Neuroscience Letters 521 (2012) 130–135

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

# Heterozygosity for the proteasomal *Psmc1* ATPase is insufficient to cause neuropathology in mouse brain, but causes cell cycle defects in mouse embryonic fibroblasts

Nooshin Rezvani [a], Jamal Elkharaz [b], Karen Lawler [b], Maureen Mee [b], R. John Mayer [b], Lynn Bedford [b,*]

[a] Department of Neuroscience, Baylor College of Medicine, Houston, USA
[b] School of Biomedical Sciences, University of Nottingham, Nottingham, UK

## HIGHLIGHTS

- Proteasomal *Psmc1* ATPase heterozygosity does not cause neuropathology in mouse brain.
- Age-related intraneuronal K48-specific polyubiquitin granular staining in mouse brain.
- Proteasomal *Psmc1* ATPase heterozygosity leads to cell cycle defects in MEFs.

## ARTICLE INFO

*Article history:*
Received 17 April 2012
Received in revised form 14 May 2012
Accepted 29 May 2012

*Keywords:*
Heterozygosity
Ubiquitin proteasome system
26S proteasome
Neurodegeneration
Lewy body
Cell cycle

## ABSTRACT

The ubiquitin proteasome system (UPS) is a fundamental cellular pathway, degrading most unwanted intracellular soluble proteins. Dysfunction of the UPS has been associated with normal aging as well as various age-related pathological conditions, including chronic human neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, leading to a significant interest in the involvement of this degradative system in neurones. We previously reported that the 26S proteasome was essential for neuronal homeostasis and survival in mouse brains following conditional genetic homozygous knockout of a key subunit of the multi-meric 26S proteasome (19S ATPase *Psmc1*). Here, we investigated the effects of *Psmc1* heterozygosity in the mouse brain and primary mouse embryonic fibroblasts. Neuropathologically and biochemically, *Psmc1* heterozygous (*Psmc1*$^{+/-}$) knockout mice were indistinguishable from wild-type mice. However, we report a novel age-related accumulation of intraneuronal lysine 48-specific polyubiquitin-positive granular staining in both wild-type and heterozygous *Psmc1* knockout mouse brain. In *Psmc1*$^{+/-}$ MEFs, we found a significant decrease in PSMC1 levels, altered 26S proteasome assembly and a notable G2/M cell cycle arrest that was not associated with an increase in the cell cycle regulatory protein p21. The disturbance in cell cycle progression may be responsible for the growth inhibitory effects in *Psmc1*$^{+/-}$ MEFs.



## 1. Introduction

The ubiquitin proteasome system (UPS) is the major degradation pathway for most unwanted intracellular soluble proteins [19,42]. Polyubiquitin chains are enzymatically assembled on unwanted protein substrates as a tag for their degradation by the 26S proteasome. Ubiquitination involves the formation of an isopeptide bond between an internal lysine (K) reside in the protein substrate (including ubiquitin) and the carboxy terminus of ubiquitin. Polyubiquitin chains using the K48 residue of ubiquitin are one of the most abundant and specify 26S proteasome degradation. Regulated degradation of proteins by the UPS is important for removing improperly folded or otherwise defective proteins, but also affects practically every fundamental cellular process, such as cell-cycle progression [8,23].

Evidence for dysfunction of the UPS in the development of neurodegenerative disease has been accumulating since the identification of ubiquitinated proteins in their hallmark neuropathological inclusions, which includes neurofibrillary tangles and Lewy bodies (LB) in Alzheimer's disease (AD), and Parkinson's disease (PD) and dementia with Lewy bodies, respectively [9,30,35,37]. Aging is associated with a decrease in proteasome activity and this is further decreased in brain tissue from degenerative conditions such as AD and PD [10,45,47]. Whilst disease-associated proteins have been shown to be degraded by the UPS, *e.g.* α-synuclein, aggregated proteins can inhibit 26S

* Corresponding author at: School of Biomedical Sciences, University of Nottingham, Queens Medical Centre, Nottingham NG2 7UH, UK. Tel.: +44 1158230512; fax: +44 1158230142.
*E-mail address:* mbzlb@exmail.nottingham.ac.uk (L. Bedford).

0304-3940/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2012.05.070

proteasome activity [13,14,43,48]. A pathogenic role for the UPS in PD is supported by mutations in Parkin, an E3 ubiquitin ligase, targeting proteins for proteasomal degradation by the UPS and mitochondrial autophagy (mitophagy), whose inactivation can be associated with familial and sporadic PD [11]. Proteasome inhibition is reported to contribute to neurodegeneration and LB-like formation in various *in vivo* models of PD [4,7,16,46]. Taken together, these observations have lead to a significant interest in the involvement of this degradative system in neurones.

Previously, we reported that the 26S proteasome was essential for neuronal homeostasis and survival. Depletion of 26S proteasomes in mouse brain neurones, by conditional genetic homozygous inactivation of a key subunit of the multi-meric proteasome complex (19S ATPase *Psmc1*), caused neurodegeneration and the formation of intraneuronal Lewy body-like protein inclusions resembling human pales bodies [4]. Here we present our characterization of *Psmc1* heterozygous knockout ($Psmc1^{+/-}$) mice and primary mouse embryonic fibroblasts (MEFs). Whilst we did not observe any differences between the $Psmc1^{+/-}$ and *Psmc1* wild-type ($Psmc1^{+/+}$) mouse brains, we found an age-related accumulation of K48-specific polyubiquitin-positive granular immunostaining in both genotypes. In $Psmc1^{+/-}$ MEFs, we found decreased PSMC1 protein expression, altered assembly of the 26S proteasome and G2/M cell cycle arrest.

