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Transgenic Mouse and Cell Culture Models Demonstrate a Lack of Mechanistic Connection Between Endoplasmic Reticulum Stress and Tau Dysfunction

M.L. Spatara and A.S. Robinson*

Department of Chemical Engineering, University of Delaware, Newark, Delaware

Abstract

In vivo aggregation of tau protein is a hallmark of many neurodegenerative disorders, including Alzheimer's disease (AD). Recent evidence has also demonstrated activation of the unfolded protein response (UPR), a cellular response to endoplasmic reticulum (ER) stress, in AD, although the role of the UPR in disease pathogenesis is not known. Here, three model systems were used to determine whether a direct mechanistic link could be demonstrated between tau aggregation and the UPR. The first model system used was SH-SY5Y cells, a neuronal cultured cell line that endogenously expresses tau. In this system, the UPR was activated using chemical stressors, tunicamycin and thapsigargin, but no changes in tau expression levels, solubility, or phosphorylation were observed. In the second model system, wild-type 4R tau and P301L tau, a variant with increased aggregation propensity, were heterologously overexpressed in HEK 293 cells. This overexpression did not activate the UPR. The last model system examined here was the PS19 transgenic mouse model. Although PS19 mice, which express the P301S variant of tau, display severe neurodegeneration and formation of tau aggregates, brain tissue samples did not show any activation of the UPR. Taken together, the results from these three model systems suggest that a direct mechanistic link does not exist between tau aggregation and the UPR.

Keywords

unfolded protein response; Alzheimer's disease; SH-SY5Y; PS19; neurofibrillary tangles; paired helical filaments

Alzheimer's disease (AD) is the most prevalent form of dementia; approximately 5.3 million Americans currently have AD, and the number increases each year as the average age in our society increases (Alzheimer's Association, 2009). Pathologically, the disease involves neuronal death accompanied by accumulation of two distinct types of protein aggregates, extracellular aggregates of amyloid β (A β) peptide, often referred to as plaques, and intracellular aggregates of tau protein, often referred to as neurofibrillary tangles (NFTs) or paired helical filaments (PHFs; Selkoe, 2001). It is unclear how A β aggregates and tau aggregates are related and which is more important in disease pathology (Lee, 2001).

*Correspondence to: A.S. Robinson, 227 Colburn Laboratory, Department of Chemical Engineering, University of Delaware, Newark, DE, 19716. asr@udel.edu.

Tau protein is a natively unfolded protein, abundant in neurons, which binds to and stabilizes microtubules. Tau has 79 potential phosphorylation sites, and its phosphorylation is regulated by phosphatases and kinases, including glycogen synthase kinase-3 β (GSK-3 β). Hyperphosphorylated tau is associated with the disease state, insofar as it shows decreased microtubule binding and an increased propensity for tangle formation (Buee et al., 2000). In contrast to A β aggregation, which is specifically associated with AD, tau aggregates form in multiple neurodegenerative disorders, collectively termed tauopathies, including Pick's disease, dementia pugilistica, and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17; Hutton et al., 1998; Lee, 2001; Brandt et al., 2005).

An increasing amount of evidence indicates that endoplasmic reticulum (ER) stress may play a significant role in AD. The ER is the first organelle in the secretory pathway of eukaryotic cells and is responsible for ensuring proper folding of newly synthesized proteins. If there is an accumulation of unfolded proteins in the ER, a set of signaling pathways known as the unfolded protein response (UPR) is activated. This adaptive response attempts to restore ER homeostasis by decreasing overall protein production while selectively increasing production of chaperones and proteases. If ER stress persists, the UPR will eventually induce apoptosis or programmed cell death (Cudna and Dickson, 2003; Szegezdi et al., 2003; Schroder and Kaufman, 2005; Lai et al., 2007; Malhotra and Kaufman, 2007). The mammalian UPR consists of three signaling pathways, each induced by an ER-membrane-resident stress sensor, inositol-requiring kinase 1 (IRE1), protein kinase-like ER kinase (PERK), or activating transcription factor 6 (ATF6; Wu and Kaufman, 2006; Malhotra and Kaufman, 2007).

