The unfolded protein response is activated in Lewy body dementias

Jean-Ha Baek[1], David Whitfield[2], David Howlett[2], Paul Francis[2], Erika Bereczki[1], Clive Ballard[2], Tibor Hortobágyi[3], Johannes Attems[4], and Dag Aarsland[1, 5]

[1] Department of Neurobiology, Care Sciences and Society, Division for Neurogeriatrics, Karolinska Institute, Stockholm, Sweden.

[2] King's College London, Wolfson Centre for Age-Related Diseases, London SE1 1UL, UK

[3] Department of Neuropathology, Institute of Pathology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

[4] Institute of Neuroscience, Newcastle University, Newcastle Upon Tyne, UK

[5] Centre for Age-Related Medicine, Stavanger University Hospital, Stavanger, Norway

Correspondence should be addressed to: Prof. Dag Aarsland, Department of Neurobiology, Care Sciences and Society, Division for Neurogeriatrics, Karolinska Institute, Novum, SE-14157 Huddinge, Sweden.

Tel: +46 8 524 35379

Fax: +46 8 585 85470

E-mail: dag.aarsland @ ki.se

Keywords: Unfolded protein response, Parkinson's disease with dementia, Dementia with Lewy bodies, GRP78/BiP, Endoplasmic reticulum stress

Number of words: 4372

Number of figures and tables: 4 figures, 3 tables

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/nan.12260

Abstract

Aim

The unfolded protein response (UPR) is a pro-survival defense mechanism induced during periods of endoplasmic reticulum stress, and it has recently emerged as an attractive therapeutic target across a number of neurodegenerative conditions, but has not yet been studied in synuclein disorders.

Methods

The level of a key mediator of the UPR pathway, glucose regulated protein 78 (GRP78), also known as binding immunoglobulin protein (BiP), was measured in post-mortem brain tissue of patients with dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD) in comparison to Alzheimer's disease (AD) and age matched controls using western blot. The UPR activation was further confirmed by immunohistochemical detection of GRP78/BiP and phosphorylated protein kinase RNA-like ER kinase (p-PERK).

Results

GRP78/BiP was increased to a greater extent in DLB and PDD patients compared to AD and control subjects in cingulate gyrus and parietal cortex. However, there were no changes in the prefrontal and temporal cortices. There was a significant positive correlation between GRP78/BiP level and α-synuclein pathology in the cingulate gyrus, while AD-type pathology showed an inverse correlation relationship in the parietal cortex.

Conclusion

Overall, these results give emphasis to the role of UPR in Lewy body dementias, and suggest that Lewy body degeneration, in combination with AD-type pathologies, is associated with increased UPR activation to a greater extent than AD alone, possibly as a consequence of the increasing load of ER proteins. This work also highlights a novel opportunity to explore the UPR as a therapeutic target in synuclein diseases.

List of Abbreviations

Abbreviation	Full name
AD	Alzheimer's disease
ATF6	Activating transcription factor 6
Aβ	Amyloid-β
BA	Brodmann area
BiP	Binding immunoglobulin protein
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
DLB	Dementia with Lewy bodies
ER	Endoplasmic reticulum
GRP78	Glucose regulated protein 78
IRE1	Inositol-requiring enzyme 1
MMSE	Mini-Mental State Examination
NIA-AA	National Institute on Aging - Alzheimer's Association
p-PERK	Phosphorylated protein kinase RNA-like ER kinase
PDD	Parkinson's disease dementia
PERK	Protein kinase RNA-like ER kinase
UPR	Unfolded protein response

Introduction

Dementia with Lewy-bodies (DLB) is a common, but clinically under-recognised neurodegenerative disease. The clinical presentation is complex with more impact on quality of life and carer-burden and higher health-related costs compared to Alzheimer's disease (AD), and also with a shorter survival [1, 2]. There is a need for better mechanistic understanding of DLB to drive development of improved disease modifying treatments.

Accumulation of disease-specific misfolded proteins in the brain and subsequent neuronal loss is a key mechanism across a number of neurodegenerative diseases. Although a great deal of effort has been made to unravel how each individual 'toxic' protein exerts its deleterious effects in specific disorders, mechanistic understanding of how these proteins cause neuronal loss has been limited and thus the development of disease modifying treatments for neurodegenerative diseases has remained as a challenge.

Endoplasmic reticulum (ER) stress is another common theme in these diseases. The ER controls many of the cellular processes, and disturbance in ER function, usually caused by accumulation of unfolded proteins and calcium homeostasis within the ER, leads to ER stress. Although the sub-cellular loci of protein misfolding can vary between diseases, the interdependence of protein folding throughout the cell implies that ER dysfunction/stress could be the final common pathway for numerous neurodegenerative diseases [3]. It is well known that upon ER stress, cells activate a series of complementary adaptive mechanisms to deal with protein-folding alterations, which together are known as the unfolded protein response (UPR). The UPR increases overall protein-folding capacity, in addition to enhancing the efficiency of quality control and protein degradation mechanisms to reduce the unfolded protein load [4]. Most cell types are relatively resistant to the accumulation of misfolded protein through continuous dilution of the ER by cell replication, but this procedure is unavailable to post-mitotic neurons, therefore neurons depend totally on the UPR for survival of such insult [5].

The UPR orchestrates adaptation to protein folding stress by modulating at least three parallel

signalling pathways, initiated by the activation of the stress sensors: protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). In resting cells, PERK, IRE1, and ATF6 are associated with an ER chaperone, GRP78/BiP, which keeps them inactive. However, under conditions of ER stress, in order to deal with the increasing load of ER proteins, GRP78/BiP dissociates from these three proteins leading to their activation. UPR acts as a protective mechanism against the accumulation of toxic misfolded proteins which combines the early inhibition of protein synthesis with a later up-regulation of genes that promote protein folding or disposal to equilibrate disturbed ER homeostasis. Both of these translational and transcriptional elements of the UPR protect neurons from being overwhelmed by misfolded ER proteins. [3, 5]. However, under chronic or irreversible stress conditions, the UPR shifts its signalling toward cell death mechanisms by activating complex pro-apoptotic programmes [6].

