The Unfolded Protein Response Is Activated in Pretangle Neurons in Alzheimer's Disease Hippocampus

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Accumulation of misfolded proteins in the endoplasmic reticulum triggers a cellular stress response called the unfolded protein response (UPR) that protects the cell against the toxic buildup of misfolded proteins. Previously, we reported that UPR activation is increased in Alzheimer's disease (AD) patients. How the UPR relates to the pathological hallmarks of AD is still elusive. In the present study, the involvement of UPR activation in neurofibrillary degeneration in AD was investigated. Immunoreactivity for the phosphorylated UPR activation markers pancreatic ER kinase (pPERK), eukaryotic initiation factor 2 α , and inositol-requiring enzyme 1 α was observed in hippocampal neurons associated with granulovacuolar degeneration. The percentage of pPERK-immunoreactive neurons was increased in AD cases compared with nondemented control cases and with the Braak stage for neurofibrillary changes. Although absent from neurofibrillary tangles, pPERK immunoreactivity was most abundant in neurons with diffuse localization of phosphorylated tau protein. Additional analyses showed that pPERK immunoreactivity was associated with ubiquitin and the ubiquitin binding protein p62. A strong co-occurrence of immunoreactivity for both pPERK and glycogen synthase kinase 3 β in neurons was also observed. Together, these data indicate that UPR activation in AD neurons occurs at an early stage of neurofibrillary degeneration and suggest that the prolonged activation of the UPR is involved in both tau phosphorylation and neurodegeneration in AD pathogenesis. (*Am J Pathol* 2009, 174:1241–1251; DOI: 10.2353/ajpath.2009.080814)

Alzheimer's disease (AD), the most common form of dementia, is a chronic neurodegenerative disease causing progressive impairment of memory and other cognitive functions. Apart from the loss of synapses and neurons, AD is characterized at the neuropathological level by neurofibrillary tangles (NFTs), and extracellular aggregates of amyloid β (A β) protein. The major component of the intracellular NFTs is hyperphosphorylated tau, a microtubule stabilizing protein. Both A β and hyperphosphorylated tau have been shown to interfere with several cellular processes, such as Ca²⁺ homeostasis and protein trafficking. These disturbances can result in stress in the endoplasmic reticulum (ER) because of accumulation of un- and misfolded proteins in the ER. ER stress triggers a cellular stress response called the unfolded protein response (UPR) intended to protect the cell against the toxic build-up of misfolded proteins.^{1,2}

Under physiological conditions the ER chaperone BiP (or GRP78) is bound to three key proteins in the ER membrane: pancreatic ER kinase (PERK),³ inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF-6). When unfolded proteins accumulate in the ER lumen, BiP is attracted to bind to these unfolded proteins to support correct protein folding and is thereby released from PERK, IRE-1, and ATF-6, which are consequently activated. UPR activation results in an overall decrease in translation, increased protein degradation, and increased levels of ER chaperones, including BiP.⁴ Once sufficient BiP protein is available for binding and inactivation of PERK, IRE1, and ATF6, the UPR will be shut down and ER functioning restored. Although the initial activation of the UPR protects the cell against the toxic build-up of misfolded proteins, prolonged activation of the UPR induces cell death and may therefore be involved in the pathogenesis of protein folding diseases, such as AD.

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Previously, we have shown activation of the UPR in AD brain.⁵ Western blot analysis showed that protein levels of BiP are increased in the temporal cortex and the hippocampus of AD cases compared with nondemented control cases. In addition, immunohistochemical detection of BiP and phosphorylated PERK (pPERK) shows activation of the UPR in neurons of AD patients. The expression levels of BiP protein in the different Braak stages suggest that the UPR is activated in an early stage of AD pathology. Recently we showed that oligomeric aggregates of A β _{1–42} peptide induce mild ER stress in neuronal cells and that oligomeric A β is more toxic to cells when they are primed for UPR activation.⁶ In addition, our data show that increased intracellular A β production, in particular A β _{1–42}, enhances UPR induction and ER stress toxicity.⁷ These *in vitro* data indicate a role for early A β forms in the induction of the UPR. How the activation of the UPR in AD pathology relates to the occurrence of tau pathology in AD is unknown yet. Interestingly, *in vitro* studies indicate a direct connection between ER stress and phosphorylation of tau, where activation of the UPR induces the activity of glycogen synthase kinase 3 β (GSK-3 β).^{8,9} GSK-3 β is the major kinase for tau and considered to be involved in the hyperphosphorylation of tau present in NFTs.¹⁰

In the current study we investigated the role of UPR activation in tau pathology in AD. UPR activation markers pPERK, pIRE1 α , and phosphorylated eukaryotic initiation factor 2 α (pEIF2 α) were investigated in AD and control hippocampus. The hippocampus has a relative early involvement of tau pathology in AD and neurons with different intermediate stages of tau pathology are present in a single hippocampal section.¹¹ Also the UPR markers are prominently present in the separate subregions of the hippocampus⁵ allowing correlative analysis with tau pathology. pPERK expression was evaluated in different Braak stages to investigate the relation with NFTs. The association of pPERK with tau pathology, ubiquitin, and the ubiquitin-binding protein p62 was analyzed using double immunohistochemistry. In addition, the co-localization between pPERK and GSK-3 β was investigated. Our data are in accordance with a model in which activation of the UPR enhances tau phosphorylation and aggregation and precedes tangle formation in the hippocampus of AD patients.