Given the important role of the ER in protein synthesis and quality control as well as the potential for ER stress to lead to apoptosis, it is not surprising that ER dysfunction may play a role in AD. Neuronal tissue from AD patients shows multiple UPR activation markers, including elevated levels of BiP (Hoozemans et al., 2005), increased phosphorylation of PERK (Hoozemans et al., 2005, 2009), increased phosphorylation of eIF2 α (Hoozemans et al., 2009), and disruption of ER Ca²⁺ regulation (Verkhatsky, 2005), indicating that the UPR is activated in AD.

ER stress could potentially provide a link between extracellular A β aggregation and intracellular tau aggregation. Treatment of cultured neuronal cells with A β aggregates has been shown to cause both UPR induction (Chafekar et al., 2007; Resende et al., 2008) and increased phosphorylation of tau (Resende et al., 2008), although the mechanism by which A β causes these effects is unclear. GSK-3 β , a known tau kinase, is activated by the UPR; thus, ER stress could be a cause of increased tau phosphorylation. Chronic ER stress resulting in ER-induced apoptosis has been suggested as a potential cause of neuronal death in AD (Katayama et al., 2004), but it is also possible that the UPR plays a neuroprotective role.

Although AD patients clearly exhibit ER stress, it remains unclear what role this stress plays in the disease. Is ER stress caused by formation of A β aggregates or tau aggregates or both? Does activation of the UPR result in increased aggregate formation? Is the UPR a cause of neuronal cell death? The current work uses well-controlled model systems in an attempt to

understand better the potential mechanistic connections between the UPR and tau dysfunction.

MATERIALS AND METHODS

Materials

All chemicals used were obtained from major chemical suppliers. Unless otherwise noted, all cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

Cell Culture

SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in a 1:1 mixture of Eagles's minimum essential medium (EMEM) and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C and 5% CO₂. Medium was replenished every 2–3 days. Cells were maintained in T-75 flasks and passaged at a ratio of 1:20 to 1:50 when flasks reached 80–90% confluence.

SH-SY5Y cells were differentiated with retinoic acid for all experiments. Cells were removed from T-75 flasks with 0.25% trypsin and 0.5 mM EDTA, and cell concentration was determined by hemocytometry. Cells were plated in six-well plates at a density of 5×10^4 cells/well. Approximately 5–7 days after plating, medium was supplemented with 10 µM retinoic acid. Cells were cultured in the presence of retinoic acid for a minimum of 8 days prior to use in UPR experiments.

Human embryonic kidney cells (HEK293) stably trans-fected with a plasmid containing the tetracycline (Tet) repressor were obtained from Invitrogen and are referred to by the commercial name "T-REx-293." T-REx-293 cells were cultured in Dulbecco's modification of Eagles's minimum essential medium (DMEM) with 4 mM L-glutamine and high glucose supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C and 5% CO₂. Medium was replenished every 2–3 days. Media also included 5 µg/ml blasticidin, the selective marker for the Tet repressor plasmid. Cells were maintained in T-75 flasks and passaged at a ratio of 1:5 to 1:8 when flasks reached 80–90% confluence.

Transfection

T-REx-293 cells were transfected with 0.5 µg/well (in six-well plates) pT-REx-DEST31 Gateway vector (Invitrogen) containing the gene for wild-type tau (4R isoform), tau P301L, or a control gene (lacZ) using the reagent Lipofectamine 2000 (Invitrogen). Some cells were also treated with Lipofectamine in the absence of DNA as a negative control for transfection.

Cells were allowed to grow in antibiotic-free media for a 48-hr recovery period after transfection before switching to selective media including 5 µg/ml blasticidin and geneticin in varying concentrations (0, 200, 500, and 800 µg/ml). After approximately 7 days of selection, nontransfected control cells grown with 500 and 800 µg/ml geneticin concentrations were no longer viable. Stably transfected cells were then propagated in 500 µg/ml geneticin.

Chemical Induction of the UPR

To determine the effect of the UPR on tau biology, the UPR was induced using chemical reagents in differentiated SH-SY5Y cells, which natively express tau. To induce the UPR, medium containing 10 µg/ml tunicamycin or 500 nM thapsigargin was added to differentiated cells in six-well plates. As a positive control for tau phosphorylation, some cells were also treated with 75 nM okadaic acid, a phosphatase inhibitor (Perez et al., 2002; Xu et al., 2004). At specified times after treatment, cell samples were taken and analyzed for both UPR activity and changes in tau phosphorylation state and solubility.

