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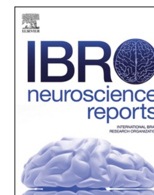

Effects of amyloid-β on protein SUMOylation and levels of mitochondrial proteins in primary cortical neurons

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A R T I C L E I N F O

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A B S T R A C T

Defining the molecular changes that underlie Alzheimer's disease (AD) is an important question in neuroscience. Here, we examined changes in protein SUMOylation, and proteins involved in mitochondrial dynamics, in an in vitro model of AD induced by application of amyloid-β 1–42 (Aβ_{1-42}) to cultured neurons. We observed Aβ_{1-42}-induced decreases in global SUMOylation and in levels of the SUMO pathway enzymes SENP3, PIAS1/2, and SAE2. Aβ exposure also decreased levels of the mitochondrial fission proteins Drp1 and Mff and increased activation of caspase-3. To examine whether loss of SENP3 is cytoprotective we knocked down SENP3, which partially prevented the Aβ_{1-42}-induced increase in caspase-3 activation. Together, these data support the hypothesis that altered SUMOylation may play a role in the mechanisms underlying AD.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative brain disorder (Breijyeh and Karaman, 2020), with aggregation of extracellular amyloid-beta peptide (Aβ) as a main histopathological hallmark. This Aβ pathology is initiated decades before the onset of clinical symptoms (Bateman et al., 2012). Given that there is no cure for AD, a key objective is to identify and understand the molecular mechanisms underlying AD, in order to intervene and restore neuronal function. Small ubiquitin-like modifier (SUMO) proteins are ~10 kDa proteins that are covalently conjugated to lysine residues in target proteins to modify their function. SUMO1 and SUMO2/3 paralogues are expressed in the mammalian brain and, in addition to their physiological roles, are implicated in many neuropathologies, including AD (Princz and Tavernarakis, 2020). Recent studies indicate that increased SUMOylation of specific substrates can be neuroprotective in response to cell stress (Guo and Henley, 2014).

An enzymatic cascade mediates SUMOylation, starting with SUMO activation by an ATP-dependent E1 enzyme, formed by a heterodimer of SAE1 (AOS1) and SAE2 (UBA2). The activated SUMO is conjugated by the E2 conjugating enzyme Ubc9, which in combination with SUMO E3 ligases (including the PIAS family proteins), mediates target recognition and conjugation of SUMO to the substrate (Chang and Yeh, 2020). This process can be reversed by SUMO-specific proteases, the best characterized of which are the SENP family of six cysteine proteases (Guo and Henley, 2014).

Global increases in SUMO2/3 conjugation are a cellular protective response to severe ischemic stress (Datwyler et al., 2011), mediated by stress-induced loss of the deSUMOylating enzyme SENP3 (Guo et al., 2013). A key target for SENP3-mediated deSUMOylation is the GTPase dynamin-related protein 1 (Drp1), which plays a major role in regulating mitochondrial morphology and integrity (Kraus et al., 2021).

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Recruitment of Drp1 to mitochondria during cell stress causes fragmentation and cytochrome *c* (cyt *c*) release, potent signals of cell death (Chang and Blackstone, 2010), which can be attenuated by enhanced Drp1 SUMOylation resulting from loss of SENP3 (Guo et al., 2017). This suggests that changes in SENP3 stability could provide new drug targets and therapeutic strategies.

Here we investigated global SUMOylation, SUMO pathway enzymes, and changes in proteins relevant to mitochondrial dysfunction and neuronal death in an in vitro $A\beta_{1\text{–}42}$ peptide challenge model of AD. Since Aβ levels in AD are extremely low and the toxic neuronal damage phenotype seen in AD generally takes decades to manifest, we applied $A\beta_{1\text{–}42}$ peptide at concentrations that do not result in acute loss of neuronal viability and death for a more realistic in vitro model of the cellular pathways disrupted in AD (Gilson et al., 2015; Hampel et al., 2021). Finally, we examined whether promoting protein SUMOylation by knocking down the SUMO-specific deSUMOylating enzyme SENP3 may promote cell survival in this in vitro AD model.

2. Material and methods

2.1. Cell culture

Cortical cultures were prepared from E18 Wistar rat embryos, using a modified protocol (Martin et al., 2007). All animal care and procedures were carried out in full compliance with University of Bristol and ARRIVE guidelines, and the UK Animals Scientific Procedures Act, 1986. In addition, all experimental protocols were approved by University of Bristol Animal Welfare and Ethics Review Body (ethics approval number UIN: UB/18/004) panel and the Biological and Genetic Modification Safety Committee (BGMSC).