Materials and Methods

Postmortem Brain Tissue

Human brain specimens of probable AD, other dementias, and age-matched nondemented control cases were obtained at autopsy with a short postmortem interval (The Netherlands Brain Bank, Amsterdam, The Netherlands). Clinical diagnosis was defined according to DSM-III-R criteria and the severity of dementia was evaluated according to the Global Deterioration Scale of Reisberg.¹² Neuropathological evaluation was performed on formalin-fixed, paraffin-embedded tissue from different sites, including the frontal cortex (F2), temporal pole cortex, pa-

Table 1. Cases Used for Immunohistochemical Analysis in This Study

Case no.	Pathological diagnosis	Sex	Age	Braak stage
1	CON	M	80	0
2	CON	M	80	0
3	CON	F	73	0
4	CON	M	57	1
5	CON	F	77	1
6	CON	M	73	1
7	CON	F	76	1
8	CON	M	90	2
9	CON	F	81	2
10	CON	M	87	2
11	CON	M	85	2
12	CON	M	74	3
13	CON	M	83	4
14	CON	F	94	4
15	CON	F	97	4
16	AD	M	86	3
17	AD	M	86	4
18	AD	M	64	4
19	AD	F	75	5
20	AD	M	87	5
21	AD	F	78	5
22	AD	M	64	5
23	AD	F	62	5
24	AD	F	69	6
25	AD	M	57	6
26	AD	F	72	6
27	AD/LBV	F	71	3
28	AD/LBV	F	95	4
29	AD/LBV	M	75	4
30	AD/LBV	F	70	6
31	AD/LBV	M	65	6
32	DEM/VASC	F	89	3
33	DEM/VASC	M	80	3
34	DEM/VASC	F	90	3

CON, control case; AD, Alzheimer's disease case; AD/LBV, Alzheimer's disease case with Lewy body variant pathology; DEM/VASC, dementia caused by vascular lesions in the brain; F, female; M, male.

rietal cortex (superior and inferior lobule), occipital pole cortex, and the hippocampus (essentially CA1 and entorhinal area of the parahippocampal gyrus). The distribution and the density of NFTs was determined using Bodian staining and immunohistochemistry for hyperphosphorylated tau. Senile plaques were stained with the methenamine silver method.¹³ Staging of AD was evaluated according to Braak and colleagues.^{11,14} Age, sex, clinical diagnosis, and Braak score for NFTs of all cases used in this study are listed in Table 1. Mean postmortem delay of all cases was 6 hours with a range from 4 to 16 hours.

Immunohistochemistry

Sections (5 μ m thick) were mounted on Superfrost plus tissue slides (Menzel-Gläser, Braunschweig, Germany) and dried overnight at 37°C. For all stainings sections were deparaffinized and subsequently immersed in 0.3% H₂O₂ in methanol for 30 minutes to quench endogenous peroxidase activity. Normal sera and antibodies were dissolved in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (Boehringer Mannheim, Mannheim, Germany). Primary antibodies and their

Table 2. Name, Epitope, Source, and References of Primary Antibodies Used in This Study

Name, type	Epitope (immunogen)	Source	References
pPERK	PERK phosphorylated at threonine 981	Santa Cruz Biotechnology, Santa Cruz, CA	15–17
pIRE1 α	IRE1 α phosphorylated at serine 724	Novus Biologicals, Littleton, CO	16
pelf2 α	eIF-2 α phosphorylated at serine 52	Sigma, St. Louis, MO	17, 18
AT8	Tau phosphorylated at serine 202	Innogenetics, Ghent, Belgium	19
AT100	Tau phosphorylated at serine 212 and threonine 214	Pierce, Rockford, IL	19
AT270	Tau phosphorylated at threonine 181	Pierce	19
Ubiquitin	Ubiquitin	Chemicon, Temecula, CA	20
p62	p62 protein	Progen Biotechnik, Heidelberg, Germany	21, 22
GSK-3 β	GSK-3 β	BD Biosciences, San Jose, CA	23
GSK-3 β pSer9	GSK-3 β phosphorylated at serine 9	Cell Signaling, Danvers, MA	23