Elucidating the role of UPR in the regulation of protein homeostasis begun almost three decades ago when Kozutsumi and colleagues showed that the presence of misfolded proteins in the ER signals the induction of GRP78 and GRP94 in simian cells [7]. A few years later, the first indication that the ER stress mechanism was involved in neurodegenerative diseases came from Hamos and colleagues, in which the level of GRP78/BiP was increased in the cytologically normal hippocampal neurons of the AD patients [8]. Since then, the number of studies investigating the role of UPR in various neurodegenerative diseases including PD and AD has grown exponentially [9-13]. Furthermore, manipulation of the UPR via genetic methods in animal models has also demonstrated that UPR activation can directly contribute to neurodegeneration [14]. Interestingly, it was shown that dysregulation of protein synthesis may play an essential role in neurodegenerative diseases by directly affecting the expression of synaptic proteins, leading to synaptic dysfunction and neuronal loss [15]. Recently, Moreno *et al*. took these findings a step further and showed that inhibiting a key mediator of the UPR pathway by a specific PERK inhibitor prevented UPR-mediated translational repression and abrogated development of clinical signs of prion disease in mice, with neuroprotection throughout the mouse brain [16]. Furthermore, Ma and colleagues

reported that conditional deletion of PERK in an APP/PS1 transgenic mouse model of AD recued synaptic protein expression and ameliorated synaptic dysfunction [17]. Together, these studies reveal that failure to restore protein homeostasis and prolonged UPR activation may be neurotoxic rather than neuroprotective, and point to a new way of treating neurodegenerative diseases.

Until now, several studies have shown promising results for UPR as a potential therapeutic target for AD and PD, but there are gaps in the knowledge regarding the involvement of UPR in cases of dementia where both AD and PD pathology are evident, such as PD with dementia (PDD) and DLB. The pathological hallmarks of PDD and DLB are accumulation of Lewy bodies and Lewy neurites, principally composed of α-synuclein [18-21]. Although these are defining abnormalities for PDD and DLB, approximately 40% of cases have additional amyloid-β (Aβ) and neurofibrillary tangle pathology [22, 23], with more pronounced cognitive dysfunction than in patients with 'pure' AD [24, 25]. Therefore, the aim of the present study is to investigate whether the UPR pathway is activated to a greater extent with co-occurrence of tau and α-synuclein pathology, in PDD and DLB, in comparison to AD and control subjects, and whether the UPR activity is associated with clinical and/or pathological measures.

Materials and methods

Post-mortem human brain tissue

Post-mortem brain tissue was obtained from several sources; University Hospital Stavanger (Norway), the MRC London Neurodegenerative Diseases Brain Bank, the Thomas Willis 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 UK National Research Ethics Service (08/H1010/4 and KI IRB) and from the Ethics Review Board of Sweden (etikprövningsnämnderna, 2013/914-31/4) . The demographic details of the patients and control subjects are shown in Table 1. Biochemical and histopathological analysis was undertaken on prefrontal cortex (Brodmann area

(BA) 9; n = 99), temporal cortex (BA21; n = 93), anterior cingulate gyrus (BA24; n = 89) and parietal cortex (BA40; n = 118). Only prefrontal cortex tissue sections were available for immunohistochemical analysis and temporal cortex tissues from AD patients were not available due to technical reasons.

Neuropathological assessment and diagnosis was performed according to standardised neuropathological scoring/grading systems, including Braak staging, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scores, Newcastle/McKeith Criteria for Lewy body disease, National Institute on Aging - Alzheimer's Association (NIA-AA) guidelines for Aβ deposition, neurofibrillary tangle, and Lewy body and neurite pathologies [26-30]. For each region, semi-quantitative assessments for each of the three pathologies were conducted, ranging from 0 (none), 1 (sparse), 2 (moderate) to 3 (severe) as previously described [31]. 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. Most patients were longitudinally followed (usually over 8-10 years) and annual rate of decline on MMSE was calculated. Final diagnoses for patients are clinico-pathological consensus diagnoses incorporating the one-year rule to differentiate DLB and PDD [27]. Hoehn & Yahr scores were also taken from the subset of PDD patients.

Immunohistochemistry

Immunolabelling of sections was undertaken as previously reported [32, 33]. Briefly, 7 μm sections from cingulate gyrus, prefrontal and parietal cortices were dewaxed and rehydrated through xylene and descending concentrations of alcohol into water. For GRP78/BiP and phosphorylated tau, antigen retrieval was carried out by microwaving for 10 minutes in citrate buffer pH6.0. For PERK, pPERK, and α-synuclein, antigen retrieval was carried out by autoclaving for 10 minutes in EDTA buffer pH8.0; this was followed by immersion for 15 minutes in 98% formic acid for α-synuclein

(only). For immunohistochemistry, endogenous peroxidases were blocked by 0.3% hydrogen peroxide in PBS (30 minutes). Primary antibodies were added (PERK 1:1500 Abcam ab115617; pPERK 1: 200 Santa Cruz sc32577; BiP 1:1000 Abcam ab21685; phosphorylated tau AT8 1:200 Thermo Scientific MN1020; α-synuclein, NCL-SYN 1:100 Novacastra Laboratories) and tissue sections incubated overnight at 4℃. Subsequent labelling for light microscopy involved appropriate biotinylated secondary antibodies (all at 1:500 Vector Labs, Peterborough, UK), ABC Elite and DAB kits (Vector Labs). Counter staining was with Mayer's haematoxylin (Sigma). When sections were double labelled, the second chromogen was Novared (Vector Labs).

Western Blotting

Western blot was performed as previously described [32, 33]. Briefly, 500mg of frozen tissue was taken from each brain region. Meninges, white matter, blood vessels and clots were dissected from the frozen tissue to leave approximately 200mg of grey matter which was homogenised in ice cold buffer (pH 7.4) containing 50mM tris-HCL, 5mM EGTA, 10mM EDTA, 'complete protease inhibitor cocktail tablets' (Roche), and 2μg/ml pepstatin A dissolved in ethanol:DMSO 2:1 (Sigma). Buffer was used at a ratio of 2ml to every 100mg of tissue and homogenisation performed using an IKA Ultra-Turrax mechanical probe (IKA Werke, Germany) until the liquid appeared homogenous. Protein concentration of each sample was measured by using BCA Protein Assay Kit (Thermo Scientific).

