Pale Body-Like Inclusion Formation and Neurodegeneration following Depletion of 26S Proteasomes in Mouse Brain Neurones are Independent of α-Synuclein

Simon M. L. Paine1,2,4, Glenn Anderson2, Karen Bedford3, Karen Lawler4, R. John Mayer4, James Lowe5, Lynn Bedford4*

1 Neural Development Unit, University College London Institute of Child Health, London, United Kingdom, 2 Department of Histopathology, Great Ormond Street Hospital for Children NHS Trust, London, United Kingdom, 3 Hull and East Yorkshire Hospitals, Hull Royal Infirmary, Hull, United Kingdom, 4 School of Biomedical Sciences, University of Nottingham, Nottingham, United Kingdom, 5 Division of Histopathology, School of Molecular Medical Sciences, University of Nottingham, Nottingham, United Kingdom

Abstract

Parkinson’s disease (PD) is characterized by the progressive degeneration of substantia nigra pars compacta (SNpc) dopaminergic neurones and the formation of Lewy bodies (LB) in a proportion of the remaining neurones. α-synuclein is the main component of LB, but the pathological mechanisms that lead to neurodegeneration associated with LB formation remain unclear. Three pivotal elements have emerged in the development of PD: α-synuclein, mitochondria and protein degradation systems. We previously reported a unique model, created by conditional genetic depletion of 26S proteasomes in the SNpc of mice, which mechanistically links these three elements with the neuropathology of PD: progressive neurodegeneration and intraneuronal inclusion formation. Using this model, we tested the hypothesis that α-synuclein was essential for the formation of inclusions and neurodegeneration caused by 26S proteasomal depletion. We found that both of these processes were independent of α-synuclein. This provides an important insight into the relationship between the proteasome, α-synuclein, inclusion formation and neurodegeneration. We also show that the autophagy-lysosomal pathway is not activated in 26S proteasome-depleted neurones. This leads us to suggest that the paranuclear accumulation of mitochondria in inclusions in our model may reflect a role for the ubiquitin proteasome system in mitochondrial homeostasis and that neurodegeneration may be mediated through mitochondrial factors linked to inclusion biogenesis.

Citation: Paine SML, Anderson G, Bedford K, Lawler K, Mayer RJ, et al. (2013) Pale Body-Like Inclusion Formation and Neurodegeneration following Depletion of 26S Proteasomes in Mouse Brain Neurones are Independent of α-Synuclein. PLoS ONE 8(1): e54711. doi:10.1371/journal.pone.0054711

Editor: Ted M. Dawson, Johns Hopkins, United States of America

Received October 22, 2012; Accepted December 14, 2012; Published January 30, 2013

Copyright: © 2013 Paine et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Parkinson’s UK and a Centenary Clinical Research Training Fellowship from the Pathological Society of Great Britain and Ireland. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: lynn.bedford@nottingham.ac.uk

Introduction

Parkinson’s disease (PD) is defined pathologically by the loss of dopaminergic (DA) neurones from the substantia nigra pars compacta (SNpc) and the accumulation of aggregated α-synuclein in Lewy bodies (LB) in a proportion of the remaining neurones. Attention is increasingly turning to the concept of proteostasis in diseases in which abnormal protein accumulation develops in association with the formation of inclusion bodies [1]. The formation of inclusions is likely to be cytoprotective [2], with neuronal death being the result of failure to compensate. Therefore, it is important to understand which cellular systems are involved in the formation of inclusion bodies and what leads to neuronal death in this context. Three elements are regarded as pivotal in the pathogenesis of neurodegeneration in PD: α-synuclein, mitochondria and protein degradation systems.

As well as being the defining protein component of LB [3], point mutations and multiplication of the Snca locus, encoding α-synuclein, lead to familial forms of the disease, and Snca variation is associated with an increased risk of developing sporadic PD [4,5]. LB are a feature of these forms of PD and are present in a wide range of neuronal populations beyond the mesencephalon. Their presence in the neocortex and limbic system is seen in the second most common form of dementia, dementia with Lewy bodies (DLB) [6,7]. LB pathology often coexists with other neurodegenerative disorders, such as Alzheimer’s disease (AD) [8]. Despite 15 years of intense study since Snca was first linked to PD [3], the pathological mechanisms that lead to neurodegeneration associated with LB formation remain unclear.