Cells were plated on poly-L-lysine-coated culture dishes at a density of 600000 per well (6-well plates) in Neurobasal medium (Gibco) containing 10% horse serum, B27 (Gibco), and 5 mM glutamine and incubated at 37 °C in humidified air supplemented with 5% CO_2. Twenty-four hours later, the media was replaced by Neurobasal medium containing B27 and 2 mM glutamine and incubated at 37 °C in humidified air supplemented with 5% CO_2 until experimental usage.

2.2. Lentivirus production

Scrambled shRNA or shRNA targeting SENP3 (target sequence TATGGACAGAACTGGCTCAATGACCAGGT) (Rawlings et al., 2019) were cloned into a modified pXLG3 lentiviral vector under the control of a U6 promoter. Lentivirus was produced in HEK293T cells using the helper vectors p8.91 and pMD2. G, as described previously (Rocca et al., 2017).

2.3. Aβ aggregation and application to cultures

$A\beta_{1\text{–}42}$ or its reverse peptide $A\beta_{42\text{–}1}$ (Bachem) were incubated in 0.2% ammonium hydroxide in Milli-Q water (1 mg/mL) at 37 °C for 4 days before use. Aliquots were stored at − 80 °C. Primary neuronal cultures were exposed to 2 μM $A\beta_{1\text{–}42}$ or $A\beta_{42\text{–}1}$ for 48 h, at 17 days in vitro (DIV) (Hoppe et al., 2013a,b). In some experiments, primary cortical neurons were infected with either scrambled shRNA or SENP3 shRNA lentivirus for 4 days prior to 2 μM $A\beta_{1\text{–}42}$ or $A\beta_{42\text{–}1}$ challenge.

2.4. Immunoblotting

Cells were lysed directly into 1x Laemmli sample buffer, collected in eppendorfs and heated to 95 °C for 10 min prior to SDS-PAGE. Western blots were immunoblotted with the following antibodies: SUMO1 (Cell Signaling Technology, 1:1000), SUMO2/3 (Cell Signaling Technology, 1:1000), SENP3 (Cell Signaling Technology, 1:1000), Ubc9 (Cell Signaling Technology, 1:1000), PIAS1/2 (Abcam, 1:1000), UBA2 (Santa Cruz Biotechnology, 1:500), Drp1 (BD Biosciences, 1:2000), Mff (Santa Cruz Biotechnology, 1:500), Fis1 (ProteinTech, 1:1000), Mfn2 (Cell Signaling Technology, 1:2000), OPA1 (Abcam, 1:1000), AMPKα (Cell Signaling Technology, 1:1000), p-AMPKα (Cell Signaling Technology, 1:1000) and cleaved caspase-3 (Cell Signaling Technology, 1:1000). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (Sigma, 1:10000) were used as secondary antibodies and GAPDH (Abcam, 1:20000) was used as loading control. Each immunoblot is representative of at least three experiments carried out using different cell populations and analysed using ImageJ software (NIH, USA).

2.5. Statistics

Statistical analyses were performed using Student's t-test or two-way ANOVA followed by Fisher *post-hoc* test when appropriate. Statistical significance was considered at $p \leq 0.05$. Values are presented as mean ± standard error of the mean (S.E.M.) and expressed as percentage of the control $A\beta_{42\text{–}1}$ value.

3. Results

3.1. $A\beta_{1\text{–}42}$ decreases SUMO1 and SUMO2/3 conjugation

We first analysed the effects of $A\beta_{1\text{–}42}$ or its inactive reverse peptide $A\beta_{42\text{–}1}$ (2 μM) on global SUMOylation in primary cortical neurons incubated with Aβ for 48 h. Exposure to $A\beta_{1\text{–}42}$ caused a global decrease in both SUMO1 and SUMO2/3 conjugation to proteins (Fig. 1). Furthermore, analysis of a selection of prominent bands revealed significant changes in individual SUMO1 and SUMO2/3 conjugates (Table 1). Distinct bands on the SUMO1 blot showed decreases at ~80 kDa and ~45 kDa, while SUMO2/3 reactive bands at ~40 kDa and ~30 kDa were also decreased by $A\beta_{1\text{–}42}$ (Table 1). Crucially, no differences were observed between cells treated with the reverse control peptide $A\beta_{42\text{–}1}$ and untreated cells (Supplementary Tables 1, 2 and 3).

