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Perk Genetic Variation and Function in Progressive Supranuclear Palsy

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Abstract
Progressive supranuclear palsy (PSP) is a neurodegenerative disorder pathologically characterized by intracellular tangles of hyperphosphorylated tau protein distributed throughout the neocortex, basal ganglia, and brainstem. A genome-wide association study identified EIF2AK3 as a risk factor for PSP. EIF2AK3 encodes PERK, part of the endoplasmic reticulum's (ER) unfolded protein response (UPR). PERK is an ER membrane protein that senses unfolded protein accumulation within the ER lumen. Recently, several groups noted UPR activation in Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis, multiple system atrophy, and in the hippocampus and substantia nigra of PSP subjects. In Chapter 2, we evaluate PERK activation in the pons, medulla, midbrain, hippocampus, frontal cortex and cerebellum in subjects with PSP, AD, and in normal controls. We found UPR activation primarily in disease-affected brain regions in both disorders. In PSP, the UPR was primarily activated in the pons and medulla and to a much lesser extent in the hippocampus. In AD, the UPR was extensively activated in the hippocampus. We also observed UPR activation in the hippocampus of some elderly normal controls, severity of which positively correlated with both age and tau pathology but not with Aβ plaque burden. Finally, we evaluated EIF2AK3 coding variants that influence PERK activation. We show that a PERK haplotype that demonstrates increased eIF2α kinase activity is genetically associated with increased PSP risk. The UPR is activated in disease affected regions in PSP and the genetic and biological evidence shows that this activation increases risk for PSP and is not a protective response.

There are two common protein coding variants of PERK, HapA and HapB, which differ by three amino acids. Recent work indicates HapB PERK has more kinase activity in response to thapsigargin treatment than does HapA in human β-lymphocytes. The goal of the work detailed in Chapter 3 was to: 1) replicate and expand upon previous findings in β-lymphocytes and 2) determine which of the three amino acid coding changes is responsible for the difference in PERK activity between HapA and HapB. This work confirms that β-lymphocytes expressing HapB PERK show more eIF2α phosphorylation than those expressing HapA. Paradoxically, HapB PERK cells also show less phosphorylated PERK. These findings were echoed in mouse embryonic fibroblasts expressing PERK variant constructs. Further work exploring the functional differences between PERK variants is warranted.

Chapter 4 discusses the implications of the work detailed in Chapters 2 and 3 and suggests future directions for this work, including examination of post-translational modifications of PERK and exploration of how PERK variants function in cell culture models of tauopathy.

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PERK GENETIC VARIATION AND FUNCTION IN PROGRESSIVE SUPRANUCLEAR PALSY

For Alphonsus Joseph Urevick: I hope you would have found this dissertation "very good."

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ABSTRACT

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Progressive supranuclear palsy (PSP) is a neurodegenerative disorder pathologically characterized by intracellular tangles of hyperphosphorylated tau protein distributed throughout the neocortex, basal ganglia, and brainstem. A genome-wide association study identified *EIF2AK3* as a risk factor for PSP. *EIF2AK3* encodes PERK, part of the endoplasmic reticulum's (ER) unfolded protein response (UPR). PERK is an ER membrane protein that senses unfolded protein accumulation within the ER lumen. Recently, several groups noted UPR activation in Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis, multiple system atrophy, and in the hippocampus and substantia nigra of PSP subjects. In Chapter 2, we evaluate PERK activation in the pons, medulla, midbrain, hippocampus, frontal cortex and cerebellum in subjects with PSP, AD, and in normal controls. We found UPR activation primarily in disease-affected brain regions in both disorders. In PSP, the UPR was primarily activated in the pons and medulla and to a much lesser extent in the hippocampus. In AD, the UPR was extensively activated in the hippocampus. We also observed UPR activation in the hippocampus of some elderly normal controls, severity of which positively correlated with both age and tau pathology but not with Aβ plaque burden.

Finally, we evaluated *EIF2AK3* coding variants that influence PERK activation. We show that a PERK haplotype that demonstrates increased eIF2α kinase activity is genetically associated with increased PSP risk. The UPR is activated in disease affected regions in PSP and the genetic and biological evidence shows that this activation increases risk for PSP and is not a protective response.

There are two common protein coding variants of PERK, HapA and HapB, which differ by three amino acids. Recent work indicates HapB PERK has more kinase activity in response to thapsigargin treatment than does HapA in human β-lymphocytes. The goal of the work detailed in Chapter 3 was to: 1) replicate and expand upon previous findings in β-lymphocytes and 2) determine which of the three amino acid coding changes is responsible for the difference in PERK activity between HapA and HapB. This work confirms that β-lymphocytes expressing HapB PERK show more eIF2α phosphorylation than those expressing HapA. Paradoxically, HapB PERK cells also show less phosphorylated PERK. These findings were echoed in mouse embryonic fibroblasts expressing PERK variant constructs. Further work exploring the functional differences between PERK variants is warranted.

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CHAPTER 1: INTRODUCTION

Progressive Supranuclear Palsy (PSP)

PSP is a devastating neurodegenerative disease that presents with gaze palsy, gait disturbance, difficulty swallowing and speaking, and dementia (Steele, Richardson, & Olszewski, 1964). Patient prognosis is usually poor, and affected individuals can expect to survive only a few years from initial diagnosis (dell'Aquila et al., 2013). There are currently no drug or surgical treatments that prevent disease onset or progression, and symptomatic treatment for PSP has minimal, sustained clinical efficacy (Bensimon et al., 2009; Chang & Weirich, 2014; Cotter, Armytage, & Crimmins, 2010; Frattali, Sonies, Chi-Fishman, & Litvan, 1999; Golbe, 2014). Thus, it is critical to identify drug targets for this disease to reduce the suffering of patients and their families.

PSP is part of a group of neurodegenerative diseases known as tauopathies, diseases characterized by intracellular aggregates of the protein tau in affected brain regions. Other diseases in this group include Alzheimer's disease (AD), corticobasal degeneration (CBD), Pick's disease, and some forms of frontotemporal dementia (FTD) (Kovacs, 2014). Though all of these diseases demonstrate similar tau pathology, the location of that pathology in the brain varies with each disorder's symptomatic presentation. For example, tau aggregates are most abundant in the frontal and temporal cortex of patients with FTD, and these patients present with difficulty in executive functioning and/or language processing (behavioral-variant FTD and primary progressive aphasia, respectively). Tau aggregates in PSP are primarily in the brainstem, the location of many nuclei that control eye movement, balance, and tongue and throat movements, and in the basal ganglia, the brain's general movement control center (Dickson, Kouri, Murray, & Josephs, 2011; Hauw et al., 1994; Irwin et al., 2014).

However, tauopathies present with a spectrum of pathology and clinical symptoms, and there is considerable overlap between them (Irwin et al., 2014; Kobylecki et al., 2015). This complicates diagnosis both pre- and postmortem, but also allows research into specific tauopathies to inform research about this class of neurodegenerative disorders in general. Thus, though PSP is relatively rare (Steele et al., 1964), investigation into its pathogenesis may be more widely applicable.

PSP tau pathology at its least severe appears in the subthalamic nucleus, substantia nigra, and globus pallidus. As pathological severity increases, tau pathology appears progressively in the posterior frontal lobe, dentate nucleus, pons, caudate and anterior frontal and parietal lobes. The more brain regions tau affects the shorter the duration of the disease. Sub classifications of PSP, including the classical Richardson's syndrome (RS), PSP-parkinsonism (PSP-P), and pure akinesia with gait freezing (PAGF) present with distinct pathological footprints in the brain, with the highest severity of tau pathology evident in RS and the lowest in PSP-P (Williams et al., 2007). The prognosis for RS is also significantly worse than for PSP-P (dell'Aquila et al., 2013). Thus, both distribution and severity of tau pathology dictate clinical presentation and disease duration.

Tau function and dysfunction in neurodegenerative disease

Microtubule-associated binding protein tau (MAPT) binds to tubulin and helps stabilize and facilitate assembly of microtubules (Hirokawa, 1994), regulate intracellular organelle transport and the attachment of motor proteins to tubulin (Ebneth et al., 1998; Sato-Harada, Okabe, Umeyama, Kanai, & Hirokawa, 1996; Trinczek, Ebneth, Mandelkow, & Mandelkow, 1999), and influence the structure of the neuron (reviewed in Shahani and Brandt 2002). Deletion of the *MAPT* gene produces relatively subtle

phenotypes in mouse models, suggesting some functional redundancy between tau and other MAPs (Dawson et al., 2001; Harada et al., 1994). In the brain, tau is abundantly expressed in axons (Binder, Frankfurter, & Rebhun, 1985), while expression in glial cells is more moderate (LoPresti, Szuchet, Papasozomenos, Zinkowski, & Binder, 1995). It is unclear whether tau's prominence as a pathological feature of so many neurodegenerative diseases is a result of a loss of function, a gain of toxic function, or some combination of the two (Shahani & Brandt, 2002).

Though it is a natively unfolded protein, many post-translational modifications (PTMs) can affect tau's conformation and function. Many of these modifications, especially phosphorylation, also serve as signposts of tau pathology (Iqbal, Liu, Gong, Alonso, & Grundke-Iqbal, 2009; Noble, Hanger, Miller, & Lovestone, 2013). Before it forms tangles, tau becomes "hyperphosphorylated" (Bancher et al., 1989) and thus more likely to aggregate. Though mature neurofibrillary tangles (NFTs) have long been recognized as the pathological hallmarks of tauopathy, it may be that these aggregates act as therapeutic sinks for hyperphosphorylated tau (htau). In this model, it is small accumulations of oligomeric htau that promote neurodegeneration, not NFTs (Brunden, Trojanowski, & Lee, 2008; Gerson et al., 2014; Iqbal et al., 2009).

Recent work supports the hypothesis that abnormal tau can move from one cell to an adjacent cell and "seed" new tau aggregation. The seeds potentially initiate aggregate formation by acting through a prion-like mechanism, recruiting soluble tau into the tangle seed (Clavaguera et al., 2009; Goedert, Clavaguera, & Tolnay, 2010; Guo & Lee, 2011; Sanders et al., 2014). This theory is supported by the observation that brain homogenates from various tauopathies can induce tangle formation when they are injected into mouse brain (Clavaguera et al., 2013). In AD, the cell non-autonomous model is consistent with the widely accepted hypothesis that tau pathology initiates in

the entorhinal cortex, and then spreads to the hippocampus and neocortex (Braak et al. 2006; Braak and Braak 1991). This non-autonomous mechanism may also occur in PSP, in which tau pathology initiates in the brainstem.

Genetics of PSP

There is considerable phenotypic variability in the clinical presentation of PSP (Respondek et al., 2014), and efforts to treat it could be hindered by the difficulty of early diagnosis. As with many neurodegenerative diseases, the symptoms that prompt a patient to seek diagnosis and treatment may come too late for effective intervention, even once such interventions are available. Thus, it will be imperative, moving forward, to identify not only the earliest symptoms of disease, but also to screen people prospectively for risk factors. This screening could be both physiological, as in biomarker analysis, and genetic.

Some mutations in the gene *MAPT*, which codes for tau, result in a PSP phenotype (Poorkaj et al., 2002; Rademakers et al., 2005; Stanford et al., 2000), further establishing tau's role as a crucial player in PSP pathogenesis. A common inversion haplotype in *MAPT* also increases risk for developing PSP (Baker et al., 1999). This haplotype, called H1, represents an inversion of several genes in the vicinity of *MAPT* on chromosome 17, including *Saitohin*, *NSF*, *IMP5*, *CRHR1*, and *LOC284058* (Pittman, 2005; Stefansson et al., 2005). The alternative haplotype, H2, is present almost exclusively in Europeans and may be protective against PSP (Pau Pastor et al., 2004). Further work is needed to determine why H1 confers PSP risk, though the likely origin is *MAPT* itself (Pittman, 2005).

Alternative splicing of exons 2, 3, and 10 of *MAPT* result in six different tau isoforms, each containing either one or two acidic inserts at the N-terminal end of tau

and three or four microtubule (MT) binding repeats near the C-terminal end of tau (3R tau and 4R tau; Gendron and Petrucelli 2009). The difference in number of MT binding repeats affects binding affinity; these tau isoforms compete for the same MT binding sites, and 4R tau can displace MT-bound 3R tau (M. Lu & Kosik, 2001). Pathological tau aggregates in PSP are composed only of 4R tau, whereas similar aggregates in Pick's disease are composed of 3R tau (Buee & Delacourte, 1999). NFTs in AD are a mix of 3R and 4R tau (Togo et al., 2004). This indicates genetic variants influencing tau expression and splicing may be critical in determining an individual's risk of developing a particular tauopathy (Pittman, 2005).

Schellenberg and colleagues recently completed a genome-wide association screen (GWAS) for additional PSP risk loci (Höglinger et al., 2011). GWASs compare common single nucleotide polymorphisms (SNPs) between affected and unaffected individuals for a particular disease or trait. "Hit" SNPs act as signposts indicating a risk-conferring genetic feature is nearby, and the actual risk locus is likely in strong linkage disequilibrium (LD) with that hit SNP. The PSP GWAS identified several genes that contribute to PSP risk, including *STX6* (encoding syntaxin 6), *MOBP* (encoding myelin-associated oligodendrocyte basic protein), and *EIF2AK3* (encoding eukaryotic translation initiation factor 2 alpha kinase 3, also called PERK). PERK is an ER membrane protein that acts as a stress sensor in the ER unfolded protein response (UPR).

PERK and the Unfolded Protein Response (UPR)

In addition to PERK, there are two other UPR stress sensors (both of which are also ER membrane proteins): inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6; Ron and Walter 2007). All arms of the UPR activate when

the chaperone BiP, normally bound on the luminal side of each protein, dissociates in order to aid in the re-folding of accumulated misfolded proteins in the ER lumen. This facilitates translocation and cleavage of ATF6 and exposes a phosphorylation site on both PERK and IRE1α (Scheper & Hoozemans, 2009). Each of the three branches of the UPR initiates discrete signaling cascades in response to the accumulation of mis- or unfolded proteins in the ER lumen. These signaling cascades can be both protective and destructive to the cell. Though the protective role of the UPR is to restore homeostasis by attenuating translation, promoting ER-associated degradation (ERAD; Travers et al. 2000), and upregulating chaperone production (Kozutsumi, Segal, Normington, Gething, & Sambrook, 1988; Matus, Glimcher, & Hetz, 2011), prolonged ER stress can trigger apoptosis (Rutkowski et al., 2006; Urra, Dufey, Lisbona, Rojas-Rivera, & Hetz, 2013).

The PERK arm of the UPR acts primarily on translation. When PERK is activated (thus becoming phosphorylated PERK, or pPERK), a kinase domain on the cytosolic side of the protein phosphorylates eukaryotic translation initiation factor 2α (eIF2α). peIF2α then acts at the ribosome to slow down general translation initiation and promote translation of ATF4. ATF4 promotes the transcription of genes that enhance import of amino acids and protect against oxidative stress (Harding et al., 2003). However, ATF4 also increases expression of CHOP, a transcription factor that promotes both dephosphorylation of eIF2α (via the phosphatase GADD34) and apoptosis (Tabas & Ron, 2011).

Elements of the PERK pathway are also involved in another important cellular response to misfolded protein accumulation: autophagy (Rouschop et al., 2010; Yorimitsu, Nair, Yang, & Klionsky, 2006). UPR activation induces formation of autophagosome-like structures that engulf portions of the ER itself, potentially acting as a negative regulator of ER expansion during stress (Bernales, McDonald, & Walter,

2006). More specifically, PERK activation significantly upregulates transcription of autophagy receptor genes (Deegan et al., 2015). This upregulation of autophagy in response to ER stress may help restore normal cell functioning and promote cell survival (Ogata et al., 2006).

Genetic variation that either alters the PERK protein or regulates the amount of this protein in the cell could perturb these crucial stress-response pathways and contribute to disease pathogenesis or progression in PSP and other neurodegenerative diseases. A developing body of literature supports PERK's role as an important player in neurodegeneration and suggests that further study of PERK genetics and protein function could yield important insights into potential treatment options for these diseases (Hetz & Mollereau, 2014).

The function and dysfunction of the UPR in disease

Previous work showed that the UPR is activated in post-mortem brains from AD (Jeroen J M Hoozemans et al., 2009), Parkinson's disease (J J M Hoozemans et al., 2007), amyotrophic lateral sclerosis (Atkin et al., 2008; Wang, Popko, & Roos, 2010), frontotemporal dementia (D. A. T. Nijholt, van Haastert, Rozemuller, Scheper, & Hoozemans, 2012) and multiple system atrophy (Makioka et al., 2010) patients. The UPR is also activated in PSP (Stutzbach et al., 2013, see Chapter 2) All of these disorders, PSP included, are characterized by pathological aggregates of misfolded proteins in the brain. The UPR tends to be activated in cells with early-stage staining for neuropathological proteins (usually a diffuse staining pattern) rather than full-blown tangles or inclusions, suggesting that activation of the UPR is an early event in disease (Jeroen J M Hoozemans et al., 2009; Makioka et al., 2010; D. A. T. Nijholt et al., 2012).

Activation of the UPR generally and PERK specifically could influence neurodegeneration in several ways. First, it is important to note that tau aggregates are primarily cytoplasmic and do not generally traffic through the ER (Congdon & Duff, 2008). This means that misfolded tau is not directly triggering the UPR via accumulation in the ER lumen. However, large protein aggregates or small, toxic accumulations of htau oligomers could interfere with cytoplasmic components of the UPR signaling machinery, particularly endoplasmic reticulum-associated degradation (ERAD) and autophagy. A "backlog" of cytoplasmic proteins targeted for degradation could interfere with the normal degradation of misfolded proteins exported from the ER and could impede upregulation of protein degradation (Abisambra et al., 2013). This downstream roadblock could initiate prolonged ER stress and bias the cell toward apoptosis (Urra et al., 2013).