Extensive data implicates mitochondrial factors in neurodegeneration [9,10]. In addition to several genetic factors known to cause familial PD linking to mitochondria (e.g. Parkin), respiratory chain defects in the SN, and other tissues, from sporadic PD cases have long been recognized and neurotoxins that act via various mechanisms to impair mitochondrial complex I have been used to model the disease [9–11]. Mitochondria are a prominent component of pale bodies (PB), the precursor of LB [12,13].
Ubiquitin is key to the two main cellular degradation systems: the ubiquitin proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) [14]. Ubiquitin-positive inclusions are a consistent feature of the majority of human neurodegenerative diseases, suggesting involvement of protein degradation systems [15–17]. The UPS and ALP have specific roles in the degradation of α-synuclein in vitro, under normal conditions the UPS is the main degradation system for α-synuclein whilst the ALP is recruited with raised α-synuclein levels [18]. Also relevant is the formation of LB has been regarded as an aggressive process, involving components of the UPS as well as the ALP [19,20].

Recent attention has focused on the importance of protein degradation systems in relation to mitochondrial function [21]. The UPS has a role in mitochondrial protein quality control [22,23], and specifically, mitophagy (the selective degradation of dysfunctional mitochondria by autophagy). Aberrant mitochondria are selected for mitophagy by PINK1-dependent recruitment of cytosolic Parkin, an E3 ubiquitin ligase implicated in PD, which mediates K48- and K63-linked polyubiquitination associated with the UPS and ALP respectively [24,25]. There is, therefore, a clear point of convergence between two elements known to be important in PD pathogenesis: mitochondria and protein degradation systems. Interestingly, α-synuclein has also recently been found to be directly involved in mitochondrial homeostasis [26–28].

We previously reported that 26S proteasomal depletion in mouse brain neurones, including SNpc DA neurones, causes neurodegeneration and the formation of Lewy-like inclusions resembling human PB [13]. The mouse inclusions are defined as α-synuclein-like because they are morphologically, neuropathologically and biochemically resembling human PB [13]. The mouse inclusions are defined as α-synuclein-like because they are morphologically, neuropathologically and biochemically resembling human PB [13]. The mouse inclusions are defined as α-synuclein-like because they are morphologically, neuropathologically and biochemically resembling human PB [13]. The mouse inclusions are defined as α-synuclein-like because they are morphologically, neuropathologically and biochemically resembling human PB [13]. The mouse inclusions are defined as α-synuclein-like because they are morphologically, neuropathologically and biochemically resembling human PB [13].

Animals
Mice lacking the first two exons and upstream untranslated region of the α-synuclein gene (Snca), encoding amino acids 1–41 of α-synuclein, were kindly provided by Prof. Vladimir Buchman, University of Cardiff, Wales UK [29]. These mice (Snca<sup>−/−</sup>) were crossed with floxed Psmc1 mice (Psmc1<sup>fl/fl</sup>) expressing Cre recombinase from the tyrosine hydroxylase (TH) promoter (TH<sup>Cre</sup>) to achieve catecholaminergic neurone-specific inactivation of Psmc1 (Psmc1<sup>fl/fl</sup>;TH<sup>Cre</sup>) [13]. TH is expressed from mid-gestation in midbrain dopaminergic neurones [30]. For forebrain neurone-specific inactivation of Psmc1, Cre recombinase was expressed under the control of the calcium calmodulin-dependent protein kinase IIβ promoter (Psmc1<sup>fl/fl</sup>;CaMKIβ<sup>Cre</sup>) [13]. CaMKIβ<sup>Cre</sup> is expressed in post-mitotic neurones from approximately post-natal week 2 [31,32]. All mice were male. Appropriate littermate mice were used as controls.

Genotyping
Genomic DNA was used in a polymerase chain reaction, 62°C annealing. Primers: Cre (sense) GTTAGACCCGGACGTGATG; Cre (antisense) CTAATCGCCATCCTTACGACAG; Psmc1 (wild-type and floxed sense) TACGAACCTCCTGTCCCAAC; Psmc1 (knock-out sense) CAGAAATACAGCCAGTGACC; Psmc1 (common antisense) CTGGAACTCAGTGGATTGAG; Snca (wild-type antisense) AAGGAAAGCCGAGTGATGTAC; Snca (knock-out antisense) ACTGGAAGGATTGGAGCTACG.