Recombinant Protein Expression

T-REx-293 cells stably transfected with the DEST31 plasmid containing the gene of interest as well as nontransfected control cells were plated in six-well plates at a density of 2×10^5 cells/well. Cells were grown in selective media for ~24 hr before protein expression was induced by addition of 1 µg/ml tetracycline. At specified times after treatment, cell samples were taken for analysis by RT-PCR and Western blotting.

Mouse Tissue Samples

Brain samples from wild-type and PS 19 transgenic mice, which express P301S tau (Yoshiyama et al., 2007), collected at 6 and 11 months were a generous gift from Dr. Virginia Lee of the University of Pennsylvania's Center for Neurodegenerative Disease Research. PS 19 mice have a neurodegenerative phenotype with limb weakness and brain atrophy after 3 months of age, progressing to paralysis at 7–10 months of age. In brain tissue samples from PS19 mice, tau is hyperphosphorylated and has decreased solubility (Yoshiyama et al., 2007). When received, samples had been sectioned into cerebral cortex, hippocampus, cerebellum, brainstem, spinal cord and olfactory bulb and frozen at -80°C .

Western Blotting

For analysis of protein levels by Western blotting, cells were lysed in M-Per Protein Extraction Reagent including Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (Pierce, Rockford, IL). Insoluble cellular debris was removed by centrifugation at 12,000g for 15 min. Total protein concentration in cleared lysates was quantified using the BCA assay (Pierce) with bovine serum albumin (BSA) as a standard. Equal amounts of protein for each sample (typically 6.5 µg) were run on 4–20% SDS gels for 2 hr at 120 V. Gels were then transferred to nitrocellulose overnight (>16 hr) at 10 V. Nitrocellulose membranes were blocked for >2 hr with a blocking solution of either powdered milk or BSA in Tris-buffered saline with 0.1% Tween (TBST). Membranes were then incubated with primary antibody overnight at 4°C. The specific supplier, blocking conditions, incubation conditions, and secondary antibody for each primary antibody used in this work are listed in Table I. After three 10-min washes with TBST, membranes were incubated with an HRP-conjugated secondary antibody (GE Healthcare), and the blots were visualized with the ECL-Plus kit (GE Healthcare) on a Typhoon 9400 scanner and quantified in ImageQuant software (GE Healthcare).

Tau Solubility

The solubility of tau in cell culture samples was determined by sequential extraction using a series of solvents (Ishihara et al., 2001). Cells were lysed in high-salt reassembly buffer (RAB; 0.1 M MES, 0.5 mM MgSO₄, 0.75 M NaCl and 0.02 M NaF at pH 7.0) to extract soluble as well as microtubule-bound tau. Insoluble material was separated from the RAB fraction by centrifugation at 13,000g for 30 min at 4°C. The resulting pellet was resuspended in the detergent containing-RIPA buffer (50 mM Tris-HCl, 150 mM NaCl₂, 1% NP-40, 15 mM EDTA, 0.5% sodium deoxycholate, and 0.1% SDS at a pH of 8.0) to solubilize small tau aggregates. Insoluble material was separated from the RIPA fraction by centrifugation at 13,000g for 30 min at 4°C. The resulting pellet was resuspended in 70% formic acid to solubilize any remaining tau, including large aggregate species. The formic acid fraction was diluted 10× with 2 M Tris (pH 8.3) to reduce the acidity of the samples prior to analysis. Fractions were assayed for the presence of tau using Western blotting with the tau-5 antibody (Biosource).

RNA Analysis

Total RNA was purified from cultured cell samples using the SV Total RNA Isolation kit (Promega, Madison, WI) according to the manufacturer's protocol. For brain tissue samples, tissue was homogenized using a PowerGen homogenizer (Fisher, Fair Lawn, NJ), and total RNA was isolated using Trizol reagent (Invitrogen), also according to the manufacturer's instructions. Total RNA yields were measured by A₂₆₀ using a NanoVue spectrophotometer (GE Healthcare).

Synthesis of cDNA from isolated RNA was performed with the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instructions and with an oligo(dT)₁₅ primer. After the reverse transcription reaction (RT-PCR), the reverse transcriptase was inactivated by incubation at 70°C for 15 min.

