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Reduction of *RPT6/S8* (a Proteasome Component) and Proteasome Activity in the Cortex is Associated with Cognitive Impairment in Lewy Body Dementia

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Abstract. Lewy body dementia is the second most common neurodegenerative dementia and is pathologically characterized by α-synuclein positive cytoplasmic inclusions, with varying amounts of amyloid-β (Aβ) and hyperphosphorylated tau (tau) aggregates in addition to synaptic loss. A dysfunctional ubiquitin proteasome system (UPS), the major proteolytic pathway responsible for the clearance of short lived proteins, may be a mediating factor of disease progression and of the development of α-synuclein aggregates. In the present study, protein expression of a key component of the UPS, the RPT6 subunit of the 19S regulatory complex was determined. Furthermore, the main proteolytic-like (chymotrypsin- and PGPH-) activities have also been analyzed. The middle frontal (Brodmann, BA9), inferior parietal (BA40), and anterior cingulate (BA24) gyrus' cortex were selected as regions of interest from Parkinson's disease dementia (PDD, $n = 31$), dementia with Lewy bodies (DLB, $n = 44$), Alzheimer's disease (AD, $n = 16$), and control ($n = 24$) brains. Clinical and pathological data available included the MMSE score. DLB, PDD, and AD were characterized by significant reductions of RPT6 (one-way ANOVA, $p < 0.001$; Bonferroni *post hoc* test) in prefrontal cortex and parietal cortex compared with controls. Strong associations were observed between RPT6 levels in prefrontal, parietal cortex, and anterior cingulate gyrus and cognitive impairment ($p = 0.001$, $p = 0.001$, and $p = 0.008$, respectively). These findings highlight the involvement of the UPS in Lewy body dementia and indicate that targeting the UPS may have the potential to slow down or reduce the progression of cognitive impairment in DLB and PDD.

Keywords: Alzheimer's disease, amyloid-beta, cognitive impairment, dementia with Lewy bodies, Parkinson's disease with dementia, RPT6, tau, ubiquitin proteasome system

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INTRODUCTION

Lewy body diseases (LBD) include dementia with Lewy bodies (DLB), Parkinson's disease (PD), and PD dementia (PDD). DLB and PDD account for 10–20% of dementias [1]. Clinically, both conditions are characterized by progressive cognitive decline, visual hallucinations, fluctuating cognition, and parkinsonism. The neuropathological hallmark lesions include α-synuclein aggregates which present as intracytoplasmic Lewy bodies and Lewy neurites in axons and dendrites in cortical and limbic areas (DLB and PDD) as well as in the substantia nigra (PDD and PD). In addition, ubiquitin-containing inclusions [2, 3] and amyloid-β (Aβ) plaques are frequently seen. The neurochemical features of DLB and PDD include severe loss of cholinergic neurons within the nucleus basalis magnocellularis [4] and extensive cortical and thalamic cholinergic deficits [5–7]. Similar to Alzheimer's disease (AD), no disease modifying treatments have been discovered for LBDs. Cholinesterase inhibitors [8, 9] and memantine [10] offer symptomatic benefit, but the development of therapies targeting the mechanisms of α-synuclein accumulation and aggregation in the cortex are at a preliminary stage.

The ubiquitin-proteasome system (UPS), the major proteolytic pathway responsible for the clearance of short lived proteins and the major non-lysosomal pathway for α-synuclein degradation [11], has in particular been a focus of research interest. Structurally, the 26S proteasome consists of a 20S proteolytic core and a 19S regulatory complex, composed of α and β subunits organized in four rings of seven subunits, three of the β-subunits in each ring containing the active sites at which proteolysis of substrate proteins occurs [12]. Each of these three subunits has distinct proteolytic activities, described as chymotrypsin-like, trypsin-like, and peptidyl glutamyl-peptide hydrolytic (PGPH) activities [13]. Together, these activities represent the best characterized peptidase activities although activities associated with cleavage of branched-chain, aromatic and small neutral amino acids have been reported [14]. The α subunits are involved in tethering of the 19S regulatory complex to the 20S proteasome and serve to maintain the structural stability of the 20S proteasome [15].

ATPase sub-units (RPT1-6) are essential for cellular survival; furthermore, the ATPase sub-units are hypothesized to recognize the polyubiquitin degradation signal and to unfold the protein substrates for their degradation by the 20S core, thereby controlling the access of substrates to the proteolytic core [16, 17]. The 20S catalytic core alone has a closed gate and requires an activator to regulate its protease activity.

Dysfunction of the 26S proteasome has been increasingly recognized as playing a fundamental role in the pathogenesis of many neurodegenerative disorders [18, 19]. Neurodegenerative disorders share a common feature which is the accumulation of misfolded proteins in the form of insoluble protein aggregates or inclusion bodies. Each of these aggregates has a specific protein component depending on the disease, such as α-synuclein in Lewy bodies or hyperphosphorylated tau in neurofibrillary tangles in AD. However, irrespective of the characteristic protein aggregate, ubiquitin has been identified as an additional component of inclusion bodies in many neurodegenerative diseases [20], suggesting that polyubiquitination and impairment of the UPS is generally involved in inclusion body formation. In particular in LBDs, several lines of evidence support the involvement of the UPS and postmortem studies using PD cohorts have shown a reduction in proteasomal activity in the substantia nigra [21]. Preliminary studies have also identified proteasomal abnormalities in cortical regions of DLB subjects [22]. Moreover, inhibition of proteasome activity in neuronal cell lines resulted in accumulation of ubiquitinated proteins [23] and α-synuclein aggregation [24, 25]. Finally, aggregated α-synuclein binds strongly to the 19S component and inhibits the UPS [26].

Studying the UPS is essential to have a better understanding of these specific pathways to enable the development of targeted therapies. We therefore investigated the protein expression of a key component of the UPS, the RPT6 subunit of the 19S regulatory complex and relevant proteasome activities (chymotrypsin-like and PGPH-like) in individuals with DLB, PDD, and AD in comparison to controls. The relationship between these changes and cognitive impairment was also explored. RPT6 is the best characterized of the ATPase proteasome subunits and has been linked by several studies to neurodegeneration, hence its use in our study as the representative proteasome subunit.