Histology
For light microscopy, mice were perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in phosphate buffered saline pH 7.4. PFA-fixed brains were processed to paraffin with chloroform as the clearing agent. General morphological examination used haematoxylin and cosin staining. Immunostaining was performed as directed in 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. Primary antibody incubation was for 1 hour at room temperature [tyrosine hydroxylase (Sigma), α-synuclein (BD Biosciences), p62 (Enzo Life Sciences), ubiquitin K63-isopeptide motif (Enzo Life Sciences), lysine 48-linkage specific polyubiquitin (Millipore), in-house ubiquitin, COX IV (Cell Signalling), cytochrome C (Santa Cruz), LC3B (Sigma) and Cathepsin D (Cell Signalling and Abcam)]. Double immunofluorescent staining used similar methods; primary (from different species) and appropriate fluorescent secondary antibodies were incubated in sequence. For electron microscopy, mice were perfused with 0.9% saline followed by gluteraldehyde fixative suitable for EM.

Stereology
Horizontal sections were cut at 7 micrometres. Two consecutive sections were collected onto a single APEX-coated slide starting rostral to the substantia nigra and continuing through to the brainstem. Every seventh slide through the SNpc was stained for TH and counterstained with haematoxylin [33]. The slides were scanned and analysed with ImageJ. The SNpc was delineated using anatomical landmarks and neuronal cytology. Neurones were counted if they fell within the outline of the SNpc, possessed the characteristic cytological features of a neurone within the SNpc, had clear cytoplasmic TH immunoreactivity and the dominant nucleus was present only in the reference (lower) section. The count was repeated three times in each of three animals for each genotype. The sum of the neurones counted for
Results

\(\alpha\)-synuclein Aggregation does not Modify Neurodegeneration and is not Essential for the Biogenesis of PB-like Inclusions in 26S Proteasome-depleted Neurones

We previously demonstrated impaired 26S proteasome function in mouse neurones, including SNpc DA neurones, caused neurodegeneration and the formation of intraneuronal PB-like inclusions [13]. Specifically 26S proteasomal dysfunction was achieved by Cre/loxP conditional genetic targeting of Psmc1, a key ATPase subunit of the 19S regulatory particle of the 26S proteasome. We showed PSMC1 was responsible for the assembly of the 20S proteolytic core particle and therefore ubiquitin-independent proteasomal degradation was not affected [13]. To study whether \(\alpha\)-synuclein directly contributes to the formation of inclusions and degeneration of DA neurones in vivo; the 20S proteolytic core particle and therefore ubiquitin-independent proteasomal degradation was not affected [13]. To study whether \(\alpha\)-synuclein directly contributes to the formation of inclusions and degeneration of DA neurones in the mouse SNpc, we generated mice constitutively lacking \(\alpha\)-synuclein (Figure 1A). Our results from 3-week-old mice because autonomic dysfunction, as a consequence of 26S proteasomal depletion in all catecholaminergic (TH) neurones, causes premature death [13]. We recognize that these are young mice, but emphasize that the neuropathology following 26S proteasomal dysfunction in SNpc neurones involves elements pivotal to PD pathways and therefore, this is an important in vivo model.

Quantification of the total number of TH-positive neurones showed that the 26S proteasome-depleted mice \((Psmc1^{fl/fl};TH^{Cre})\) possessed approximately 85% fewer neurones when compared with control mice \((Psmc1^{+/+};TH^{Cre})\), irrespective of the presence \((\text{Snca}^{+/+})\) or absence \((\text{Snca}^{-/-})\) of \(\alpha\)-synuclein (Figure 1B). In keeping with the analysis of TH-positive neurone number, there was a significant reduction in the volume of the SNpc when either group of control mice \((Psmc1^{fl/fl};TH^{Cre})\) was compared with either group of 26S proteasome-depleted mice \((Psmc1^{fl/fl};TH^{Cre})\) (Figure 1C). That is, the absence of \(\alpha\)-synuclein \((\text{Snca}^{-/-})\) had no significant effect on the volume of the SNpc.