Twenty micrograms of each sample was loaded on 7.5% SDS-polyacrylamide gel for protein separation then transferred to nitrocellulose membrane (Immobilon-P, Millipore). After blocking non-specific binding, the membranes were incubated with anti-GRP78/BiP (rabbit polyclonal, 1:1000, Abcam) primary antibody followed by an anti-rabbit HRP conjugated secondary antibody (GE healthcare, 1:5000). Bands were visualised using chemiluminescent substrate (Millipore) and a LAS-3000 luminescent image reader (Fujifilm). Western blot data were evaluated and quantified using Multi Gauge Image Analyzer (version 3.0). In order to control for any inconsistency in

loading samples, 'house-keeping' proteins were used as internal controls to check whether the differences in the amount of protein of interest could be due to an initial difference in the amount of sample loaded. The most commonly used protein to serve this purpose is β-actin, as actin is a cytoskeletal filament that forms the internal scaffolding of a cell, and therefore its expression is ubiquitous in neuronal and non-neuronal tissue [34]. Each membrane was therefore probed for actin (1:10000 dilution, Sigma-Aldrich) to normalise the level of immunolabelling of the GRP78/BiP protein to actin, so that any potential variations in protein loading could be eliminated.

Statistical analysis

Statistical analyses were performed using SPSS. Shapiro-Wilk and Levene's tests were used to ensure that the parametric assumptions of normality and homogeneity of variance respectively, were not violated. Where assumptions were found to be violated, a natural log (Ln) data transformation was applied and the data were retested for normality and equal variance [35]. One-way ANOVA followed by Scheffe post-hoc test was performed where data could be normalised, otherwise Kruskal-Wallis non-parametric analysis was performed. Spearman correlation analyses were used to determine the association between the level of GRP78/BiP protein and clinical and pathological measures.

Results

There were no significant associations between age and GRP78/BiP protein levels in any of the four regions (r between -0.15 and 0.18, $P > 0.2$ for all regions). However, there was a significant difference in the average age of death between diagnostic groups (One-way ANOVA, $F_{(3,126)} = 6.044$, $P = 0.001$), in which AD patients had significantly higher average age of death compared to control subjects ($P = 0.003$) or patients with DLB ($P = 0.016$) or PDD ($P = 0.002$). There were no significant differences in gender, pH, MMSE, or annual rate of MMSE decline between diagnostic groups (Table 1).

Protein levels of GRP78/BiP are increased in cingulate gyrus and parietal cortex of PDD and DLB patients

In the cingulate gyrus, there was a significant increase in the level of GRP78/BiP in PDD and DLB patients compared to AD and control subjects ($P < 0.001$, Figure 1A, E). A similar pattern was observed in the parietal cortex, in which the GRP78/BiP level in the PDD and DLB patients were significantly higher compared to AD patients ($P = 0.002$ for PDD; $P = 0.001$ for DLB, Figure 1B). Interestingly, there was a significant decrease in the GRP78/BiP level in AD patients compared to control subjects in parietal cortex ($P < 0.001$, Figure 1B). There were no significant changes in the level of GRP78/BiP between different disease groups in either prefrontal (Figure 1C), or temporal cortices (Figure 1D).

Localisation of GRP78/BiP, PERK, p-PERK, and α-synuclein in neurons in the prefrontal cortex

The prefrontal cortex was selected for immunohistochemical analysis as this region is proposed to have a role in executive function and cognition, a deterioration of which is a cardinal symptom of DLB and PDD. The GRP78/BiP localised in the cytoplasm of neurons (Figure 2). Although there was a slight increase in the labeling intensity of GRP78/BiP in AD, PDD, and DLB patient samples compared to normal control, this intensity was similar across the different disease groups (Figure 2). Since there were changes in the GRP78/BiP levels in the cingulate gyrus and parietal cortex region, GRP78/BiP labelling was also done in these areas (Supplementary material, Figure S1). The number of GRP78/BiP-positive cells in AD, PDD, and DLB patients appeared to be slightly greater than control subjects, but again was similar across the different disease groups in both brain regions. In both PDD and DLB patient samples, GRP78/BiP was co-localised with α-synuclein only in some of the neurons (arrows, Figures 3A–D). In AD patient samples, GRP78/BiP-positive neurons were seldom co-localised with phosphorylated Tau (green arrow, Figure 3E), and majority of the GRP78/BiP-positive neurons had no phosphorylated Tau (blue arrow, Figure 3E, F). Furthermore,

phosphorylated Tau-positive neurofibrillary tangles did not show GRP78/BiP immunoreactivity (arrowhead, Figure 3F). Similarly, in DLB patient samples, there were no apparent neurons expressing both the GRP78/BiP and phosphorylated Tau (arrow, Figure 3G, H), and neurofibrillary tangles had no GRP78/BiP expression either (arrowhead, Figure 3G, H). Granular staining was observed for p-PERK in AD, PDD, and DLB patient samples (Figures 4B–D), in which the intensity appeared to be a little higher in the PDD and DLB patients compared to AD patients (Figure 4B). The staining intensity of p-PERK was higher in all cases compared to controls (Figure 4A). PERK and p-PERK staining on sequential sections showed that a greater number of neurons were positive for PERK (arrows, Figure 4E) than p-PERK (arrows, Figure 4F).

Association between GRP78/BiP level and clinical and pathological measures

The average regional pathological scores for each disease group are presented in Table 2, and the association between GRP78/BiP level and pathological measures are shown in Table 3. There was a significant positive correlation between GRP78/BiP level and α-synuclein in the cingulate gyrus (R = 0.629, $P < 0.001$), and inverse correlation with plaques and tangles in the parietal cortex (R = -0.364 and -0.301 respectively, $P < 0.001$ for both) (Table 3). However, there were no significant associations between the GRP78/BiP level and both Braak stages and Newcastle/McKeith criteria for Lewy body disease, MMSE scores before death or annual rate of MMSE decline in all patients in each disease group (data not shown). Hoehn & Yahr scores of a subset of PDD patients (n = 22) also showed no correlation with GRP78/BiP level (data not shown).

Discussion

The present study is the first study to report UPR activation in PDD and DLB. The novel finding of the current study is that the level of GRP78/BiP was greatly increased in the PDD and DLB patients compared to people with AD in cingulate gyrus (Figure 1A) and parietal cortex (Figure 1B), suggesting that the UPR pathway may be activated to a greater extent in cases with cortical Lewy

body pathology or where both Lewy body and plaque pathologies are present. In the cingulate gyrus, GRP78/BiP level in PDD and DLB patients were also significantly higher compared to controls (Figure 1A). However, the level of GRP78/BiP did not change in the prefrontal or temporal cortices of all disease groups (Figures 1C, D), which may imply that the UPR is activated at a different level in different areas of the brain depending on the severity of the pathology in that particular region at the time of death.