To determine the extent of XBP1 splicing, a segment of the XBP1 gene containing the region spliced by IRE1 during UPR activation was PCR amplified from cDNA. The primers used were mouse 5'-TTACGGGAGAAAACCTCACGGA-3' and 5'-GGGTCCAACCTTGTCAGAAATGC-3' and human 5'-ACGAGAGAAAACCTCATGGC-3' and 5'-GGGTCCAAGTTGTCAGAAATGC-3'. PCR conditions were 95°C for 5 min; 35 cycles of 95°C for 1 min, 49°C for 30 sec, and 72°C for 30 sec; and a final incubation at 72°C for 5 min. PCR products were separated on 2.5% agarose gels containing 0.00013% ethidium bromide for 2 hr at 100 V to visualize spliced and unspliced XBP1 fragments.

To determine levels of BiP and calreticulin (CRT) mRNA, quantitative PCR (qPCR) was performed on an ABI Prism 7000 Real-Time PCR instrument (Applied Biosystems, Foster City, CA) using DyNAmo HS SYBR Green dye (New England Biolabs, Boston, MA). Rpl 19, a ribosomal protein not regulated by ER stress (Lin et al., 2007), was used as an internal normalization standard. Primers used were mouse Rpl 19 5'-CTGATCAAGGATGGGCTGAT-3' and 5'-TCAATCTTCTTGGATTCCCG-3', mouse BiP 5'-GTCTGCTTCGTGTCTCCTCC-3' and 5'-GGAATAGGTGGTCCCCAAGT-3', and mouse calreticulin 5'-AAGAGCAGTTCTTGGACGGA-3' and 5'-

CACCAGTGTCTGGCCCTTAT-3'. PCR conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. Each 30- μ l qPCR included 1 μ l of reverse transcription reaction and a primer concentration of 0.33 μ M.

Quantification and Statistical Analysis of Gels

Western blots and DNA gels were quantified in Image-Quant software, and statistical analysis was performed in Minitab software. A one-way ANOVA test with a 95% confidence level was used to determine whether a statistically significant difference existed among the samples. In cases when a difference was found, Dunnett's method (Dunnett, 1955) was used to identify samples that were significantly different from the DMSO-treated control.

RESULTS

Chemical Induction of UPR in SH-SY5Y Cells Did Not Affect Tau Biology

The effect of endoplasmic reticulum (ER) stress on tau biology was examined using SH-SY5Y cells as a model neuronal system (Biedler et al., 1978). Previous studies on SH-SY5Y cells have shown that addition of Ab oligomers can cause tau aggregation, but the mechanism of action was not explored (Ferrari et al., 2003). For the following experiments, cells were differentiated with retinoic acid to promote neuron-like morphology for at least 8 days prior to induction of UPR. The effect of cell differentiation on tau in SH-SY5Y cells has been well described in the literature. In undifferentiated cells, tau is localized predominantly in the nucleus and tau does not bind to microtubules in situ or in vitro (Tanaka et al., 1995; Uberti et al., 1997; Zhong et al., 1999). After differentiation, tau is localized primarily in the cytosol and along neuritic processes (Uberti et al., 1997). Differentiation has also been shown to change the pattern of expression of different tau isoforms (Uberti et al., 1997) and the phosphorylation of tau (Jamsa et al., 2004).

The UPR was induced in SH-SY5Y cells by treatment with either tunicamycin (TM), which inhibits N-linked glycosylation (Heifetz et al., 1979), or thapsigargin (TG), which disrupts calcium homeostasis (Thastrup et al., 1990; Sagara and Inesi, 1991). These chemical treatments have been used in many ER stress studies and are known to activate the UPR (Lin et al., 2007; Gonzales et al., 2008; Cho et al., 2009; Kim et al., 2009; Zhang et al., 2009). Cells were also treated with okadaic acid (OA), a phosphatase inhibitor, as a positive control for tau phosphorylation (Perez et al., 2002; Xu et al., 2004).

To confirm that the UPR was activated in our experimental system, we monitored several UPR markers following addition of TM, TG, and OA. Results for splicing of XBP1 mRNA (an early event in the IRE1 branch of the UPR) are shown in Figure 1. As expected, treatment with TM or TG caused splicing of XBP1 beginning at 1 hr for TG and 2.5 hr for TM and for times up to 6 hr, indicating that the IRE1 branch of the UPR is activated in these samples. Attenuation of splicing was seen at 24 hr, as expected (Lin et al., 2007). The percentage of spliced XBP1 was determined from quantification of band intensities in the gel, and results with statistically significant levels of splicing (compared with DMSO

control) are indicated in Figure 1B. No significant XBP1 splicing was observed in cells treated with OA alone.