We previously demonstrated the formation of intraneuronal cosinophilic PB-like inclusions following 26S proteasomal depletion in surviving mouse SNpc neurones \((Psmc1^{fl/fl};TH^{Cre};\text{Snca}^{+/+})\), exhibiting \(\alpha\)-synuclein, ubiquitin, p62 and cytochrome oxidase IV (COX IV) immunoreactivity (Figure 2Ai,iii,v,ix,xi) [13]. Careful morphological and immunohistochemical examination revealed, with the exception of \(\alpha\)-synuclein (Figure 2Aiv), that the inclusions in SNpc neurones lacking \(\alpha\)-synuclein \((Psmc1^{fl/fl};TH^{Cre};\text{Snca}^{-/-})\) were similarly cosinophilic and immunoreactive to p62, ubiquitin and COX IV (Figure 2Aii,vi,ix,x,xii). We sought to determine whether the number of PB-like inclusions in TH-positive neurones resulting from loss of 26S proteasomes was modified by \(\alpha\)-synuclein. The total number of cosinophilic inclusions in the SNpc was counted and divided by the total number of neurones present in this region with the potential to form inclusions, i.e. TH-positive neurones. Figure 2B shows that the proportion of neurones bearing inclusions was similar in both groups of 26S proteasome-depleted mice, irrespective of the presence \((Psmc1^{fl/fl};TH^{Cre};\text{Snca}^{+/+})\) or absence \((Psmc1^{fl/fl};TH^{Cre};\text{Snca}^{-/-})\) of \(\alpha\)-synuclein.

In order to assess whether this was a specific response of mesencephalic DA cells, we conditionally deleted P onc1 in mouse forebrain neurones by expressing Cre recombinase from the calcium calmodulin-dependent protein kinase II promoter (CaMKII-Cre), causing PB-like inclusions in cortical neurones \((Psmc1^{fl/fl};CaMKII-Cre)\) [13]. Significantly, we found here too the absence of \(\alpha\)-synuclein \((\text{Snca}^{-/-})\) does not modify the processes leading to cortical PB-like inclusion formation (Figure 2C). Intraneuronal inclusions were not identified in any of the mice.

Accumulation of Aberrant Mitochondria

We used ultrastructural analysis to investigate more subtle pathological differences between PB-like inclusions in 26S proteasome-depleted neurones with \((Psmc1^{fl/fl};TH^{Cre};\text{Snca}^{+/+})\) and without \((Psmc1^{fl/fl};TH^{Cre};\text{Snca}^{-/-})\) \(\alpha\)-synuclein. This revealed that the intraneuronal inclusions in SNpc neurones were indistinguishable (Figure 3). The inclusions were composed predominantly of morphologically abnormal mitochondria, which, in general, had disrupted or disintegrated cristae (Figures 3D-G). The mitochondria within the inclusions were interspersed with membrane in numerous small vesicles and granular material (Figures 3E and 3F). We observed the presence of occasional autophagosome-like structures containing electron-dense material (Figure 3F) as well as recognizable cytoplasmic elements, including mitochondria (Figure 3H), but the majority of the vesicles were empty.

The ALP is not Activated in 26S Proteasome-depleted Neurones

Several studies have shown compensatory activation of the ALP with proteasome inhibition [18,34,35]. To investigate the involvement of the ALP following 26S proteasomal depletion in our model and whether this was associated with the membranous vesicles seen in inclusions, we carried out LC3 and cathepsin D immunostaining and Western blotting. LC3 is a marker of autophagosomes and post-translational modification of cytosolic LC3-I to LC3-II that is associated with autophagosomal membranes is an index of autophagy activation [36,37]. Punctate endogenous LC3 immunostaining has also been associated with LC3-II and activation of autophagy with proteasome inhibition [35]. Overall, we did not consistently detect LC3-positive...
inclusions or the formation of LC3 puncta in 26S proteasome-depleted neurones, irrespective of the presence or absence of α-synuclein (Figures 4A and 4B). Further, LC3-cytochrome C double-immunofluorescent staining did not demonstrate co-localization of LC3 and mitochondria in inclusions following 26S proteasomal depletion, but LC3 staining was intermixed with dispersed mitochondria in both 26S proteasome-depleted and control neurones (Figure S1A). We report that variable LC3 staining was evident in some inclusion bodies and is shown in Figure S1B. Interestingly, a recent study of Parkin-mediated mitophagy showed LC3 was associated with dispersed mitochondria, but absent from paranuclear mitochondrial aggregates, and that 26S proteasome function may facilitate their dispersal and uptake by the autophagic machinery [38].