In the cellular and animal models of PD, an increase in the proteins that are involved in the UPR pathway such as GRP78/BiP and PERK has been shown, suggesting UPR activation and the involvement of ER stress in the pathophysiology of the disease [12, 36, 37]. Similar results were observed in human post-mortem tissue from PD patients [9, 38]. However, there have been discrepancies in the level of GRP78/BiP in AD patients. In some of the studies, it was reported that the expression level of GRP78/BiP is increased [8, 10, 39], whereas in other studies either a decrease or no changes at all between control subjects and AD patients were reported [40, 41]. These differences may be explained by the use of different antibodies (a generic anti-KDEL antibody versus GRP78/BiP specific antibody) or stages of the disease, or different regions studied. Consistent with aforementioned studies, the present study has also shown varying results in different brain areas, in which the level of GRP78/BiP in AD patients did not change in cingulate gyrus or in prefrontal cortex, but was significantly decreased in the parietal cortex (Figure 1). A decrease in the GRP78/BiP level in AD patients in the parietal cortex may not be due to their significantly higher age compared to other disease groups, but could be the result of pathological differences between AD and Lewy body diseases such as DLB and PDD. It has been shown that prominent atrophy and degenerative changes occur in the parietal cortex region in AD [42-44]. Therefore, it can be hypothesised that the decrease in the GRP78/BiP in parietal cortex in AD patients may be due to the absence of and/or limited number of “healthy” cells present in this region to activate the UPR pathway. This attenuated UPR signalling may in turn exacerbate atrophy and

neurodegeneration by increasing vulnerability to ER stress in which a cell is forced to take the apoptotic route without having any chance to repair itself.

GRP78/BiP immunohistochemical results from the current study were also consistent with previous studies [8, 10], in which GRP78/BiP was localised in the neuronal cytoplasm, and the labeling intensity was slightly higher in the AD, PDD, and DLB patients compared to control subjects (Figure 2). Furthermore, in AD patients, only a very few GRP78/BiP-positive neurons expressed phosphorylated Tau (Figure 3E), and neurofibrillary tangles did not show GRP78/BiP immunoreactivity (Figure 3F). As described previously [10, 39], p-PERK immunoreactivity was observed as granules in the cytoplasm of neurons in the prefrontal cortex (Figure 4B–D). More neurons were positive for PERK than p-PERK (Figure 4E, F), which may suggest that the majority of neurons are primed for UPR activation, but only some of them actually become activated.

Although the present study investigated the regions with known pathological involvement for AD, PDD, and DLB, small sample size and analysis of only four cortical regions may have been limitations to finding greater changes in the GRP78/BiP level. Furthermore, there may be larger group differences in GRP78/BiP level with other quantitative approach looking at protein distribution and expression at cellular level as opposed to analysing proteins in tissue homogenates by western blotting. Finally, the correlation analysis between the age and GRP78/BiP level was carried out to determine whether the age was a confounding factor. This analysis was conducted regardless of the diagnostic groups. Although there was no association between the GRP78/BiP level and age (data not shown), the fact that the age may have influenced the comparison between AD and other groups cannot be totally excluded as AD patients were significantly older in age at the time of death (Table 1). The DLB and PDD patients were well age-matched to the control group.

While the UPR is normally a neuroprotective response, it seems that chronic activation, and the conversion of cytoprotective to cytotoxic signaling may be the contributing factors to disease. Greater increase in GRP78/BiP level in PDD and DLB compared to AD observed in the current study suggests that UPR may be activated at a greater degree because both α-synuclein and Aβ

pathology are present in PDD and DLB. In other words, in patients with PDD or DLB, there may be a greater build-up of misfolded 'toxic' protein in the ER compared to 'pure' AD patients due to co-occurrence of synuclein and Aβ pathologies. Therefore, as a consequence, the UPR may become activated at a higher degree to cope with a greater level of disturbance in proteostasis. On the other hand, it was interesting that only synuclein pathology was positively correlated with GRP78/BiP level in the cingulate gyrus (Table 3), which coincides well with the increase in GRP78/BiP level in the same region only in PDD and DLB patient groups, but not in AD compared to control (Figure 1A). The α-synuclein influence on the level of GRP78/BiP in the cingulate gyrus may be related to the fact that this is the region with highest α-synuclein pathology (Table 2). There may be an effect in other areas also, but this may not be evident in the samples due to less severe pathology. This suggests that the Lewy body pathology in itself may be sufficient to chronically activate the UPR system. A decrease in GRP78/BiP level only in AD patients in the parietal cortex (Figure 1B) and inverse correlation between AD-type pathology (plaques and tangles) and GRP78/BiP level in the same region (Table 3) further indicate that the AD-type pathology alone may not activate the UPR system as much. Therefore, this co-occurrence of synuclein pathology with AD-type pathology may be a trigger to change cytoprotective properties of the UPR system to cytotoxic. There has been ongoing debate as to whether it is α-synuclein pathology or AD-type pathology that drives cognitive decline in DLB, and emerging evidence suggests that there may be a synergism between α-synuclein, amyloid and tau pathologies [45]. The data from the present study are also in line with this, and suggest that activated UPR and subsequent protein misfolding is a common mechanism leading to these changes, and may particularly be important amongst individuals with cortical Lewy bodies or a combination of pathologies.

However, since the current study only measured the changes in the GRP78/BiP level, there are still some unresolved questions before any firm conclusions can be made. For example, does higher GRP78/BiP level suggest chronic UPR activation or does it simply suggest transient higher UPR activation? If the disease-specific toxic proteins are prevented from building up in the ER

lumen, will UPR still be activated? Is UPR activation a response to neurodegeneration or does it contribute to disease initiation? To answer these questions further studies are inevitable. However, the results from the present study serve as a stepping stone for future studies as this study has clearly shown that the UPR is involved in Lewy body dementias for the first time. In addition, now that the UPR is emerging as a promising therapeutic target for neurodegenerative diseases [15, 16, 46], further studies, possibly using cellular or animal models of Lewy body dementia, are required to investigate whether the detrimental effect of neurodegeneration can be saved by manipulating the UPR pathway. Based on previous studies, using compounds to inhibit UPR appears to be a viable therapy [16, 37, 47]. Another important implication to take from the present study is that since UPR is activated at a different level in patients with both α-synuclein and tau pathology (PDD and DLB patients) compared to patients with single pathology (AD patients), it is possible that there may be specific mechanisms acting in people with both pathologies. This may become an important fact to bear in mind when selecting samples for further investigations, evolving diagnostic criteria, and stratifying populations for clinical trials.