Phosphorylation of eIF2 α (an early event in the PERK branch of the UPR) was also monitored. Western blots of total eIF2 α levels and phosphorylated eIF2 α levels are shown in Figure 2A. Phosphorylation of eIF2 α was higher 1 and 3 hr after treatment with TM or TG, indicating that the PERK branch of the UPR is activated in these cases. Increased eIF2 α phosphorylation was not observed in samples treated with OA alone. Figure 2B shows quantification of phosphorylated eIF2 α levels, and statistically significant differences from DMSO control are indicated. Total eIF2 α levels were also quantified, and, as expected, no statistically significant differences were observed among samples.

It has been reported that the activity of GSK-3 β , a known tau kinase, is increased by the UPR (Song et al., 2002; Brewster et al., 2006). Because this provides a potential mechanistic link between the UPR and tau biology, we examined GSK-3 β levels in TM-, TG-, and OA-treated samples. Figure 3A shows Western blots of total GSK-3 β levels and GSK-3 β phosphorylated at serine 9. Phosphorylation of GSK-3 β at this site inhibits kinase activity; thus, an increase in phosphorylation would decrease activity. Quantification of the phosphorylated GSK-3 β levels is shown in Figure 3B. In the blot shown, a small decrease in GSK-3 β phosphorylation was observed in samples treated with TM at 6 hr and samples treated with TG at 3 and 6 hr. This difference was not apparent in the replicate blot, and, when quantified and averaged, the change was not significant. This indicates that GSK-3 β was not significantly activated by the UPR in this system. Samples treated with OA showed increased phosphorylation of GSK-3 β at 3 and 6 hr resulting from nonspecific phosphatase inhibition, as expected.

To determine what effect UPR activation has on tau biology, tau solubility and extent of phosphorylation were tested. Samples of cells treated with TM, TG, and OA were analyzed by Western blotting using tau-5 antibody, which recognizes all tau species, and phosphospecific antibodies, which recognize only tau that is phosphorylated at specific sites. Representative Western blots of total tau and tau phosphorylated at serine 199 or 202 are shown in Figure 4A. Quantifications of Western blots for phosphorylated tau species are shown in Figure 4B (serine 199/202), Figure 4C (serine 262), and Figure 4D (serine 396). Hyperphosphorylation of tau at these sites is associated with increased propensity to form tangles (Buee et al., 2000). Treatment with OA, a phosphatase inhibitor, caused an increase in levels of tau phosphorylation at serine 199/202, as previously reported (Perez et al., 2002), but no significant increase in phosphorylation of tau at serine 262 or serine 396. However, this is not surprising, insofar as large variability in OA-induced phosphorylation among different tau sites has been observed previously. A recent study in cultured neurons reported a 450% increase in phosphorylation at serine 199/202 but only a 20% increase in phosphorylation at serine 396 upon treatment with 100 nM OA. Interestingly, samples treated with only TM or TG did not show any significant change in tau phosphorylation levels (Rahman et al., 2009). Total tau levels were also quantified, and no statistically significant differences existed among samples. When tau solubility was assayed by sequential extraction using RAB, RIPA, and 70% formic acid, tau was observed only in the RAB fraction (data not shown), indicating that no insoluble aggregates were formed.

Overexpression of WT and P301L Tau Did Not Induce the UPR

To determine whether tau dysfunction could be a cause of ER stress, both wild-type (WT) tau and a mutant form of tau (P301L), along with a control protein, β -galactosidase (β -gal), were overexpressed in HEK293 cells. The commercial tetracycline-regulated T-REx expression system (Invitrogen) was used to allow control of tau expression. Figure 5 shows Western blots of tau levels in uninduced cells and cells 24 and 48 hr after protein expression was induced with 1 μ g/ml tetracycline. Tau was observed in the WT and P301L samples following induction but not in the uninduced samples or the nontransfected and β -gal control samples, confirming that these cell lines express tau only under control of the tetracycline-induced promoter. Two tau bands are observed on the Western blot. This has been previously observed when tau was recombinantly expressed in CHO cells, and the difference in mobility was attributed to different degrees of phosphorylation (Vogelsberg-Ragaglia et al., 2000). The increased expression level of the P301L tau compared with that of WT tau most likely was due to stochastic differences in transfection efficiency. Previous work using this system to express human transferrin has shown that the transfection generates expression clones with a wide range of expression levels (Jones et al., 2005).