To further evaluate the activation of autophagy, we measured the levels of LC3-II (ratio with β-actin) following Western blotting. Due to the small size and accuracy of reproducibly dissecting specifically the mouse SNpc, we used cortical tissue from control (Psmc1fl/fl, CaMKIIa-Wt) and 26S proteasome-depleted mice (Psmc1fl/fl, CaMKIIa-Cre). Supporting our immunohistochemical observations, the levels of LC3-II were not significantly different between 26S proteasome-depleted and control mice (Figure 4C). The levels of p62, a substrate of macroautophagy [39], were also similar (data not shown). However, the presence of p62 in inclusion bodies in 26S proteasome-depleted neurones is consistent with previous studies demonstrating it is required for aggregation of polyubiquitinated proteins and mitochondria [40,41].

Following 26S proteasomal dysfunction we did not identify any inclusions in the SNpc or cortex that immunostained for cathepsin D, an essential lysosomal protease (Figures 5A and 5B). These observations are analogous to an in vitro study in cortical neurones modeling proteasomal dysfunction by applying pharmacological inhibitors that showed globular cathepsin D staining with activation of the ALP, but this was associated with dissolution of inclusions and did not co-localize with inclusion bodies [34]. Western blot and densitometric analysis showed the levels of mature cathepsin D were not significantly different between 26S proteasome-depleted and control mice (Figure 5C). Taken together, these results suggest that the ALP is not activated in 26S proteasome-depleted neurones.

Discussion

In the present study we have investigated an in vitro model system of neurodegeneration and inclusion formation that brings together several of the pivotal elements implicated in PD. We
have shown that α-synuclein is not essential for the biogenesis of PB-like inclusions and that it does not modify the neurodegeneration that follows in vivo depletion of 26S proteasomes in DA and cortical neurones. Additionally, we have shown that 26S proteasomal dysfunction does not lead to activation of the ALP.

In synucleinopathies such as PD and DLB, extensive research has focused on the over-expression of α-synuclein and its ability to aggregate [4,5,42]. Many transgenic mice have been created to over-express human PD variants of α-synuclein, but these models have not shown region-specific neuronal loss in the pattern seen in PD [4,42-44]. The relevance of the 26S proteasomal depletion...
Figure 3. The absence of α-synuclein does not modify the ultrastructure of PB-like inclusions in mouse SNpc neurones. Representative electron micrographs of control (A; Psmc1^{fl/fl};TH^{Wt}) and 26S proteasome-depleted SNpc neurones with (B; Psmc1^{fl/fl};TH^{Cre};Snca^{+/+}) and without (C; Psmc1^{fl/fl};TH^{Cre};Snca) α-synuclein. Enlarged views of the boxed areas are shown in D-F respectively. The inclusions contain mainly abnormal mitochondria (E-G; m) interspersed with numerous small vesicles (E and F; v). Autophagosome-like structures containing electron-dense material (E, F and I; arrows) as well as recognizable cytosolic elements including mitochondria (H) are present. n, nucleus; i, PB-like inclusion; m, mitochondria; v, vesicle and er, endoplasmic reticulum. Scale bar, 500 nm.

Figure 4. Autophagy is not activated in 26S proteasome-depleted mouse neurones. (A) Absence of LC3 immunoreactivity in representative neurones of the SNpc (Psmc1^{fl/fl};TH^{Cre}, nigral) and cortex (Psmc1^{fl/fl};CaMKIIα-Cre, cortical) in the presence (Snca^{+/+}) and absence (Snca^{−/−}) of α-synuclein. The arrows indicate PB-like inclusions. Scale bar, 10 μm. (B) The normal pattern of LC3 in nigral and cortical neurones shows a fine punctate cytoplasmic staining. Neurones from Psmc1^{fl/fl};TH^{Cre};Snca^{+/+} (nigral) and Psmc1^{fl/fl};CaMKIIα-Wt;Snca^{+/+} (cortical) mice are shown, but the pattern of LC3 immunostaining was similar in the absence of α-synuclein. Scale bar, 10 μm. (C) Representative Western blot of LC3-I and LC3-II in total cortical homogenates from control (Psmc1^{fl/fl};CaMKIIα-Wt) and 26S proteasome-depleted (Psmc1^{fl/fl};CaMKIIα-Cre) mice. Graph depicts LC3-II levels normalized to β-actin, n = 4, no significant difference. Error bars indicate SEM.