3.2. $A\beta_{1\text{–}42}$ reduces levels of important enzymes involved in the SUMOylation pathway

We next evaluated the effects of $A\beta_{1\text{–}42}$ on key enzymes in the SUMOylation pathway. Total levels of the SUMO2/3-selective protease SENP3 were dramatically reduced by $A\beta_{1\text{–}42}$ (Fig. 2A), whereas levels of the only SUMO E2 conjugating enzyme, Ubc9, remained unchanged (Fig. 2B). Interestingly, however, levels of both the SUMO E3 ligases PIAS1/2 (Fig. 2C) and the SUMO activating enzyme SAE2 (Fig. 2D) were reduced by $A\beta_{1\text{–}42}$.

3.3. Effects of $A\beta_{1\text{–}42}$ on mitochondrial proteins

Mitochondria are highly dynamic organelles that constantly undergo repeated cycles of fission and fusion to maintain appropriate numbers and to mediate quality control (Herst et al., 2017). Mitochondrial fission involves the recruitment of the GTPase and SUMO substrate Drp1 from the cytosol to the mitochondrial surface via binding to adaptor proteins, one of which is mitochondrial fission factor (Mff) (Loson et al., 2013). Mitochondrial dysfunction or damage in neurons is closely associated with neurodegeneration (Bock and Tait, 2019), and altered mitochondrial dynamics have been observed in AD patients and models of AD (Wang et al., 2009), so we tested the effects of $A\beta_{1\text{–}42}$ on the mitochondrial fission machinery. As shown in Fig. 3, $A\beta_{1\text{–}42}$ reduces total levels of Drp1 and also leads to an increase in caspase-3 cleavage, a marker of apoptosis (Figs. 3A and 3B).

Mff is the dominant mitochondrial adaptor protein for Drp1 (Loson et al., 2013) and its levels are also decreased by $A\beta_{1\text{–}42}$ (Fig. 3C). It has been proposed that another Drp1 adaptor protein, mitochondrial fission protein 1 (Fis1), plays an ancillary role, acting after Drp1 and Mff initiate fission (Shen et al., 2014). We found that Fis1 levels are unaltered by $A\beta_{1\text{–}42}$ (Fig. 3D). We also investigated the levels of adenosine