METHODS AND MATERIALS

Participants, diagnosis, and assessment

Postmortem brain tissue was obtained from: University Hospital Stavanger (Norway), the MRC

Table 1
Patient demographic data

Diagnosis	Gender (M/F) %	Age at death (mean)	PMD (mean hours)	pH (mean)	MMSE (last assessment)
Control (25)	60/40	79.7 ± 7.6	39.1 ± 22.9	6.47 ± 0.28	n/a
PDD (34)	53/47	79.9 ± 6.0	33.5 ± 15.6	6.44 ± 0.34	13 (0–27)
DLB (55)	58/42	81.7 ± 6.5	41.3 ± 28.0	6.37 ± 0.41	13 (0–30)
AD (16)	31/69	88.0 ± 7.8	34.9 ± 23.9	6.30 ± 0.33	10.5 (0–19)

Data are means ± SD age in years; PMD, postmortem delay; DLB, dementia with Lewy bodies; PDD, Parkinson's disease dementia; AD, Alzheimer's disease. PMD and pH were not significantly different between the groups in the one-way analysis of variance (ANOVA) ($p < 0.05$).

London Neurodegenerative Diseases Brain Bank, the Thomas Willis Oxford Brain Collection, and the Newcastle Brain Tissue Resource. The UK brain banks are part of the Brains for Dementia Research Network. All participants gave informed consent for their tissue to be used in research and the study had ethics approval from the National Research Ethics Service (08/H1010/4). Table 1 shows the demographic details of the patients and controls. Biochemical and histopathological analysis was undertaken on prefrontal cortex (BA9), anterior cingulate gyrus (BA24), and parietal cortex (BA40). BA9 was selected due to its proposed role in executive function and cognition [27], decline of which is characteristic of DLB and PDD. BA24 was selected since α-synuclein pathology in BA24 develops early in DLB and PDD [28], while BA40 was selected because it shows severe AD and comparatively low LBD pathology, respectively [29].

Neuropathological assessment was performed according to standardized neuropathological scoring/grading systems, including phases of amyloid-β (Aβ) deposition (Aβ-phases), neurofibrillary tangle Braak stages, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scores, Newcastle/McKeith Criteria for Lewy body disease, and National Institute on Aging - Alzheimer's Association (NIA-AA) guidelines [1, 30–33]. Controls were neurologically normal, with only mild age associated neuropathological changes (e.g., neurofibrillary tangle Braak stage ≤II) and no history of neurological or psychiatric disease.

Cognitive impairment data consisted of the last Mini-Mental State Examination (MMSE) scores a maximum of two years prior to death [34]. Patients and controls were categorized according to cognitive impairment in the following manner: 'unimpaired cognition' for individuals classified by the brain bank(s) as being clinical controls; 'neurodegenerative disease without dementia' for individuals with MMSE scores of 25 to 30 and no antemortem diagnosis of dementia; 'mild dementia' for individuals with MMSE scores from 17 to 24; 'moderate dementia' for individuals with MMSE scores of 10 to 16; and 'severe dementia' for individuals with MMSE scores of 9 or less [35]. Table 2 shows how the clinical diagnoses were divided between these categories. Final diagnoses for patients are clinico-pathological consensus diagnoses incorporating the one-year rule to differentiate DLB and PDD [1].

Preparation of tissue samples for western blotting

Preparation of tissue for western blotting was performed as previously described [35]. Briefly, cortical grey matter was dissected free of the meninges and white matter at 0–4°C. Approximately 200 mg tissue was homogenized in 4 ml ice-cold buffer containing 50 mM tris-HCL, 5 mM EGTA, 10 mM EDTA, 'complete protease inhibitor cocktail tablets' (Roche, 1 tablet per 50 ml of buffer), and 2 μg/ml pepstatin A dissolved in ethanol:DMSO 2:1 (Sigma). An IKA Ultra-Turrax mechanical probe (IKA Werke,

Table 2
Demographic data for cognitive impairment categories

MMSE Category	Diagnosis	Gender	Age at death
Control (1)	Control $n = 25$, PDD $n = 1$	M = 61.5%	79.2 ± 1.6
MCI (2)	DLB $n = 5$, PDD $n = 4$	M = 55.6%	80.3 ± 1.7
Mild (3)	DLB $n = 7$, PDD $n = 5$, AD $n = 3$	M = 73.3%	79.9 ± 1.8
Moderate (4)	DLB $n = 14$, PDD $n = 11$, AD $n = 4$	M = 55.2%	81.8 ± 1.5
Severe (5)	DLB $n = 10$, PDD $n = 12$, AD $n = 8$	M = 50%	81.6 ± 1.1

Age at death is mean ± SEM.

Germany) was used for homogenization. Aliquots were immediately frozen on dry ice and stored at –70°C. Protein concentration was assessed in triplicate using the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, USA); briefly 10 μl of crude homogenate was diluted 1:50 and read in triplicate at 595 nm using a FlexStation 3 (Molecular Devices).

Western blotting

Western blotting was undertaken as previously described [35]. Briefly, crude brain homogenate was diluted 4:5 with 5x sample buffer (Genscript MB01015, USA), boiled for 5 min then stored at –20°C. Samples were loaded at 20 μg/ml total protein on 10% SDS-polyacrylamide gel for protein separation, transferred to nitrocellulose membrane (Hydrobond C, Amersham Biosciences, Amersham, UK), and probed with Proteasome 19S ATPase subunit RPT6 (p45-110, Enzo Life Sciences, Exeter, UK, 1:2000), Proteasome 20S α3 subunit (MCP257, Enzo Life Sciences, Exeter, UK, 1:2000) Proteasome 20S α6 subunit, (MCP20, Enzo Life Sciences, Exeter, UK, 1:2000). Bands were detected using an Odyssey infrared fluorescent scanner, the integral of intensity quantified using Odyssey infrared imaging systems application software version 3.0.16 and expressed as ratios to rat cortex run on the same gel in arbitrary units. All samples were run in duplicate.

Proteasome enzyme activity assay

Proteasome activity was assessed in postmortem brain tissue using fluorogenic synthetic peptide substrates (for chymotrypsin-like activity, Suc-Leu-Leu-Val-Tyr-AMC; for PGPH-like activity, Z-Leu-Leu-Glu-AMC) as described previously [36]. In brief, frozen grey matter from patient and control subjects were immediately homogenized by ultra turrax in ice-cold buffer (50 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 2 mM ATP, 0.5 mM DTT, pH 7.5). Protein concentration was determined by the Bradford method. Brain lysates (100 μl per well) containing 1 mg/ml of protein were incubated with 1 μl (5 mM substrate III, Suc-Leu-Leu-Val-Tyr-AMC) or 10 μl (1 mM substrate II, Z-Leu-Leu-Glu-AMC)1 mM PGPH substrates for 60 min at 37°C. Measurements were performed in 96-well plates (total volume 100 μl per well) and all samples were assayed in triplicate for both activities. In a separate well, lysates were also pre-incubated with 5 mM carbobenzoxyl-leucinyl-leucinyl-leucinal (MG-132; a final concentration of 50 μM for chymotrypsin-like activity) and 150 μM for PGPH-like activity, or 100% DMSO for 30 min at room temperature. The background fluorescence values obtained by incubating the lysates with the proteasome inhibitors were subtracted from activity values. Proteasomal activity rates are expressed as fluorescence units (FU)/mg protein/h. The substrate hydrolysis was determined by measuring the fluorescence intensity of the AMC released using a FlexStation 3 (Molecular Devices LTD, UK) at an emission wavelength of 355 nm and an excitation wavelength of 460 nm. The specificity of the proteasomal assay was confirmed by the ability of the proteasome inhibitor to inhibit chymotrypsin-like and PGPH-like activities.