Another way PERK activity may influence neurodegeneration is *via* translational inhibition. Though this global decrease in protein translation may be helpful in the short term, prolonged lack of protein synthesis is ultimately detrimental. Work from Moreno et al. (2012) showed that prion infected mice demonstrated prolonged phosphorylation of eIF2α along with synaptic deficits and neurodegeneration. However, overexpression of GADD34, an eIF2α phosphatase (Ron & Walter, 2007), restored translation and rescued several prion disease phenotypes, increasing survival. Conversely, treatment with salubrinal, an inhibitor of eIF2α phosphatases (Boyce et al., 2005), exacerbated neuron loss and decreased survival. This could indicate that translational repression mediated by sustained eIF2α phosphorylation interferes with normal neuronal function and may bias the cell toward degeneration.

Though moderate PERK depletion might prove beneficial in the case of neurodegeneration, complete loss of PERK could be detrimental. The developmental

disorder Wolcott-Rallison syndrome results from PERK insufficiency (Delépine et al., 2000). Wolcott-Rollin presents with skeletal dysplasia, growth retardation, and diabetes mellitus (Stöss, Pesch, Pontz, Otten, & Spranger, 1982). PERK knockout in a mouse model results in a similar phenotype (Peichuan Zhang et al., 2002). Thus, any treatment targeting PERK would need to modulate rather than eliminate its activity. Indeed, a follow-up study from Moreno et al.(2013) showed that though treatment with a selective PERK inhibitor rescued several disease phenotypes in prion-infected mice, it also increased glucose levels and resulted in a 20% decrease in body weight.

Phosphorylation of eIF2α also plays a role in synaptic plasticity and memory (Hetz & Mollereau, 2014). Conditional PERK knockout in an APP-PS1 mouse line rescues Aβ-related defects in spatial memory and long-term potentiation (LTP; Ma et al. 2013). The effect is similar for knockout of GCN2, another eIF2α kinase. Without the de novo protein synthesis that is largely blocked by prolonged eIF2α phosphorylation, memory formation and consolidation are severely impaired. Thus, stress conditions in the cell that continuously trigger eIF2α kinases like PERK and GCN2 may be partially responsible for memory-related symptoms of neurodegeneration. Likewise, any alterations to PERK that increase its eIF2α kinase activity could directly contribute to neurodegenerative disease pathogenesis or progression.

Recent work from Liu et al. (2012) demonstrated that there are two common PERK coding haplotypes, designated Haplotype A (HapA; more common) and Haplotype B (HapB; less common). In human β-lymphocytes, cells homozygously expressing HapB demonstrated significantly more eIF2α phosphorylation in response to treatment with the ER stress-inducing drug thapsigargin (Rogers, Inesi, Wade, & Lederer, 1995) than did cells expressing HapA. Interestingly, the three SNPs that confer

the coding differences between HapA and HapB are in strong LD with the PSP GWAS SNP giving the strongest signal in the PERK gene (Höglinger et al., 2011; Stutzbach et al., 2013). This presents a potential pathogenic mechanism for PERK's involvement in PSP and is the focus of this thesis.

Novel questions addressed by this thesis

Is PERK activated in disease-affected brain regions in PSP?

There is evidence of UPR activation in Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis, multiple system atrophy, and in the hippocampus and substantia nigra of PSP subjects (Atkin et al., 2008; J J M Hoozemans et al., 2007; Jeroen J M Hoozemans et al., 2009; Makioka et al., 2010; D. A. T. Nijholt et al., 2012; Wang et al., 2010). In Chapter 2 of this thesis, I evaluate PERK activation in the pons, medulla, midbrain, hippocampus, frontal cortex and cerebellum in subjects with PSP, AD, and in normal controls. UPR activation may be an early event in neurodegenerative pathogenesis (Jeroen J M Hoozemans et al., 2009). Therefore, I also determine whether this activation co-localizes with pre-tangle tau pathology, both in disease and normal controls.

Are PERK variants associated with PSP?

Schellenberg and colleagues recently completed a genome-wide association screen (GWAS) for PSP risk loci (Höglinger et al., 2011). One of the genes identified was eukaryotic translation initiation factor 2 alpha kinase 3 (*EIF2AK3* or PERK). The

SNP identified in the PSP GWAS is in strong LD with two common PERK haplotypes that differ at three amino acids: S136-R166-A704 (low risk allele, called HapA) or C136-Q166-S704 (higher risk allele, called HapB). In the second part of Chapter 2, I examine whether these PERK coding haplotypes are associated with PSP risk.

How do variations in the PERK protein affect activity?

These coding changes are potentially functional variants of PERK and may affect its role in the UPR. Work from Liu et al. (2012) showed that HapB PERK has a stronger response to drug-induced ER stress than HapA PERK. Also, at least one of the three coding changes, S136C (rs867529), is predicted by several methods to be a deleterious substitution for PERK (Burke et al., 2007). Thus, the goal of the second part of my thesis is to replicate the findings of Liu et al. (2012) and to determine which of the three amino acid variations that comprise the PERK haplotypes are responsible for this functional difference. To do this, I first examine phosphorylation of PERK and eIF2α in human β-lymphocytes. I then express artificial PERK variant constructs with single amino acid alterations in mouse embryonic fibroblasts (MEFs) derived from PERK null mice and assess phosphorylation of PERK and eIF2α as well as ATF4.

CHAPTER 2: THE UNFOLDED PROTEIN RESPONSE IS ACTIVATED IN DISEASE-AFFECTED BRAIN REGIONS IN PROGRESSIVE SUPRANUCLEAR PALSY AND ALZHEIMER'S DISEASE

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ABSTRACT

Background: Progressive supranuclear palsy (PSP) is a neurodegenerative disorder pathologically characterized by intracellular tangles of hyperphosphorylated tau protein distributed throughout the neocortex, basal ganglia, and brainstem. A genome-wide association study identified *EIF2AK3* as a risk factor for PSP. *EIF2AK3* encodes PERK, part of the endoplasmic reticulum's (ER) unfolded protein response (UPR). PERK is an ER membrane protein that senses unfolded protein accumulation within the ER lumen. Recently, several groups noted UPR activation in Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis, multiple system atrophy, and in the hippocampus and substantia nigra of PSP subjects. Here, we evaluate UPR *PERK* activation in the pons, medulla, midbrain, hippocampus, frontal cortex and cerebellum in subjects with PSP, AD, and in normal controls. **Results:** We found UPR activation primarily in disease-affected brain regions in both disorders. In PSP, the UPR was primarily activated in the pons and medulla and to a much lesser extent in the hippocampus. In AD, the UPR was extensively activated in the hippocampus. We also observed UPR activation in the hippocampus of some elderly normal controls, severity of which positively correlated with both age and tau pathology but not with Aβ plaque burden. Finally, we evaluated *EIF2AK3* coding variants that influence PERK activation. We show that a haplotype associated with increased PERK activation is genetically associated with increased PSP risk. **Conclusions:** The UPR is activated in disease affected regions in PSP and the genetic evidence shows that this activation increases risk for PSP and is not a protective response.

Keywords: Progressive supranuclear palsy; PERK; Unfolded protein response; EIF2AK3; Alzheimer's disease

INTRODUCTION

Progressive supranuclear palsy (PSP) is a late-onset neurodegenerative movement disorder clinically characterized by vertical gaze palsy, poor balance and frequent falls, as well as cognitive impairment and dementia (Litvan, 1998; Steele et al., 1964). The primary symptoms of PSP are consistent with the observed neuropathology, mainly neuronal degeneration in the brainstem, particularly the pons and medulla (Dickson, Rademakers, & Hutton, 2007). Postmortem pathological analysis of these brain regions in PSP patients reveals numerous intracellular neurofibrillary and glial tangles comprised of hyperphosphorylated protein tau (htau). Thus PSP, along with Alzheimer's disease (AD), belongs to a group of disorders collectively known as tauopathies, as all these disorders show abundant tau aggregates or inclusions as prominent neuropathologic features. Other tauopathies include frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), corticobasal degeneration (CBD), and Pick's disease (Ballatore, Lee, & Trojanowski, 2007). Some mutations in the gene *MAPT*, which encodes tau, can result in a PSP phenotype (P Pastor et al., 2001; Poorkaj et al., 2002; Ros et al., 2005; Rossi et al., 2004; Stanford et al., 2000), while common variants in the *MAPT* region are associated with PSP susceptibility (Baker et al., 1999; Conrad et al., 1997; Cruchaga et al., 2009; Höglinger et al., 2011). Thus, genetic studies as well as our data here indicate that tau is clearly linked to PSP pathogenesis.

Schellenberg and colleagues recently completed a genome-wide association study (GWAS) for PSP risk loci (Höglinger et al., 2011). One of the genes identified was eukaryotic translation initiation factor 2 alpha kinase 3 (*EIF2AK3*), which encodes the protein pancreatic endoplasmic reticulum kinase (PERK). PERK is an endoplasmic reticulum (ER) membrane protein that acts as a stress sensor in the ER unfolded protein response (UPR). In addition to PERK, there are two other stress sensors (both of which

are also ER membrane proteins): inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6; Ron & Walter, 2007).

All three arms of the UPR activate when the chaperone immunoglobulin binding protein (BiP), normally bound on the luminal side of each protein, dissociates in order to aid in the folding of accumulated unfolded proteins in the ER lumen. Dissociation of BiP from PERK and IRE1α facilitates their activation by promoting homodimerization and *trans*-autophosphorylation (Walter & Ron, 2011). ATF6 is then activated *via* a cleavage event and subsequently translocated from the ER to the nucleus (Scheper & Hoozemans, 2009). Each of the three branches of the UPR initiates discrete signaling cascades in response to the accumulation of unfolded proteins in the ER lumen. The role of the UPR is to restore protein homeostasis by upregulating chaperone production (Kozutsumi et al., 1988; Matus et al., 2011), attenuating translation, promoting degradation of misfolded proteins *via* ER-associated degradation (ERAD; Travers et al., 2000), and promoting autophagy (D. a T. Nijholt et al., 2011). Prolonged ER stress can trigger apoptosis (Ron & Walter, 2007; Rutkowski et al., 2006).

The PERK arm of the UPR acts primarily on translation. When PERK is activated (thus becoming phosphorylated PERK, or pPERK), a kinase domain on the cytosolic side of the protein phosphorylates eukaryotic translation initiation factor 2α (eIF2α or peIF2α when phosphorylated). peIF2α is a less active form of the protein, and its decreased efficiency slows general translation initiation and promotes translation of activating transcription factor 4 (ATF4). ATF4 promotes transcription of genes that enhance amino acid uptake and protect against oxidative stress (Harding et al., 2003). Elements of the PERK pathway are also involved in regulating autophagy, a process that degrades misfolded proteins (Bernales et al., 2006; Rouschop et al., 2010). Thus, genetic variation that results either in alteration of PERK protein function or significant

changes in the amount of PERK would perturb several crucial stress-response pathways.

Several neurodegenerative disorders, including PSP, are characterized by pathological aggregates of misfolded proteins in the brain. Previous work showed that the UPR is activated in post-mortem AD brains (Jeroen J M Hoozemans et al., 2009), as well as in the brains of patients with frontotemporal lobar degeneration with tau inclusions (FTLD-tau) (D. A. T. Nijholt et al., 2012), PD (J J M Hoozemans et al., 2007), amyotrophic lateral sclerosis (ALS; Atkin et al., 2008; Wang et al., 2010), and multiple system atrophy (MSA; Makioka et al., 2010). Nijholt *et al*. (2011) reported evidence of UPR activation in the hippocampus and, to a lesser extent, the *locus ceruleus* and putamen of PSP patients.

We investigated activation of PERK and eIF2α in postmortem brains from subjects with PSP and AD, as well as from normal elderly subjects. We used antibodies that recognize the phosphorylated species of PERK and eIF2α, *i.e.* the activated forms of these 2 proteins (pPERK and peIF2α, respectively). Our primary goal was to investigate the brain regions most affected by tau pathology in PSP. We searched for evidence of PERK and eIF2α activation in the pons, medulla and midbrain, regions affected in PSP, in the hippocampus and frontal cortex, which are regions affected in AD, and in the cerebellum, a brain region which is relatively spared in both diseases, although the deep cerebellar nuclei and cerebellar cortex may harbor modest amounts of tangles and plaques, in PSP and AD, respectively. We also looked at PERK and eIF2α activation in young controls to determine if ER stress is activated in normal aging. Our results indicated that PERK and eIF2α activation parallels the pattern of neuropathology in PSP and AD. In normal hippocampus, activation increases with age and correlates with tau but not Aβ amyloid pathology. We also examined coding haplotypes that were

previously shown to affect PERK activation (Liu et al., 2012). We found that the haplotype that corresponds to the highest PERK activation is in linkage disequilibrium (LD) with the high risk allele of the top PSP GWAS marker, indicating that UPR activation increases PSP risk and is not a protective response in PSP.

MATERIALS AND METHODS

Human Tissue

We obtained postmortem human pons, medulla, midbrain, frontal cortex, hippocampus, and cerebellum samples from the Center for Neurodegenerative Disease Research (CNDR; University of Pennsylvania School of Medicine, Philadelphia, PA) using the CNDR Integrated Neurodegenerative Disease Database (Xie et al., 2011) and from the Michigan Alzheimer's Disease Research Center Brain Bank (MADRC; University of Michigan, Ann Arbor, MI). We chose PSP and AD cases for lack of co-morbid diagnoses and availability of fixed tissue-- PSP and AD cases with a secondary neuropathological diagnosis (NPDx; for instance, PD) were excluded from the present study. All PSP and AD cases were evaluated by a neurologist pre-mortem and referred to the CNDR or MADRC brain donation programs, where a neuropathologist made a NPDx according to established criteria (Hyman & Trojanowski, 1997; Litvan et al., 1996). Controls had no clinical history or postmortem diagnosis of a neurodegenerative disease. One control displayed a moderate amount of Lewy body pathology in the medulla and another displayed a mild amount of tau deposition in the midbrain (though not in the substantia nigra). All control cases were free of Lewy bodies in the hippocampus. We age-matched all cases and controls (See Table 2.1 and Table 2.S1 for demographic information). Tissue used for immunohistochemical and immunofluorescence studies was fixed in either ethanol (70%) or 10% neutral buffered

formalin overnight and then processed for paraffin embedding and 6μm thick sections were generated as described (Heiko Braak et al., 2006) using a Leitz 1512 microtome. The average age of PSP, AD, and normal controls was approximately 75 years. Average disease duration for PSP patients was 6.7 years, while the average duration for AD patients was 10.8 years. The average post-mortem interval (PMI) for all cases was 10.2 hours (Table 2.1, Table 2.S1). Pontine sections included the locus coeruleus and surrounding tegmentum, midbrain sections included the substantia nigra, medulla sections included the olivary nucleus, hippocampal sections included the CA and dentate regions, frontal cortex sections included both white and gray matter, and cerebellar sections included the folia.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Arnold et al., 2010; Lippa et al., 2009). We deparaffinized brain sections on slides using xylene (Mallinckrodt Baker Inc., Phillipsburg, NJ), and then hydrated them through a series of ethanol washes, and quenched endogenous peroxidases by immersing sections in a mixture of hydrogen peroxide and methanol. Following a wash in running water, we performed antigen retrieval by microwaving sections immersed in citrate buffer (Thermo Shandon Limited, Astmoor, WA). We then washed sections in 0.1M Tris (pH 7.6; Fisher Scientific), blocked in 0.1M Tris (pH 7.6)/2% fetal bovine serum (FBS), and applied primary antibody (incubated overnight at 4^{o}). This wash/block procedure was identical for secondary antibody application, with an incubation time of one hr. Following another wash, we applied avidin/biotin complex (Vector Labs) to each section and incubated the sections for one hr. Finally, we developed sections with DAB chromagen (Biogenex), counterstained with hematoxylin, dehydrated through a series of ethanol and xylene

washes. Cover slips were sealed with Cytoseal (Richard Allen Scientific, Kalamazoo, MI). Antibodies used are listed in Table 2.2.

Immunofluorescence (IF)

We deparaffinized, hydrated, quenched, and performed antigen retrieval on slide-mounted sections as described above. We then blocked sections in 0.1M Tris/2% FBS, and applied mouse and rabbit primary antibodies (diluted in 0.1M Tris/2% FBS). Primary antibody incubation time was 2 hr at room temperature. Following a wash in 0.1M Tris and transfer to a "dark" chamber, we applied secondary antibodies (goat-anti-mouse and goat-anti-rabbit; Vector Labs) and let sections incubate for another two hours at room temperature. We then washed the sections again and applied 0.3% Sudan Black in 70% ethanol (Romijn et al., 1999) for five min to quench endogenous lipofuscin related flourophores. After another wash, the sections were coverslipped using Vectashield (with DAPI; Vector Labs; Uryu et al., 2003).

Slide Scoring and Analysis

pPERK and peIF2 antibodies both stained cells in a characteristic punctate pattern (Fig. 2.1a and 2.2a; (J J M Hoozemans et al., 2007; Jeroen J M Hoozemans et al., 2009). We scored each tissue section for pPERK or peIF2α IHC staining according to the following scale: *negative (-)*: no cells stained, *rare (R)*: 1-3 cells stained, +: 4-20 cells stained, ++: 20+ cells stained, could have diffuse distribution of stained cells, may have high density of stained cells in some fields of the section, +++: high density of stained cells in almost every field of the section. A second rater confirmed scores in 20% of randomly selected slides (see Fig. 2.S1). For double IF of hyperphosphorylated tau (htau) and pPERK, we visualized and photographed 10 fields per section and manually counted the number of htau positive cells, the number of pPERK positive cells, and the number of cells positive

for both pPERK and htau. We scored all sections blind to disease group on an Olympus CHBS microscope (IHC) or an Olympus BX60 Transmitted-Reflected Light Microscope with BF/DF/DIC/Polarized Light and a SPOT RT Color digital microscope camera (IF).