Once expression of tau was confirmed, cell samples were tested for UPR activation. Figure 6 shows splicing of XBP1 mRNA with a positive control from treatment of SH-SY5Y cells with TG for 1 hr. None of the samples show any splicing of XBP1, indicating that the IRE1 branch of the UPR is not activated by WT or P301L tau expression in T-REx 293 cells. Figure 7A shows a representative Western blot of total and phosphorylated eIF2 α in these samples, and quantification of phosphorylated eIF2 α levels is shown in Figure 7B. No statistically significant changes in total or phosphorylated eIF2 α levels were observed, indicating the PERK branch of the UPR was not activated by WT or P301L expression in T-REx 293 cells. Taken together, the XBP1 and eIF2 α results indicate that overexpression of WT or P301L tau in this system did not activate the UPR.

The UPR Was Not Induced in PS19 Transgenic Mice

Although the UPR was not observed in T-REx 293 cells expressing P301L or WT tau, transient overexpression of tau in a cell culture system is significantly different from the physiological progression of tauopathies, in which tau accumulates and aggregates over long periods of time. To test further for the ability of tau dysfunction to induce the UPR, brain tissue samples from transgenic mice expressing P301S tau were tested for XBP1 splicing. PS19 mice have mild neurological pathology at 6 months and severe pathology at 11 months (Yoshiyama et al., 2007). Figure 8 shows representative XBP1 splicing results for both WT and PS19 transgenic mice at 6 and 11 months of age. XBP1 splicing was not observed in any of the samples, indicating that the UPR was not activated in this transgenic mouse model. In addition, the levels of BiP and calreticulin mRNA were measured by qPCR in samples from mice at 11 months of age. The relative -fold increase in mRNA levels for PS19 mice compared with a nontransgenic control are plotted in Figure 9. BiP and calreticulin are both ER-resident chaperones that are up-regulated during ER stress. BiP mRNA levels have been observed to increase more than tenfold upon treatment of cultured cells with TM and more than twofold during protein overexpression (Lin et al., 2007). Calreticulin mRNA levels have been reported to increase more than twofold upon during

TM treatment of *Caenorhabditis elegans* (Lee et al., 2007), and increased expression of the calreticulin gene has been observed in cultured cells treated with TG (Waser et al., 1997). Interestingly, in the tissue samples from PS19 mice examined here, BiP and calreticulin mRNA levels were lower than levels in the nontransgenic control. The cause of this down-regulation of BiP and calreticulin is not known. However, BiP and calreticulin were clearly not up-regulated, providing further conformation that the UPR was not activated in the PS19 transgenic mice.

DISCUSSION

ER stress and activation of the UPR have been implicated in several diseases, including Alzheimer's disease, although the role of the stress response in disease pathologies is not known (Lindholm et al., 2006; Yoshida, 2007; Scheper and Hoozemans, 2009). ER stress has been observed in brain tissue of Alzheimer's disease patients (Hoozemans et al., 2005, 2009) and in combination with tau aggregation when cultured neurons are treated with A β oligomers (Resende et al., 2008). However, it is unclear whether formation of tau aggregates induces ER stress or whether activation of the UPR contributes to tau aggregation. It is also possible that tau aggregation and ER stress are separate events and are not directly linked. This work tests the relationship between tau biology and the UPR in several model systems in an attempt to find a direct, mechanistic link between the two. Surprisingly, no correlation between UPR activation and tau was observed in any of the systems tested.

The first model system described here was used to test the hypothesis that activation of the UPR could cause changes in tau biology such as increased phospho-rylation and propensity to form tangles. This hypothesis is consistent with recent experimental results from other groups. In a study by Resende and colleagues (2008), cultured neuronal cells were treated with A β oligomers, resulting in hyperphosphorylation of tau, activation of GSK-3 β , and increases in BiP levels. Treatment of cells with a selective inhibitor of GSK-3 β or dantrolene, an inhibitor of ER calcium release, decreased the extent of A β -induced phosphorylation of tau, indicating that GSK-3 β activation and ER stress were involved in tau phosphorylation in this system. The authors propose that A β oligomers cause ER stress, which in turn activates GSK-3 β , leading to increased phosphorylation of tau. A significant unanswered question in this study is how extracellular A β oligomers trigger ER stress. However, if A β effects on tau are the result of ER stress, then induction of ER stress by other methods should result in a similar sequence of events. The results described here indicate that this is not the case.