Statistical preparation and analysis

Statistical analysis of the biochemical data was undertaken as described previously [35, 37]. The normality of the data for each protein was determined using the Shapiro-Wilk test and normalized where necessary. In each case, the protein values were subsequently expressed as residuals (unstandardized) created from the multivariable regression analysis, to eliminate the confounding effect of the demographic variables (gender, postmortem delay (PMD), age at death, length of brain storage) on the protein values. RPT6 values were significantly predicted by age at death (in BA9 and 40) and PMD (in BA24 and 40), PGPH-like activity values were significantly predicted by PMD in BA40, and so a residual variable was created for this protein and this activity to statistically remove this effect. This variable was then normalized using a log10 transformation. We tested for differences in protein levels between groups using one-way ANOVA and Bonferroni *post-hoc* test. Intercorrelations of neurochemical variable and correlations with demographic and clinical features were examined using Pearson product moment (r) and regression analysis. Statistical analyses were conducted using SPSS version 20.

RESULTS

Patient demographic data

Demographic variables for the study cohort are summarized in Table 1. There were no significant differences in PMD, tissue pH, or gender between diagnostic groups. AD patients were significantly

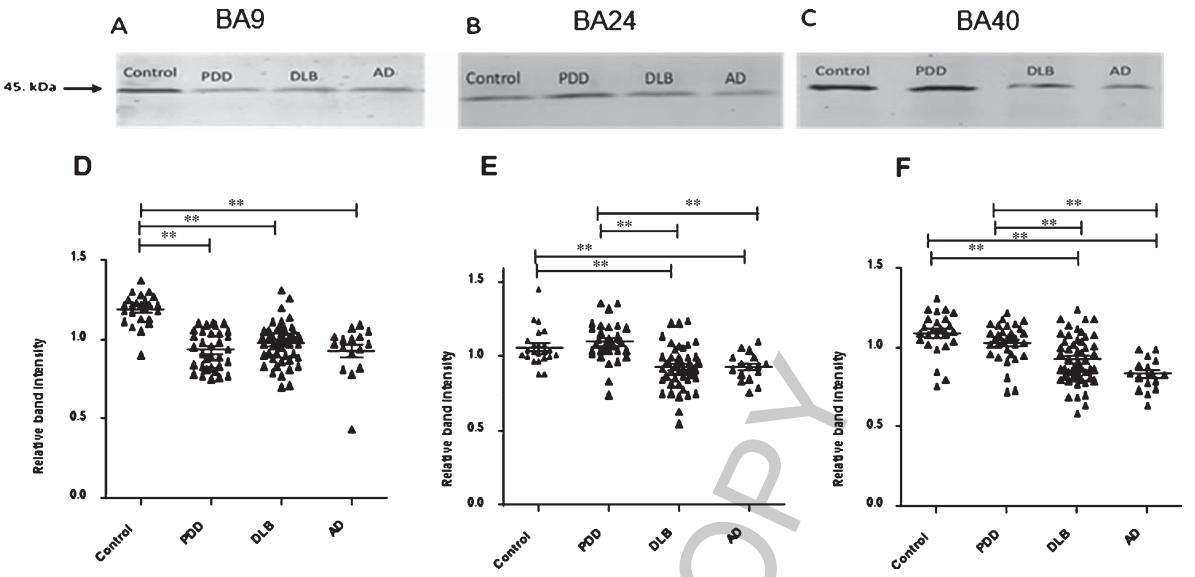

Fig. 1. 19S ATPase RPT6, proteasome sub-unit values, from semi-quantitative western blotting in PDD, DLB, AD, and control in BA9, BA24, and BA40. The image is a representative western blot showing an example of the diagnosis-specific reductions of the 19S ATPase RPT6 in BA9 (A), BA24 (B), and BA40 (C). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Bonferroni *post hoc* test. BA9 (D): Mean RPT6 values from controls ($n = 24$) were significantly higher than Parkinson's disease dementia (PDD) ($p = 0.001$, $n = 33$), dementia with Lewy bodies (DLB) ($p = 0.001$, $n = 50$), and AD ($p = 0.001$, $n = 16$) groups, one-way ANOVA ($F = 24.303$, d.f. = 3, 119, $p = 0.001$ followed by Bonferroni *post hoc* test). BA24 (E): Mean RPT6 values for the control ($n = 24$) and PDD ($n = 33$) groups were significantly higher than DLB ($p < 0.05$, $n = 52$) and AD ($p < 0.05$, $n = 16$) groups. There was no difference between the control and PDD groups, one-way ANOVA ($F = 13.56$, d.f. = 3 and 113, $p = 0.001$; Bonferroni *post hoc* test). BA40 (F): There was no significant difference in RPT6 levels between controls and PDD, but RPT6 levels for the control group ($n = 24$) and PDD ($n = 33$) groups were significantly higher than DLB ($p < 0.05$, $n = 52$) and AD ($p = 0.001$, $n = 16$) groups, one-way ANOVA ($F = 16.333$, d.f. = 3 and 121, $p = 0.001$; Bonferroni *post hoc* test). The horizontal bars within the data points in the graphs represent the mean values. (**$p < 0.01$).

older at death (one-way ANOVA $F(3;126) = 6.044$, $p = 0.001$) than controls ($p = 0.001$), patients with DLB ($p = 0.008$), or PDD ($p = 0.001$). Therefore, residuals were calculated.