sources are listed in Table 2. Negative controls for all single and double immunostainings were generated by omission of primary antibodies. For description of antibody-specificity we refer to previous reports (Table 2).^{15–23} Except for the detection of pPERK, sections were treated in 10 mmol/L pH 6.0 sodium citrate buffer heated by autoclave for 10 minutes for antigen retrieval. For detection of pPERK and pelf2 α sections were pre-incubated for 10 minutes with normal swine-serum (1:20 dilution; DAKO, Glostrup, Denmark), and for the detection of AT8 sections were pre-incubated for 10 minutes with normal rabbit-serum (1:50 dilution, DAKO). Rabbit anti-pPERK (1:800 dilution), rabbit anti-pelf2 α (1:500 dilution), or mouse anti-AT8 (1:200 dilution) was incubated overnight at 4°C. After washing with PBS, sections were incubated for 30 minutes with biotin-conjugated swine anti-rabbit F(ab')₂ (1:300 dilution, DAKO) for the detection of primary rabbit antibodies or biotin-conjugated rabbit anti-mouse F(ab')₂ (1:500 dilution, DAKO) for the detection of primary mouse antibodies. Subsequently, sections were incubated with streptavidin-biotin horseradish peroxidase complex (streptABComplex/HRP, 1:200 dilution; DAKO) for 60 minutes. Color was developed using 3,3'-diaminobenzidine (0.1 mg/ml, 0.02% H₂O₂, 5 minutes; Sigma, St. Louis, MO) as chromogen. Sections were counterstained with hematoxylin and mounted using Depex (BDH Laboratories Supplies, Poole, UK).

For the detection of pIRE1 α , GSK-3 β , and GSK-3 β pSer9, sections were incubated overnight at 4°C with rabbit anti-pIRE1 α (1:51,200 dilution), mouse anti-GSK-3 β (1:1000 dilution), and rabbit anti-GSK-3 β pSer9 (1:50 dilution), respectively. After washing with PBS sections were incubated with EnVision solution (goat anti-mouse/rabbit HRP, undiluted; DAKO). Color was developed using 3,3'-diaminobenzidine (DAB) (EnVision Detection system/HRP, 1:50 dilution, 10 minutes; DAKO) as chromogen. Sections were counterstained with hematoxylin and mounted using Depex.

Evaluation of Immunohistochemical Stainings and Statistics

A quantitative analysis of the pPERK-, AT8-, GSK-3 β -, and GSK-3 β pSer9-immunoreactive neurons was performed in the different regions of the hippocampus. The total number of neurons, assessed by morphology and

size of nucleus, were counted, as well as neurons containing pPERK, AT8, GSK-3 β , and GSK-3 β pSer9 immunoreactivity. Data are expressed as mean $\pm$ SEM. SPSS 14.0 for Windows (SPSS Inc., Chicago, IL) was used for statistical analysis of the data. One-way analysis of variance followed by Bonferroni's test for multiple comparisons was used to test for differences between groups. A *P* value of <0.05 was taken as significant. Correlation analysis was done using the Pearson parametric test.

Double Immunohistochemistry: pPERK with AT8, AT100, and AT270

To determine co-localization of pPERK with AT8 and AT100 sections were pre-incubated with serum-free protein blocking (SFPB, DAKO) for 10 minutes and subsequently incubated with rabbit anti-pPERK (1:800 dilution) for 60 minutes. After washing with PBS sections were incubated with EnVision solution (goat anti-rabbit HRP, undiluted; DAKO) for 30 minutes. Color was developed using DAB as chromogen. Sections were treated in 10 mmol/L pH 6.0 sodium citrate buffer heated by autoclave for 10 minutes. After pre-incubation with SFPB for 10 minutes, sections were incubated overnight at 4°C with AT8 (1:200 dilution), AT100 (1:1600 dilution), or AT270 (1:1600 dilution). Sections were washed with PBS and incubated with biotin-conjugated rabbit anti-mouse F(ab')₂ (1:500 dilution, DAKO) for 30 minutes and subsequently with alkaline-conjugated streptavidin (1:100 dilution, DAKO) for 60 minutes. Color was developed using Liquid Permanent Red (LPR, DAKO) as chromogen. Sections were counterstained with hematoxylin and mounted using Aquamount (BDH Laboratories Supplies).

Double Immunohistochemistry: pPERK with Ubiquitin and p62

To determine co-localization of pPERK with ubiquitin and p62 sections were pre-incubated with SFPB for 10 minutes and subsequently incubated with rabbit anti-pPERK (1:400 dilution) for 60 minutes. After washing with PBS sections were incubated with biotin-conjugated swine anti-rabbit F(ab')₂ (1:300 dilution, DAKO) for 30 minutes

and subsequently with streptavidin-biotin horseradish peroxidase complex (streptABComplex/HRP, 1:200 dilution; DAKO) for 60 minutes. Color was developed using DAB as chromogen. Subsequently, sections were treated in 10 mmol/L pH 6.0 sodium citrate buffer heated by autoclave for 10 minutes for antigen retrieval and in addition to remove all bound antibodies. After pre-incubation with SFPB for 10 minutes, sections were incubated overnight at 4°C with mouse anti-ubiquitin (1:1600 dilution) or mouse anti-p62 (1:1000 dilution). Sections were washed with PBS and incubated with biotin-conjugated rabbit anti-mouse F(ab')₂ (1:500 dilution, DAKO) for 30 minutes and subsequently with alkaline-conjugated streptavidin (1:100 dilution, DAKO) for 60 minutes. Color was developed using LPR as chromogen. Sections were counterstained with hematoxylin and mounted using Aquamount.