In our studies, ER stress was induced by two different chemical treatments, thapsigargin and tunicamycin, and activation of the UPR was confirmed by the UPR signaling markers XBP1 and eIF2 α . However, no changes in tau phosphorylation or solubility were observed. In addition, activation of GSK-3 β was not conclusively demonstrated. It is possible that ER stress is one of multiple results of A β treatment. For example, there is evidence that A β addition causes inhibition of the proteasome (Oddo, 2008). The combination of ER stress and other factors may be necessary to cause tau hyperphosphorylation and aggregation. It is also possible that ER stress is a result of A β treatment but does not play a causative role in tau hyperphosphorylation. Although the mechanism by which A β increases tau

phosphorylation remains unclear, the results presented here indicate that UPR activation alone is not sufficient to lead to hyperphosphorylation of tau.

One limitation of these experiments is that the chemical reagents used cause acute ER stress, which causes sustained UPR activation and eventual apoptosis. Under conditions of low or moderate stress, the UPR can result in a return to cellular homeostasis. The specific factors that determine the balance between proapoptotic and prosurvival responses to ER stress and the effects of prolonged or repeated low-level ER stress are not yet well understood (Rutkowski et al., 2006; Lin et al., 2007). However, chronic, low-level stress may play a significant role in neurodegeneration and other protein folding disorders and warrants increased study in the future.

The second hypothesis examined here is that tau dysfunction could lead to ER stress and UPR activation. To test this hypothesis, an experimental system that models aggregation-prone tau states was needed. Researchers have identified at least 37 mutations in the tau gene that are associated with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) and other, similar disorders (Wolfe, 2009). To mimic the disease state, two of the FTDP-17 variants of tau (P301L and P301S) were used. The P301L variant was recombinantly overexpressed in cultured cells, and the P301S variant was expressed in transgenic mice. In both of these model systems, no evidence of ER stress or UPR activation was observed, indicating that these disease-like tau variants do not lead to ER stress. This result is particularly convincing in the case of the trans-genic mouse model, for which tau is known to aggregate and cause neurological impairment.

The inability of tau to induce the UPR in the model systems described here in combination with previous work demonstrating that extracellular A β alone can cause UPR induction in cultured neurons indicates that A β is the most likely cause of the ER stress that is observed in AD patients. However, ER stress has also been observed in other neurodegenerative disorders (Scheper and Hoozemans, 2009), including Parkinson's disease (Hoozemans et al., 2007) and amyotrophic lateral sclerosis (Atkin et al., 2008), indicating that activation of this response is not unique to aggregation of A β . This is the first report of which the authors are aware that suggests no link between tau aggregation and ER stress in neurodegenerative disorders.

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Fig. 1.

SH-SY5Y cells, 8 days following differentiation by retinoic acid, were untreated (DMSO) or treated with tunicamycin (TM), thapsigargin (TG), okadaic acid (OA), or a combination of OA and TM or TG as described in Materials and Methods. **A:** A segment of the XBP1 gene containing the region spliced by IRE1 was amplified from cDNA of cell lysates by PCR. PCR products were separated on a 2.5% agarose gel to resolve unspliced (U) and spliced (S) XBP1. **B:** Quantification of XBP1 splicing from the agarose gels was performed as described in Materials and Methods. Error bars represent the standard deviation from the mean of two biological replicates. Asterisks indicate a statistically significant difference from DMSO control with a 95% confidence level.

Fig. 2.

A: Levels of total eIF2α and phosphorylated eIF2α in lysates from SH-SY5Y cells, which were treated as described in Figure 1, were measured by Western blotting.

Glyceraldehyde-3-phosphate de-hydrogenase (GAPDH) was used as a protein loading control. **B:** Quantification from Western analysis of phosphorylated eIF2α normalized to GAPDH levels. Error bars represent the standard deviation from the mean of two biological replicates. Asterisks indicate a statistically significant difference from DMSO control with a 95% confidence level.

Fig. 3.