Differences in the levels of 19S ATPase RPT6 proteasome sub-unit between diagnostic groups

Significant reductions in RPT6 proteasome sub-unit were detected in the prefrontal cortex (BA9) in DLB (–17%, $p = 0.001$), PDD (–21%, $p = 0.001$), and AD (–22%, $p = 0.001$) compared with controls (one-way ANOVA, $F = 24.303$, d.f. = 3, 119; $p = 0.001$; Bonferroni *post hoc* test) (Fig. 1). In BA 40, there was a significant reduction in RPT6 proteasome sub-unit in DLB (–14%, $p = 0.001$) and AD (–23%, $p = 0.001$) compared with controls (one-way ANOVA, $F = 16.33$, d.f. = 3 and 121, $p = 0.001$; Bonferroni *post hoc* test). There was no significant alteration in the level of RPT6 protein in patients with PDD compared with the control groups (Fig. 1). Furthermore, significant reductions in RPT6 sub-unit levels were seen in DLB (–13%, $p = 0.001$) and AD (–13%, $p = 0.001$) compared with the PDD. In BA24 mean RPT6 levels were significantly elevated in patients with PDD by 15%, $p = 0.001$ compared with AD and DLB groups (one-way ANOVA $F = 13.5$, d.f. = 3 and 113, $p = 0.001$; Bonferroni *post hoc* test). The *post hoc* test revealed that there was no significant difference between the control and PDD groups ($p > 0.05$) (Fig. 1).

Assessment of PGPH-like proteasome activity

In BA9, a significant reduction in PGPH-like activity was seen in AD patients compared with controls (–23%, $p = 0.012$) (one-way ANOVA, $F = 3.816$, d.f. = 3 and 34, $p = 0.019$; Bonferroni *post hoc* test). There was no significant difference between DLB (–13%, $p > 0.05$) and PDD (–14%, $p > 0.05$) groups compared to controls (Fig. 2).

In BA40, there was a significant reduction in PGPH-like activity in the AD (–45%, $p = 0.001$), DLB (–39%, $p = 0.001$), and PDD (–29%, $p = 0.02$) groups compared with controls (Fig. 2). The differences between the patient groups and the control were

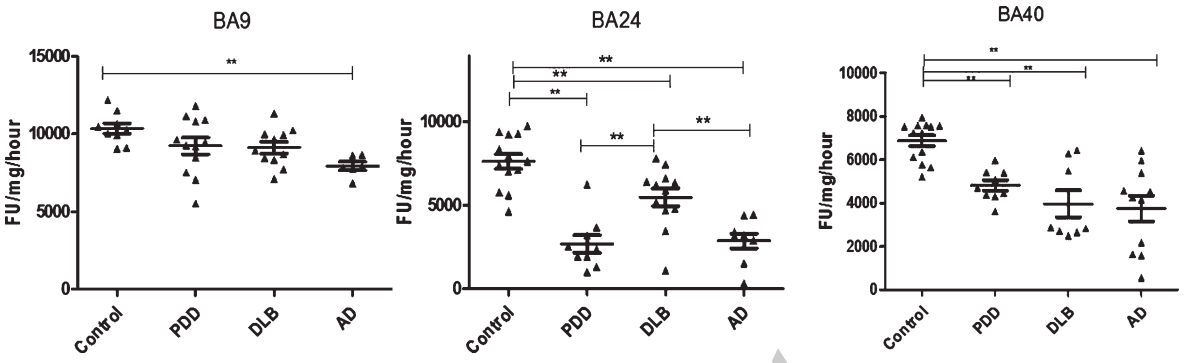

Fig. 2. Analysis of PGPH-like activities in brain homogenates from BA9, BA40, and BA24 of DLB, PDD, AD, and controls. Scatter plots are shown of PGPH-like activity measurement in BA9, BA40, and BA24 homogenates from DLB, PDD, AD, and normal control samples using the fluorogenic substrate assay. Activities are expressed as fluorescence units (FU)/mg protein/hour. BA9: PGPH-like activity was significantly decreased only in AD patients ($p = 0.012$, $n = 6$) compared with the control ($n = 9$); DLB and PDD groups were lower compared with the control subjects, but there was no statistically significant difference between them. The values for the ANOVA for PGPH-like activity measurement in BA9 were: $F = 7.897$, d.f. = 3, 34, $p = 0.001$). BA40; the differences between the patients' groups (PDD, DLB, and AD) and the control were statistically different (one-way ANOVA, $F = 10.263$, d.f. = 3 and 42, $p = 0.001$;). The reduction in PGPH-like activity was higher in the AD group with a mean ± SEM value of 3741.8 ± 587.5, $n = 11$, compared with 1.28 ± 0.028, $n = 24$ for the controls. The reduction in both DLB and PDD were also significant with a mean ± SEM value of 4133.7 ± 640, $n = 10$ and 4809 ± 240, $n = 9$ compared with control (Bonferroni *post hoc* test). In BA24, there was a significant difference between DLB ($p = 0.013$, $n = 12$), PDD ($P = 0.001$, $n = 9$) and AD ($P = 0.001$, $n = 9$) compared with the control ($n = 13$) (one-way ANOVA, $F = 23.087$, d.f. = 3 and 39, $p = 0.001$; Bonferroni *post hoc* test). PGPH-like activity measurements were significant lower in both AD ($p = 0.004$, $n = 9$) and PDD ($p = 0.002$, $n = 9$) compared with DLB subjects. The horizontal bars within the data points in the graphs represent the mean values. (**$p < 0.01$).

statistically different (one-way ANOVA, F = 10.263, d.f. = 3 and 42, $p = 0.001$; Bonferroni *post hoc* test).

In BA24, there was a significant reduction in PGPH-like activity in DLB (–28%, $p = 0.013$), PDD (–64%, $p = 0.001$), and AD (–62%, $p = 0.001$) groups compared with control subjects (one-way ANOVA, F = 23.087, d.f. = 3 and 39, $p = 0.001$; Bonferroni *post hoc* test). The reduction in PGPH-like activity in PDD (–51%, $p = 0.002$) and AD (–47%, $p = 0.004$) was also significant different compared with DLB (Fig. 2).

Assessment of chymotrypsin-like proteasome activity

In BA9, chymotrypsin-like activity was significantly reduced in PDD (–27%, $p = 0.004$), DLB (–24% $p = 0.013$), and AD (–38%, $p = 0.001$), compared with control values (one-way ANOVA, F = 7.897, d.f = 3 and 34, $p = 0.001$; Bonferroni *post hoc* test) (Fig. 3). Chymotrypsin-like activity was lowest in the AD group compared with DLB and PDD; however, there were no significant differences among the three groups, PDD, DLB, or AD. In BA40, analysis of data indicated a significant reduction in chymotrypsin-like activity in the AD, DLB, and PDD (1415.85 ± 9.9, $n = 12$, 1453.09 ± 11.77, $n = 9$ and 1436.88 ± 20.61, $n = 10$) groups compared with the control groups (1568.53 ± 10.2, $n = 13$) (one-way ANOVA, F = 30.033, d.f. = 3 and 40, $p = 0.001$; Bonferroni *post hoc* test).