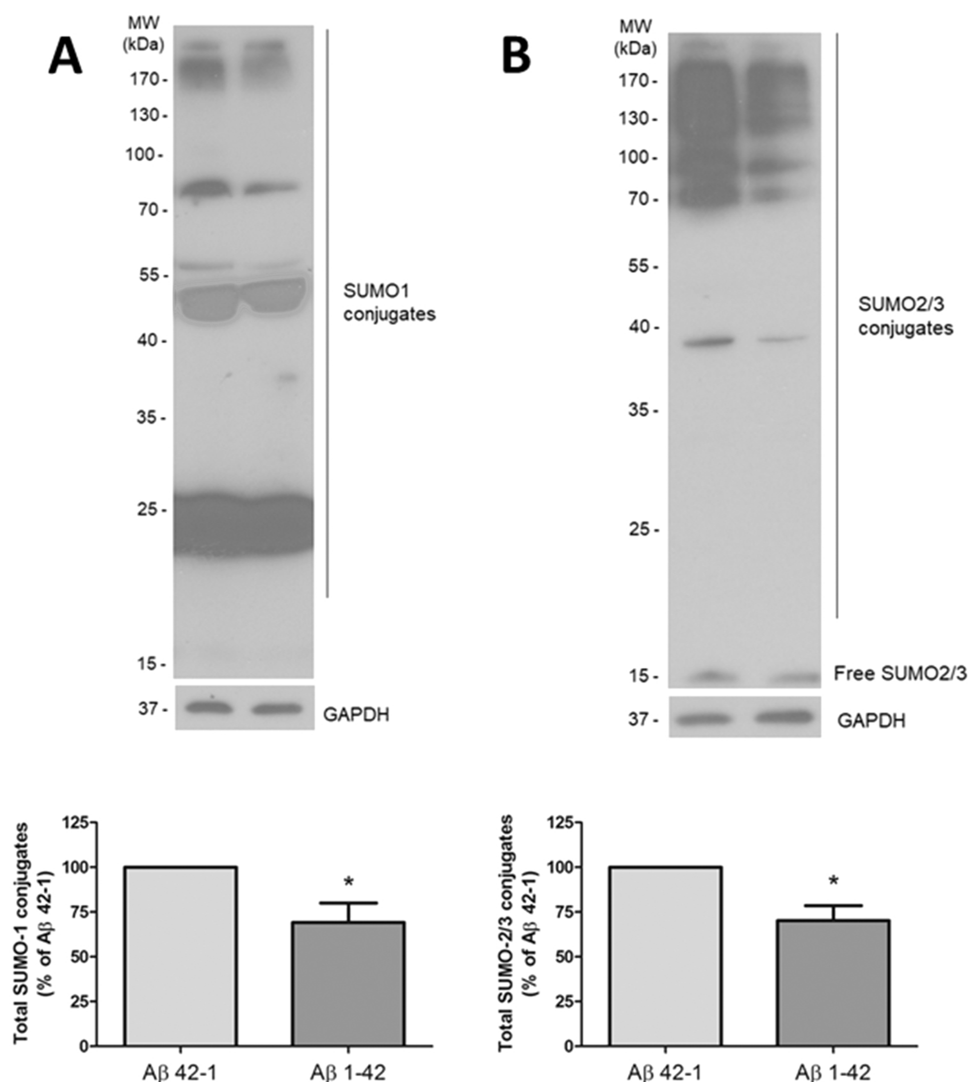

Fig. 1. Aβ_{1-42} decreases SUMO1 and SUMO2/3 conjugation. DIV17 rat cortical neuronal cultures were incubated with 2 μM Aβ_{1-42} or its reverse peptide Aβ_{42-1} for 48 h and cell lysates Western blotted for SUMO1 (A) or SUMO2/3 (B). In each case, the left hand lane represents cells treated with reverse peptide (Aβ_{42-1}) and the right hand lane cells treated with Aβ_{1-42}. GAPDH was used as a loading control. N = 4. *p ≤ 0.05, Student's t-test, compared to Aβ_{42-1} group.

Table 1
Analysis of prominent bands reveals significant changes in SUMO1 and SUMO2/3 conjugation in response to Aβ_{1-42}.

	SUMO1 conjugation			SUMO2/3 conjugation		
	High MW	~80 kDa	~45 kDa	High MW	~40 kDa	~30 kDa
Aβ_{42-1}	100	100	100	100	100	100
Aβ_{1-42}	81.78 ± 17.3	72.63 ± 11.5 *	40.08 ± 7.9 *	71.98 ± 19.4	53.00 ± 8.5 *	54.46 ± 14.1 *

Results are presented as mean ± S.E.M. and expressed as percentage of Aβ42-1 value. N = 4 (for high MW SUMO1 conjugation and all SUMO2/3 analysis), N = 5 for the ~80 kDa SUMO1 band, and N = 6 for the ~45 kDa SUMO1 band. Statistical analyses were performed using Student's t-test. *p < 0.05.
Aβ_{1-42}: amyloid-beta peptide; **MW:** Molecular Weight; **kDa:** kilodaltons; **S.E.M.:** Standard Error of the Mean.

monophosphate (AMP)-activated protein kinase (AMPK), which is rapidly activated by mitochondrial stress and triggers mitochondrial fission, at least in part, via phosphorylation of Mff (Toyama et al., 2016). Again, Aβ_{1-42} treatment did not change the levels of total AMPK or its active phosphorylated form (Fig. 3E).

Since we observed changes in levels of the fission GTPase Drp1 in response to Aβ_{1-42}, we also tested whether levels of the fusion GTPases Mfn2, which is responsible for fusion at the mitochondrial outer membrane, or OPA1, which mediates inner membrane fusion (Mishra and Chan, 2014), were altered by Aβ_{1-42} application. However, neither Mfn2 nor OPA1 levels were altered by Aβ_{1-42} (Figs. 3F and 3G).

3.4. SENP3 depletion partially protects against the increase of cleaved caspase-3 induced by *Aβ_{1-42}*

Previous studies have demonstrated that loss of SENP3 is neuroprotective against ischemic stress and heavy metal-induced toxicity (Guo et al., 2013; Guo et al., 2017). We therefore, wondered if loss of SENP3 could reverse the decrease in SUMOylation observed after application of A$\beta_{1-42,}$ and potentially promote cell survival. Consistent with this hypothesis, shRNA-mediated knockdown of SENP3 counteracted the decreased levels of protein SUMO2/3-ylation induced by Aβ_{1-42} (Fig. 4 A and 4B). Moreover, SENP3 knockdown partially rescued the Aβ_{1-42}-induced increase in cleaved caspase-3 (Fig. 4 C), suggesting that loss of SENP3 may promote cell survival in this in vitro model of AD.