On the whole, the results from the current study highlight the role of UPR in Lewy body dementias, and suggest that Lewy body disease and the combination of Lewy body and AD-type plaque pathologies are associated with increased UPR activation to a greater extent than it is in pure AD, possibly as a consequence of the increasing load of ER proteins. This work highlights a novel opportunity to explore the UPR as a therapeutic target in synuclein diseases.

Acknowledgements

The authors thank all the donors and brain banks for the tissue used in this study, in particular Dr. Claire Troakes at the MRC London Neurodegenerative Diseases Brain Bank. Much appreciation goes to Dr. Alan Thomas (National Institute of Health Research Biomedical Research Centre for Mental Health, King's College London) and Dr. John O'Brien (Department of Psychiatry, University of Cambridge) for their collaboration in collecting the pathological and clinical data used

in this study. This research was supported by The David and Astrid Hagelén Foundation. Tissue for this study was provided by several brain banks. The Newcastle Brain Tissue Resource which is funded in part by a grant from the UK Medical Research Council (G0400074), by NIHR Newcastle Biomedical Research Centre and Unit awarded to the Newcastle upon Tyne NHS Foundation Trust and Newcastle University, and by a grant from the Alzheimer's Society and Alzheimer's Research Trust as part of the Brains for Dementia Research Project. The London Neurodegenerative Diseases Brain Bank at King's College London, which receives funding from the UK MRC and Brains for Dementia Research. We would also like to thank the Thomas Willis Brain Collection. C.B. would like to thank the National Institute for Health Research (NIHR) Mental Health Biomedical Research Centre and Dementia Unit at South London and Maudsley NHS Foundation Trust and [Institute of Psychiatry] King's College London. This article also presents independent research supported/funded by the National Institute for Health Research (NIHR). TH received salary support from Grant No. KTIA_13_NAP-A-II/7, Hungary. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Author contributions

J.-H.B., P.F., and D.A. conceived and designed the experiments. J.-H.B., D.W., and D.H. carried out the experiments. All authors were involved in analysing the data. J.-H.B. and D.A. prepared the manuscript. All authors have read and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Oesterhus R, Soennesyn H, Rongve A, Ballard C, Aarsland D, Vossius C. Long-term mortality in a cohort of home-dwelling elderly with mild Alzheimer's disease and Lewy body dementia. Dement Geriatr Cogn Disord. 2014;38(3-4):161-9.

2. Rongve A, Vossius C, Nore S, Testad I, Aarsland D. Time until nursing home admission in people with mild dementia: comparison of dementia with Lewy bodies and Alzheimer's dementia. Int J Geriatr Psychiatry. 2014;29(4):392-8.
3. Hetz C, Mollereau B. Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. Nat Rev Neurosci. 2014;15(4):233-49.
4. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science. 2011;334(6059):1081-6.
5. Jager R, Bertrand MJ, Gorman AM, Vandenabeele P, Samali A. The unfolded protein response at the crossroads of cellular life and death during endoplasmic reticulum stress. Biol Cell. 2012;104(5):259-70.
6. Urra H, Dufey E, Lisbona F, Rojas-Rivera D, Hetz C. When ER stress reaches a dead end. Biochim Biophys Acta. 2013;1833(12):3507-17.
7. Kozutsumi Y, Segal M, Normington K, Gething MJ, Sambrook J. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature. 1988;332(6163):462-4.
8. Hamos JE, Oblas B, Pulaski-Salo D, Welch WJ, Bole DG, Drachman DA. Expression of heat shock proteins in Alzheimer's disease. Neurology. 1991;41(3):345-50.
9. Hoozemans JJ, van Haastert ES, Eikelenboom P, de Vos RA, Rozemuller JM, Scheper W. Activation of the unfolded protein response in Parkinson's disease. Biochem Biophys Res Commun. 2007;354(3):707-11.
10. Hoozemans JJ, Veerhuis R, Van Haastert ES, Rozemuller JM, Baas F, Eikelenboom P, Scheper W. The unfolded protein response is activated in Alzheimer's disease. Acta Neuropathol. 2005;110(2):165-72.
11. Roussel BD, Kruppa AJ, Miranda E, Crowther DC, Lomas DA, Marciniak SJ. Endoplasmic reticulum dysfunction in neurological disease. Lancet Neurol. 2013;12(1):105-18.
12. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci. 2002;22(24):10690-8.
13. Abisambra JF, Jinwal UK, Blair LJ, O'Leary JC, 3rd, Li Q, Brady S, Wang L, Guidi CE, Zhang B, Nordhues BA, Cockman M, Suntharalingham A, Li P, Jin Y, Atkins CA, Dickey CA. Tau accumulation activates the unfolded protein response by impairing endoplasmic reticulum-associated degradation. J Neurosci. 2013;33(22):9498-507.
14. Cornejo VH, Pihan P, Vidal RL, Hetz C. Role of the unfolded protein response in organ physiology: lessons from mouse models. IUBMB Life. 2013;65(12):962-75.
15. Moreno JA, Radford H, Peretti D, Steinert JR, Verity N, Martin MG, Halliday M, Morgan J, Dinsdale D, Ortori CA, Barrett DA, Tsaytler P, Bertolotti A, Willis AE, Bushell M, Mallucci GR. Sustained translational repression by eIF2alpha-P mediates prion neurodegeneration. Nature. 2012;485(7399):507-11.
16. Moreno JA, Halliday M, Molloy C, Radford H, Verity N, Axten JM, Ortori CA, Willis AE, Fischer PM, Barrett DA, Mallucci GR. Oral treatment targeting the unfolded protein response prevents neurodegeneration and clinical disease in prion-infected mice. Sci Transl Med. 2013;5(206):206ra138.
17. Ma T, Trinh MA, Wexler AJ, Bourbon C, Gatti E, Pierre P, Cavener DR, Klann E. Suppression of eIF2alpha kinases alleviates Alzheimer's disease-related plasticity and memory deficits. Nat Neurosci. 2013;16(9):1299-305.
18. Forno LS. Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol. 1996;55(3):259-72.
19. Pollanen MS, Dickson DW, Bergeron C. Pathology and biology of the Lewy body. J Neuropathol Exp Neurol. 1993;52(3):183-91.
20. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997;388(6645):839-40.