In BA24, chymotrypsin-like activity was found to be significantly lower in PDD (878 ± 62, $n = 9$) and AD (906 ± 72, $n = 9$) samples compared with both control (1100 ± 39, $n = 13$) and DLB (1027 ± 23, $n = 12$) subjects (one-way ANOVA, F = 4.663, d.f. = 3 and 39, $p = 0.007$; Bonferroni *post hoc* test).

Correlations between proteasome activity and expression level of RPT6 subunit

To test whether or not proteasome activity was associated with the protein levels of the proteasome subunits, Spearman's rank correlation was determined between PGPH- and chymotrypsin-like activities, and the semi-quantitative protein values of RPT6. In BA9, significant positive correlations were found between RPT6 and both chymotrypsin-like activity (Rs 0.418, $p = 0.009$, $n = 38$) and PGPH-like activity (Rs 0.363, $p = 0.025$, $n = 38$), while in BA40, there was a significant positive correlation with only chymotrypsin-like activity (Rs 0.409, $p = 0.006$, $n = 44$).

Reduction in RPT6 were associated with cognitive impairment

The reduced levels of RPT6 detected in BA9 were found to be associated with cognitive impairment

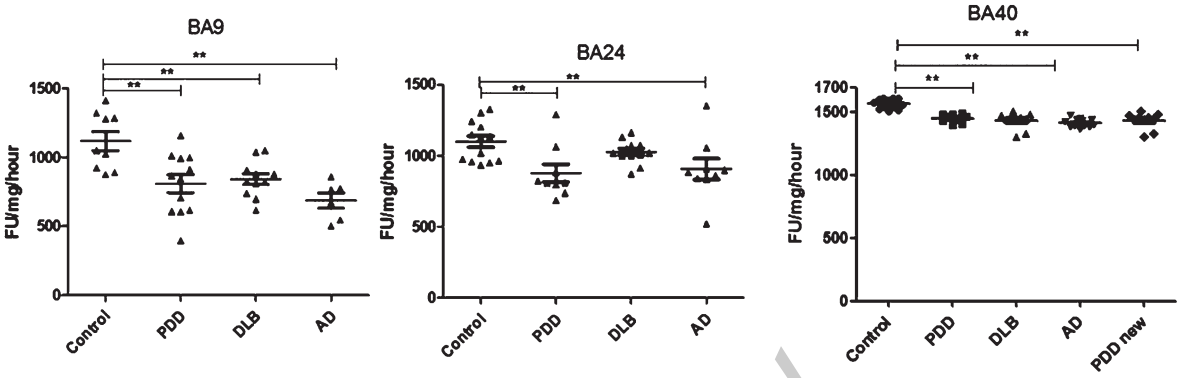

Fig. 3. Analysis of chymotrypsin-like activities in brain homogenates from BA9, BA40, and BA24 of DLB, PDD, AD, and controls. Scatter plots are shown of chymotrypsin-like activity measurement in BA9, BA40, and BA24 homogenates from DLB, PDD, AD, and normal control samples using the fluorogenic substrate assay. Activities are expressed as fluorescence units (FU)/mg protein/hour. BA9; the activities' values for the control group were significantly higher than the PDD ($p = 0.004$, $n = 12$), DLB ($p = 0.013$, $n = 11$) and AD ($p = 0.001$, $n = 6$) groups. The ANOVA values for chymotrypsin-like activity measurement in BA9 are: F = 7.897, d.f. = 3 and 34, $p = 0.001$) BA40; the activities' values for the control group ($n = 13$) were significantly higher than the PDD ($p = 0.001$, $n = 10$), DLB ($p = 0.001$, $n = 9$), and AD ($p = 0.001$, $n = 12$) groups. The ANOVA for chymotrypsin-like activity measurement in BA40 (one-way ANOVA, F = 30.033, d.f. = 3 and 40, $p = 0.001$; Bonferroni *post hoc* test) BA24; there was a significant difference between the PDD ($p = 0.015$, $n = 9$) and AD ($p = 0.044$, $n = 9$) groups compared with the control ($n = 13$) (one-way ANOVA, F = 4.664, d.f. = 3 and 39, $p = 0.007$; Bonferroni *post hoc* test). (**$p < 0.01$).

(Fig. 4A, $R^2 = 0.297$, beta = –0.545, degree of freedom [df] = 1, 102, $t = –6.571$, $p = 0.001$), and this analysis included all control, DLB, PDD, and AD subjects. In addition, in pairwise comparisons, patients with mild dementia had mean RPT6 levels that were significantly lower than individuals with unimpaired cognition (41%, $p = 0.001$), and RPT6 levels were significantly lower in people with moderate dementia (both 20%, $p = 0.001$) and with severe dementia (both 23%, $p = 0.001$) in BA9 compared with controls. Reduced levels of RPT6 in BA40 were also associated with cognitive impairment (Fig. 4C, R2 = 0.180, beta = –0.425, df = 1, 105, $t = –4.807$, $p = 0.001$) according to regression analysis of all controls and dementia patients. In BA40, RPT6 levels were significantly lower in people with moderate cognitive impairment (both 10%, $p = 0.033$) and with severe cognitive impairment (both 19%, $p = 0.001$) compared with controls. The association between reduced levels of RPT6 and cognitive impairment in BA24 was weaker (Fig. 4B, $R^2 = 0.04$, beta = –0.206, df = 1, 99, $t = –2.09$, $p = 0.039$) and not significantly different between people with different severities of cognitive impairment (one-way ANOVA $p > 0.05$).