Statistical analysis

We used Spearman correlations to examine correlations between level of tau pathology vs. pPERK staining and age vs. pPERK staining, Fisher's exact test to examine association between disease condition and pPERK/peIF2α staining, Chi Square to examine sex distribution among disease/normal groups, ANOVA to examine the mean difference among disease/normal groups for average age at death and post-mortem interval, and a Student's t-test to examine the mean difference between disease groups for average age of onset. All statistical analyses were two-sided. Statistical significance was set at the 0.05 level unless otherwise indicated.

Analysis of linkage disequilibrium around rs7571971*.*

In a recent GWAS for PSP risk loci (Höglinger et al., 2011), a significant association was established between PSP risk and rs7571971. This SNP falls in an intron of *EIF2AK3*, the gene encoding PERK. While it is reasonable to assume the SNP somehow affects risk for PSP by affecting expression of *EIF2AK3*, it remains to be proven. To garner genetic evidence for this hypothesis, we first evaluated the pattern LD in sequence data from the 1000 Genomes project (Abecasis et al., 2012) and pairwise LD evaluated using SNAP (Suite of Nucleotide Analysis Programs, Johnson et al., 2008). Based on these results, we genotyped 1043 PSP patients using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) for the following four SNPs: rs7571971, rs867529 (S136C), rs1805165 (S704A), and rs13045 (R166Q; Table 2.3). All cases were autopsied and had a neuropathologic diagnosis of PSP (Hauw et al., 1994). Genotyping

was done according to manufacturer's protocol. PCR conditions were as follows: 95°C for 10 minutes, then 50 cycles of 92°C for 15 seconds, 60°C for 1 minute, 4°C for 2 minutes. Genotypes were visualized and called using a 7900HT Fast Real Time PCR System and the allelic discrimination function of Sequence Detection System V.2.4 (Applied Biosystems, Foster City, CA). Finally, we phased the resulting four-SNP genotypes using eHap software (Seltman, Roeder, & Devlin, 2003), which provides maximum likelihood estimates of haplotype frequencies.

RESULTS

The PERK arm of the UPR is activated in PSP

To determine whether the UPR is activated in PSP, we stained post-mortem human brain tissue from PSP and AD patients as well as normal elderly controls using antibodies against pPERK and peIF2α, the activated forms of these proteins. We chose six brain areas to stain for PERK and eIF2α activation: the pons, medulla, and midbrain (affected in PSP), the hippocampus and frontal cortex (affected in AD), and the cerebellum, which is relatively spared in both diseases.

In PSP cases, of the regions tested, the pons, medulla, and midbrain demonstrated the highest degree of pPERK and peIF2α staining (Fig. 2.1b, Fig. 2.2b, Fig. 2.3a-c, and Fig. 2.4a-c) as measured by number of cells showing staining per field (Fig. 2.S1). These are the brain areas most affected by tau pathology in PSP. pPERK and peIF2α staining was punctate and cytoplasmic with some non-specific nuclear staining (Fig. 2.1a and Fig. 2.2a), a pattern observed by others in AD and PD (J J M Hoozemans et al., 2007; Jeroen J M Hoozemans et al., 2009). In the pons, all PSP cases showed some cells positive for both pPERK and peIF2α. pPERK was observed in the medulla and midbrain in all but one case for each region. For peIF2α, all cases

showed positive cells in the medulla and all but one case showed positive cells in the midbrain.

PSP cases as a group showed significantly more pPERK and peIF2α staining in the pons, medulla, and midbrain compared to elderly controls. For pPERK, only one control subject (age 63) showed "rare" positive cells in the pons and medulla. This is not the same control subject that displayed Lewy body pathology in the medulla. In the midbrain, very few controls were positive for pPERK. For peIF2α, most controls were negative in these brain areas except for a single subject with rare positive cells in the medulla. For AD, there were more positive cases with a higher density of positive cells compared to controls but less than found in PSP (Fig. 2.3 and 2.4, a-c, Table 2.3).

In the hippocampus and frontal cortex, AD cases as a group scored significantly higher than PSP or normal elderly controls for both pPERK and peIF2α staining (Table 2.3). pPERK and peIF2α staining was especially strong in the AD hippocampus, with nearly all cases demonstrating high levels of positive cells. All PSP cases had mild to moderate pPERK staining in the hippocampus, though not all cases demonstrated peIF2α staining. Surprisingly, many normal elderly controls demonstrated at least a mild level of pPERK and peIF2α positive cells in the hippocampus (Fig. 2.1c, Fig.2.2c, Fig. 2.3d, and Fig. 2.4d). Staining was generally milder in the frontal cortex than in the hippocampus, although AD cases still scored significantly higher than PSP cases or normal controls (Fig. 2.3e and Fig. 2.4e). PSP cases scored significantly higher than normal controls for pPERK staining but not for peIF2α staining (Table 2.3). Notably, the pons, medulla, and midbrain are severely affected in PSP [2] but only moderately or mildly affected in AD (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). Conversely, the hippocampus and frontal cortex are strongly affected in AD (Serrano-Pozo et al., 2011),

but only mildly affected or unaffected in PSP. Thus, PERK activation is strongest in areas of the brain highly affected by pathology in PSP and AD. Nearly all cases were negative for pPERK and peIF2α in the folia of the cerebellum (Fig. 2.1d, Fig. 2.2d, Fig. 2.3f, and Fig. 2.4f), although one AD case showed rare staining in this area. Regardless, there is generally little to no pathology in this area in PSP or AD, and thus our findings are consistent with the inference that pathology and PERK activation occur in the same disease-affected brain areas.

Activation of pPERK in hTau Positive Cells

We were interested in whether the UPR is activated in cells affected by tau pathology. We performed double immunofluorescence staining for pPERK and htau on sections of pons and hippocampus in PSP, AD, and normal controls (Fig. 2.5a). In PSP pons, an average of 72% of pPERK positive cells were also positive for htau. However, only 43% of htau positive cells were also positive for pPERK (Fig. 2.3c). This substantial overlap is in contrast to AD hippocampus, in which only 20% of pPERK positive cells also stained for htau and only 12% of htau positive cells stained for pPERK (Fig. 2.3d). Overlap between htau and pPERK staining was also low in PSP and normal hippocampus (data not shown). In the pons, overlap between pPERK puncta and htau occurred mostly in cells with diffuse, cytoplasmic htau staining rather than dense, fibrillar staining (Fig. 2.3b). This suggests that PERK is activated in pre-tangle neurons. Hoozemans et al. (2009) described similar distribution of htau/pPERK staining in AD hippocampus.

PERK is activated in normal hippocampus

Unexpectedly, we found pPERK and peIF2α staining in the hippocampus of age-matched elderly normal controls as described above. To follow up on this finding, we expanded our initial control hippocampus sample to include cases with a wide range of

ages (range: 16-92, mean: 52.4; see Table 2.S1). We found that age significantly correlated with the pPERK staining score (Fig. 2.6a). The older the subject, the more likely they were to have high levels of PERK activation in the hippocampus. However, not all aged normal controls demonstrated hippocampal pPERK activation although some subjects at all ages examined here were negative for pPERK staining.

We also found that the level of tau pathology correlated with pPERK staining. The more tau pathology (as measured by PHF-1 staining) in a normal hippocampus, the more likely that the hippocampus was also positive for activated PERK (Fig. 2.6b). All controls negative for pPERK staining were also negative for htau staining; cases with severe pPERK staining scores also scored high for htau. This correlation was significant (Spearman R: 0.7523, p = .0002). There was no correlation between pPERK staining in the hippocampus and Aβ amyloid plaque pathology (as measured by Thioflavin S staining to detect senile plaques); all normals with high pPERK scores and relatively high tau scores in the hippocampus were negative for Aβ amyloid plaques and Lewy bodies (data not shown).

PERK protein coding variants are associated with PSP risk

Alleles at rs7571971 are significantly associated with PSP risk [10]. To identify other SNPs in high linkage disequilibrium with rs7571971, we evaluated 1000 Genomes data for subjects of European ancestry. As assessed by LD measure r^2 (Devlin & Risch, 1995), 14 SNPs were in high LD with rs7571971 ($r^2 > 0.8$), including the 3 non-synonymous coding variants. Of these 14, none fell in the coding region of any gene besides *EIF2AK3* and all but 5 fell within *EIF2AK3* (Table 2.5).

The 3 non-synonymous coding variants in *EIF2AK3* were Ser136Cys, Arg166Gln, and Ser704Ala. When haplotypes were constructed from 1000 Genome

data, there were two common haplotypes Ser-Arg-Ser (haplotype A) and Cys-Gln-Ala (haplotype B) with predicted frequencies of 0.64 and 0.29, respectively; one uncommon haplotype, Ser-Gln-Ser (haplotype D), with a frequency of 0.06; and 4 rare haplotypes of frequency close to 1/1000 . The top PSP GWAS SNP for this gene is rs7571971, a 2-allele polymorphism in *EIF2AK3* intron 2 with a minor allele frequency of 0.25-0.28 (Höglinger et al., 2011). From the 1000 genome analysis, the minor allele for rs7571971 is almost perfectly correlated with haplotype B and the major allele with haplotypes A and D.

To confirm the relationship of LD amongst SNP alleles in PSP subjects, we genotyped 1,043 PSP cases for rs7571971, and the 3 coding variant SNPs. The genotypes for these four SNPs were then phased using maximum likelihood. We observed that, in PSP cases, haplotype frequencies were almost identical to those from 1000 Genomes data: for A, 0.645 versus 0.642; for B, 0.288 versus 0.301; and for D, 0.061 versus 0.053. Again haplotypes A and D are completely correlated with rs7571971 allele C (Fig. 2.7), the protective PSP allele. Haplotype B is completely correlated with allele T, the high risk PSP allele. Recently Liu et al (2012) showed that when lymphoblastoid cell lines are treated with thapsigarin to induce ER stress, cells homozygous for the B haplotype showed stronger activation than cells homozygous for the A haplotype. Thus B is the high-risk haplotype for PSP suggesting that activation is not a protective response, but rather increases risk for PSP.

DISCUSSION

We found that PERK is activated in disease-affected brain regions in PSP, including the pons, medulla, and midbrain. We also found that PERK's downstream effector, eIF2α, is activated similarly in PSP brainstem areas. In contrast, PERK and eIF2 are not activated or only weakly activated in normal and AD brainstem,

respectively. We confirmed that AD cases have strong immunoreactivity for pPERK and peIF2α in the pyramidal cells of the hippocampus (Jeroen J M Hoozemans et al., 2009) and in the frontal cortex. In contrast, PSP cases show mild to moderate pPERK staining in these regions (D. A. T. Nijholt et al., 2012). PERK and eIF2α were not activated in the cerebellum in either disease. In AD and PSP, the pattern of UPR activation parallels the regional distribution of pathology in these two disorders.

We explored the relationship between abnormal tau deposits and UPR activation in the PSP brainstem. Although there is some overlap between cells with activated PERK and cells with htau, at least half of htau-positive cells do not have concurrent PERK activation. A greater proportion of pPERK positive cells were also positive for tau, but 25% stained for pPERK alone. Thus, although PERK is activated in brain regions highly affected by tau pathology, htau and pPERK do not necessarily overlap at the single cell level. One potential explanation for lack of complete overlap may be that PERK activation precedes tangle formation, and is no longer activated in cells with mature tangles. We found that overlap between pPERK and htau mostly occurred in cells with diffuse htau staining rather than dense tau staining, supporting this hypothesis. Similarly, Hoozemans *et al*. (2009) found that cells in the AD hippocampus that were positive for pPERK also stained for markers of early tau aggregation (Jeroen J M Hoozemans et al., 2009). This evidence suggests that PERK activation may temporally precede overt tau aggregation, and could be triggered by immunohistochemically undetectable levels of abnormal tau. The genetic data implicating both PERK and tau in PSP supports a plausible temporal relationship between PERK activation and tau aggregation.

Genetic findings (Höglinger et al., 2011) and the data presented here implicate PERK as well as the UPR in the pathogenesis of PSP. Genetic findings also associate *MAPT* with PSP (Höglinger et al., 2011; Poorkaj et al., 2002; Rademakers et al., 2005;

Tsuboi, Josephs, Cookson, & Dickson, 2003), and along with the presence of aggregated tau as the key neuropathologic feature of PSP, these data clearly establish that tau is intimately linked to PSP pathogenesis. While the UPR is activated by misfolded proteins within the ER, and aggregated misfolded tau occurs in PSP, AD, and other tauopathies, tau is a cytosolic protein and does not appear to traffic through the ER as part of a secretory pathway. In normal neurons, most tau protein is intracellular and attached to microtubules. In tauopathies, tau aggregates in the cytoplasm of cells, in cellular processes, and at nerve terminals, but there is no evidence that tau aggregates in the ER. Recent work in mouse models of α-synucleinopathies and studies on PD autopsy material (E. Colla et al., 2012; Emanuela Colla et al., 2012) suggest that small amounts of α-synuclein can be found in ER, and that in the disease state, these levels are elevated, thereby activating the ER stress response. Still, since there is no direct evidence that tau traffics through the ER, or evidence of tau aggregates in the ER, it is unlikely that misfolded tau directly activates the canonical UPR. This view is supported by the fact that in PSP, pPERK and pEIF2α are activated in cells with no observable tau pathology, but we cannot exclude the possibility that very low or undetectable levels of aggregated tau are present. Rather, a more likely explanation is that tau-induced cytoplasmic events act to trigger the UPR by an unknown mechanism, which in turn influences the degradation of tau. A possible mechanism is that cytoplasmic aggregated tau may inhibit processes such as the ERAD-proteosome pathway used by cells to degrade misfolded ER proteins, and thus preventing the normal degradation of these proteins, stimulating ER stress (Abisambra et al., 2013).

PERK and eIF2α are also activated in pathology-associated regions of a number of other neurodegenerative diseases, including another tauopathy, AD (Jeroen J M Hoozemans et al., 2009). The UPR is also activated in non-tau diseases that include ALS where UPR activation is observed in the spinal cord in sporadic cases (Atkin et al.,

2008), and in PD where UPR activation occurs in the substantia nigra (J J M Hoozemans et al., 2007). Expanded-repeat huntingtin, the pathological protein in Huntington's disease, induces ER stress in culture (Lajoie & Snapp, 2011). Notably, these diseases share a common pathology, *i.e*. the accumulation of abnormal aggregated proteins in the CNS. Thus, there may be a common mechanism by which aggregated cytoplasmic proteins activate the UPR. The genetic association between PERK and PSP suggests that this UPR activation can influence the disease process, at least in the case of PSP.

Surprisingly, we found that 10/14 normal controls over 50 years of age had at least minimal activation of pPERK in the hippocampus. This is in contrast to previous studies that report no pPERK staining in this region in normal controls (D. A. T. Nijholt et al., 2012). In these subjects, the degree of pPERK immunoreactivity correlated positively with both the degree of htau immunoreactivity and age, but did not correlate with amyloid pathology. The presence of at least some tau pathology in the hippocampus of normal subjects is consistent with work by others (Heiko Braak, Thal, Ghebremedhin, & Del Tredici, 2011), and could potentially indicate either pre-clinical AD or early neurofibrillary tangle predominant dementia (NFTD). However, in the absence of clinical symptoms, it is not possible to make either diagnosis. These findings in normal controls are consistent with the idea that the activation of the UPR is due to the tau pathology and not the amyloid pathology.

We reported strong genetic evidence that *EIF2AK3* genotypes confer risk for PSP (Höglinger et al., 2011). The strongest signal comes from single nucleotide polymorphism (SNP) rs7571971 that is within *EIF2AK3*. There are several non-synonymous coding polymorphisms in *EIF2AK3* that track with risk and *EIF2AK3* appears to be the gene in this region involved in PSP. However, another less likely but still plausible explanation is that PSP risk in this region comes from a regulatory element

that is intronic, within *EIF2AK3*, or in a close by intergenic region and that this element controls expression of another gene. Also, the true PSP association could be from nearby genes (*e.g. FOXI3* or *RPIA*) though this is less likely since the signal from SNPs in highlighting these genes are not as significant as SNPs within *EIF2AK3*. The work presented here clearly demonstrates that in PSP, PERK is activated in a region-specific pattern that matches regions where neurodegeneration occurs. Thus this functional evidence along with the strength of the genetic evidence indicates that *EIF2AK3* and not an adjacent locus is the gene that confers risk for PSP.

The SNP giving the strongest *EIF2AK3* signal in the PSP GWAS (rs7571971) is intragenic in intron 2. This SNP is in strong disequilibrium with 3 *EIF2AK3* exonic SNPs which are non-synonymous. This relationship was predicted using 1,000 Genomes data and confirmed here in PSP subjects (Fig. 2.7). These coding variants form two common (A and B) and one rare haplotype (D). In PSP subjects, the low risk allele [C] at rs7571971 completely correlates with haplotypes A and D while the high risk rs7571971 allele [T] completely correlates with haplotype B (Fig. 2.7).

Work in lymphoblastoid cell lines (Liu et al., 2012) with different haplotypes show that expression of EIF2AK3 is not altered by these haplotypes. However, when the PERK arm of the UPR is activated by thapsigargin, PERK from haplotype B homozygote cells is more active in phosphorylating eIF2α when compared to PERK from cells homozygous for haplotype A. The haplotype that confers high risk for PSP produces the more active form of PERK, suggesting that activation of the UPR is pathogenic in PSP and not a protective response. This is consistent with observations in prion protein induced neurodegeneration. Moreno et al., showed that during prion replication, synaptic failure and neuronal loss is temporally associated with UPR activation and inhibition of translation. When translation is restored using over-expression of GADD34

to dephosphorylate eIF2α, survival of infected animals is prolonged. In contrast, when the UPR is activated using salubrinal, survival is decreased (Moreno et al., 2012). Both observations are consistent with activation of the PERK/eIF2α arm of the UPR enhancing neurodegeneration, as proposed here for PSP.