21. Ballard C, Ziabreva I, Perry R, Larsen JP, O'Brien J, McKeith I, Perry E, Aarsland D. Differences in neuropathologic characteristics across the Lewy body dementia spectrum. Neurology. 2006;67(11):1931-4.
22. Galpern WR, Lang AE. Interface between tauopathies and synucleinopathies: a tale of two proteins. Ann Neurol. 2006;59(3):449-58.
23. Vekrellis K, Xilouri M, Emmanouilidou E, Rideout HJ, Stefanis L. Pathological roles of alpha-synuclein in neurological disorders. Lancet Neurol. 2011;10(11):1015-25.
24. Olichney JM, Galasko D, Salmon DP, Hofstetter CR, Hansen LA, Katzman R, Thal LJ. Cognitive decline is faster in Lewy body variant than in Alzheimer's disease. Neurology. 1998;51(2):351-7.
25. Aarsland D, Litvan I, Salmon D, Galasko D, Wentzel-Larsen T, Larsen JP. Performance on the dementia rating scale in Parkinson's disease with dementia and dementia with Lewy bodies: comparison with progressive supranuclear palsy and Alzheimer's disease. J Neurol Neurosurg Psychiatry. 2003;74(9):1215-20.
26. Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del Tredici K. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol. 2006;112(4):389-404.
27. McKeith IG, Dickson DW, Lowe J, Emre M, O'Brien JT, Feldman H, Cummings J, Duda JE, Lippa C, Perry EK, Aarsland D, Arai H, Ballard CG, Boeve B, Burn DJ, Costa D, Del Ser T, Dubois B, Galasko D, Gauthier S, Goetz CG, Gomez-Tortosa E, Halliday G, Hansen LA, Hardy J, Iwatsubo T, Kalaria RN, Kaufer D, Kenny RA, Korczyn A, Kosaka K, Lee VM, Lees A, Litvan I, Londos E, Lopez OL, Minoshima S, Mizuno Y, Molina JA, Mukaetova-Ladinska EB, Pasquier F, Perry RH, Schulz JB, Trojanowski JQ, Yamada M, Consortium on DLB. Diagnosis and management of dementia with Lewy bodies: third report of the DLB Consortium. Neurology. 2005;65(12):1863-72.
28. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology. 1991;41(4):479-86.
29. Montine TJ, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Dickson DW, Duyckaerts C, Frosch MP, Masliah E, Mirra SS, Nelson PT, Schneider JA, Thal DR, Trojanowski JQ, Vinters HV, Hyman BT, National Institute on A, Alzheimer's A. National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. Acta Neuropathol. 2012;123(1):1-11.
30. Thal DR, Rub U, Orantes M, Braak H. Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology. 2002;58(12):1791-800.
31. Howlett DR, Whitfield D, Johnson M, Attems J, O'Brien JT, Aarsland D, Lai MK, Lee JH, Chen C, Ballard C, Hortobagyi T, Francis PT. Regional Multiple Pathology Scores Are Associated with Cognitive Decline in Lewy Body Dementias. Brain Pathol. 2014.
32. Whitfield DR, Vallortigara J, Alghamdi A, Howlett D, Hortobagyi T, Johnson M, Attems J, Newhouse S, Ballard C, Thomas AJ, O'Brien JT, Aarsland D, Francis PT. Assessment of ZnT3 and PSD95 protein levels in Lewy body dementias and Alzheimer's disease: association with cognitive impairment. Neurobiol Aging. 2014;35(12):2836-44.
33. Whitfield DR, Vallortigara J, Alghamdi A, Hortobagyi T, Ballard C, Thomas AJ, O'Brien JT, Aarsland D, Francis PT. Depression and Synaptic Zinc Regulation in Alzheimer Disease, Dementia with Lewy Bodies, and Parkinson Disease Dementia. Am J Geriatr Psychiatry. 2014.
34. Lecuit T, Lenne PF. Cell surface mechanics and the control of cell shape, tissue patterns and morphogenesis. Nat Rev Mol Cell Biol. 2007;8(8):633-44.
35. Rice J. Mathematical statistics and data analysis. Belmont, California: Duxbury Press; 2007.

36. Bellucci A, Navarria L, Zaltieri M, Falarti E, Bodei S, Sigala S, Battistin L, Spillantini M, Missale C, Spano P. Induction of the unfolded protein response by alpha-synuclein in experimental models of Parkinson's disease. J Neurochem. 2011;116(4):588-605.
37. Colla E, Coune P, Liu Y, Pletnikova O, Troncoso JC, Iwatsubo T, Schneider BL, Lee MK. Endoplasmic reticulum stress is important for the manifestations of alpha-synucleinopathy in vivo. J Neurosci. 2012;32(10):3306-20.
38. Selvaraj S, Sun Y, Watt JA, Wang S, Lei S, Birnbaumer L, Singh BB. Neurotoxin-induced ER stress in mouse dopaminergic neurons involves downregulation of TRPC1 and inhibition of AKT/mTOR signaling. J Clin Invest. 2012;122(4):1354-67.
39. Hoozemans JJ, van Haastert ES, Nijholt DA, Rozemuller AJ, Eikelenboom P, Scheper W. The unfolded protein response is activated in pretangle neurons in Alzheimer's disease hippocampus. Am J Pathol. 2009;174(4):1241-51.
40. Katayama T, Imaizumi K, Sato N, Miyoshi K, Kudo T, Hitomi J, Morihara T, Yoneda T, Gomi F, Mori Y, Nakano Y, Takeda J, Tsuda T, Itoyama Y, Murayama O, Takashima A, St George-Hyslop P, Takeda M, Tohyama M. Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. Nat Cell Biol. 1999;1(8):479-85.
41. Sato N, Urano F, Yoon Leem J, Kim SH, Li M, Donoviel D, Bernstein A, Lee AS, Ron D, Veselits ML, Sisodia SS, Thinakaran G. Upregulation of BiP and CHOP by the unfolded-protein response is independent of presenilin expression. Nat Cell Biol. 2000;2(12):863-70.
42. Guadagna S, Esiri MM, Williams RJ, Francis PT. Tau phosphorylation in human brain: relationship to behavioral disturbance in dementia. Neurobiol Aging. 2012;33(12):2798-806.
43. Jacobs HI, Van Boxtel MP, Uylings HB, Gronenschild EH, Verhey FR, Jolles J. Atrophy of the parietal lobe in preclinical dementia. Brain Cogn. 2011;75(2):154-63.
44. Kirvell SL, Esiri M, Francis PT. Down-regulation of vesicular glutamate transporters precedes cell loss and pathology in Alzheimer's disease. J Neurochem. 2006;98(3):939-50.
45. Compta Y, Parkkinen L, Kempster P, Selikhova M, Lashley T, Holton JL, Lees AJ, Revesz T. The significance of alpha-synuclein, amyloid-beta and tau pathologies in Parkinson's disease progression and related dementia. Neurodegener Dis. 2014;13(2-3):154-6.
46. Halliday M, Mallucci GR. Targeting the unfolded protein response in neurodegeneration: A new approach to therapy. Neuropharmacology. 2014;76 Pt A:169-74.
47. Saxena S, Cabuy E, Caroni P. A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice. Nat Neurosci. 2009;12(5):627-36.