Reduced proteasome activity is associated with cognitive impairment

Chymotrypsin-like and PGPH proteasome activities had a significant inverse association with cognitive impairment in BA9 (according to regression analysis of all dementia patients and controls) (Fig. 5A and D, $R^2 = 0.337$, beta = –0.581, [df] = 1, 29, $t = –3.84$, $p = 0.001$, $R^2 = 0.275$, beta = –0.524, [df] = 1, 29, $t = –3.315$, $p = 0.002$), in BA24 (Fig. 5B and E, $R^2 = 0.151$, beta = –0.388, [df] = 1, 38, $t = –2.599$, $p = 0.013$, $R^2 = 0.371$, beta = –0.609, [df] = 1, 38, $t = –4.733$, $p = 0.001$), and in BA40 (Fig. 5C and F, $R^2 = 0.531$, beta = –0.728, [df] = 1, 40, $t = –6.72$, $p = 0.001$, $R^2 = 0.217$, beta = –0.466, [df] = 1, 38, $t = –3.244$, $p = 0.002$). Pairwise comparisons in BA9 indicated high chymotrypsin-like activity in the control compared with moderate ($p = 0.014$) and severe scores ($p = 0.01$) (one-way ANOVA F = 5.009, d.f. = 4 and 26, $p = 0.004$; Bonferroni *post hoc* test). The difference in PGPH-like activity between cognitive impairment groups was significant between individuals with unimpaired and moderately impaired cognition (one-way ANOVA F = 3.616, d.f. = 4 and 26, $p = 0.004$; Bonferroni *post hoc* test). In BA24, there was no significant difference in chymotrypsin-like activity in unimpaired cognition group compared with all other groups. The difference in PGPH-like activity between cognitive impairment groups was significantly different between unimpaired cognition groups and MCI ($p = 0.03$), mild ($p = 0.024$), moderate ($p = 0.001$), and severe scores ($p = 0.001$) (one-way ANOVA F = 9.839, d.f. = 4 and 35, $p = 0.001$; Bonferroni *post hoc* test). In BA40, the level of chymotrypsin-like activity was significantly higher in the unimpaired cognition group compared with MCI ($p = 0.001$), mild ($p = 0.001$),

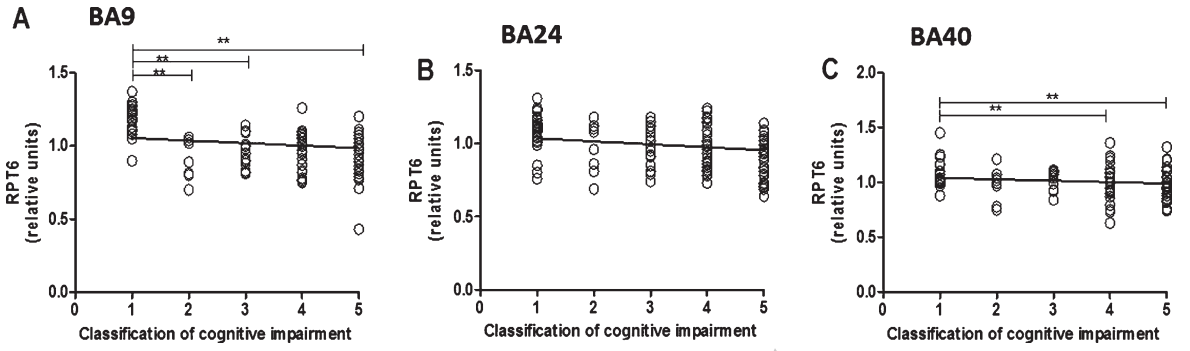

Fig. 4. Relationship between RPT6 expression levels and cognitive impairment based upon MMSE classification. 19S ATPase RPT6, proteasome sub-unit values protein levels in BA9, BA24, and BA40 predicted cognitive impairment. Regression analysis showed RPT6 levels in BA9, BA24, and BA40 of control, DLB, PDD, and AD to be significant predictors of the cognitive impairment category ([BA9] $R^2 = 0.297$, beta = –0.545, degree of freedom [df] = 1, 102, $t = –6.571$, $p = 0.001$, [BA24] $R^2 = 0.04$, beta = –0.206, df = 1, 99, $t = –2.09$, $p = 0.039$, [BA40] R2 = 0.180, beta = –0.425, df = 1, 105, $t = –4.807$, $p = 0.001$]. The analysis of variance (ANOVA) for the model was significant ($p = 0.0001$). The difference in mean RPT6 levels between cognitive impairment groups was analyzed by one-way ANOVA and the Bonferroni *post hoc* test, which revealed RPT6 levels in BA9 to be significantly higher in controls compared with the other groups (one-way ANOVA F = 17.82, d.f. = 4 and 99, $p = 0.001$; Bonferroni *post hoc* test). In BA40, RPT6 levels were significantly lower in people with moderate dementia (10%, $p = 0.033$) and with severe dementia (19%, $p = 0.001$) compared with controls. The difference in mean RPT6 levels between cognitive impairment groups in BA24 was not found to be significant (one-way ANOVA $p > 0.05$). (**$p < 0.01$).

moderate ($p = 0.001$), and severe scores ($p = 0.001$) (one-way ANOVA F = 21.845, d.f. = 4 and 37, $p = 0.001$; Bonferroni *post hoc* test). PGPH-like activity was significantly higher between unimpaired cognition group compared with MCI ($p = 0.02$), mild ($p = 0.001$), moderate ($p = 0.001$), and severe scores ($p = 0.034$) (one-way ANOVA F = 8.851, d.f. = 4 and 35, $p = 0.001$; Bonferroni *post hoc* test).

We assessed the levels of alpha3 and alpha6, essential subunits of the 20S catalytic unit (Supplementary Figures 1 and 2, respectively). We also expressed the measurement of both chymotrypsin-like and PGPH-like activity as a ratio to both subunits to determine if the changes we saw represented a change in rate of activity per catalytic unit or of the number of catalytic units (Supplementary Figures 3–6). It can be seen that there was no overall pattern with high variation according to brain region.

DISCUSSION

The main finding of the present study was the reductions of the RPT6 ATPase 19S regulatory subunit in DLB and AD in the frontal lobe neocortical area BA9, anterior cingulate gyrus BA24, and parietal cortex BA40. Furthermore, the reduction in RPT6 levels was associated with changes in two proteasome proteolytic activities. Finally, both measurements were associated with cognitive scores prior to death. In this study, for the first time, an association between cognitive decline and both the reduction of RPT6 and the proteolytic activity of the proteasome has been demonstrated.

RPT6 is one of the six ATPase subunits (RPT 1–6) of the 19S regulatory complex; it is a 45kDa subunit. Degradation of ubiquitinated substrate proteins by the 26S proteasome is dependent upon ATP [13], which binds to the six ATPase subunits of the 19S regulatory complex. All six of the ATPase subunits contain the same substantial main functional domains: an N-terminus coiled-coil domain important for formation of the 19S base, and a C-terminus ATPase domain that is involved in ATP-dependent substrate unfolding and 20S CP opening [38]. These ATPases provide the energy necessary for the degradation of multi-ubiquitin conjugated proteins by the 26S proteasome, and it is also believed that ATPase subunits participate in the substrate-unfolding step of the degradation pathway [39].