The two low risk haplotypes (haplotype A, Ser-Arg-Ser; and haplotype B, Ser-Gln-Ser) differ only at the middle amino acid, 166— this amino acid is unlikely to functionally influence PERK activation. The low and high risk (Haplotype B, Cys-Gln-Cys) haplotypes differ at both positions 166 and 704, and one or both may influence PERK activity. Amino acid 166 is on the portion of PERK that is in the ER lumen and positioned where this protein senses mis-folded proteins. Position 704 is on the cytoplasmc side of PERK, a segment of the protein that is phosphorylated when activated and that has the active site for phosphorylating eIF2α. Additional work is needed to confirm that haplotype B PERK is the more active protein and to determine if mis-folded protein sensing or activation via auto phosphorylation is affected.

Conclusions

The PERK protein and its downstream effector eIF2α are phosphorylated in disease-affected regions in both PSP and Alzheimer's disease. A previous study using PSP samples described UPR activation primarily in the hippocampus, a brain region not affected in this disease (D. A. T. Nijholt et al., 2012). In contrast, we examined a large panel of brain areas (pons, medulla, midbrain, hippocampus, frontal cortex, and folia of the cerebellum) from PSP and AD cases as well as normal controls to show that this activation is disease-specific in its geographic distribution in the brain. In contrast to previous reports, we also found UPR activation in the hippocampus of a subset of our normal controls, a completely novel finding. This activation positively and significantly

correlated with both age and amount of tau pathology. This suggests that tau and UPR activation are linked. We also demonstrated a genetic association between an EIF2AK3 protein coding haplotype and PSP, indicating that variation in the PERK protein affects PSP risk.

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Table 2.1 Subject information.

	n	Avg Age of Onset	Avg Age at Death	Avg Disease Duration	% Female	Avg PMI
Normal – age-matched	15	N/A	71.7(8.4)	N/A	46.7	12.7(8.5)
Normal – non age-matched	12	N/A	54.5(24.9)	N/A	66.7	9.1(3.4)
Normal - total	27	N/A	64.0(19.4)	N/A	56.7	11.1(6.9)
PSP	17	66.8(5.8)	73.7(5.2)	6. 7(2.2)	64.7	11.7(6.0)
AD	9	65.0(6.3)	75.8(5.1)	10. 8(3.4)	44.4	9.4(5.8)

There were no significant differences between groups for average age at death (ANOVA, p = 0.33), post-mortem interval (ANOVA, p = 0.54), average age of onset (PSP and AD only, Student's t-test, p = .45) or percent female (Chi Square, p = 0.89). Average disease duration for AD was significantly longer than for PSP (Student's *t*-test, p = 0.002). Standard deviations indicated inside parenthesis

Table 2.2 Anitbodies used.

	Antigen	Epitope	Source	Dilution
Primary	pPERK	pThr981	Santa Cruz	1:4000
	peIF2α	pSer51	Sigma-Aldrich	1:2000
	AT8	pSer202/pThr205	Thermo Scientific	1:7500
Secondary	goat α rabbit IgG	biotin	Vector Labs	1:1000
	goat α rabbit IgG	AlexaFluor 488	Alexa	1:500
	goat α mouse IgG	AlexaFluor 594	Alexa	1:500

Table 2.3 P-values for comparison of pPERK and pEIF2α immunoreactivity in PSP, AD, and normal controls for different brain regions.

Comparison groups	Pons		Medulla		Midbrain	
	pPERK	peIF2α	pPERK	peIF2α	pPERK	peIF2α
AD vs. Normal	3.4E-5	0.0045	0.034	0.041	0.0026	0.0037
PSP vs. AD	3.8E-4	0.00049	1.7E-4	0.028	4.0E-7	0.009
PSP vs. Normal	3.8E-9	4.1E-5	6.1E-7	6.0E-6	6.0E-6	1.4E-4

Comparison groups	Hippocampus		Frontal Cortex		Cerebellum	
	pPERK	peIF2α	pPERK	peIF2α	pPERK	peIF2α
AD vs. Normal	0.0006	0.0042	4.1E-5	4.1E-5	0.4	1
PSP vs. AD	0.000021	8.2E-5	1.6E-4	0.0049	0.5	1
PSP vs. Normal	0.0034	0.073	0.0045	0.1	1	1

P-values are from a Fisher exact test. "E" indicates "x 10^". pPERK and peIF2α staining in PSP brainstem areas (pons, medulla, midbrain) were significantly greater than in AD or normal brainstem areas. AD brainstem areas had significantly more pPERK and peIF2α staining than normal brainstem areas. In contrast, primary AD-affected brain areas (hippocampus, frontal cortex) had significantly more pPERK and peIF2α staining than PSP or normal hippocampus and frontal cortex. There was no difference in staining between AD, PSP, or normal brains in the cerebellum

Table 2.4 PERK haplotypes.

			Alleles (%)	
	rs867529-rs13045-rs1805165	**Affected Amino Acids**	**PSP (*n*=994)**	
Haplotype A	GCA	Ser136-Arg166-Ser704	1233	(62.5)
Haplotype B	CTC	Cys136-Gln166-Ala704	626	(31.7)
Haplotype D	GTA	Ser136-Gln166-Ser704	113	(5.7)

Table 2.5 SNPs in high LD with rs7571971.

SNP	Gene	Distance in base pairs from GWAS hit SNP	RSquared	DPrime	Coordinate_HG18
rs1805165	EIF2AK3	20460	0.889	1	88656006
rs6739095	EIF2AK3	15287	0.886	0.96	88661179
rs11898161	EIF2AK3	13703	0.925	1	88662763
rs1913671	EIF2AK3	4532	0.886	0.96	88680998
rs11681299	EIF2AK3	6381	0.889	1	88682847
rs867529	EIF2AK3	17922	0.889	1	88694388
rs6731022	EIF2AK3	21684	0.886	0.96	88698150
rs11684404	EIF2AK3	29271	0.886	0.96	88705737
rs11680549	EIF2AK3	30997	0.813	0.957	88707463
rs6547787		34385	0.886	0.96	88710851
rs1606803		37965	0.889	1	88714431
rs62157778		38739	0.889	1	88715205
rs13003510		46139	0.925	1	88722605
rs13001657		51260	0.888	0.96	88727726

Table 2.S1 Individual Case Information

Case Demographics						
Case #	NPDx	Sex	Age at Death	Disease Duration	Post-Mortem Interval	*EIF2AK3* haplotype
1	PSP	M	73	6	20.5	A/B
2	PSP	F	70	7	4.5	A/A
3	PSP	F	79	11	17	B/B
4	PSP	M	73	4	10.5	
5	PSP	F	72	5	20	
6	PSP	F	71	6	6.5	A/A
7	PSP	F	81	7	17	A/A
8	PSP	F	77	11	12.5	A/B
9	PSP	M	85	3	-	
10	PSP	F	75	5	12	B/B
11	PSP	F	76	8	8	A/A
12	PSP	M	67	10	12	A/A
13	PSP	M	70	6	11	B/B
14	PSP	F	78	8	8	A/D
15	PSP	F	64	7	21	A/B
16	PSP	M	72	7	3.5	A/A
17	PSP	F	60	3	3	B/D
18	Normal	M	60	-	14	A/B
19	Normal	F	75	-	3.5	A/D
20	Normal	F	70	-	10.5	A/A
21	Normal	M	67	-	10.5	
22	Normal	M	90	-	6	A/B
23	Normal	M	74	-	7.5	
24	Normal	F	83	-	3	A/B
25	Normal	F	75	-	15	
26	Normal	M	83	-	14.5	A/A
* 27	Normal	M	63		5	
* 28	Normal	M	70		19	
* 29	Normal	F	67		5.5	
†* 30	Normal	M	65	-	26	
† 31	Normal	F	89	-	7	
† 32	Normal	F	72	-	4	
† 33	Normal	F	51	-	5	
† 34	Normal	F	92	-	5	
† 35	Normal	F	68	-	32	
† 36	Normal	F	85	-	14	
†* 37	Normal	F	65	-	19	A/B
† 38	Normal	F	56	-	12	
† 39	Normal	F	46	-	12	
† 40	Normal	M	47	-	11	
† 41	Normal	M	38	-	12	
† 42	Normal	M	16	-	8	
† 43	Normal	F	33	-	7	
† 44	Normal	M	29	-	12	
45	AD	F	71	12	17	

46	AD	M	85	13	6.5	
47	AD	F	72	17	12	A/A
48	AD	F	81	11	4	A/B
49	AD	M	70	10	5	A/A
50	AD	M	77	6	4	B/B
51	AD	M	74	12	7.5	A/B
52	AD	M	73	10	9	A/B
53	AD	F	79	6	20	A/A

Neuropathological diagnosis, sex, age at death, disease duration, post-mortem interval, Braak stage, and hippocampal Aβ amyloid plaque score for all cases and controls used

† = hippocampus only

* = pons, cerebellum and/or hippocampus only.

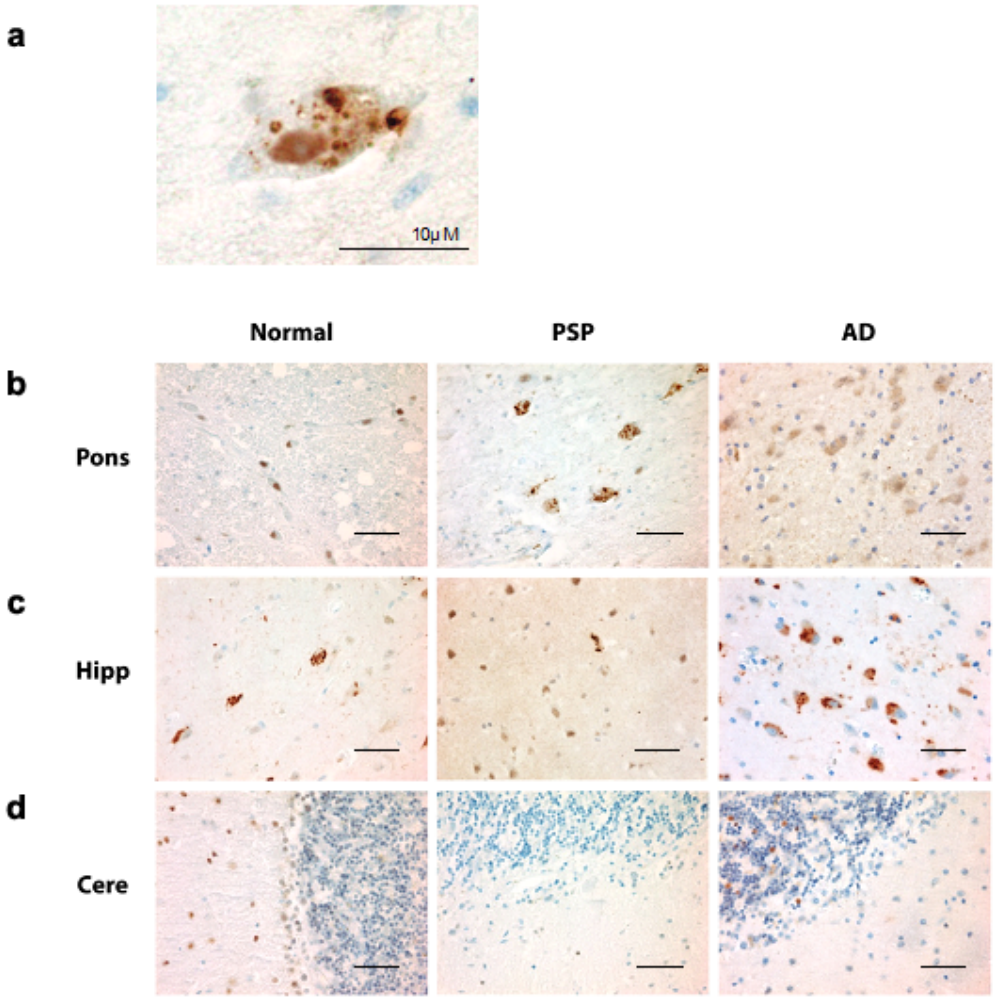


Figure 2.1 pPERK is activated in PSP, AD, and normal brain. a. Example of a cell with pPERK immunoreactive puncta in the pons of a PSP case. **b-d.** Representative fields showing pPERK staining of pons, hippocampus, and cerebellum in normal, PSP, and AD cases. Scale bars are 50μm unless otherwise indicated

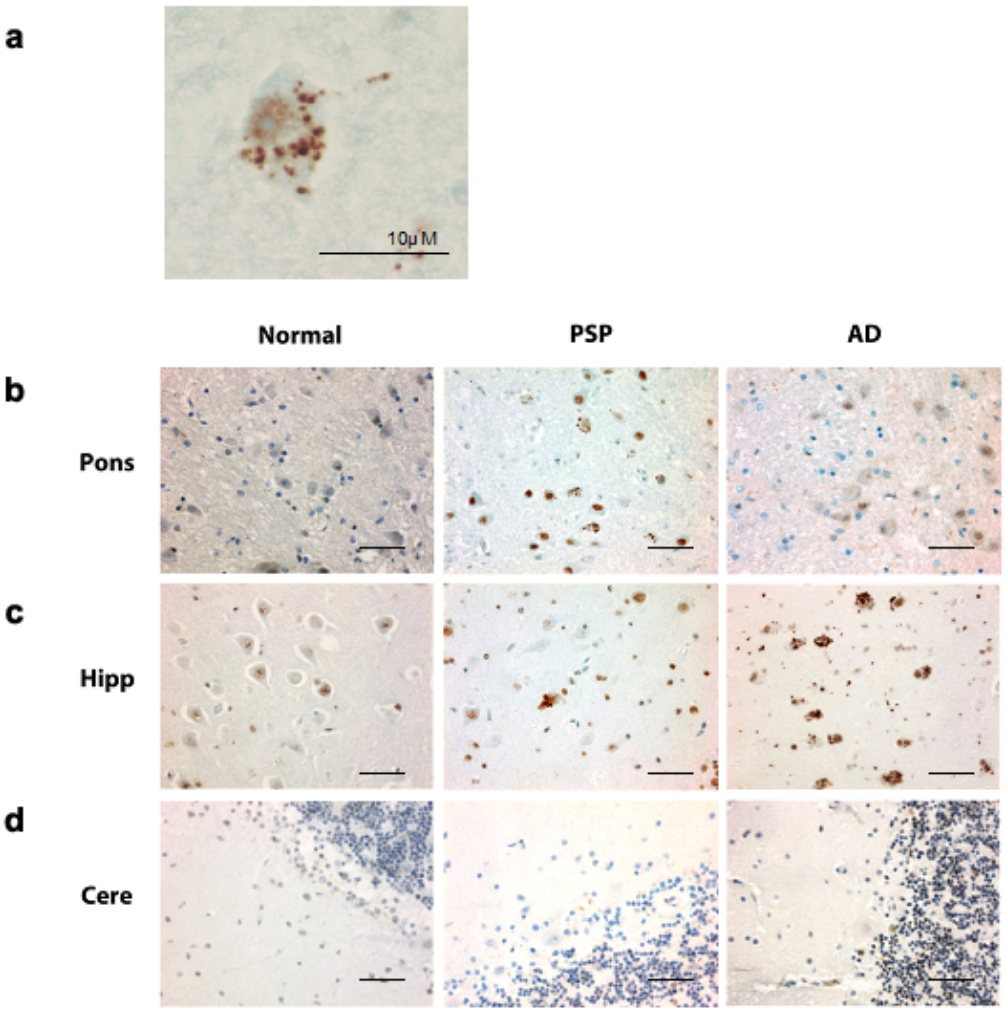


Figure 2.2 peIF2α is activated in PSP, AD, and normal brain. a. Example of a cell with peIF2α puncta in the pons of a PSP case. **b-d.** Representative fields from peIF2α staining of pons, hippocampus, and cerebellum in normal, PSP and AD cases. Scale bars are 50μm unless otherwise indicated

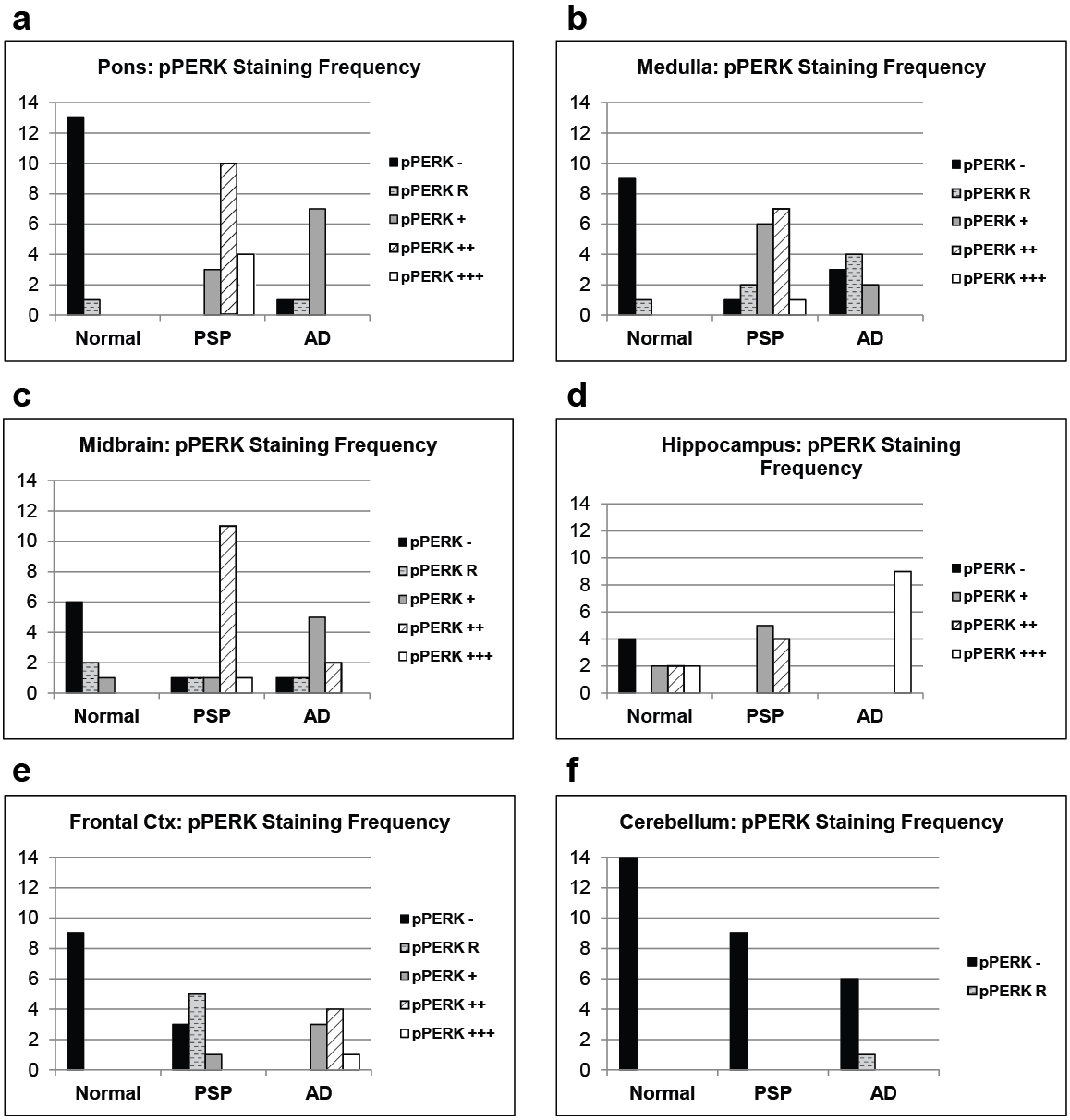


Figure 2.3 Frequency of pPERK staining scores in PSP, AD, and normal brain. Distribution of pPERK staining scores. +++ = widespread activation, ++ = moderate activation, + = diffuse activation, R = rare activation, - = no activation. Y-axis indicates number of cases with a particular pPERK staining score. All P-values obtained using Fisher exact test. **a-c.** PSP cases had the strongest pPERK staining in the pons (PSP vs. Normal: p = 3.8E-9) and the medulla (PSP vs. Normal: p = 6.1E-7), as well as moderate staining in the midbrain (PSP vs. Normal: p = 6.0E-6) which was affected in all PSP cases. **d-e.** AD cases had the strongest pPERK staining in the hippocampus (AD vs. Normal: p = 0.0006) and moderate staining in the frontal cortex (AD vs. Normal: p = 4.1E-5) both of which were affected in AD. **f.** No cases had significant pPERK staining in the cerebellum