Double Immunohistochemistry: pPERK with GSK-3 β and pGSK-3 β

To determine co-localization of pPERK with GSK-3 β and pGSK-3 β sections were pre-incubated with SFPB for 10 minutes and subsequently incubated with rabbit anti-pPERK (1:800 dilution) for 60 minutes. After washing with PBS sections were incubated with biotin-conjugated swine anti-rabbit F(ab')₂ (1:300 dilution, DAKO) for 30 minutes and subsequently with alkaline-conjugated streptavidin (1:100 dilution, DAKO) for 60 minutes. Color was developed using LPR as chromogen. Sections were treated in 10 mmol/L pH 6.0 sodium citrate buffer heated by autoclave for 10 minutes. After pre-incubation with SFPB for 10 minutes, sections were incubated overnight at 4°C with mouse anti-GSK-3 β (1:50 dilution) or rabbit anti-GSK-3 β pSer9 (1:10 dilution). Sections were washed with PBS and incubated with EnVision solution (goat anti-mouse HRP (or goat anti-rabbit HRP, undiluted; DAKO) for 30 minutes. Color was developed using DAB as chromogen. Sections were counterstained with hematoxylin and mounted using Aquamount.

Analysis of Double-Immunohistochemical Stainings

In this study we used the Nuance spectral imaging system (CRi, Woburn, MA) for the analysis of double-stained specimens. Spectral imaging unmixes colors based on their spectral characteristics, enabling visualization of the different colored reaction products.²⁴ Spectral imaging data cubes were taken from 460 to 660 nm at 10-nm intervals and analyzed with the Nuance software. Spectral libraries of single-brown (DAB), single-red (LPR), and hematoxylin were obtained from control slides. The resulting library was applied to the double-stained slides and the different reaction products were then spectrally unmixed into individual black and white images, representing the localization of each of the reaction products, and reverted to fluorescence-like images composed of pseudo-colors using the Nuance software.

Results

UPR Markers pPERK, pelf2 α , and pIRE1 α Are Associated with GVD in AD Hippocampus

The immunohistochemical localization of pPERK, pelf2 α , and pIRE1 α was investigated in the hippocampus of AD and nondemented controls. As described previously,⁵ pPERK immunoreactivity was observed as granules in pyramidal neurons (Figure 1A). In this study a similar granular staining pattern was detected for pelf2 α and pIRE1 α (Figure 1, B and C). pPERK-, pelf2 α -, and pIRE1 α -positive granules were surrounded by a clear

Figure 1. Immunohistochemical localization of pPERK, pelf2 α , and pIRE1 α in AD hippocampus. **A:** pPERK is detected by immunohistochemistry in pyramidal neurons in the hippocampus. In this case a part of the subiculum of an AD case is shown. The **inset** shows the cellular localization of pPERK, which is present in granules that can be defined as GVD. **B:** pelf2 α immunohistochemistry on the same AD case and area as shown in **A**. pelf2 α can also be detected in GVD granules (**inset**). **C:** pIRE1 α can also be detected in pyramidal neurons in the hippocampus of AD cases (shown is subiculum). Similar granular structures were detected as observed with pPERK and pelf2 α immunohistochemistry (**inset**). Scale bars: 200 μ m (**A–C**); 20 μ m (**insets**).

Figure 2. Quantitative analyses of pPERK immunoreactivity. **A:** Neurons immunoreactive for pPERK were quantified in different regions of the hippocampus. CA1-CA4 denote the Cornu Ammonis areas; DG, dentate gyrus; Sub, subiculum. **Arrows** indicate the borders between different hippocampal regions. **B:** The percentages of neurons immunoreactive for pPERK were quantified in nondemented control cases, AD cases, AD cases with Lewy body variant (AD/LBV), and dementia cases with a vascular insufficiency (DEM/VASC). Data are expressed as mean $\pm$ SEM. * $P < 0.05$ compared with control levels in same region.

halo and were morphologically similar to the classic granules of granulovacuolar degeneration (GVD). GVD is characterized by basophilic granules surrounded by a clear zone measuring 1 to 5 μ m in diameter, occurring predominantly in hippocampal neurons in AD.²⁵ In addition to granular structures that can be defined as GVD also smaller granules (<1 μ m in diameter) positive for pPERK, pelf2 α , and pIRE1 α could be observed.

UPR Activation Is Associated with AD Pathology in the Hippocampus

In AD cases, a marked increase in the number of neurons immunoreactive for pPERK, pelf2 α , and pIRE1 α was noted. To investigate this in more depth, the percentages of neurons immunoreactive for pPERK throughout the different regions of the hippocampus were quantified in nondemented control cases and AD cases as well as AD cases with mixed Lewy body pathology and cases with vascular dementia (Table 1). In the CA4, CA1, and subiculum (SUB) a significant increase in pPERK-positive neurons was observed in AD cases compared with nondemented control cases (Figure 2). Several clinically nondemented cases have been staged with Braak stage 4 for neurofibrillary changes. Analyses of the data with these cases as a separate group showed intermediate numbers of pPERK-positive neurons in relation to control and AD cases (data not shown).