It has been shown previously that the 19S RPT6 expression level decreased when α-synuclein was increased in mouse PD models [40] and a study of 9 PD, 7 PDD, and 9 controls revealed a decrease in the 19S RPT3/S6 subunit in the inferior frontal gyri of PDD although the expression was similar in control and PD [41]. Inactivation of the 19S regulatory particle (RPT2) subunit prevented the formation of the 26S proteasome, leaving the 20S proteasome subunit, which is ubiquitin-independent, unaffected [42]. Therefore, the reduction in RPT6 subunit expression identified in DLB, PDD, and AD patients in three

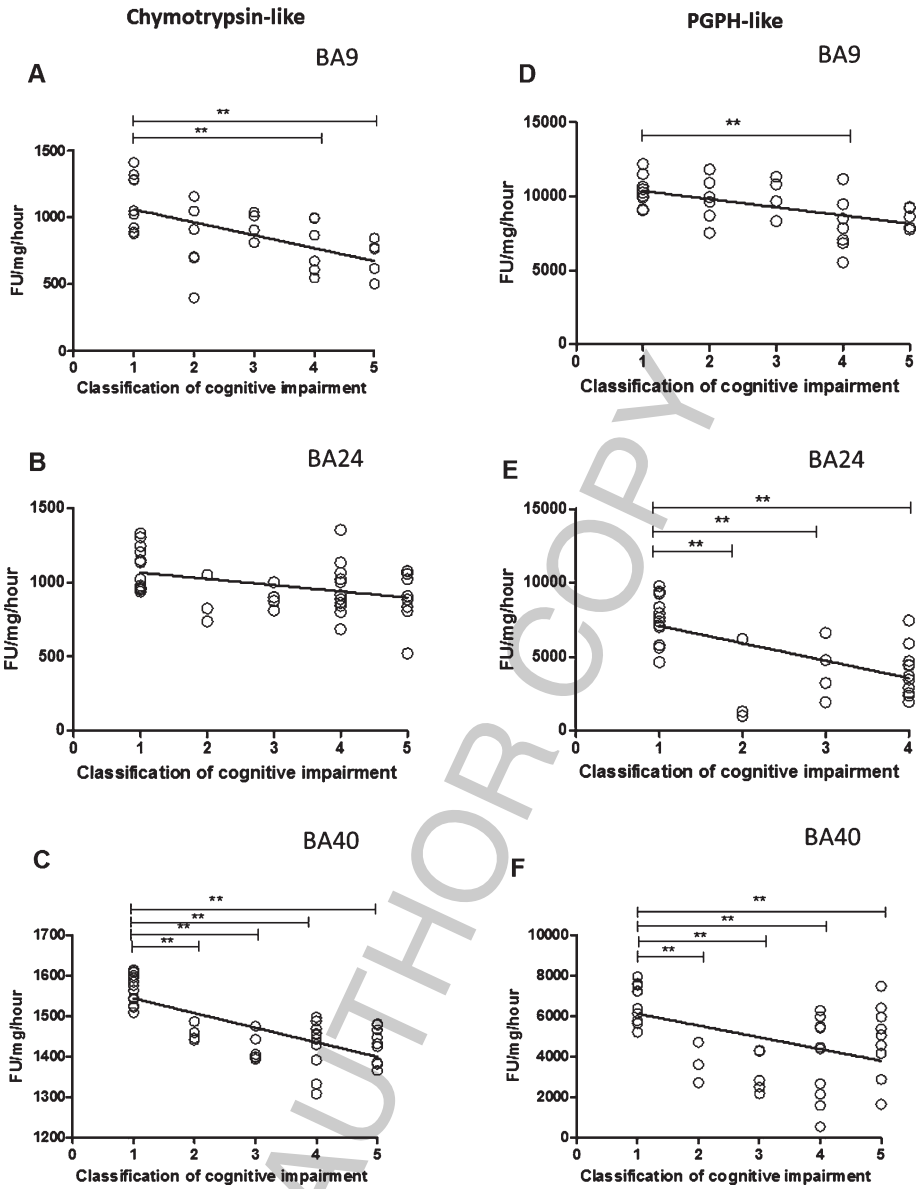

Fig. 5. The relationship between Chymotrypsin- and PGPH-like proteasome activity measurement levels and cognitive impairment based upon MMSE classification. Chymotrypsin- and PGPH-like proteasome activity measurement levels, using fluorogenic substrate assay, predicted cognitive impairment in BA9, BA24, and BA40. Regression analysis showed Chymotrypsin-like and PGPH proteasome activity to be a significant predictor of cognitive impairment in BA9 (A and D, $R^2 = 0.337$, beta = –0.581, [df] = 1, 29, $t = –3.84$, $p = 0.001$, $R^2 = 0.275$, beta = –0.524, [df] = 1, 29, $t = –3.315$, $p = 0.002$), in BA24 (B and E, $R^2 = 0.151$, beta = –0.388, [df] = 1, 38, $t = –2.599$, $p = 0.013$, $R^2 = 0.371$, beta = –0.609, [df] = 1, 38, $t = –4.733$, $p = 0.001$), and in BA40 (C and F, $R^2 = 0.531$, beta = –0.728, [df] = 1, 40, $t = –6.72$, $p = 0.001$, $R^2 = 0.217$, beta = –0.466, [df] = 1, 38, $t = –3.244$, $p = 0.002$). The difference in mean Chymotrypsin-like and PGPH proteasome activity measurement levels between different cognitive impairment groups was analyzed by one-way ANOVA and the Bonferroni *post hoc* test, which revealed high chymotrypsin-like activity in the controls compared with moderate ($p = 0.014$) and severe scores ($p = 0.01$) (one-way ANOVA F = 5.009, d.f. = 4 and 26, $p = 0.004$; Bonferroni *post hoc* test). There was a high PGPH-like activity in unimpaired cognition compared with the moderate groups (one-way ANOVA F = 3.616, d.f. = 4 and 26, $p = 0.004$; Bonferroni *post hoc* test). In BA24, there was higher PGPH-like activity in unimpaired cognition group compared with MCI ($p = 0.03$), mild ($p = 0.024$), moderate ($p = 0.001$) and severe scores ($p = 0.001$) (one-way ANOVA F = 9.839, d.f. = 4 and 35, $p = 0.001$; Bonferroni *post hoc* test). In BA40 the level of chymotrypsin-like activity was significantly higher in the controls compared with MCI ($p = 0.001$), mild ($p = 0.001$), moderate ($p = 0.001$) and severe scores ($p = 0.001$) (one-way ANOVA F = 21.845, d.f. = 4 and 37, $p = 0.001$; Bonferroni *post hoc* test). There was a higher level of PGPH-like activity in unimpaired cognition group compared with MCI ($p = 0.02$), mild ($p = 0.001$), moderate ($p = 0.001$) and severe scores ($p = 0.034$) (one-way ANOVA F = 8.851, d.f. = 4 and 35, $p = 0.001$; Bonferroni *post hoc* test). (**$p < 0.01$).