doi:10.1371/journal.pone.0054711.g003
doi:10.1371/journal.pone.0054711.g004
model to PD is still under debate because of conflicting data using 20S proteasomal inhibitors in vivo [45–50]. One of the strengths of our model system is that reproducible depletion of 26S proteasomes in mesencephalic DA neurones and non-catecholaminergic cortical neurones is achieved using conditional gene deletion, not the work of an inhibitor, which may have other effects [51]. However, we recognize that while the inclusions that form in 26S proteasome-depleted neurones have the characteristics of PB, we do not know if these would develop into LB if the neurones survived. Importantly, our work adds to the data on the pathological significance of the aggregated 26S proteasomal depletion in total cortical homogenates from control (Psmc1fl/fl;CaMKIko-Wt) and 26S proteasome-depleted (Psmc1fl/fl;CaMKIko-Cre) mice. Graph depicts mature cathepsin D levels normalized to β-actin. n = 4, no significant difference. Error bars indicate SEM. doi:10.1371/journal.pone.0054711.g005

Figure 5. The lysosomal pathway is not activated in 26S proteasome-depleted neurones. (A) Representative immunostaining for cathepsin D in neurones of the SNpc (Psmc1fl/fl;THCre, nigral) and cortex (Psmc1fl/fl;CaMKIko-Cre, cortical) in the presence (Snca+/+) and absence (Snca−/−) of α-synuclein. Scale bar, 10 μm. The arrows indicate PB-like inclusions. (B) The normal pattern of cathepsin D in nigral and cortical neurones shows a fine punctate cytoplasmic staining. Neurones from Psmc1fl/fl;THCre;Snca−/− (nigral) and Psmc1fl/fl;CaMKIko-Wt;Snca−/− (cortical) mice are shown, but cathepsin D staining was similar in the absence of α-synuclein. Scale bar, 10 μm. (C) Representative Western blot and densitometric analysis of mature cathepsin D in total cortical homogenates from control (Psmc1fl/fl;CaMKIko-Wt) and 26S proteasome-depleted (Psmc1fl/fl;CaMKIko-Cre) mice. Graph depicts mature cathepsin D levels normalized to β-actin. n = 4, no significant difference. Error bars indicate SEM.

α-Synuclein Independent Pale Body-Like Inclusions

α-synuclein Independent Pale Body-Like Inclusions
as the consequence of activation of a cytoprotective pathway, e.g. an aggresome-like mechanism following proteasomal inhibition [19], and apart from α-synuclein, LB contain over 250 different proteins from diverse cellular processes [71]. However, in this context α-synuclein is not the downstream mediator of neurodegeneration because we show α-synuclein plays a redundant role in the formation of inclusion bodies and neurodegeneration. This has implications for all cases of LB in which there is no known mutation of the α-synuclein gene. We emphasize that our data does not exclude an important role for α-synuclein in PD and events that initiate neurodegeneration, but provides evidence that proteasomal dysfunction may be a relevant factor immediately responsible for neuronal death, which may be amplified by, as well as accompanied by, aggregation of α-synuclein.

Several in vitro studies, including primary cortical neurones, modeling proteasomal dysfunction by applying pharmacological inhibitors have shown 20S proteasome inhibition activates the ALP [34,35]. Using similar methods to evaluate this pathway, we did not observe activation of the ALP in 26S proteasome-depleted mouse neurones in vivo. Our findings are consistent with a recent elegant in vivo study that only found cross-talk between the UPS and ALP in neurones with increased α-synuclein burden, i.e. in human wild-type α-synuclein transgenic mice. In normal mice, induction of the ALP was not observed following proteasome inhibition [18]. There are intrinsic differences between in vitro and in vivo experimental model systems as well as different species, but our findings extend the limited in vivo knowledge between the UPS and ALP degradative systems in SNpc and cortical neurones.