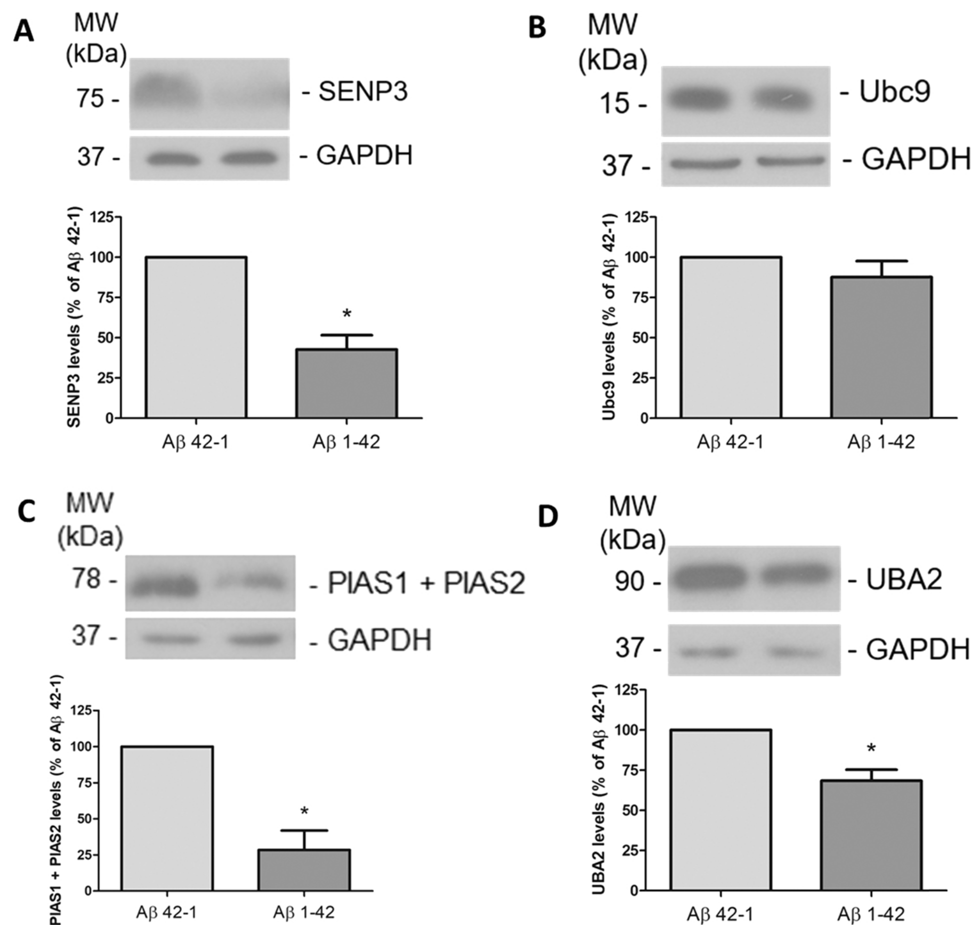

Fig. 2. A$\beta_{1–42}$ decreases SENP3 (A), PIAS1/2 (C) and UBA2 (D) levels. The levels of Ubc9 (B) were unchanged. DIV17 rat cortical neuronal cultures were incubated with 2 μM A$\beta_{1–42}$ or its reverse peptide for 48 h. GAPDH was used as a loading control. N = 6 (SENP3), N = 5 (Ubc9 and UBA2), and N = 3 (PIAS1/2). *$p \leq 0.05$, Student's t-test, compared to A$\beta_{42–1}$ group.

4. Discussion

Our hypothesis was that since SUMOylation is an important regulator of synaptic and neuronal function and dysfunction (Guo and Henley, 2014), it likely plays key roles in the neuronal deficits that lead to AD. Indeed, altered SUMOylation dynamics have been observed in both human AD brain, and in animal models of AD (McMillan et al., 2011). Furthermore, perturbed mitochondrial dynamics are a central factor in AD pathology (Lin and Beal, 2006), but the mechanisms and pathways involved are poorly characterized.

Here, we used an in vitro A$\beta_{1–42}$ application model to examine changes in SUMOylation, SUMO pathway enzymes and essential mitochondrial proteins in AD. We used 2 μM A$\beta_{1–42}$ that, while still a high concentration compared to the pathophysiological levels measured in in vivo transgenic mouse models (Maia et al., 2015), has been used previously in studies from our group and others (Hoppe et al., 2013a, 2013b; Tarczyluk et al., 2015; Wälti et al., 2018). Both SUMO1 and SUMO2/3 conjugation were decreased by 2 μM A$\beta_{1–42}$. Consistent with this, the SUMO-activating enzyme SAE2 and the SUMO E3 ligase PIAS1/2 were reduced by A$\beta_{1–42}$. Somewhat counterintuitively, given that total SUMO2/3 conjugation is reduced, the SUMO2/3-specific deSUMOylating enzyme SENP3 was also decreased by A$\beta_{1–42}$. At first sight, this would lead to the prediction of increased SUMO2/3-ylation. However, SUMOylation pathways are highly dynamic and complex, and the increase that may result from SENP3 loss may be offset by our observed decreases in the E1 and E3 enzymes. Furthermore, reliable antibodies are not yet available for several rat SUMO proteases, so we cannot currently exclude the possibility that reduced SENP3 may also be accompanied by increases in other SUMO proteases. Nonetheless, it has previously been reported that SENP3 expression is downregulated in microarray analyses of sporadic AD tissues (Weeraratna et al., 2007), and the brains of human Down Syndrome (DS) patients (Binda et al., 2017). Since amyloid precursor protein (APP) is encoded on the trisomic chromosome in DS, DS patients exhibit advanced amyloid pathology and early-onset dementia, further supporting our observed link between Aβ deposition and SENP3 levels.