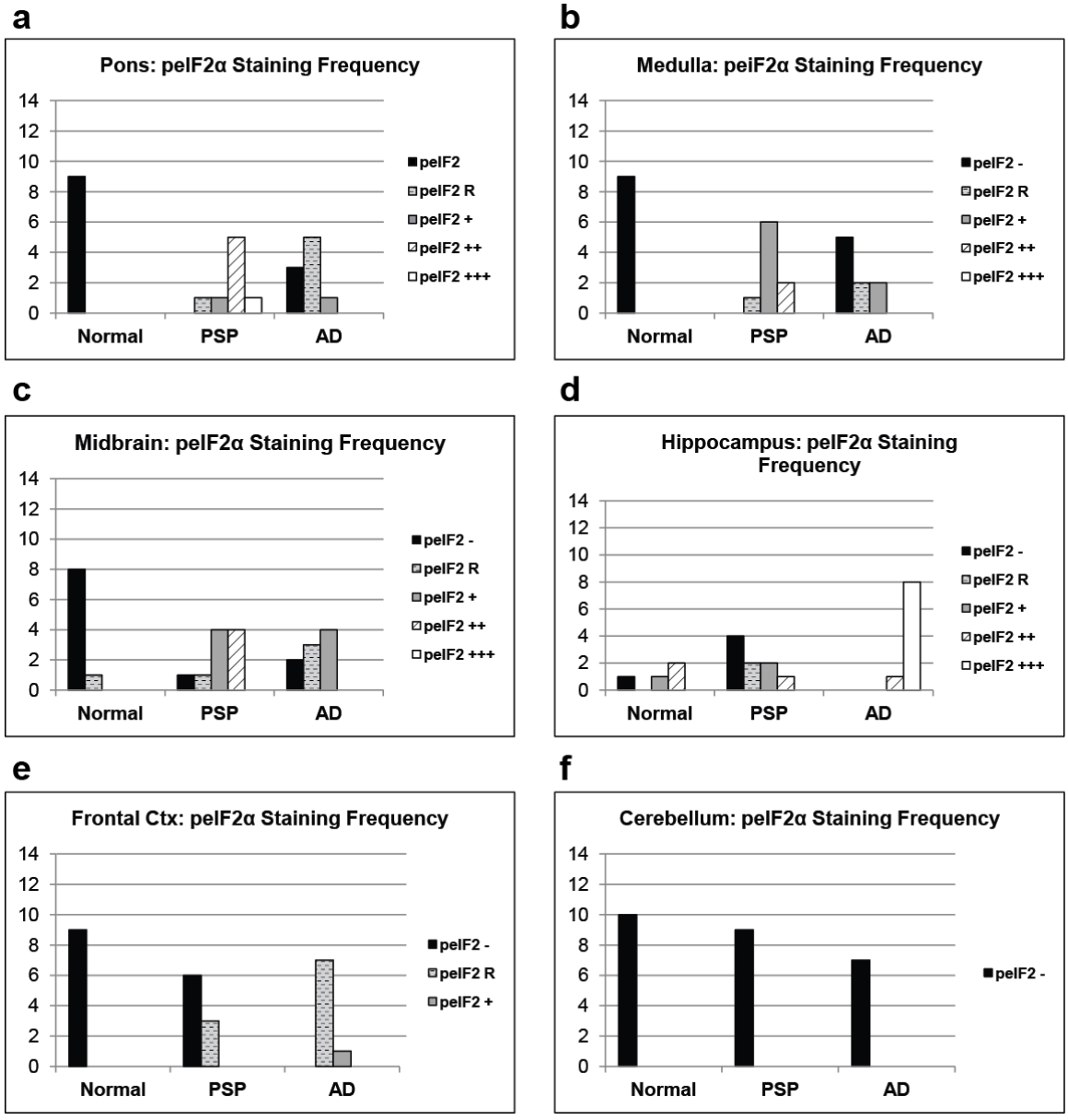


Figure 2.4 Frequency of peIF2α staining scores in PSP, AD, and normal brain. Distribution of peIF2α staining scores. +++ = widespread activation, ++ = moderate activation, + = diffuse activation, R = rare activation, - = no activation. Y-axis indicates number of cases with a particular peIF2α staining score. All P-values obtained using Fisher exact test. **a-c.** PSP cases had the strongest peIF2α staining in the pons (PSP vs. Normal: p = 4.1E-5) and the medulla (PSP vs. Normal: p = 6.0E-6), as well as moderate staining in the midbrain (PSP vs. Normal: p = 0.00041) which was affected in all PSP cases. **d-e.** AD cases had the strongest peIF2α staining in the hippocampus (AD vs. Normal: p = 0.0042) and moderate staining in the frontal cortex (AD vs. Normal: p = 4.1E-5) both of which were affected in AD. **f.** No cases had significant peIF2α staining in the cerebellum

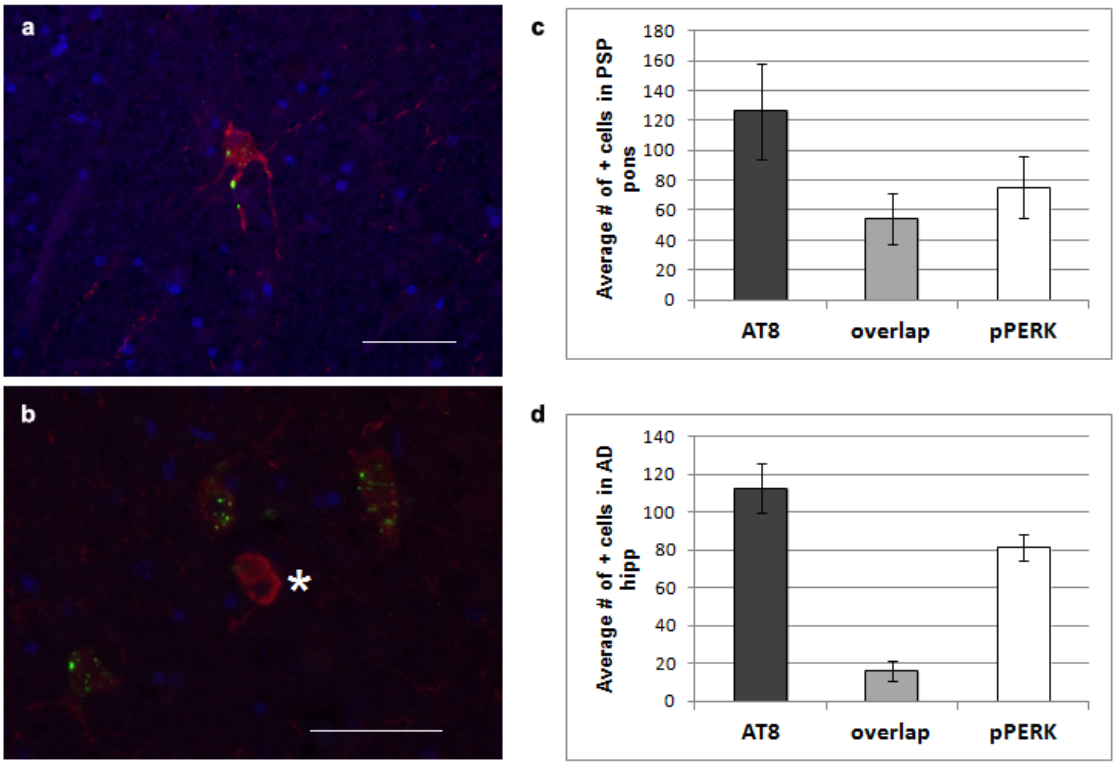
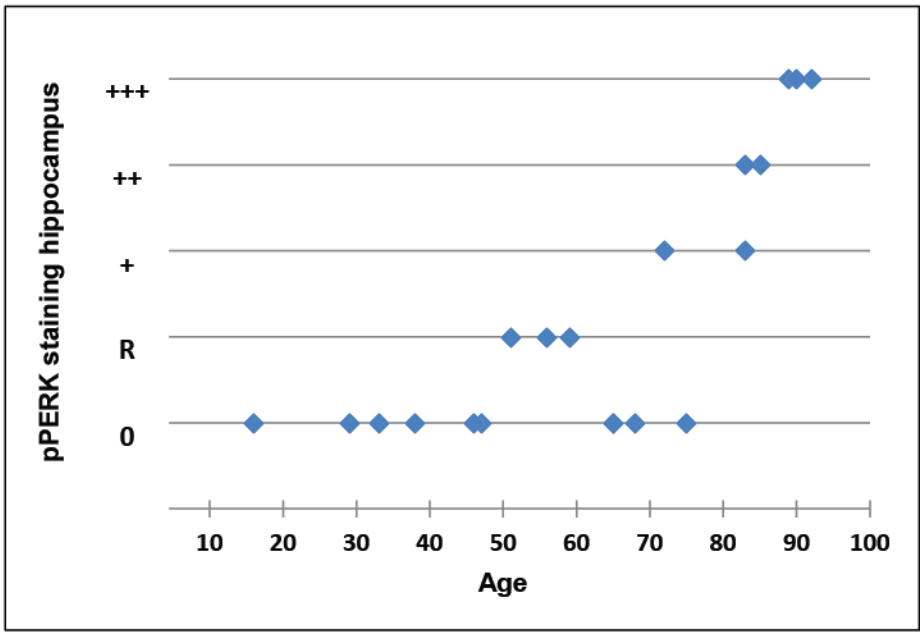


Figure 2.5 Hyperphosphorylated tau and pPERK partially co-localize in PSP pons and AD hippocampus. a. Example of a neuron co-stained for htau (red) and pPERK (green). Tau staining is widespread and diffuse. pPERK staining is punctate and localized to the soma and proximal neurites. **b.** pPERK staining occurred mostly in cells with diffuse, non-fibrillar htau staining. Cells with dense, fibrillar htau staining did not stain for activated PERK (*). **c.** In PSP pons, most pPERK positive cells also stained for htau (72%), whereas fewer than half of htau stained cells (43%) also stained positive for pPERK. **d.** htau and pPERK staining overlapped very little in AD hippocampus (14% and 20%). Scale bars are 50μm unless otherwise indicated

a



b

		pPERK score				
		-	R	+	++	+++
tau score	-	9	3	1		
	R			1		
	+					1
	++				2	
	+++					2

Figure 2.6 Severity of PERK activation in normal hippocampus correlates with age and tau pathology. a. Plot of pPERK staining score (X-axis) *versus* subject age at death (Y-axis). Each diamond represents one normal subject. Some individuals both young and old were negative for pPERK staining. Of those that stained positive for pPERK (including those showing rare through +++ levels of immunoreactivity), older individuals tended to have more severe pPERK staining scores. **b.** Frequency table plotting htau score against pPERK staining score in normal hippocampus. Htau score and pPERK score were positively correlated (Spearman R: .7523; p=0.0002). The higher the htau score of an individual hippocampus, the higher the pPERK staining score tended to be. Htau scores were obtained from the CNDR Integrated Neurodegenerative Disease Database[42] using antibody PHF-1

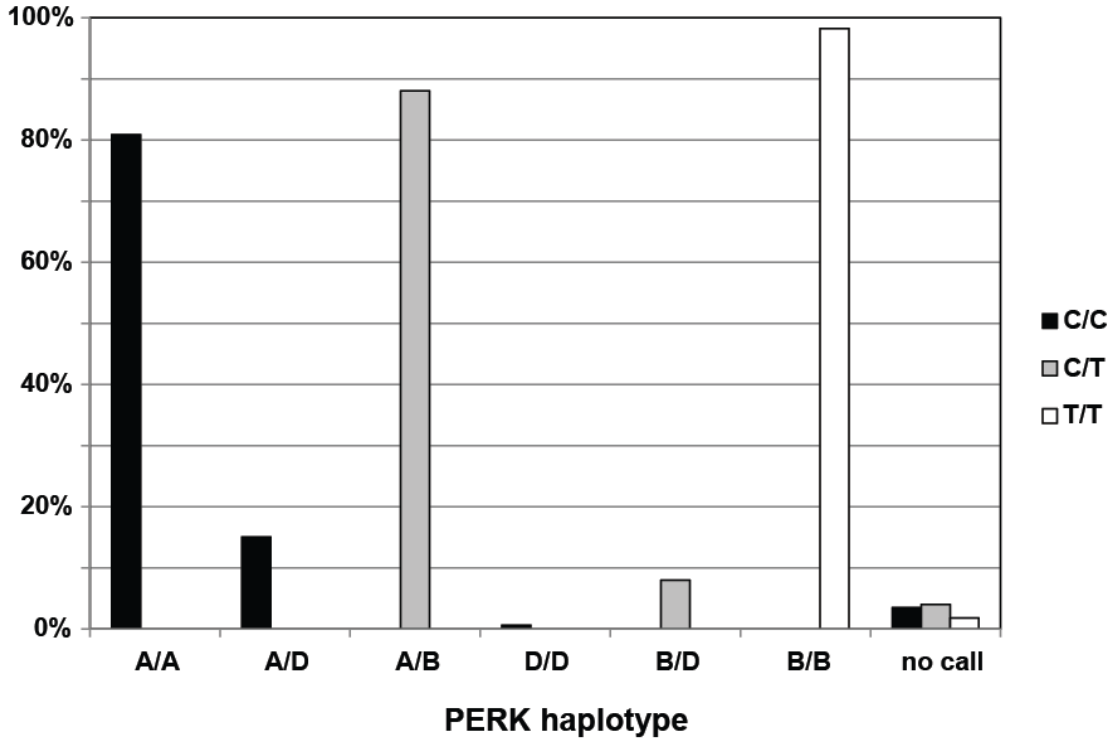


Figure 2.7 Comparison of PERK haplotype with GWAS risk allele. A GWAS for PSP identified a risk locus on chromosome 2 (rs7571971). The common, low risk allele at this locus is cytosine (C) and the PSP risk allele is thymine (T) [10]. Among individuals homozygous for C at this locus, all harbor PERK haplotype A or D in some combination. Individuals heterozygous (C/T) at this locus were heterozygous for haplotypes A, B, and/or D. Individuals homozygous for T at the GWAS risk locus were always homozygous for PERK haplotype B. This demonstrates that one of the two amino acid changes conferred by the B PERK haplotype that are not shared by the D haplotype may be responsible for the PSP risk evident on Chr. 2

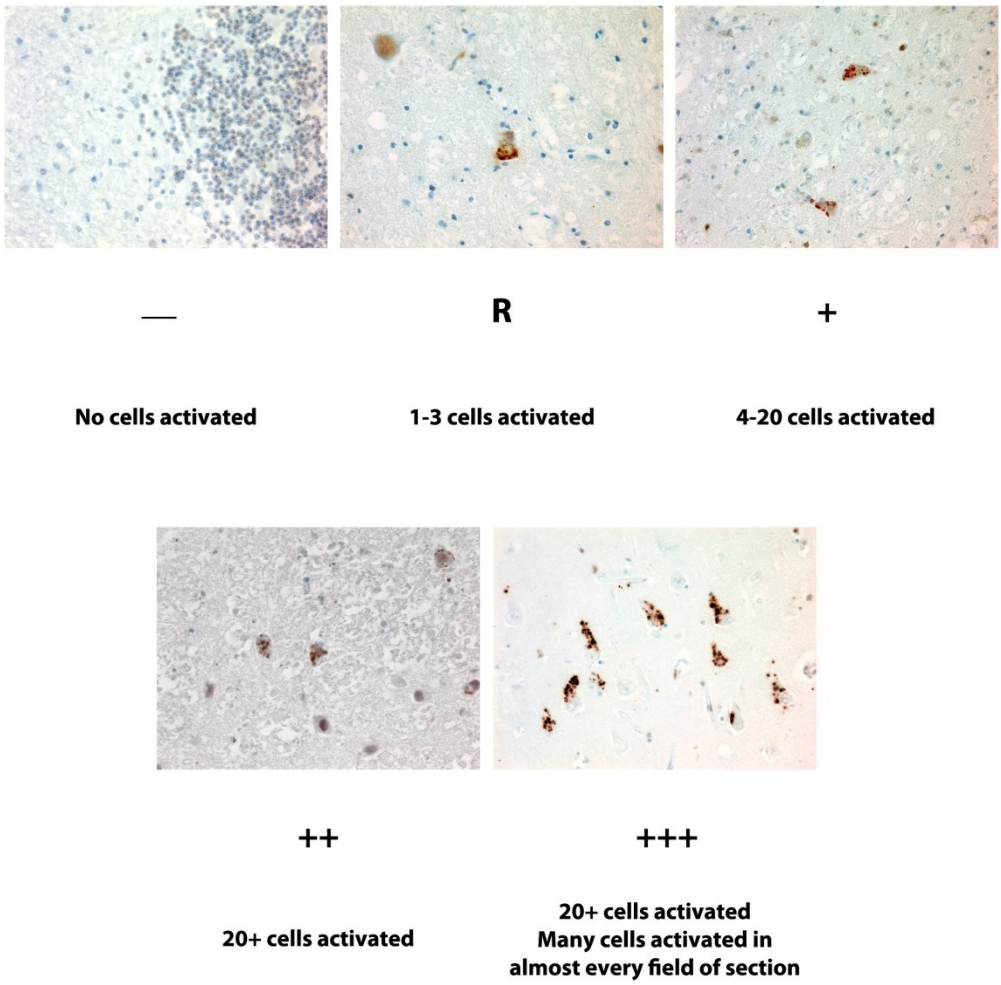


Figure 2.S1 Scoring system examples. Representative fields from brain areas that scored "-" (negative), "R" (rare), "+" (mild staining), "++" (moderate staining), and "+++" (heavy staining), along with scoring criteria.