Although aggregation of undegraded proteins in inclusions may be an obvious non-specific consequence of disrupted neuronal homeostasis, the overt paranuclear accumulation of morphologically-impaired mitochondria in 26S proteasome-depleted neurones is significant. Models of proteasomal dysfunction using pharmacological 20S inhibitors have described neuronal death and the formation of cytoplasmic inclusions containing proteins that are present in LB, i.e. ubiquitin and α-synuclein, but not shown accumulation of mitochondria [34,35]. The UPS has emerged as an important mechanism by which mitochondrial proteins can be degraded whilst preserving mitochondrial function. Key molecules that regulate mitochondrial membrane dynamics are proteasome substrates, supporting a model of perturbed fission/fusion following 26S proteasomal dysfunction [22,25]. The accumulation of aberrant mitochondria in 26S proteasome-depleted neurones is also redolent of Parkin-mediated mitophagy, a process that was recently shown to be facilitated by the UPS [25,72]. Significantly, one study functionally linked the UPS and ALP for mitophagy by showing that 26S proteasome-mediated degradation of outer mitochondrial membrane proteins was essential for mitophagy [38]. In this context our findings are important because 26S proteasomal dysfunction did not lead to activation of the ALP. The mitochondria-containing inclusions in 26S proteasome-depleted neurones may reflect impaired mitophagy. Our study supports a role for the UPS in mitochondrial homeostasis in mammalian neurones in vivo.

The molecular events regulating mitochondrial homeostasis in neurones, including why impairment of the UPS leads to mitochondrial compromise and how this may signal for neuronal death, require further characterization to understand their relevance to neurodegeneration and diseases such as PD. We suggest subtle disturbances in mitochondrial homeostasis through impairment of critical cellular systems such as the UPS with aging may lead to the accumulation of dysfunctional mitochondria and neurodegeneration. Pale bodies contain numerous mitochondria and it is essential to understand the reasons behind their presence in paranuclear inclusions [13]. Interestingly, recent publications suggest α-synuclein may also be directly involved in mitochondrial homeostasis [26–28,73–76]. Therefore, mutations or multiplications of Snca may act, similarly to products of PD-related genes linked to mitochondria and well-known environmental factors, as well as proteasomal dysfunction, to negatively influence mitochondrial homeostasis and lead to neurodegeneration.

In summary, our data highlight the importance of considering broader mechanistic models, which also have connections to α-synuclein, in events leading to neurodegeneration. For example, one hypothetical model that may explain our findings and connect the elements implicated in PD is failure of mitochondrial homeostasis, with degradative systems (UPS, aggresome, ALP, mitophagy) downstream of several factors known to be involved in maintaining mitochondrial function, including α-synuclein. Proteasomal inhibition and downstream effects on mitochondrial homeostasis may be a critical feature leading to neurodegeneration worthy of further investigation.

Supporting Information

Figure S1 (A) Double immunofluorescent staining for cytochrome C (mitochondria, green) and LC3 (autophagosomes, red) of sections from control (Poneαδ/δ;CaMKII-Wt) and 26S proteasome-depleted (Poneαδ/δ;CaMKII-αδ) mice. Enlarged views of the boxed areas are shown (zoom). Scale bar, 10 µm. (B) LC3-immunopositive inclusions were evident in some nigral (Poneαδ/δ;TIF1δ;Snca+/+) and cortical (Poneαδ/δ;CaMKII-Wt;Snca+/+) neurones, irrespective of the presence or absence of α-synuclein (data not shown). Scale bar, 10 µm. (TIF)

Acknowledgments

We thank Prof. V. Buchman for the α-synuclein knock-out mice.

Author Contributions

Conceived and designed the experiments: SP RJM JL LB. Performed the experiments: SP GA KB KL LB. Analyzed the data: SP RJM JL LB. Wrote the paper: SP RJM JL LB.