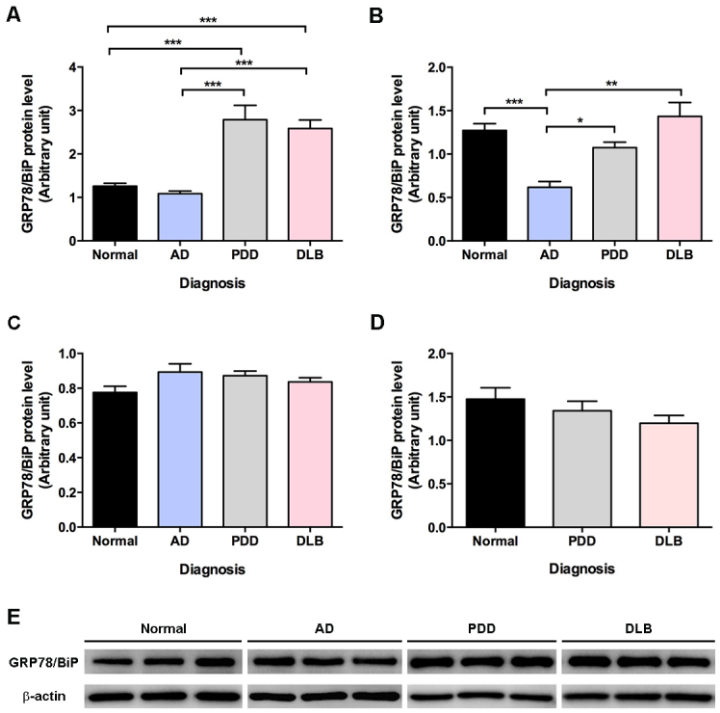
Figure legends

Figure 1. GRP78/BiP protein expression levels in various regions of the brain from healthy control, AD, PDD, and DLB subjects. (A) In cingulate gyrus (BA 24) region, there was a significant increase in the level of GRP78/BiP protein in PDD and DLB patients compared to AD and control subjects. (B) In parietal cortex (BA 40) region, there was a significant decrease in the level of GRP78/BiP protein in AD patients compared to control subjects, while its level was significantly higher in the PDD and DLB patients compared to AD patients. (C, D) There were no significant changes in the GRP78/BiP protein level in prefrontal cortex (BA 9) (C) or temporal cortex (BA 21) (D) regions. (E) A representative western blot image showing GRP78/BiP protein expression in cingulate gyrus region in control, AD, PDD, and DLB subjects. * $P = 0.002$, ** $P = 0.001$, *** $P < 0.001$.

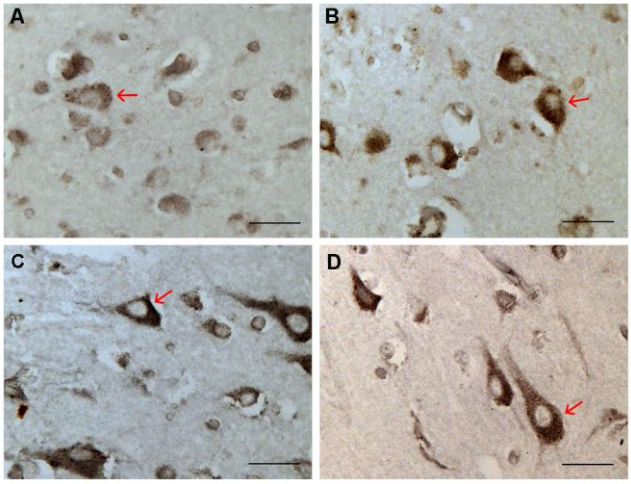
Figure 2. Immunohistochemical detection of GRP78/BiP in prefrontal cortex region (BA 9) of control (A), AD (B), PDD (C), and DLB (D) subjects. GRP78/BiP protein expression was found in the cytoplasm of neurons (arrows). Although there was a slight increase in the labelling intensity in AD, PDD, and DLB patient samples compared to healthy control, this intensity was more or less similar amongst different disease groups. Scale bars represent 25 μm.

Figure 3. Double immunohistochemical labeling of GRP78/BiP with either α-synuclein or phosphorylated Tau in prefrontal cortex region (BA 9) of various neurodegenerative diseases. (A–D) In both PDD and DLB patient samples, only a few neurons expressed both GRP78/BiP and a-synuclein (arrows). Majority of neurons only expressed GRP78/BiP in the cytoplasm (arrowheads). (E–H) In AD (E, F) and DLB (G, H) patient samples, GRP78/BiP was rarely co-localised with phosphorylated Tau (green arrow), while most of neurons only expressed GRP78/BiP in the cytoplasm (blue arrow). Neurofibrillary tangles did not show any immunoreactivity against GRP78/BiP (arrowheads).