Previously, we reported that UPR activation is increased in Parkinson's disease with α -synuclein pathology in the substantia nigra.¹⁷ No significant increase in pPERK immunoreactivity was observed in the hippocampus of AD cases with Lewy body pathology (AD/LBV) as compared with nondemented control cases. In these cases no Lewy bodies or α -synuclein immunoreactive neurons were observed in the hippocampal areas investigated in this study (results not shown). Although a possible contribution of α -synuclein in UPR activation in the mixed AD/LBV cases examined in this study cannot be excluded, our data indicate that UPR activation is associated with AD pathology. In addition, in cases with vascular dementia no significant difference was detected in pPERK immunoreactivity in the hippocampus compared with nondemented control cases (Figure 2).

pPERK Correlates with Early Ubiquitin Pathology in AD Hippocampus

Because GVD inclusions have been reported to be immunoreactive for ubiquitin,²⁵ co-localization of pPERK with ubiquitin and p62 was investigated in AD hippocampus. Both proteins are part of the ubiquitin proteasome system (UPS) and are involved in the accumulation of misfolded proteins.²⁶ Ubiquitin co-localizes with NFTs, neuritic plaques, and neuropil threads in AD brain.²⁷ In this study, ubiquitin immunoreactivity was detected in extra- and intracellular inclusions with a diameter between 1 and 5 μ m or smaller, however these could not be defined as GVD granules because of the absence of a clear zone surrounding the immunoreactive inclusion. Therefore, although most neurons with pPERK-immunoreactive granules contain ubiquitin-positive inclusions, these were different from GVD granules (Figure 3A). No co-localization was observed between pPERK and ubiquitin-immunoreactive NFTs (Figure 3B). P62 is a ubiquitin-binding protein, involved in the accumulation of ubiquitin-conjugated proteins into cytoplasmic inclusions.^{21,22} As previously reported,²² p62 immunoreactivity was present in NFTs and weakly observed in neuropil threads and almost absent in neuritic plaques (Figure 3C). No pPERK immunoreactivity was observed in neurons containing p62-positive NFTs (Figure 3D). Most of pPERK-positive neurons were free of p62 immunoreactivity, although a small part of the pPERK-positive neurons (10 to 20%) contained some p62 immunoreactivity, which appeared diffuse in the cytoplasm.

pPERK Is Associated with Phosphorylated Tau

To further investigate the association of pPERK with tau pathology, the percentage of pPERK-positive neurons in the different subregions of the hippocampus in controls and AD cases was determined. The mean levels were determined for the different Braak stages for neurofibrillary changes (Figure 4A). A significant increase compared with Braak stage 0 was observed in Braak stages 3 to 4 and 5 to 6 in the CA1 and subiculum. The average levels of pPERK-immunoreactive neurons show a similar distribution along the Braak stage as neurons immunoreactive for AT8, which specifically detects tau phosphor-

Figure 3. Co-localization of pPERK with markers of the ubiquitin proteasome system. **A:** Double immunohistochemistry for pPERK (red) and ubiquitin (brown). **B:** Magnification of **inset** indicated in **A**. No co-localization is present between pPERK with ubiquitin inclusions. There is an increased association of pPERK-positive neurons with intra- and extracellular ubiquitin immunoreactive granules. **C:** Double immunohistochemistry for pPERK (red) and p62 (brown). There is no co-localization between pPERK- and p62-immunoreactive inclusions. **D:** Magnification of **inset** as indicated in **C**. Scale bars: 100 μ m (**A**, **C**); 50 μ m (**B**, **D**).

ylated at serine 202 (Figure 4B). Analysis of the correlation between the number of pPERK- and AT8-positive neurons shows that these markers significantly correlate in the hippocampal areas investigated (Table 3). The subiculum, CA1, and CA4 show the highest correlation coefficient.

Figure 4. pPERK and AT8 immunoreactivity in different stages of pathology. **A:** pPERK immunoreactivity in different regions of the hippocampus increases with the Braak score for NFT pathology. **B:** Similar results are observed with AT8 immunoreactivity. AT8-immunoreactive neurons included immunoreactive NFT as well as diffuse appearance of AT8 immunoreactivity in neurons. Data are expressed as mean $\pm$ SEM. * P < 0.05 compared with Braak stage 0 in the same region.