CHAPTER 3: FUNCTIONAL CONSEQUENCES OF PERK GENETIC VARIATION

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ABSTRACT

EIF2AK3, the gene that codes for the PERK protein, is associated with PSP disease risk. There are two common protein coding variants of PERK, HapA and HapB, which differ by three amino acids. Recent work indicates HapB PERK has more kinase activity in response to thapsigargin treatment than does HapA in human β-lymphocytes (Liu et al., 2012). This project had two goals: 1) replicate and expand upon previous findings in β-lymphocytes and 2) determine which of the three amino acid coding changes is responsible for the difference in PERK activity between HapA and HapB. This work confirms that β-lymphocytes expressing HapB PERK show more eIF2α phosphorylation than those expressing HapA. Paradoxically, HapB PERK cells also show less phosphorylated PERK. These findings were echoed in mouse embryonic fibroblasts expressing PERK variant constructs. Further work exploring the functional differences between PERK variants is warranted.

INTRODUCTION

The PERK protein is part of the cell's unfolded protein response (UPR). The PERK arm of the UPR acts primarily on translation. When PERK is activated (thus becoming phospho-PERK, or pPERK), a kinase domain on the cytosolic side of the protein phosphorylates eukaryotic translation initiation factor 2α (eIF2α). peIF2α then acts at the ribosome to slow down general translation initiation and promote translation of ATF4 (Ron & Walter, 2007). ATF4 promotes the transcription of genes that enhance import of amino acids and protect against oxidative stress (Harding et al., 2003).

Three single nucleotide polymorphisms (SNPs) that confer coding changes to the PERK protein are linked with disease risk for progressive supranuclear palsy (PSP; Stutzbach et al., 2013). The more common coding variant, “Haplotype A,” is lower risk, while the less common variant “Haplotype B” is higher risk (for amino acid sequences, see Table 3.2). Work in human β-lymphoblastoid cell lines shows that the Haplotype B form of PERK is more active in response to treatment with the UPR-inducing drug thapsigargin (Liu et al., 2012).

The goal of this thesis project is to determine what role PERK and the UPR play in PSP pathogenesis. I have done this by determining the distribution of activated PERK in the post-mortem PSP-affected brain, examining the association between pPERK and tau, and analyzing the genetic association between common variations to the PERK protein and PSP (Stutzbach et al., 2013). The present analysis aims to expand on those findings by determining the biological effects of PERK protein coding variants on PERK function.

MATERIALS AND METHODS

Generation of PERK constructs

Human PERK cDNA clones were obtained from Transomic Technologies (Huntsville, AL; Fig 3.1A). To create PERK variants, we performed site-directed mutagenesis using the QuikChange Multi kit (Agilent Technologies, Santa Clara, CA). For primers, see Table 3.1. All PERK variants were sequence-confirmed using CMV primers (pTCN promoter for PERK insert; Fig. 3.1A and 3.1B).

Cell Culture

We obtained human β-lymphocyte cell lines from Coriell Cell Repository (Camden, NJ). Cell lines expressing PERK haplotype A were NA06985, NA06991, NA06993, NA07029, NA07055; cell lines expressing PERK haplotype B were NA07348, NA07357, NA10835, NA11993, and NA12249. We cultured β-lymphocytes in RPMI-1640 media (Corning Cellgro, Manassas, VA) supplemented with 15% fetal bovine serum (FBS), 1% L-glutamine, and 1% Pen Strep antibiotic. Drug treated cells were dosed with 0.1μM thapsigargin (reconstituted in DMSO) for 30 minutes.

Mouse embryonic fibroblasts (MEFs) negative for the PERK protein were the generous gift of the Constantinos Koumenis laboratory (University of Pennsylvania, Philadelphia, PA) and originated from PERK knockout mice generated by the Douglas R. Cavener laboratory (Pennsylvania State University, State College, PA). PERK knockout was carried out using Cre-Lox recombination as described in Zhang P. et al (2002).

MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% L-glutamine, and 1% Pen-strep. PERK constructs and the pTCN empty vector were tranfected into MEFs using Lipofectamine LTX with PLUS reagent (Invitrogen, Carlsbad, CA) and cells were harvested 24 hours later. Drug-treated cells were dosed with 0.1μM thapsigargin (reconstituted in DMSO) for 75 minutes before harvest.

At harvest, all cells were washed twice with 1x PBS then (with the exception of cells harvested for nuclear protein isolation) lysed using cell lysis buffer formulated by

the David Ron laboratory in Cambridge, UK (http://ron.cimr.cam.ac.uk/protocols/IP.IMMUNOBLOT.html), consisting of 1% TritonX 100, 150mM NaCl, 20mM Hepes pH 7.5, 10% glycerol, 1mM EDTA, 1x Halt phosphatase inhibitor (Life Technologies, Thermo-Fisher Scientific, Rockville, IL), and cOmplete protease inhibitor cocktail (Roche, Basel, Switzerland). Cells targeted for nuclear protein isolation were harvested using the Biovision cell fractionation kit (Milpitas, CA) according to manufacturer protocol. Cells harvested for PERK expression analysis were lysed in TRI reagent (Ambion, Thermo Scientific, Rockford, IL) and RNA isolated according to manufacturer protocol. Protein concentration for all cell lysates was calculated using the Pierce BCA assay (Thermo Scientific, Rockford, IL) according to manufacturer protocol.

PERK expression analysis

PERK null MEFs were transfected with 2.5 ug PERK cDNA using Lipofectamine LTX with PLUS reagent (Invitrogen, Carlsbad, CA) and cells were harvested 24 hours later using Tri reagent (Ambion, Thermo Scientific, Rockford, IL). RNA was isolated according to TRI reagent manufacturer protocol. RNA from PERK-transfected cells was reverse-transcribed into cDNA using High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and transcript levels quantified using qPCR TaqMan Assays designed to detect PERK (Hs00178128_m1 and Hs00984006_m1; Applied Biosystems, Foster City, CA) according to manufacturer protocol (50^{o} C 2min, 95^{o}C 10min, 40x: 95^{o}C 15sec, 60^{o}C 1min). PCR results were quantified and analyzed using the $\Delta\Delta$CT (cycle threshold) relative quantification method in Expression Suite software (Applied Biosystems, Foster City, CA). Endogenous control was *Hprt*

(Mm01545399_m1). Relative Quantification (RQ) values (fold change compared to calibrator sample [HapA]) greater than 2 or less than 0.5 were considered significant (https://genomique.iric.ca/resources/files/How_to_deal_with_qPCR_results.pdf).

Western blotting

Cell lysates were combined with Lane Marking Reducing Sample Buffer (Thermo Scientific, Rockford, IL) and boiled for 10 minutes before loading onto 4-20% Tris-HCL Criterion gels (BioRad, Hercules, CA). Proteins were transferred to PVDF membrane, blocked in 5% non-fat milk for one hour or 5% bovine serum albumin (for peIF2α detection only) for one hour, then incubated in primary antibody overnight. Blots were washed in Tris-buffered saline with 1% Triton-X 100 (Roche Boehringer Mannheim, Basel, Switzerland; TBS-T), incubated in secondary antibody for two hours, washed again, then incubated in Luminata Forte or Crescendo Western substrate (Millipore, Bilerica, MA) for five minutes. Each blot was exposed to film for a variable length of time and developed in a Xomat 200A Professional developer (Kodak, Rochester, NY). For antibody sources and concentrations, see Table 3.2.

Western blot analysis

Western bands were quantified using ImageJ image analysis software (http://imagej.nih.gov/ij/). Each band was normalized first to its corresponding loading control: total eiF2α was the loading control for peIF2α, and a 125 kDA protein band from a Fast Green protein stain was the loading control for total PERK, pPERK, and ATF4. In the MEF experiment, each pPERK, ATF4, and peIF2α band was then normalized to its corresponding total PERK band to correct for variability in PERK expression. All values reported represent normalized relative density values for each band.

RESULTS

Thapsigargin treatment

To determine the optimal treatment duration for thapsigargin treatment, I performed a time course analysis of eIF2α phosphorylation in PERK null (P -/-) MEFs expressing human PERK. I transfected cells with HapA PERK and treated with 0.1μM thapsigargin (tg) for 5, 15, 30, 45, 75, 90, 105, and 120 minutes before harvest. peIF2α levels increased steadily with longer treatment times, peaking at 75 minutes and remaining strong through 105 minutes (Fig. 3.2 A-B). For all subsequent experiments, treatment time in P -/- MEFs expressing human PERK constructs was 75 minutes.

PERK expression analysis

To confirm uniform transfection and expression, I transfected P-/- MEFs with each PERK construct and harvested RNA at 24 and 48 hours to perform qPCR. The results of two Taqman Gene Expression Assays (Life Technologies) for PERK demonstrated no significant differences in PERK transcript level between constructs (for construct information, see Fig. 3.1 and Table 3.2); RQ values were less than 2 and greater than 0.5 (Fig. 3.3; see Methods section). However, PERK transcript levels in general were higher at 24 hours post-transfection (ptf) than at 48 hours ptf. Therefore, cells were harvested at 24 hours ptf for all subsequent PERK transfection experiments.

PERK haplotype comparison in human β-lymphocytes

Liu et al. (2012) showed that human β-lymphocytes (β-L) homozygous for PERK HapB have higher levels of eIF2α phosphorylation in response to tg treatment than β-Ls homozygous for haplotype A. In order to replicate and expand upon this finding, I

harvested cell lysates from four β-L lines homozygous for HapA and four homozygous for HapB, with and without 0.1μM tg treatment (30 min). All cell lines were sequence-confirmed homozygotes for HapA or HapB (see Methods for cell line information). I then probed for total PERK, pPERK, total eIF2α, peIF2α, and β-actin (loading control) by Western blot (Fig. 3.4A).

Total PERK levels did not vary between HapA and HapB, with or without tg treatment (Fig. 3.4B). However, phosphorylated PERK (pPERK) levels were significantly lower in HapB lines in comparison to HapA lines in both untreated and tg treated cells (Fig. 3.4C; for total PERK and pPERK antibody information, see Table 3.3). This is in contrast to significantly higher peIF2α levels in HapB lines in comparison to HapA lines (Fig. 3.4D). While these peIF2α findings replicate the results from Liu et al (2012), this decrease in pPERK in HapB is a novel finding. This indicates that the increase in peIF2α levels may be independent of PERK's autophosphorylation status.

There are a number of potential explanations for this seemingly contradictory finding. First, the changes to the PERK protein that make up HapB PERK may interfere with the pPERK antibody epitope (Thr982), indicating a difference in detection of pPERK rather than a difference in actual pPERK levels. However, it is also possible that the amino acid differences between HapA and HapB on the N-terminal end of the protein (S136**C** and R166**Q**) act to promote and stabilize PERK dimerization (thereby increasing the likelihood of eIF2α kinase activity), while the change to the C-terminal, cytoplasmic end of PERK (S704A, the change nearest to a protein kinase domain) decreases the likelihood of PERK transautophosphorylation. Further investigation into how the protein

coding differences between HapA and HapB affect post-translational modifications would be needed to determine which of these explanations is more likely.

Another caveat to this finding is that the lower pPERK levels for HapB +tg seem to be driven by only half of the Hap B cell lines (compare the last four lanes for pPERK in Fig. 3.4A). Thus, there may be additional factors at work in these cell lines and further study of additional HapB homozygotes would be necessary to confirm and expand upon this finding.

PERK variant comparison in P-/- MEFs:

To determine which of the three protein coding variants that make up HapB is responsible for the difference in eIF2α phosphorylation between HapA and HapB, I created several PERK constructs with the goal of expressing each in PERK knockout cells and measuring activity. These PERK constructs are listed in Table 3.2.

I expressed each of these constructs in P-/- MEFs obtained from the Koumenis laboratory (Zhang et al., 2002)and harvested cell lysates 24 hours post-transfection (ptf). I then probed for total PERK, pPERK, total eIF2α, peIF2α, ATF4 (ATF4 probed in nuclear lysates only), and β-actin by Western blot (Fig. 3.5, Fig. 3.10). I then performed relative quantification analysis using ImageJ software (http://imagej.nih.gov) and normalized each Western band first to a loading control (a band at approximately 125 kDa from a fast green protein stain) and then to PERK expression for that run. Quantifications in Figures 3.6-3.9 represent the mean values from 4 independent replicates of this experiment. To determine statistical significance, I performed unpaired t-tests between each “test” construct (HapB, 136C, 166Q, 704A) and HapA for all

measures. I also performed paired t-tests within each construct to compare expression levels with and without thapsigargin treatment.

PERK levels were not statistically different between constructs in the absence of tg treatment (Fig. 3.6A). However, tg treatment resulted in a dip in total PERK levels in HapB ($p=0.02$) and 136C ($p=.05$) in comparison to HapA (Fig 3.6B). Because total PERK levels for both HapB and 136C also tended to be lower in the absence of tg treatment, these differences were likely just slightly magnified in the drug treatment condition. Total PERK levels did not significantly change within each construct after tg treatment (Fig 3.6C).

pPERK levels for HapB, 136C, 166Q, and 704A were not statistically different from Hap A in the absence of tg treatment (Fig. 3.7A). However, pPERK levels were slightly, albeit significantly, lower in 704A relative to HapA ($p=.01$; Fig. 3.7B). pPERK for all constructs increased significantly with tg treatment (all p-values < 0.05; Fig. 3.7C). To determine whether the magnitude of response to tg treatment differed between each construct and HapA, I calculated the ratio of tg treated to untreated for pPERK levels. The ratio of tg treated to untreated for each construct was not significantly different from the tg treated to untreated for HapA, though tg treatment tended to have a greater magnitude of effect on HapB (Fig 3.7D).

peIF2α levels for HapB, 136C, 166Q, and 704A were not statistically different from HapA with or without tg treatment, and tended to be variable (Fig. 3.8A). HapB peIF2α levels did tend to be slightly higher than those of HapA, but this difference was nonsignificant ($p=0.253$; Fig 3.8B). peIF2α for all constructs did not increase significantly with tg treatment (all p-values > 0.05), again likely due to variability (Fig 3.8C). Likewise,

the ratio of tg treated to untreated for each construct was not significantly different from the tg treated to untreated for HapA (Fig. 3.8D).

ATF4 levels for HapB, 136C, 166Q, and 704A were not statistically different from HapA with or without tg treatment. However, as with peIF2α, HapB levels of ATF4 tended to be highest and HapA levels lowest (tg treatment condition), with the individual amino acid variants showing an intermediate level (Fig. 3.9A-B). ATF4 for all constructs did not increase significantly with tg treatment (all p-values > 0.05; Fig 3.9C), though this may, again, be due to variability. The ratio of tg treated to untreated for each construct was not significantly different from the tg treated to untreated for HapA (Fig. 3.9D).

DISCUSSION

In this study, I was able to replicate the finding from Liu et al (2012) that human β-lymphocytes homozygous for PERK haplotype B demonstrate higher levels of eIF2α phosphorylation in response to tg treatment than do β-Ls homozygous for PERK haplotype A. I also found that HapB B-Ls showed lower pPERK levels in response to the same treatment. Bearing in mind the newness of the pPERK antibody and the potential for this antibody to have a weaker affinity for HapB PERK than HapA PERK, this finding seems ripe for a follow-up study with a larger sample size comparing the two haplotypes. If this effect can be replicated and expanded, the next step could be to examine how the HapB version of the protein differs from HapA in either structure (perhaps a difference in disulfide bonding conferred by the 136 S->C variation) or in post-translational modifications (notably, potential phosphorylation sites S136 and S704, which are ablated by HapB).