Interestingly, a recent paper reported no significant changes in SUMO1 or SUMO2/3 conjugation in response to A$\beta_{1–42}$ treatment of cultured cortical neurons, in contrast to the decrease we observed here (Maruyama et al., 2018). While the reasons for this discrepancy are currently unclear, this may be due to differences in the A$\beta_{1–42}$ oligomerization protocol used. We looked at the effects of aggregated A$\beta_{1–42}$ peptide preparations, which are predominantly fibrillar rather than soluble or oligomeric (Ferreira et al., 2015). Moreover, the decrease in conjugation observed here may be a result of the higher concentration of A$\beta_{1–42}$ (2 μM versus 1 μM) and longer duration of insult (48 h versus 1 or 24 h) than that used by Maruyama et al. (2018), which likely results in a more severe model of AD-like stress.

Impaired mitochondrial dynamics have been reported to result in excessive mitochondrial fragmentation in AD (Wang et al., 2009). In particular, alterations in the GTPase Drp1 have been proposed to contribute to abnormal mitochondria function (Wang et al., 2009;

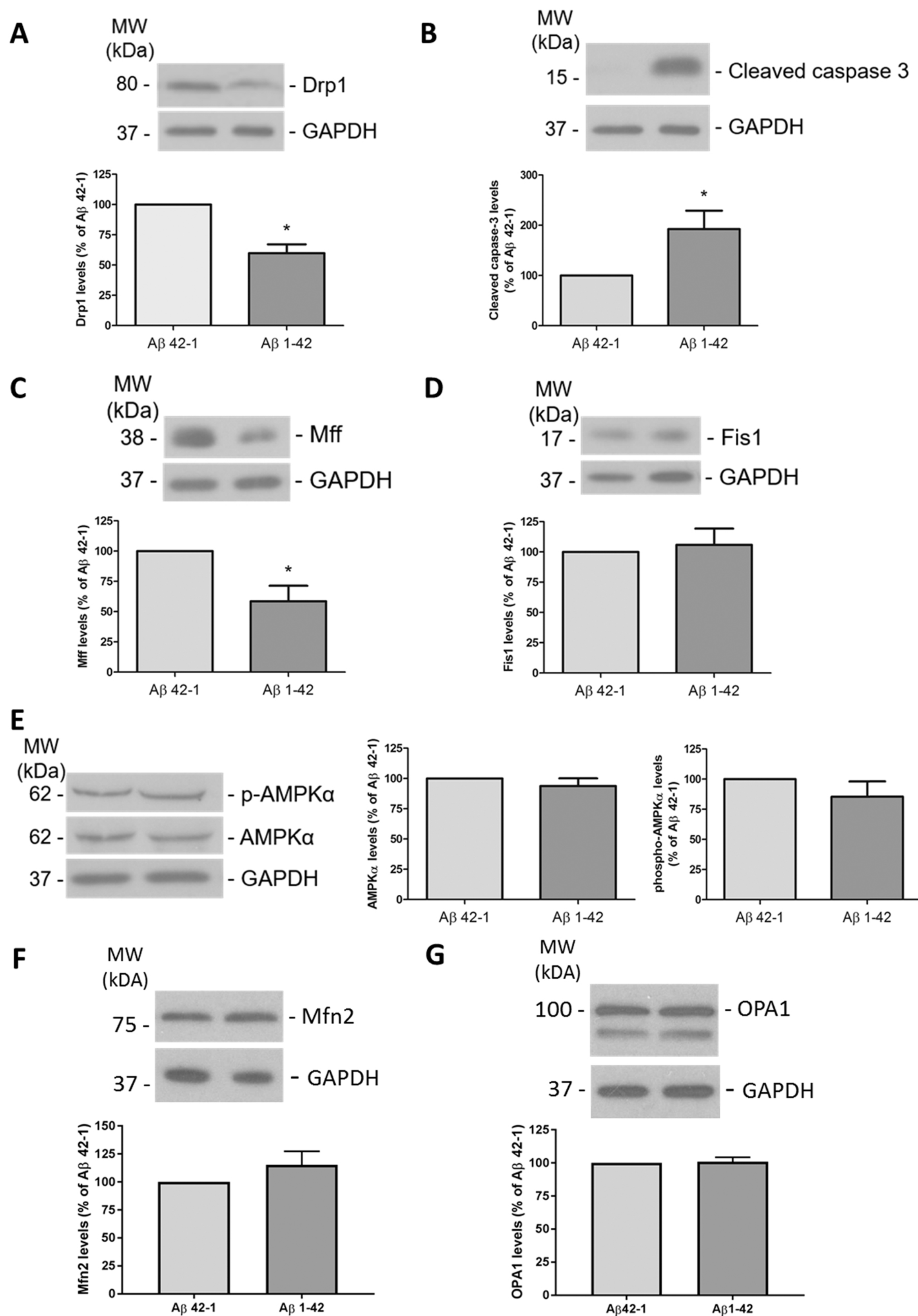