The correlation between pPERK and phosphorylated tau was investigated in more detail at the immunohistochemical level. Previously we showed that AT8 partly co-localizes with pPERK but that pPERK does not co-localize with NFTs.⁵ In this study we confirm these data using double immunohistochemistry and spectral image analysis (Figure 5, A–D). Approximately 50% of the neurons that were immunoreactive for pPERK showed moderate to intense immunostaining for AT8. Although pPERK could hardly be detected in tangle-bearing neurons (Figure 5D, arrow), neurons with diffuse AT8 immunoreactivity did show pPERK immunoreactivity. Corresponding results were observed with AT100 and AT270 (Figure 5, E–L). AT100 detects tau phosphorylated at serine 212 and threonine 214, whereas AT270 detects tau phosphorylated at threonine 181. No or very few pPERK-immunoreactive granules were observed in neurons with NFTs immunoreactive for AT100 or AT270 (Figure 5, H and L, respectively; arrows). In contrast, in neurons containing pPERK-positive granules a diffuse staining for AT100 or AT270 could be detected. These data indicate that UPR activation increases with tau-associated pathology. However, pPERK can hardly be detected in association with NFTs, suggesting an inverse correlation between UPR activation and the presence of NFTs. This indicates that the UPR is activated at a pretangle stage in pyramidal neurons in the hippocampus.

Co-Localization of pPERK with GSK-3 β in AD Hippocampus

In vitro studies have shown that activation of the UPR induces the activity of the major tau kinase GSK-3 β , providing a connection between ER stress and phosphorylation of tau.⁹ In addition, it has been described previously that GSK-3 β and phosphorylated GSK-3 β are associated with GVD.²³ With specific antibodies directed against GSK-3 β and GSK-3 β pSer9 (inactive form) the co-localization with pPERK in neurons was analyzed. Both GSK-3 β and GSK-3 β pSer9 were present as granules in GVD-containing neurons (Figure 6). Almost all neurons with pPERK-positive GVD granules also contained GSK-3 β and GSK-3 β pSer9 immunoreactivity (Figure 6, A and B). GSK-3 β -positive granules could be identified as GVD granules, however GSK-3 β in these granules did not co-localize with pPERK (Figure 6, C–F; arrow). GSK-3 β pSer9 was detected in granules in neurons, however these granules could morphologically not be identified as GVD granules (Figure 6, G–J). Both markers occurred as granules in the same neuron. No co-localization was observed between GSK-3 β pSer9 and pPERK inside these granules.

The percentages of GSK-3 β - and GSK-3 β pSer9-positive neurons in the different subregions of the hippocampus in controls and AD cases were determined. Both markers are significantly increased in Braak stages 3 to 4 and 5 to 6 in the CA1 and subiculum as compared with the levels in these regions in Braak stage 0 (Figure 7, A and B). The percentages of GSK-3 β - and GSK-3 β pSer9-immunoreactive neurons show a similar distribution along

Table 3. Correlation Analyses of pPERK, GSK-3 β , and AT8 Immunoreactivity

Area	Pearson correlation coefficient (<i>P</i> value)		
	pPERK and AT8	pPERK and GSK-3 β	pPERK and GSK-3 β pSer9
Subiculum	0.771 (<0.001)	0.883 (<0.001)	0.916 (<0.001)
CA1	0.859 (<0.001)	0.882 (<0.001)	0.858 (<0.001)
CA2	0.539 (0.001)	0.454 (0.009)	0.406 (0.021)
CA3	0.642 (<0.001)	0.728 (<0.001)	0.866 (<0.001)
CA4	0.922 (<0.001)	0.852 (<0.001)	0.904 (<0.001)

the Braak stages as neurons immunoreactive for pPERK and AT8 (Figure 4, A and B). Both GSK-3 β - and GSK-3 β pSer9-immunoreactive neurons show a significant correlation with the number of neurons that are pPERK-positive (Table 3).

Discussion

In this study we have shown that UPR activation occurs early during neurofibrillary degeneration. Previously we

have shown that the UPR is activated in AD brain. Increased levels of BiP, indicative of UPR activation,⁴ are present in temporal cortex and hippocampus in AD cases compared with nondemented control cases.⁵ Because protein expression levels of BiP are increased at an early stage of AD pathology in the temporal cortex, it is suggested that the UPR is involved in AD pathology before the widespread occurrence of neurofibrillary degeneration. pPERK is present in pyramidal neurons in AD hippocampus and almost absent in neurons in neocorti-

Figure 5. Double immunohistochemistry and spectral imaging analyses for pPERK and phosphorylated tau protein. **A:** Double immunolabeling for pPERK (brown) and AT8 (red). **B** and **C:** Immunohistochemical signals were spectrally unmixed and shown as red for AT8 and green for pPERK. **D:** Merge of these signals indicates no spatial co-localization. **Arrow** indicates the absence of pPERK in tangle-bearing neuron. **E:** Double immunohistochemistry for pPERK (brown) and AT100 (red). **F** and **G:** Immunohistochemical signals for AT8 (red) and pPERK (green) were spectrally unmixed. **H:** Merge of signals for pPERK and AT100 indicate no co-occurrence of pPERK and AT100 in tangle-bearing neuron (**arrow**). **I:** Double immunolabeling for pPERK (brown) and AT270 (red). **J** and **K:** Immunohistochemical signals for AT270 (red) and pPERK (green) were spectrally unmixed. **L:** Merge of signal for AT270 and pPERK. **Arrow** indicates the low amount of pPERK in tangle-bearing neuron. Sections were counterstained with hematoxylin indicated as blue color in merged pictures.