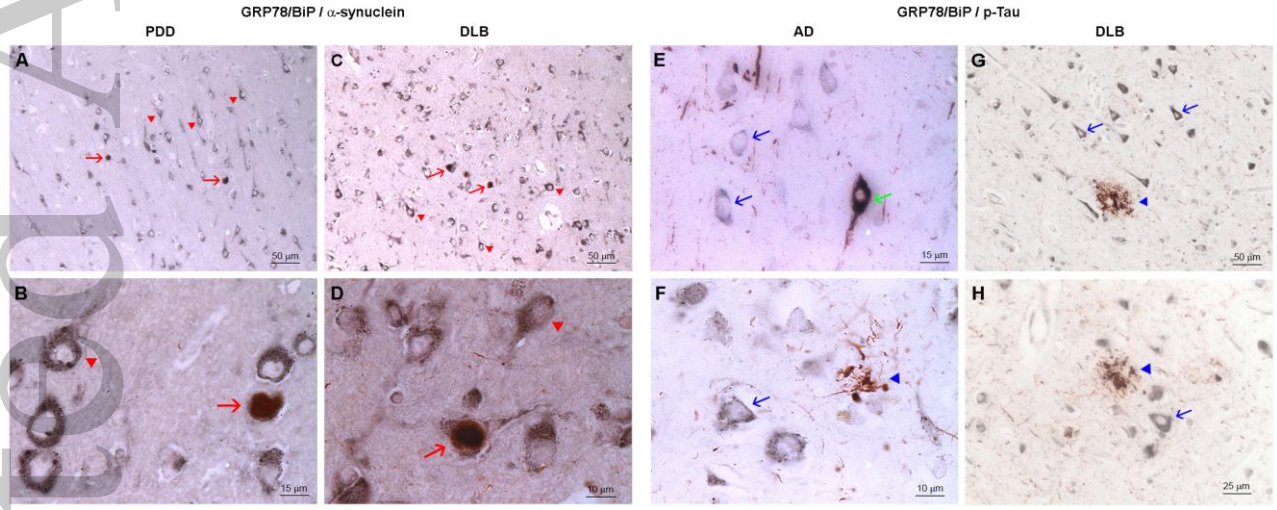
Figure 4. Immunohistochemical expression of p-PERK in prefrontal cortex (BA 9) region of control (A), AD (B), DLB (C), and PDD (D) subjects. (A–D) Granular staining pattern was observed in the cytoplasm of a neuron in all cases. The labeling intensity appeared to be greater in the AD, PDD, and DLB patient samples compared to control. (E, F) As can be seen from the adjacent sections from DLB patient samples, a greater number of neurons express PERK (E) than p-PERK (F).



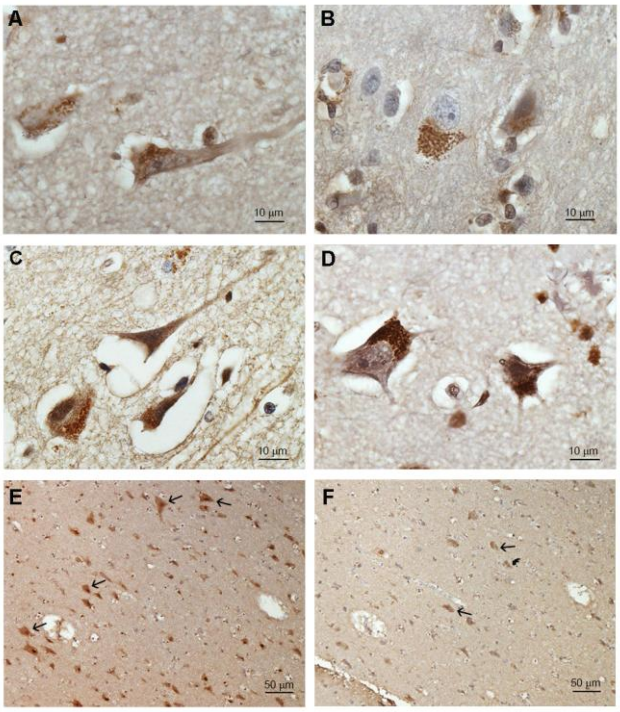
NAN_12260_F1



NAN_12260_F2



NAN_12260_F3



NAN_12260_F4

Tables

Table 1. Demographic characteristics of subjects used in this study in each diagnostic group.

Diagnosis	Gender (M/F) (%)	Age at death	pH	MMSE before death	MMSE decline per year
Control (n = 25)	60/40	79.8 ± 1.5	6.47 ± 0.06	N/A	N/A
AD (n = 16)	31/69	88.0 ± 2.0	6.30 ± 0.08	10.5 (0-19)	3.8 ± 1.0
PDD (n = 34)	53/47	79.9 ± 1.0	6.44 ± 0.06	13 (0-27)	2.0 ± 0.3
DLB (n = 55)	58/42	81.7 ± 0.9	6.37 ± 0.06	14 (0-30)	3.0 ± 0.4

"MMSE before death" is the score at the last interview before death. "MMSE decline" is the decline per year averaged over the period of clinical observation and was usually 8–10 years. No MMSE data were available for control group. MMSE is presented with a median score with range in brackets. Other measures are presented as mean ± SEM.

Table 2. Average regional pathological scores for each disease group

Brain region	Diagnosis	N	α-Synuclein	Plaques	Tangles
Prefrontal cortex (BA 9)	Control	14–16	--	0.35 ± 0.14	0.17 ± 0.08
	AD	11	0.13 ± 0.09	2.81 ± 0.14	2.56 ± 0.13
	PDD	26–28	0.79 ± 0.14	1.39 ± 0.20	0.53 ± 0.10
	DLB	41–44	1.62 ± 0.15	1.68 ± 0.15	0.93 ± 0.11
Temporal cortex (BA21)	Control	16–25	--	0.57 ± 0.18	0.30 ± 0.15
	PDD	30-33	0.94 ± 0.15	0.74 ± 0.10	0.44 ± 0.11
	DLB	34–35	1.75 ± 0.15	1.73 ± 0.14	1.31 ± 0.13
Cingulate gyrus (BA 24)	Control	20	--	0.29 ± 0.17	0.10 ± 0.07
	AD	15–16	0.31 ± 0.18	1.47 ± 0.27	1.44 ± 0.32
	PDD	10	1.85 ± 0.18	0.97 ± 0.17	0.53 ± 0.11
	DLB	36–40	2.28 ± 0.13	1.28 ± 0.15	1.23 ± 0.14
Parietal cortex (BA 40)	Control	18–23	--	0.39 ± 0.16	0.04 ± 0.04
	AD	15	0.13 ± 0.09	2.63 ± 0.18	2.81 ± 0.10
	PDD	29–33	0.59 ± 0.13	1.23 ± 0.19	0.48 ± 0.10
	DLB	46–47	1.39 ± 0.13	1.47 ± 0.15	0.98 ± 0.12

Pathology scores ranged from 0 (none), 1 (sparse), 2 (moderate) to 3 (severe). Pathology scores for α-Synuclein in the control group were not available for all brain regions. Values are presented as mean ± SEM.

Table 3. Correlations between GRP78/BiP level and pathology scores in different brain regions.

Brain region	N	α-Synuclein	Plaques	Tangles
Prefrontal cortex (BA 9)	92–95	-0.009	0.094	-0.017
Temporal cortex (BA21)	80–91	-0.091	0.031	-0.107
Cingulate gyrus (BA 24)	81–85	0.629	0.163	0.154
Parietal cortex (BA 40)	108–115	0.010	-0.364	-0.301

Values are *R*s and those in red indicate statistically significant correlations ($P < 0.001$) between that variable and GRP78/BiP level.