## 2. Materials and methods

### 2.1. Generation and genotyping of Psmc1 knockout mice

All animal experiments were approved by the Home Office Animals (Scientific Procedures) Act 1986. Generation of *Psmc1* (Gene ID 19179) knockout mice was essentially as previously described [4]. Targeted embryonic stem cell clones were used to establish heterozygous ($Psmc1^{+/-}$) knockout mice. $Psmc1^{+/-}$ mice were intercrossed to generate control or wild-type ($Psmc1^{+/+}$), heterozygous ($Psmc1^{+/-}$) and homozygous ($Psmc1^{-/-}$) *Psmc1* knockout mice. PCR amplification of ear biopsy DNA was used for genotyping as described previously [4].

### 2.2. Preparation and culture of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were prepared from individual embryos at 14.5 days post-coitus following intercrosses of $Psmc1^{+/-}$ mice. MEFs were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (PAA Laboratories), L-glutamine, MEM non-essential amino acids, β-mercaptoethanol, penicillin and streptomycin (Gibco) at 37 °C and 5% $CO_2$, in a humidified incubator.

### 2.3. Western analysis

Proteins were extracted in lysis buffer [30 mM Tris, 8 M Urea, 4% CHAPS] containing protease inhibitor cocktail, separated by SDS-PAGE and transferred to PVDF or nitrocellulose membrane. Blocking used 5% Marvel in tris buffered saline–0.1% Tween-20. Incubation in primary [1:1000 all ubiquitin (in-house), K48-specific polyubiquitin (Millipore), proteasome subunits (Enzo), p21 (Santa Cruz); 1:10,000 GAPDH (Sigma)] and appropriate horseradish peroxidise-conjugated secondary antibodies (Sigma) was for 1 h, room temperature. Proteins were visualized with enhanced chemiluminescent substrate (Pierce).

### 2.4. Histology

Histology was performed as described previously [4]. Hematoxylin (Harris) and eosin staining was used for general morphological examination. Immunostaining was performed according to Vector Laboratories M.O.M. immunodetection or Vectastain Elite rabbit IgG ABC kits. Antigen retrieval used 0.01 M citrate buffer containing 0.05% Tween-20, pH 6. Sections were incubated for 1 h at room temperature in the following primary antibodies: 1:100 all ubiquitin, Lys48-specific polyubiquitin, GFAP (Sigma), CoxIV (Cell Signalling); 1:500 p62 (Enzo Life Sciences).

### 2.5. Purification of 20S and 26S proteasomes

This was performed as previously described [4].

### 2.6. Protein quantification

Quantity one 1-D analysis Software was used to calculate band intensity from Western blots. Protein levels were normalized to GAPDH and compared to the wild-type sample.

### 2.7. Cell cycle analysis

$Psmc1^{+/+}$ and $Psmc1^{+/-}$ MEFs were harvested by centrifugation, dispersed in 70% ice-cold ethanol, washed in PBA buffer [phosphate buffered saline, 0.1% BSA and 1 mM sodium azide] and re-suspended in 1 ml PBA containing RNaseA and propidium iodide. Cells were incubated at 37 °C for 20 min prior to fluorescent activated cell sorting. Data was analyzed using WEASEL software.

## 3. Results and discussion

We previously reported that PSMC1 was essential for development in mice [4]. Early embryonic lethality has also been shown for other 19S subunit knockout mice [22,39]. Here we report the genotypic distribution of litters from crosses of heterozygous *Psmc1* knockout mice is 1 ($Psmc1^{+/+}$): 1.6 ($Psmc1^{+/-}$): 0 ($Psmc1^{-/-}$) ($n = 281$). Whilst this is suggestive of a slightly reduced number of $Psmc1^{+/-}$ mice than expected, these mice are viable, fertile and survive to adulthood with no obvious phenotype compared to wild-type mice. Because of the evidence for proteasome impairment in human chronic neurodegenerative disease, we investigated if there were any biological consequences of heterozygous *Psmc1* deletion in the mouse brain.

To quantify if there was an effect on PSMC1 protein levels in $Psmc1^{+/-}$ mice, total brain protein was subjected to Western blot analysis. Fig. 1A shows that the levels of PSMC1 protein were lower in 8-week-old $Psmc1^{+/-}$ compared to $Psmc1^{+/+}$ mice, but this was not significantly different. Next, since PSMC1 is essential for the formation and activity of the 26S proteasome [4], we examined the distribution of 20S and 26S proteasomes following glycerol density gradient centrifugation. As shown in Fig. 1B, proteasomal activity did not differ significantly between 8-week-old $Psmc1^{+/+}$ and $Psmc1^{+/-}$ brains and this was similar in aged mice (Supplementary Fig. S1A). To support these observations, given that the 26S proteasome is the central protease for ubiquitin-dependent proteolysis, ubiquitin Western blots of total brain protein showed comparable ubiquitin profiles, including K48-linked polyubiquitin, which represents the canonical signal targeting proteins for degradation by the 26S proteasome, in $Psmc1^{+/+}$ and $Psmc1^{+/-}$ mice up to 18 months of age, confirming normal UPS activity (Supplementary Fig. S1B and data not shown). Whilst the total ubiquitin profiles were similar in both genotypes independent of age, consistent with previous reports, we observed an age-related decrease in proteasomal activity (Supplementary Fig. S1C).

Previously, we showed homozygous *Psmc1* deletion in mouse brain neurones caused neurodegeneration and the formation of intraneuronal Lewy-like inclusions resembling human pale bodies [4]. Therefore, we looked for neuropathological changes in the