Figure 6. Double immunohistochemistry and spectral imaging analyses for pPERK, GSK-3 β , and pGSK-3 β . **A:** Double immunohistochemistry for pPERK (red) and GSK-3 β (brown). **B:** Double immunohistochemistry for pPERK (red) and pGSK-3 β (brown). Immunohistochemical signals for pPERK (red) and GSK-3 β (brown) (**C**) were spectrally unmixed in green for GSK-3 β (**D**) and red for pPERK (**E**). **F:** Colors were merged with the addition of spectrally unmixed hematoxylin staining indicated in blue. **Arrow** indicates that GSK-3 β in these granules does not co-localize with pPERK. Immunohistochemical signals for pPERK (red) and pGSK-3 β (brown) (**G**) were spectrally unmixed in red for pGSK-3 β (**H**) and green for pPERK (**I**). **J:** Colors were merged with the addition of spectrally unmixed hematoxylin staining indicated in blue. Scale bars: 50 μ m (**A**, **B**); 30 μ m (**C**–**J**).

cal areas.⁵ The hippocampus is affected by neurofibrillary degeneration at an earlier pathological stage compared with neocortical areas.¹¹ These findings support the suggestion that UPR activation in the hippocampus is involved with neurofibrillary degeneration in AD.

Immunoreactivity for pPERK, pelf2 α , and pIRE1 α was observed as a granular staining. These granules could morphologically be characterized as GVD. GVD is characterized by basophilic granules surrounded by a clear zone measuring 1 to 5 μ m in diameter. GVD is primarily found within pyramidal cells of the hippocampus. It is reported that GVD occurs in adult control brains and increases slightly with increasing age, however the occurrence of GVD in AD brain is increased compared with age-matched control brain.^{28,29} In line with these reports, we found that the percentage of neurons immunoreactive

for pPERK is increased in AD cases as compared with control cases. Although the molecular events in GVD-containing neurons are poorly understood, histochemical and ultrastructural observations suggest that GVD might correspond to a special type of autophagosome.²⁵ The occurrence of UPR activation markers pPERK, pIRE1 α , and pelf2 α in GVD granules is interesting in the light of reports indicating that ER stress triggers or mediates autophagy.^{30–32} There are indications that autophagosomes selectively include the ER membrane and that the ER can serve as a membrane source for autophagosome formation.^{32,33} This could explain the presence of UPR activation markers in GVD granules. The sequestration of unfolded proteins by autophagy may help to protect the neurons against the toxic build-up of unfolded proteins in the ER.

Figure 7. GSK-3 β and GSK-3 β pSer9 immunoreactivity in different stages of pathology. GSK-3 β immunoreactivity (A) and GSK-3 β pSer9 immunoreactivity (B) in different regions of the hippocampus increases with the Braak score for NFT pathology. Data are expressed as mean $\pm$ SEM. * P < 0.05 compared with Braak stage 0 in the same region.

Although it is assumed that GVD inclusions are ubiquitin-positive, initial reports on the localization of ubiquitin in GVD granules are contradictory.^{25,34,35} Also, in the present study no ubiquitin immunoreactivity was observed in GVD granules. However, granules immunoreactive for pPERK were present in neurons with ubiquitin inclusions. In addition we observed that pPERK-immunoreactive neurons were associated with extracellular ubiquitin granules, which have been reported previously as perisomatic granules.³⁶ Perisomatic granules are preferentially associated with pretangle neurons and start to occur as from Braak stage 3. It is suggested that perisomatic granules represent degenerating perisomatic neurites and are a pathological entity distinct from GVD.³⁶ P62 is a ubiquitin-binding protein and is involved in the accumulation of ubiquitin-conjugated proteins into cytoplasmic inclusions.²¹ It has been reported that p62 appears early during neurofibrillary pathogenesis.²² P62 is found in several types of pathological inclusions composed of phosphorylated tau or α -synuclein, which suggests that p62 might contribute to the aggregation process. Because p62 has been reported to interact with several signaling proteins,^{37–40} entrapment of p62 into NFTs could disrupt vital signaling pathways, thereby contributing to dysfunction or death of NFT-containing neurons. In this study we found that pPERK was associated with small ubiquitin-positive inclusions and diffuse p62 immunoreactivity, whereas pPERK was absent from neurons containing ubiquitin or p62-immunoreactive tangle-bearing neurons. These data suggest that activation of the UPR is co-occurring with early UPS activation in pretangle neurons.