A: Levels of total GSK-3 β and phosphorylated GSK-3 β in lysates from SH-SY5Y cells, which were treated as described in Figure 1, were measured by Western blotting. GAPDH was used as a protein loading control. **B:** Quantification from Western analysis of phosphorylated GSK-3 β levels normalized to GAPDH levels. Error bars represent the standard deviation from the mean of two biological replicates. Asterisks indicate a statistically significant difference from DMSO control with 95% confidence level.

Fig. 4.

A: Levels of total tau and tau phosphorylated at serine 199 or 202 in lysates from SH-SY5Y cells, which were treated as described in Figure 1, were measured by Western blotting. GAPDH was used as a loading control. Quantification from Western analysis of phosphorylated tau levels normalized to GAPDH levels for phosphorylation at serine 199/202 (**B**), serine 262 (**C**), and serine 396 (**D**). Error bars represent the standard deviation from the average of two (B,C) or three (D) biological replicates. Asterisk indicates statistically significant difference from DMSO control with a 95% confidence level.

Fig. 5. T-REx 293 cells, which were either untransfected or stably transfected with a plasmid containing the gene for either WT tau, P301L tau, or β -galactosidase (β -gal), were treated with tetracycline to induce protein expression as described in Materials and Methods. Expression of recombinant tau was confirmed by Western blotting for tau and phosphorylated tau (serines 199/202).

Fig. 6.

Protein expression was induced in T-REx 293 cells as described for Figure 5. A segment of the XBP1 gene containing the region spliced by Ire1 was amplified via PCR from cDNA collected from T-REx 293 cell lysates. PCR products were separated on a 2.5% agarose gel to resolve unspliced (U) and spliced (S) XBP1. SH-SY5Y cells treated with TG for 1 hr were used as a positive control. Results shown are representative of two biological replicates.

Fig. 7.

Protein expression was induced in T-REx 293 cells as described in Figure 5. **A:** Levels of total eIF2α and phosphorylated eIF2α in T-REx 293 cell lysates were measured by Western blotting. GAPDH was used as a protein loading control. **B:** Quantification from Western analysis of phosphorylated eIF2α levels normalized to GAPDH levels. Error bars represent the standard deviation from the average of two biological replicates.

Fig. 8.

A segment of the XBP1 gene containing the region spliced by Ire1 was amplified via PCR from cDNA of WT and PS19 mouse tissue samples. PCR products were separated on a 2.5% agarose gel to resolve unspliced (U) and spliced (S) XBP1. SH-SY5Y cells treated with TM for 2.5 hr (6 months and 11 months PS19) or TG for 1 hr (11 months WT) were used as a positive control. Results shown for PS19 mice are representative of three replicate specimens. Ctx, cerebral cortex; HP, hippocampus; Cereb, cerebellum; B. Stem, brainstem; S.C., spinal cord; Olf. Bulb, olfactory bulb.

Fig. 9. Levels of BiP and calreticulin mRNA in tissue samples from the cerebral cortex, hippocampus, and cerebellum were measured by qPCR and normalized to levels of Rpl19, a ribosomal protein not regulated by ER stress. The relative -fold changes in mRNA levels for PS19 mice compared with a nontransgenic control are plotted. Error bars represent standard deviation from the mean of samples from three transgenic animals.

TABLE I

Resources and Conditions for Western Blotting

Antigen	Supplier	Catalogue no.	Dilution	Secondary antibody	Blocking solution	Antibody incubation solution
eIF2 α	Biosource	AH01182	1:500	Rabbit	5% Milk	1% BSA
Phos eIF2 α	Invitrogen	44728G	1:1,000	Rabbit	5% BSA	1% BSA
GAPDH	Invitrogen	39-8600	1:15,000	Mouse	5% Milk	1% BSA
GSK-3 β	Cell Signaling	9315	1:1,000	Rabbit	5% Milk	5% BSA
Phos GSK-3 β	Cell Signaling	9336	1:500	Rabbit	5% Milk	5% BSA
Tau p262	Invitrogen	44750G	1:500	Rabbit	5% BSA	3% BSA
Tau p396	Invitrogen	355300	1:500	Mouse	5% Milk	3% Milk
Tau p199/202	Invitrogen	44768G	1:1,000	Rabbit	5% BSA	3% BSA
Tau 5	Biosource	AHB0042	1:1,000	Mouse	5% Milk	3% BSA