Fig. 3. $A\beta_{1-42}$ decreases Drp1 (A) and Mff (C) levels and increases cleaved caspase-3 (B). Levels of Fis1 (D), AMPK, phospho-AMPK (E), Mfn2 (F) and OPA1 (G) were unchanged. DIV17 rat cortical neuronal cultures were incubated. with 2 μM $A\beta_{1-42}$ or its reverse peptide for 48 h. GAPDH was used as a loading control. N = 6 (Drp1, Mff, cleaved caspase-3, Fis1, phospho-AMPK and total AMPK) and N = 4 (Mfn2 and OPA1). *$p \leq 0.05$, Student's t-test, compared to $A\beta_{42-1}$ group.

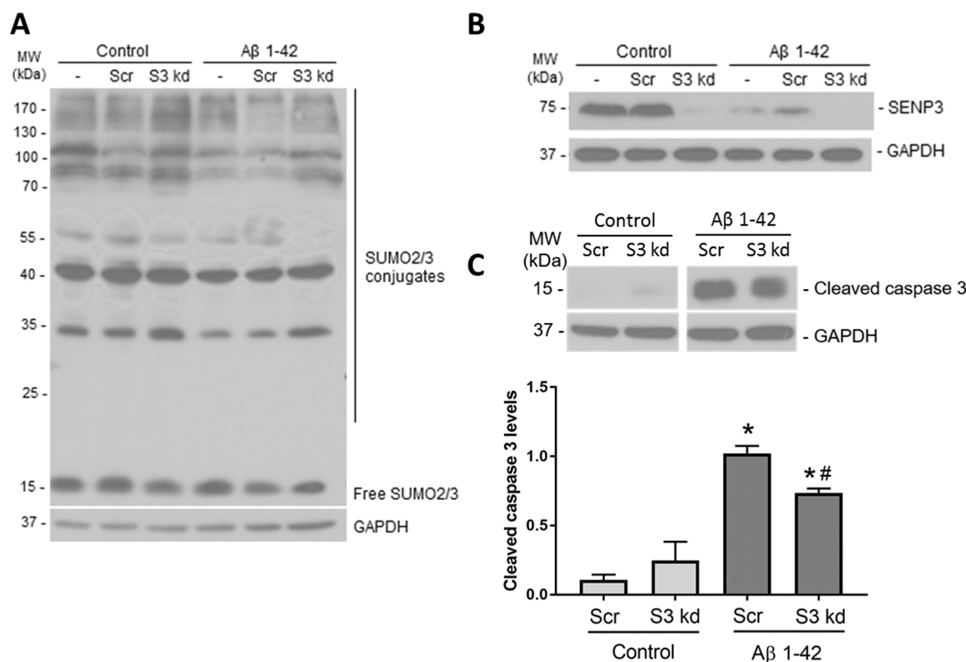

Fig. 4. SENP3 knockdown rescues the decrease in SUMO2/3 conjugation and partially rescues the increase in cleaved caspase-3 induced by $A\beta_{1-42}$. DIV13 rat cortical neuronal cultures were infected with either scrambled shRNA (Scr) or SENP3 shRNA (S3 kd) expressing lentivirus for 4 days, before incubation at DIV17 with 2 μM $A\beta_{1-42}$ for 48 h. A) SENP3 knockdown enhances SUMO2/3-ylation and partially rescues the $A\beta_{1-42}$-induced decrease in global SUMOylation. B) Validation of SENP3 knockdown in cultured neurons. C) Knockdown of SENP3 partially protects against $A\beta_{1-42}$-induced caspase-3 cleavage. N = 4. *$p \leq 0.05$ compared to control group + Scr; # $p \leq 0.05$ compared to $A\beta_{1-42}$ group + Scr (two-way ANOVA followed by Fisher's test).