The number of pPERK-positive neurons in the hippocampus is increased in Braak stages 3 to 4 for NFTs and correlates with the number of neurons immunoreac-

tive for phosphorylated tau. pPERK immunoreactivity is observed in neurons containing diffuse phosphorylated tau and is almost absent from tangle-bearing neurons. These data suggest that UPR activation occurs in pretangle neurons. Because postmortem studies are limited in studying the temporal sequence of events, we cannot exclude the possibility that UPR activation occurs in neurons that will not form NFTs. Recent studies on tau transgenic mouse models indicate that diffusible tau contributes to disease pathology.^{41–43} Data from these studies imply that NFTs themselves do not lead to synaptic dysfunction and neurotoxicity, but that nonfibrillar tau is responsible for these events.⁴⁴ In light of these studies it is interesting to find activation of the UPR, as detected by pPERK immunostaining, co-occurring with diffuse nonfibrillar phosphorylated tau. The presence of diffusible tau or nonfibrillar tau aggregates may present a highly stressful situation for the neuron, whereas the deposition of tau into insoluble aggregates may serve as a mechanism to reduce the levels of the toxic soluble species. This could explain why tangle-bearing neurons show no or low levels of pPERK immunoreactivity.

Although several reports have shown that the UPR is activated in AD,^{5,45,46} it is still elusive what triggers UPR activation in AD neurons. Amyloid plaques are not located in the ER, however *in vitro* studies have demonstrated that $A\beta_{1-42}$ can be generated in the ER of neuronal cells.^{47,48} Extracellular oligomeric aggregates of $A\beta_{1-42}$ enhance the ER stress response and intracellular $A\beta_{1-42}$ sensitizes neuroblastoma cells for ER stress toxicity.^{6,7} Oligomeric aggregates of $A\beta_{1-42}$ are readily internalized⁴⁹ and induce Ca^{2+} release from the ER.⁵⁰ Disturbance of Ca^{2+} homeostasis in the ER is a well known cause of UPR activation. In models for α -synuclein pathology, ER stress is caused by inhibition of protein trafficking from the ER.⁵¹ Interestingly, increased levels of the microtubule-binding protein tau inhibits anterograde vesicle transport in neurons.⁵² The resulting accumulation of proteins in the cell body is likely to induce stress in the ER. In addition, disturbances in the degradation machinery may contribute to ER stress and UPR activation. Decreased proteasomal activity is found in specific brain regions of AD patients⁵³ and neurons affected by neurofibrillary pathology accumulate immature forms of autophagosomes indicating impaired transport and maturation of autophagosomes.⁵⁴ Beclin 1 has a key role in autophagy and is decreased in affected brain regions of patients with AD,⁵⁵ which indicates impaired autophagy in AD neurons. In case of proteasome impairment or of increased demand for degradation during ER stress, autophagy is triggered, indicating that the two degradation pathways used by the ER are tightly coupled.^{56–58} More importantly, autophagy protects against ER stress toxicity.^{58,59} The overall reduction in protein degradation in AD neurons could induce ER stress and promote UPR activation.

In vitro studies have shown that activation of the UPR induces the activity of GSK-3 β .^{8,9} GSK-3 β is involved in a remarkably broad spectrum of AD-associated events, especially in the phosphorylation of tau, a prerequisite for the formation of NFTs. This suggests that UPR activation

in neurons might directly contribute to tau phosphorylation. In this study we demonstrate a striking co-occurrence of GSK-3 β - and pPERK-immunoreactive granules in AD neurons, and in addition, a strong correlation between the occurrence of pPERK-immunoreactive neurons with GSK-3 β - or GSK-3 β pSer9-immunoreactive neurons was observed. These data support the hypothesis that UPR activation is related to GSK-3 β activity as reported in previous *in vitro* studies. However, a direct co-localization of pPERK and GSK-3 β was not observed. This is in agreement with a previous report, which describes the localization of GSK-3 β in a part of all GVD granules and the absence of GSK-3 β pSer9 in GVD granules.²³ GSK-3 β is no direct substrate for pPERK, making co-localization between PERK and GSK-3 β not a prerequisite for a functional connection. Although underlying mechanisms remain elusive, there is considerable evidence from *in vitro* studies that GSK-3 β promotes intrinsic apoptotic signaling.^{8,9} The presence of GSK-3 β in granules could represent an attempt to sequester GSK-3 β , to prevent further tau phosphorylation and promote cell survival.^{10,23}

In conclusion, UPR activation markers pPERK, pIRE1 α , and pelf2 α are observed in granules associated with GVD in pyramidal neurons in the hippocampus of AD patients. The co-occurrence of pPERK with diffuse phosphorylated tau and markers for pretangles indicates that UPR activation occurs early during neurofibrillary degeneration. The direct connection between UPR activation and GSK-3 β activation as previously shown *in vitro* is supported by data derived from human AD brain tissue. These data support the hypothesis that UPR activation and GVD are involved at the early stages of tau phosphorylation. The initial activation of the UPR in AD neurons might work as neuroprotective to restore homeostasis and increase neuronal survival. Sustained activation of the UPR in AD neurons could accelerate phosphorylation of tau through activation of GSK-3 β . Future studies need to address the therapeutic opportunities of this pathway for the treatment of AD and other tauopathies.

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