References

1. Douglas PM, Dillin A (2010) Protein homeostasis and aging in neurodegeneration. J Cell Biol 190: 719–729.
2. Wakabayashi K, Tanji K, Odagiri S, Miki Y, Mori F, et al. (2012) The Lewy Body in Parkinson’s Disease and Related Neurodegenerative Disorders. Mol Neurobiol DOI: 10.1007/s12035-012-0280-y.
3. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Lakos R, et al. (1997) Alpha-synuclein in Lewy bodies. Nature 388: 839–840.
4. Devine MJ, Goom K, Singleton A, Hardy J (2011) Parkinson’s disease and alpha-synuclein expression. Mov Disord 26: 2160–2168.
5. Martin I, Dawson VL, Dawson TM (2011) Recent advances in the genetics of Parkinson’s disease. Annu Rev Genomics Hum Genet 12: 301–325.
6. Beach TG, Adler CH, Lue L, Sue LJ, Bachalakuri J, et al. (2009) Unified staging system for Lewy body disorders: correlation with nigrostriatal degeneration, cognitive impairment and motor dysfunction. Acta Neuropathol 117: 613–634.
7. Halliday GM, Holton JL, Revesz T, Dickson DW (2011) Neuropathology underlying clinical variability in patients with synucleinopathies. Acta Neuropathol 122: 187–204.
8. Duyckaerts C, Delattre B, Potier MC (2009) Classification and basic pathology of Alzheimer disease. Acta Neuropathol 118: 5–39.
9. Schapira AH, Gregg M (2011) Mitochondrial contribution to Parkinson’s disease pathogenesis. Parkinsons Dis DOI: 10.4061/2011/719610.
10. Keane PC, Kurzawa M, Blain PG, Morris CM (2011) Mitochondrial dysfunction in Parkinson’s disease. Parkinsons Dis DOI: 10.4061/2011/716871.
39. Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, et al. (2007) Homeostatic
36. Mizushima N (2004) Methods for monitoring autophagy. Int J Biochem Cell
33. Baquet ZC, Williams D, Brody J, Smeyne RJ (2009) A comparison of model-
31. Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, et al. (1996) Control
29. Abeliovich A, Schmitz Y, Farinas I, Choi-Lundberg D, Ho WH, et al. (2000)
28. Sai Y, Zou Z, Peng K, Dong Z (2012) The Parkinson's disease-related genes act
27. Nakamura K, Nemani VM, Azarbal F, Skibinski G, Levy JM, et al. (2011)
26. Devi L, Raghavendran V, Prabhu BM, Avadhani NG, Anandatheerthavarada
25. Chan NC, Chan DC (2011) Parkin uses the UPS to ship off dysfunctional
18. Ebrahimi-Fakhari D, Cantuti-Castelvetri I, Fan Z, Rockenstein E, Masliah E, et
17. Wong E, Cuervo AM (2010) Autophagy gone awry in neurodegenerative
diseases. Nat Neurosci 13: 805–811.
16. Cook C, Petruccioli (2009) A critical evaluation of the ubiquitin-proteasome
15. Abraham-Fakhari D, Cantuti-Castelvetri I, Fan Z, Rockenstein E, Masliah E, et
14. Ng SC, Craig R, Urbanek S, Cardoso de Melo M, Lindquist S (2010) The
13. Ng SC, Craig R, Urbanek S, Cardoso de Melo M, Lindquist S (2010) The
12. Dale GE, Probst A, Luthert P, Martin J, Anderton BH, et al. (1992)
11. Schapira AH (2010) Complex I: inhibitors, inhibition and neurodegeneration.
10. Kordower JH, Kanaan NM, Chu Y, Suresh Babu R, Stansell J, 3rd, et al. (2006)
9. Kordower JH, Kanaan NM, Chu Y, Suresh Babu R, Stansell J, 3rd, et al. (2006)
8. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, et al. (2004) The
7. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, et al. (2004) The
6. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, et al. (2004) The
5. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, et al. (2004) The
4. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, et al. (2004) The
3. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, et al. (2004) The
2. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, et al. (2004) The
1. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, et al. (2004) The
71. Leverenz JB, Umar I, Wang Q, Montine TJ, McMillan PJ, et al. (2007) Proteomic identification of novel proteins in cortical lewy bodies. Brain Pathol 17: 139–145.

72. Yoshii SR, Kishi C, Ishihara N, Mizushima N (2011) Parkin mediates proteasome-dependent protein degradation and rupture of the outer mitochondrial membrane. J Biol Chem 286: 19630–19640.

73. Nakamura K, Nemani VM, Wallender EK, Kehlcke K, Ott M, et al. (2008) Optical reporters for the conformation of alpha-synuclein reveal a specific interaction with mitochondria. J Neurosci 28: 12305–12317.

74. Kamp F, Exner N, Lutz AK, Wender N, Hegermann J, et al. (2010) Inhibition of mitochondrial fusion by alpha-synuclein is rescued by PINK1, Parkin and DJ-1. Embo J 29: 3571–3589.

75. Zigoneanu IG, Yang YJ, Krois AS, Haque ME, Pielak GJ (2011) Interaction of alpha-synuclein with vesicles that mimic mitochondrial membranes. Biochim Biophys Acta 1818: 512–519.

76. Martin LJ, Pan Y, Price AC, Sterling W, Copeland NG, et al. (2006) Parkinson’s disease alpha-synuclein transgenic mice develop neuronal mitochondrial degeneration and cell death. J Neurosci 26: 41–50.