Grohm et al., 2012). Here, we observed a decrease in Drp1 levels in response to $A\beta_{1-42}$, while levels of the fusion GTPases Mfn2 and OPA1 were unchanged. At first sight, these results may seem surprising, given previous reports of mitochondrial fragmentation resulting from excessive fission in AD (Shen et al., 2014; Baek et al., 2017). However, it is important to note that levels of fission are dependent on mitochondrial recruitment of Drp1 (Wang et al., 2009), and thus changes in total Drp1 levels may not entirely be reflected in changes in the active mitochondrial pool of Drp1. Indeed, a previous study examining the mitochondrial association of Drp1 in human AD brain samples observed that although total levels of Drp1 were decreased in AD, the mitochondrial pool of Drp1 was increased, resulting in a fragmented phenotype (Wang et al., 2009). While we observed significant changes in the expression levels of SUMOylation and mitochondrial proteins, specific $A\beta_{1-42}$-induced changes in the levels of SUMOylated Drp1 remain to be demonstrated. Further work will therefore be required to determine how mitochondrial recruitment of Drp1, and post-translational modifications of Drp1 which control this process, such as SUMOylation (Guo et al., 2013; Guo et al., 2017), are altered by $A\beta_{1-42}$ treatment.

Interestingly, and consistent with previous studies, we show that shRNA knockdown of SENP3 increased SUMO2/3-ylation, and partially prevented $A\beta_{1-42}$-induced caspase-3 cleavage. We have shown previously that SENP3 degradation during ischemia represents a cellular protective response and that knockdown of SENP3 before the insult promotes cell survival (Guo et al., 2013; Guo et al., 2017). Our current data suggest that SENP3 loss also occurs in AD models and that promoting SENP3 loss before AD-like stress favours cell survival. Further work will be required to determine exactly how SENP3 loss enhances cell survival in AD models. A likely possibility is that it functions, at least in part, by promoting SUMOylation of its substrate Drp1, to reduce excessive mitochondrial fission and resulting apoptosis.

Overall, our data demonstrate that $A\beta_{1-42}$ decreases global SUMO1 and SUMO2/3 conjugation to target proteins in cultured neurons. Since SUMOylation is proposed to be neuroprotective, we hypothesize that this reduction in target protein SUMOylation could contribute to synaptic, mitochondrial and neuronal dysfunction in AD. Consistent with this concept, $A\beta_{1-42}$ perturbed mitochondrial proteins and increased caspase-3 cleavage, a core component of the apoptotic pathway. While much more work remains to be done, our results indicate that protein SUMOylation, and SENP3 in particular, could represent a tractable target for beneficial manipulation to reduce neuronal dysfunction in AD.

4.1. Limitations of the study and future work

In our study, we have examined levels of protein SUMOylation, SUMOylation-associated proteins, and levels of mitochondrial proteins after application of $A\beta_{1-42}$. Furthermore, we have investigated the potential for SENP3 knockdown to reduce the induction of cell death markers after $A\beta_{1-42}$ treatment. While we believe these findings provide important information that will form the basis of future work by ourselves and others, we are mindful of a number of limitations of our study that should be considered when interpreting our findings.

We have used Western blotting to assess levels of our proteins of interest, and to measure levels of the established apoptotic marker cleaved caspase-3. However, we note that the veracity of these immunoblot data would be complemented by additional immunofluorescence studies. Additionally, it would be useful to perform additional cell viability assays to confirm that the activation of caspase-3 we observe upon $A\beta_{1-42}$ application accurately reports cell death. Moreover, since mitochondrial recruitment of Drp1 dictates its function in fission, further studies examining colocalization of Drp1 with mitochondria will be informative in determining how the $A\beta_{1-42}$-induced changes in total Drp1 levels observed here impact upon Drp1 recruitment to mitochondria, and how $A\beta_{1-42}$ effects mitochondrial morphology more generally. Finally, we are aware our findings report the effects of one concentration and exposure time of fibrillar $A\beta_{1-42}$ on cultured cortical neurons. Since $A\beta_{1-42}$ concentrations, exposure times, oligomerisation strategies, and neuronal preparation protocols differ widely in the field, further work will also be required to determine whether our findings are universally reproduced in these various model systems. Notwithstanding these areas for future research, we content that our data provide compelling evidence for key roles of SUMOylation and SENP3-mediated deSUMOylation of mitochondrial proteins in the regulation of neuronal viability in a model of AD.

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Declaration of interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2022.01.003.

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