondrial dysfunction, which could also be explained by the autoinhibitory nature of the SARM1 protein. Overexpression of SARM1 is just a condition to increase neuronal vulnerability to experimental processing (Gerds et al., 2013). In an early study, SARM1 was reported to be involved in induction of axonal degeneration and was associated with intra-axonal mitochondrial dysfunction in the condition of rapid loss of NMNAT2, an enzyme that synthesizes NAD (Carimalo et al., 2005). Further studies of the role of SARM1 in prion disease are warranted. Our finding that NAD deficiency, associated with PrP<sub>106–126</sub> incubation, NAD+ supplementation decreased the stain intensity; SARM1 overexpression limited the effect of NAD+ supplementation. Scale bars: 50 μm. We also found that NAD+ supplementation reduced the expression of TUNEL-stained cells; NARM1 overexpression limited the effect of NAD+ supplementation. Scale bars: 50 μm. (B) Analysis of apoptosis rate for the data shown in A. (C) N2a cell viability was assayed using the cell counting kit-8 kit with control and SARM1-overexpressed N2a cells incubated with or without NAD+ treatment. Data are expressed as means ± SEM. All assays were repeated three times.

Further studies of the role of SARM1 in prion disease are warranted. Our finding that NAD deficiency is associated with PrP<sub>106–126</sub> incubation, NAD+ supplementation decreased the stain intensity; SARM1 overexpression limited the effect of NAD+ supplementation. Scale bars: 50 μm. We also found that NAD+ supplementation reduced the expression of TUNEL-stained cells; NARM1 overexpression limited the effect of NAD+ supplementation. Scale bars: 50 μm. (B) Analysis of apoptosis rate for the data shown in A. (C) N2a cell viability was assayed using the cell counting kit-8 kit with control and SARM1-overexpressed N2a cells incubated with or without NAD+ treatment. Data are expressed as means ± SEM. All assays were repeated three times.

**Figure 6** | NADase activity of SARM1 is associated with cell survival following incubation with PrP<sub>106–126</sub>.

(A) TUNEL staining results in control, SARM1-silenced, SARM1-overexpressed, and SARM1(548A)-expressed N2a cells incubated with or without PrP<sub>106–126</sub> incubation. Incubation with PrP<sub>106–126</sub> resulted in increased TUNEL stain intensity. SARM1-overexpression enhanced this increase while SARM1-silencing and SARM1(548A) expression decreased it.

Scale bars: 50 μm. (B) Analysis of apoptosis rate for the data shown in A. (C) N2a cell viability was assayed using the cell counting kit-8 kit with control and SARM1-overexpressed N2a cells incubated with or without PrP<sub>106–126</sub> Data are expressed as means ± SEM. All assays were repeated three times.

**Figure 7** | NAD supplementation is protective against the deleterious effects of PrP<sub>106–126</sub> incubation.

(A) TUNEL staining results of control and SARM1-overexpressed N2a cells incubated with and without PrP<sub>106–126</sub> and with and without NAD treatment. Incubation with PrP<sub>106–126</sub> resulted in increased TUNEL stain intensity. In the condition of PrP<sub>106–126</sub> incubation, NAD+ supplementation decreased the stain intensity; SARM1 overexpression limited the effect of NAD+ supplementation. Scale bars: 50 μm. (B) Analysis of apoptosis rate for the data shown in A. (C) N2a cell viability was assayed using the cell counting kit-8 kit with control and SARM1-overexpressed N2a cells incubated with or without NAD+ treatment. Data are expressed as means ± SEM. All assays were repeated three times.

NAD supplementation is protective against the deleterious effects of PrP<sub>106–126</sub> incubation.
rescued PrP\textsuperscript{106–126}-induced apoptosis and increased cell survival, while SARM1 overexpression aggravated cell death. However, previous studies have found more muted effects of SARM1 deficiency. In one, SARM1 knockout protected against axonal degeneration only in the early stage of experimental allergic encephalomyelitis, and loss of SARM1 did not promote zebrafish axon rescaling nor provide motor neuron protection in an amyotrophic lateral sclerosis animal model (Tian and López-Schier, 2020). Another recent study that used a transgenic mouse model reported that SARM1 deficiency failed to change the prion-induced lesion pattern and did not alter PrP\textsuperscript{106–126} accumulation; instead, SARM1 deficiency upregulated XAF1 and promoted neuronal apoptosis (Zhu et al., 2019). The above findings indicate that the effect of SARM1 deficiency on axons is protective but not restorative and that SARM1 is involved in the early stage of axonal degeneration.

PrP\textsuperscript{106–126} peptide used as a tractable tool of prion disease study may have limitations. Though PrP\textsuperscript{106–126} peptide recapitulated biological features of PrPSc, but was not infectious. There might be unknown differences in the cell response between the peptide and real prion strain. The effectiveness on the basis of studies (Sharma et al., 2021) can gain consistent results in clinical trial is a very first step to reveal the role of SARM1 NAD\textsuperscript{+} hydrolase activity in prion disease and the utilization of a new therapeutic strategy. More study in vivo with real stains is needed in our future work to confirm our findings.

In summary, our findings demonstrate that prion-induced neuronal damage is partially dependent on the NADase activity of SARM1. We showed that depletion of SARM1, dysfunctional mutation of SARM1, and replenishment of NAD\textsuperscript{+} protect against axonal degeneration and mitochondrial dysfunction and promote cell survival in an in vitro model. Considering that the JNK-c-Jun pathway is activated in prion disease (Murata et al., 2005), SARM1 phosphorylation at S548 activates NADase function in injury-induced axonal degeneration (Murata et al., 2018), and SARM1(S548A) is an invalid mutation in PrP\textsuperscript{106–126}-induced NAD\textsuperscript{+} depletion, JNK-SARM1 phosphorylation might be induced in prion disease pathogenesis. The next step is to investigate the mechanisms of SARM1 activation and involvement in prion disease. SARM1 may represent a potential therapeutic target in prion disease.

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