brain regions and the associated reduction in proteasome activity confirms and extends previous studies by demonstrating this phenomenon in the human brain and suggests that reduced subunit expression may directly lead to proteasome impairment. The reason for the reduction in RPT6 ATPase subunit expression remains unexplained. It is possible that the reduction could be related to oxidative stress; indeed, proteasome subunits were demonstrated to be sensitive to oxidative stress [43, 44]. Furthermore, Sun et al. reported that proteasome subunits (RPT5, Rpn10, and Rpn2) can be cleaved by caspase-3 following caspase activation during apoptosis; they found decreased proteasome activity to be associated with the cleavage of these subunits [45].

RPT6 phosphorylation enhances proteolysis by promoting the assembly of the 26S proteasome, and RPT6 dephosphorylation promoted the dissociation of 26S into 19S and 20S components [46]. It is proposed that the reduction of proteasome activity is due to the decrease in the RPT6 level as there was a correlation between lower RPT6 protein levels and proteasome activity in BA9 and BA40. It could also be due to the important role of RPT6 in promoting the activity of proteasomes. The reduction of the proteolytic activity could also arise from the blockading of the entry pore to the 20S proteasome by protein aggregates, such as α-synuclein, which may in turn impede degradation of this and other proteins [47–49]. Inhibition of the 26 S proteasome with soluble oligomeric species of mutant and wild-type α-synuclein in PC12 cells has been emonstrated [47]. It is clear that these oligomers are degraded by the proteasomes, as they accumulate when proteasome function is inhibited. Proteasome inhibitors have been reported to induce α-synuclein aggregation and Lewy body-like inclusions, leading to neuronal loss among *in vitro* and *in vivo* models [42]. However, it is not clear whether the aggregation results from the impairment of the proteasomes or vice versa [50]. Results from experimental studies have indicated that inhibition of the proteasomes causes the formation of aggregates [42, 51] and protein aggregation inhibits the proteasome activity [47].

In this study, we found a reduction in RPT6 and the proteasome activity in relation to cognitive decline. It is not clear how proteasome impairment, specifically reduction in RPT6, could result in cognitive impairment. A possible mechanistic explanation for this is that the proteasome activity is regulated by protein such as Calcium/calmodulin-dependent protein kinase II (CaMKII), which plays an essential role in long-term synaptic plasticity and cognitive function [52, 53]. Consistent with this, Jarome et al. showed that phosphorylation of RPT6 by CaMKII increased proteasome activity *in vivo* and proteasome activity was necessary for long-term memory function [54, 55]. Reductions in CaMKII affect signaling pathways, including phosphorylation of RPT6, and thus the proteasome activity [54, 55], which could in turn impair synaptic plasticity and contribute to cognitive dysfunction. CaMKII has been shown to mediate proteasome activity and act as a scaffold to recruit proteasomes to dendritic spines and regulate its activity by phosphorylation of the RPT6 subunits [56]. Activation of NMDA receptors has been shown to induce this movement of the proteasome to the dendritic spine compartment [57].

In view of the above, our data strongly suggest that proteasome activation may be a target for disease modification of both DLB and PDD. In support of this view, compounds which enhance proteasome activity have been suggested to be neuroprotective. For example, pre-treatment with trans-retinoic acid protected against cell death induced by epoxomicin (a proteasome inhibitor) in SH-SY5Y cells [58]. Furthermore, altered insulin and insulin-like growth factor (IGF-1) signaling have been reported to influence the proteasome activity [59, 60], and both insulin and IGF-1 receptor expression was reduced in DLB [61]. Supporting this, treatment with IGF-1 prevented the apoptotic effects of epoxomicin on SH-SY5Y cells [62]. Therefore, compounds that are able to ameliorate insulin signaling may have disease-modifying activity in PDD and DLB. Of these, the glucagon-like pepide-1 (GLP-1) analogues are the most clinically advanced. These synthetic GLP-1 analogues such as exendin-4, liraglutide, albiglutide, and lixisenatide are resistant to degradation by dipeptidyl peptidase, pass the blood-brain barrier similar to GLP-1, (except for albiglutide), and bind to the GLP-1 receptor, bringing about an increase in insulin biosynthesis and release [63]. Both exenatide (exendin-4) and liraglutide are currently in phase II clinical trials for the treatment of AD (NCT01255163 and NCT01843075, respectively [64]. Furthermore, exenatide is currently in a phase II trial for the treatment of PD (NCT01971242). These studies suggest that compounds acting on GLP-1 receptors may translate well for the treatment of people with DLB or PDD. It is noteworthy that both retinoic acid and GLP-1 analogues have previously been suggested from drug repositioning studies for AD [65]. Many of these compounds have additional mechanisms of action,

including lowering plasma glucose concentrations for GLP-1 analogues. However, key candidate therapies are likely to involve more than one mechanism of action.

The strengths of this study are the large number of LBD cases, our access to all the clinical data and the number of projects undertaken on the same cohort, which provide chemical information on synaptic functions in addition to pathological and clinical data [35, 37, 66–69]. Furthermore, this study examined three brain regions and compared the results from each of the regions separately to determine whether the biochemical changes are specific to a particular brain area or if all of the regions have the same alteration. However, despite these advantages, there are also a number of limitations regarding the use of postmortem tissues which need to be taken into consideration. These include antemortem factors such as medication history, meaning we could not identify whether or not the medication had any effect on our observations. Postmortem factors, including PMD, and the handling and storage of tissue are further problems that also should be addressed when performing studies with postmortem tissue reviewed in [70]. Furthermore, alteration of brain tissue pH, as a consequence of agonal state can affect sample quality for genetic and biochemical measurements. These factors were taken into consideration when planning the studies in this study. Post/antemortem factors for controls, DLB, PDD, and AD were matched as closely as possible for PMD and pH, and any relationships found between protein measurements and demographics/post-mortem factors were controlled for via the creation of unstandardized residuals.

In conclusion, the present study has demonstrated that, in PDD, DLB, and AD, the activity of the RPT6 ATPase 19S regulatory subunit of the proteasome is decreased and associated with cognitive decline. The present study provides support for enhancement of the proteasome activity as a therapeutic target and current leading candidate are GLP-1 analogues.

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-160946.

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