Though PERK null MEFs transiently expressing different variants of the PERK protein (HapA, HapB, 136C, 166Q, 704A) largely did not statistically differ from one another in pPERK, peIF2α, or ATF4 levels, these results suggest a trend toward HapB-expressing cells having a stronger response to tg treatment. HapB expressing cells showed a bigger difference between baseline levels of pPERK, peIF2α, and ATF4 and these same levels after tg treatment. This difference in the magnitude of response to thapsigargin between HapA and HapB echoes the findings in β-lymphocytes discussed above. Interestingly, MEFs expressing the single amino-acid variant PERK constructs may have an "intermediate" effect on thapsigargin response—this is suggested by ATF4 levels quantified in Figure 8. Note that ATF is highest for HapB +tg, followed by 136C +tg and then 166Q +tg. This dampening of the effect of HapB in the single amino acid variant constructs may indicate that these protein coding variations act synergistically to change PERK's biological activity. However, as these results were not statistically significant, further study is needed (perhaps in a less variable model) to confirm this finding. Additionally, further work should examine the effect on PERK function of pseudophosphorylation at position 136 and position 704 (see PERK construct creation breakdown under "PERK variant comparison in P-/- MEFs" above). Another PERK construct that would be valuable in this future analysis is a 136A variant—this construct would negate possible modifications at that site present in HapA (potential S136 phosphorylation) and HapB (potential disulfide bond formation at 136C).

Further study of PERK variants and both their differences in activity and their effect on disease pathogenesis and progression seems warranted. Our previous work demonstrated that PERK HapB is associated with risk for PSP, a neurodegenerative disease. This work also showed that PERK is activated in post-mortem brain regions

affected by PSP and Alzheimer's disease. Other studies have shown PERK activation in the brain in Parkinson's disease (Hoozemans et al. 2007), ALS (Atkin et al. 2001, Wang et al. 2010), AD (Hoozemans et al 2009), multiple system atrophy (Makioka et al. 2010) and other tauopathies (Nijholt et al. 2012). Therefore, it may be that differences in PERK function are magnified in the brain or that neurons are more sensitive to these differences.

Further study into functional variations of PERK should also examine how post-translational modifications affect differences in activity between HapA and HapB. HapA contains two serines (S136 and S704) not present in HapB. Phosphorylation at either of these sites could potentially affect activity. Thus, future studies could incorporate PERK constructs pseudophosphorylated at these sites (136E and 704E). Conversely, this work could also examine activity of PERK that is unphosphorylatable and incapable of disulfide bonding at residue 136 (136A). Another way to approach this question would be to determine by mass spectrometry whether either of these serines are phosphorylated on endogenously expressed HapA PERK, either at baseline or in response to stress.

It may be that PERK -/- MEFs are not an ideal model to study PERK variation and its contribution to disease pathogenesis. Because PERK plays a role in neurodegeneration, future work could benefit from using neuronal cultures to explore this question. The specialized functions of neurons and their heavy reliance on protein translation may make them especially vulnerable to sustained PERK activation. Though there are, as of yet, no neuronal cell culture models of tau aggregation (Guo & Lee, 2014), this type of system would be ideal for studying the interaction between PERK variation and tau pathogenesis.

Table 3.1 Primers for Site-Directed Mutagenesis

Primer Name	Primer Sequence (5' to 3')
166Q: g497a	5'-ctcttccagtgggaccaagaccgtgaaagcatg-3'
136C: c407g	5'-atgtgggatccggttgcttggtgtcatccag-3'
704A: t2110g_antisense	5'-gatttcaatatgttcttttgtagcgaaaggatccattctgcgtatt-3'
136C: c407g_antisense	5'-ctggatgacaccaagcaaccggatcccacat-3'
704A: t2110g	5'-aatacgcagaatggatcctttcgctacaaaagaacatattgaaatc-3'
166Q: g497a_antisense	5'-catgctttcacggtcttggtcccactggaagag-3'
Additional Primers: Phosphomimetic	
t406g_c407a_c408g	5'-atttggatgtgggatccggtgagttggtgtcatccagccttag-3'
t2110g_c2111a_t2112g	5'-cagttaaaatacgcagaatggatcctttcgagacaaaagaacatattgaaat

	catagctc-3'
t406g_c407a_c408g_antisense	5'-ctaaggctggatgacaccaactcaccggatcccacatccaaat-3'
t2110g_c2111a_t2112g_antisense	5'-gagctatgatttcaatatgttcttttgtctcgaaaggatccattctgcgtattttaactg-3'

Table 3.2 PERK Constructs

Construct Name	Amino Acid at position 136-166-704
PERK Haplotype A	S136-R166-S704
PERK Haplotype B	C136-Q166-A704
PERK 136C	C136-R166-S704
PERK 166Q	S136-Q166-S704
PERK 704A	S136-R166-A704
PERK 136E (phospho-mimetic)	E136-R166-S704
PERK 704E (phospho-mimetic)	S136-R166-E704

Table 3.3 Antibodies

Antibody Name	Company	Dilution	Origin
PERK C33E10	Cell Signaling Technologies	1:500	Rabbit
pPERK	Eli Lilly	1:1000	Rabbit
eIF2α L57A5	Cell Signaling Technologies	1:1000	Mouse
peIF2α S51	Cell Signaling Technologies	1:250	Rabbit
ATF4 (Creb-2) SC-200	Santa Cruz	1:1000	Rabbit
β-actin	Cell Signaling Technologies	1:50,000	Mouse

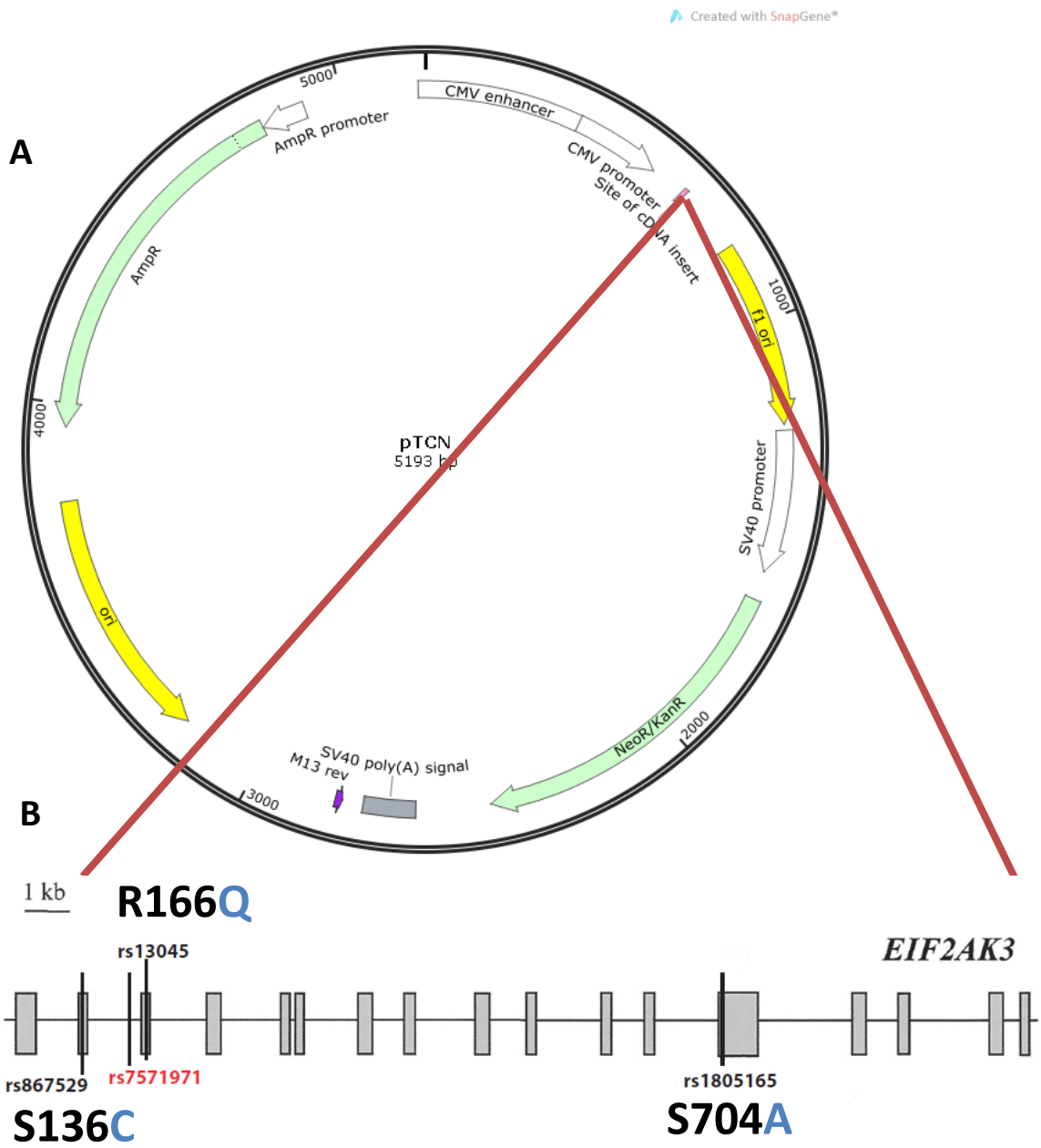


Figure 3.1. PERK construct vector map and schematic. A. Human PERK cDNA clones were obtained from Transomic Technologies (Huntsville, AL; vector: pTCN; image obtained from www.transomic.com). **B.** To create PERK variants, we performed site-directed mutagenesis using the QuikChange Multi kit (Agilent Technologies, Santa Clara, CA) at three sites: rs867529 (S136C), rs13045 (R166Q), and rs1805165 (S704A). Together, these three variations make up “Haplotype B” (HapB). We mutated PERK at all three HapB sites individually to determine whether single amino acid variations affect PERK function. For a full list of PERK variant constructs, see Table 3.2. SNP rs7571971, shown here in red, is associated with PSP risk (REF) and is in LD with all three HapB SNPs.

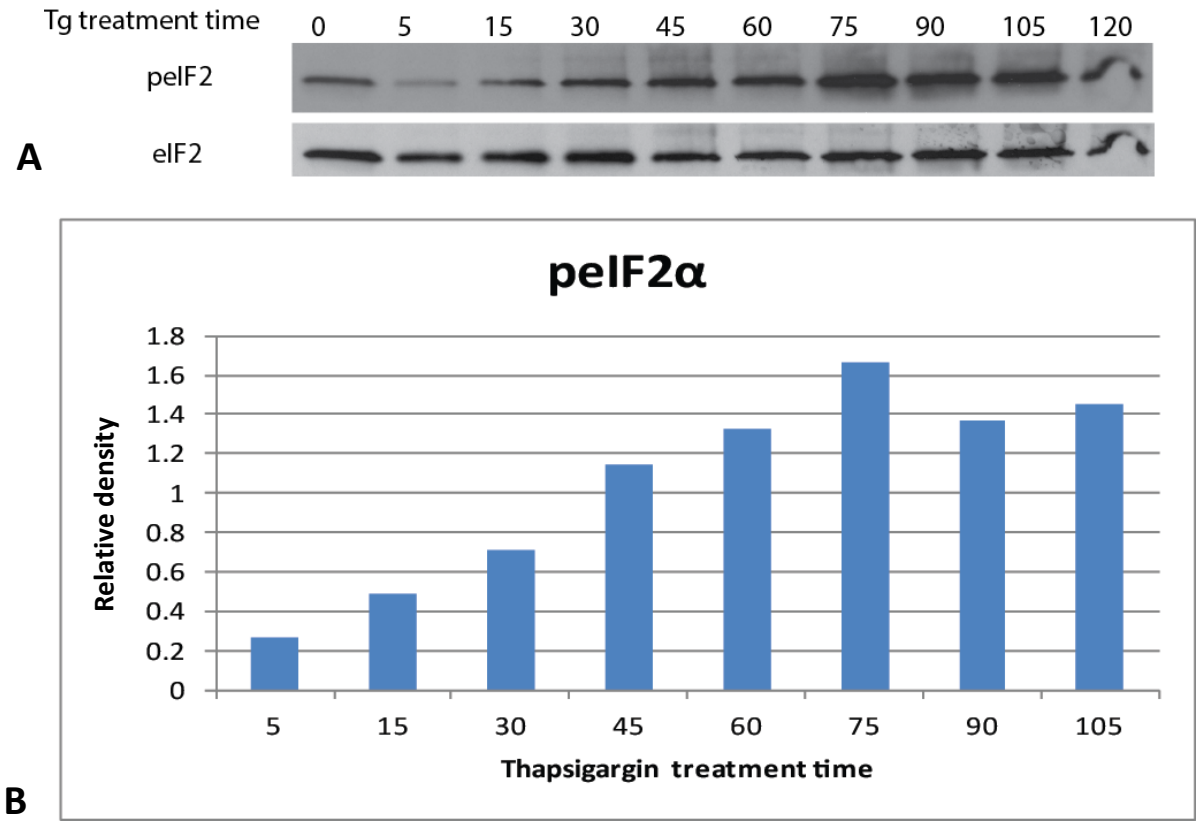


Figure 3.2. Thapsigargin treatment time course for peIF2α. A. Western blot bands of total eIF2α and peIF2α from PERK null MEFs expressing PERK HapA construct (Table 3.2). Phosphorylation of eIF2α peaked at 75 min/tg treatment. B. Quantification of Western blots in A. Relative density for each peIF2α band normalized to corresponding total eIF2α band.

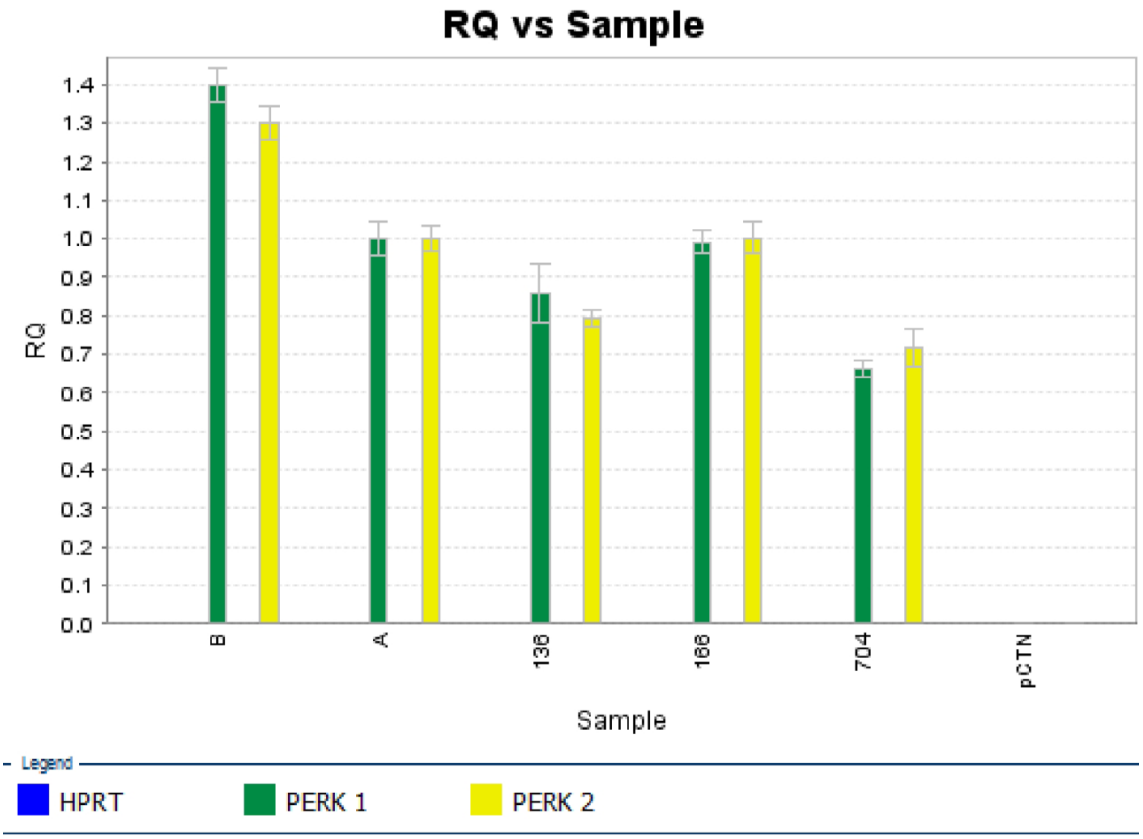
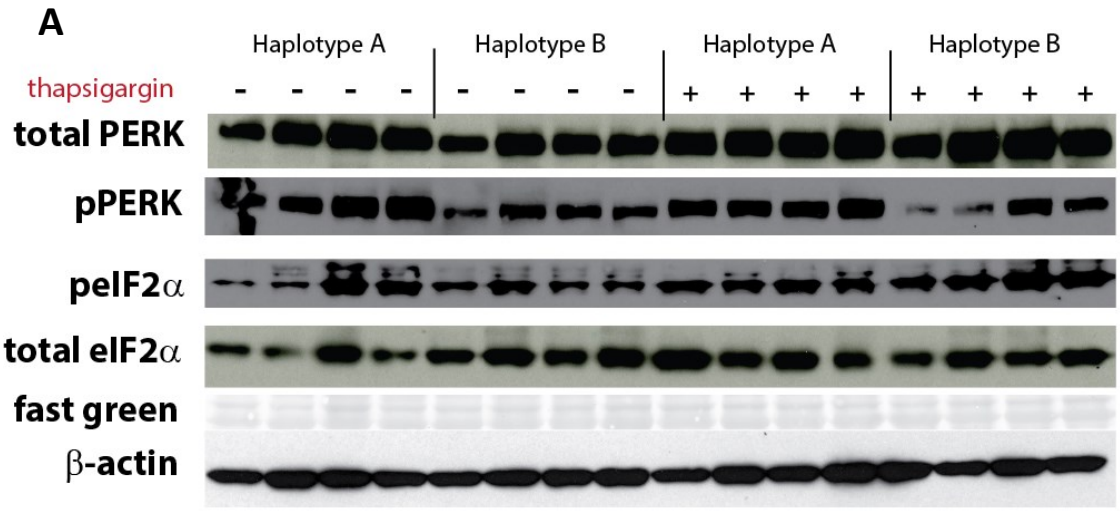


Figure 3.3. Relative quantification of PERK expression by qPCR. Each PERK construct (B: HapB, A: HapA, 136: 136C, 166: 166Q, 704: 704A, pCTN: empty vector; see Table 3.2) demonstrated less than twofold change in expression (relative quantification score [RQ] of greater than 2 or less than 0.5). PERK constructs were transfected into PERK null (-/-) MEFs and RNA harvested 24 hours later. PERK expression detected by Taqman Gene Expression Assays (Life Technologies) designed to detect PERK transcripts (PERK 1 and PERK 2). Endogenous control: HPRT1.


A
Haplotype A
Haplotype B
Haplotype A
Haplotype B
thapsigargin
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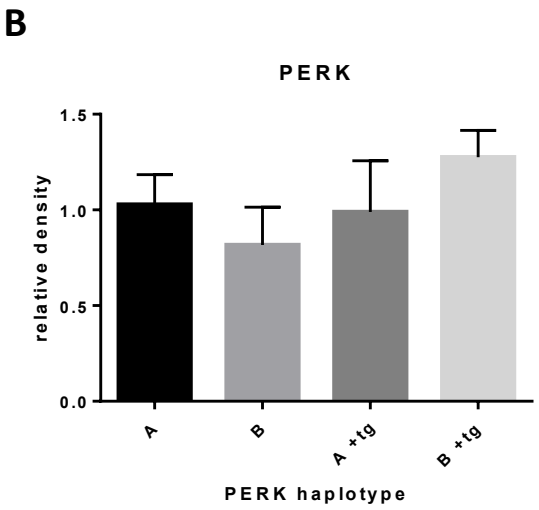
total PERK
pPERK
peIF2α
total eIF2α
fast green
β-actin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relative density
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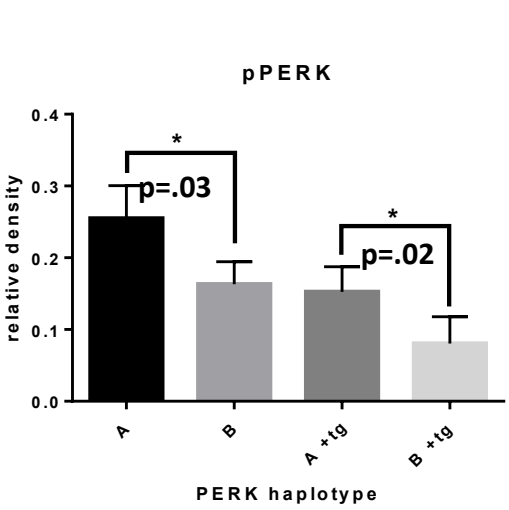
1.0
0.5
0.0
A
B
A +tg
B +tg
PERK haplotype


C
pPERK
relative density
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0.3
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*
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*
p=.02
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A +tg
B +tg
PERK haplotype

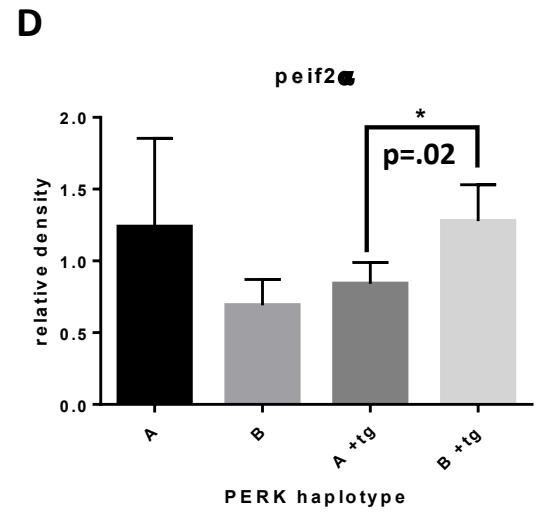

D
peif2
relative density
2.0
1.5
1.0
0.5
0.0
*
p=.02
A
B
A +tg
B +tg
PERK haplotype

Figure 3.4. Human β-lymphocytes endogenously expressing PERK haplotype A and PERK haplotype B. A. Western blots comparing levels of total PERK, pPERK, peIF2α, and total eIF2α between HapA and HapB, with and without 0.1μM tg treatment (30min). Band quantifications were normalized to fast greenCell lines expressing PERK haplotype A were NA06985, NA06991, NA06993, NA07029 (lanes 1-4 and 9-12, respectively; cell lines expressing PERK haplotype B were NA07348, NA07357, NA10835, NA11993 (lanes 5-8 and 13-16, respectively). Each lane represents one cell line homozygous for HapA (n=4) or HapB (n=4). Loading controls: fast green and β-actin. **B-D.** Quantification of Western blots in A. **B.** PERK levels did not vary significantly between haplotypes or with drug treatment. **C.** peIF2α levels were significantly higher in HapB cells after tg treatment. **D.** pPERK levels were significantly lower in HapB cells with or without tg treatment. *$p<0.05$. Error bars represent standard deviation.

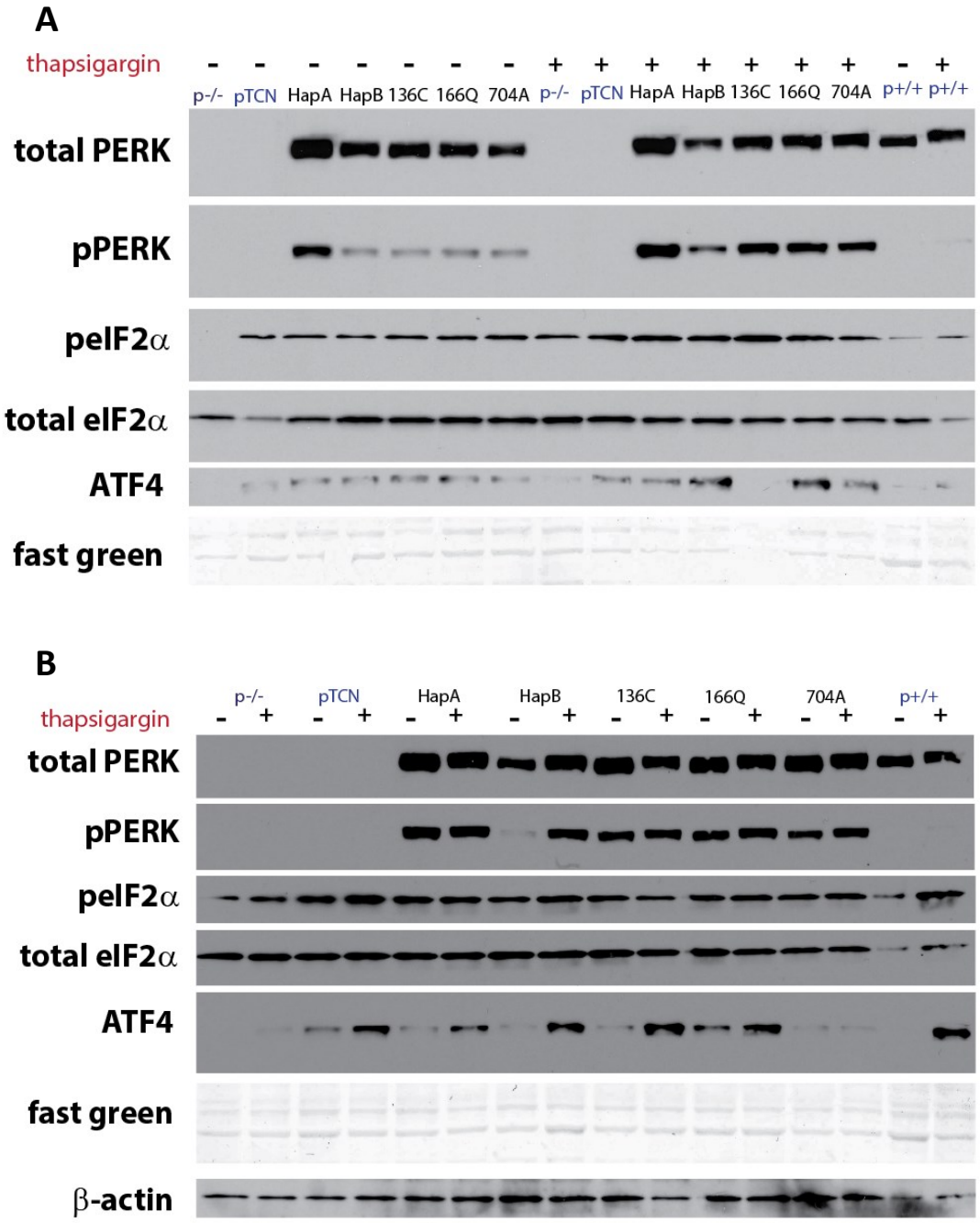


Figure 3.5 Western blots of PERK -/- MEFS expressing human PERK constructs. **A.** Western blot loaded for visual comparison between constructs. **B.** Western blot loaded for visual comparison between each construct with and without tg treatment. P-/-=PERK null MEFs, pTCN=empty vector. For quantification, see Fig 3.6-3.9.

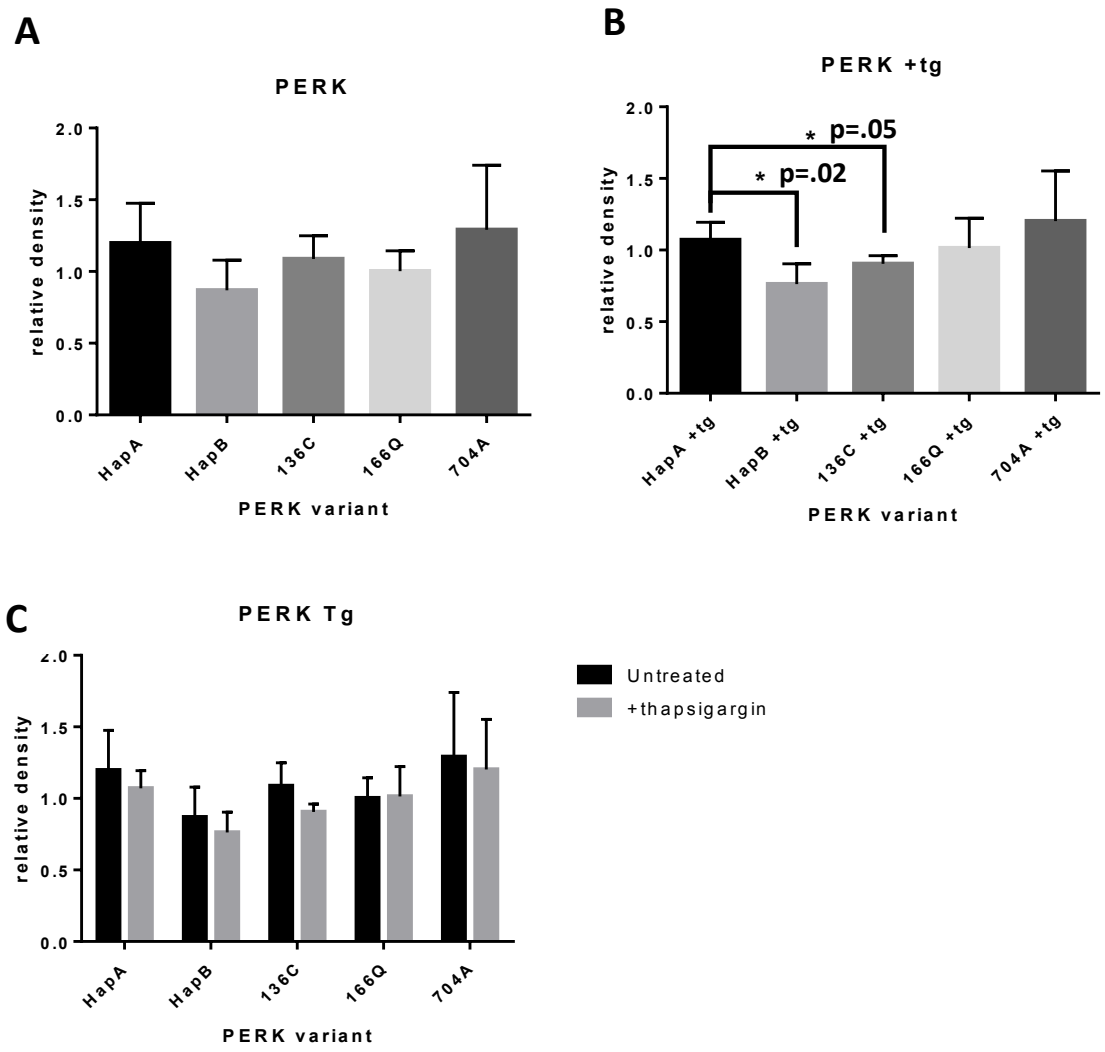
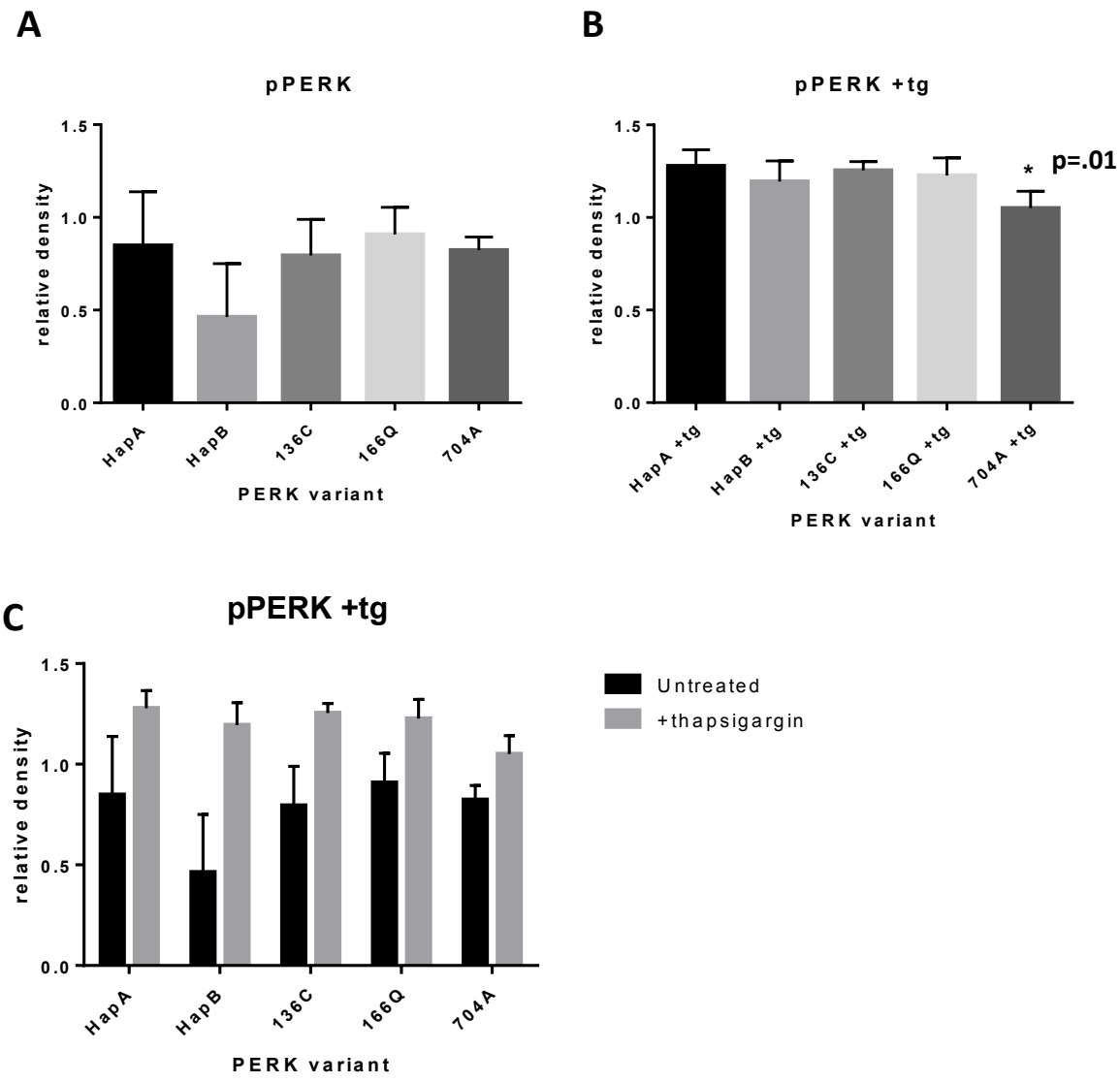


Figure 3.6 Quantification of PERK from Western blots. A-B. Comparison of PERK levels between constructs. P-values are comparisons between each construct and HapA. **A.** PERK levels were not statistically different between constructs in the absence of tg treatment. **B.** Tg treatment resulted in a dip in total PERK levels in HapB (p=0.02) and 136C (p=.05) in comparison to HapA. **C.** Comparison of PERK levels with and without tg treatment for each construct. P-values are comparisons within each construct with and without tg. PERK levels did not significantly change within each construct with tg treatment. All PERK bands normalized to fast green protein bands (loading control). Error bars represent standard deviation.


A
pPERK
relative density
1.5
1.0
0.5
0.0
HapA
HapB
136C
166Q
704A
PERK variant
B
pPERK +tg
relative density
1.5
1.0
0.5
0.0
*
p=.01
HapA +tg
HapB +tg
136C +tg
166Q +tg
704A +tg
PERK variant
C
pPERK +tg
relative density
1.5
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704A
PERK variant
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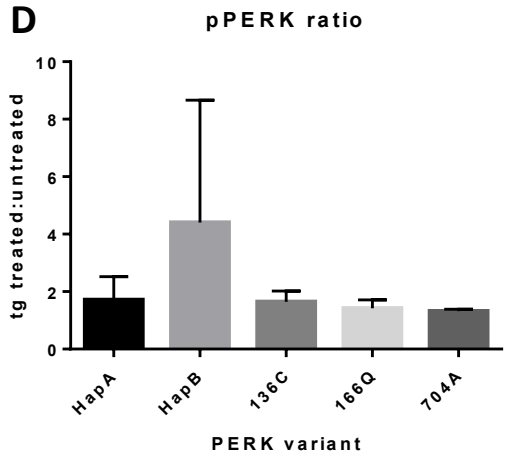
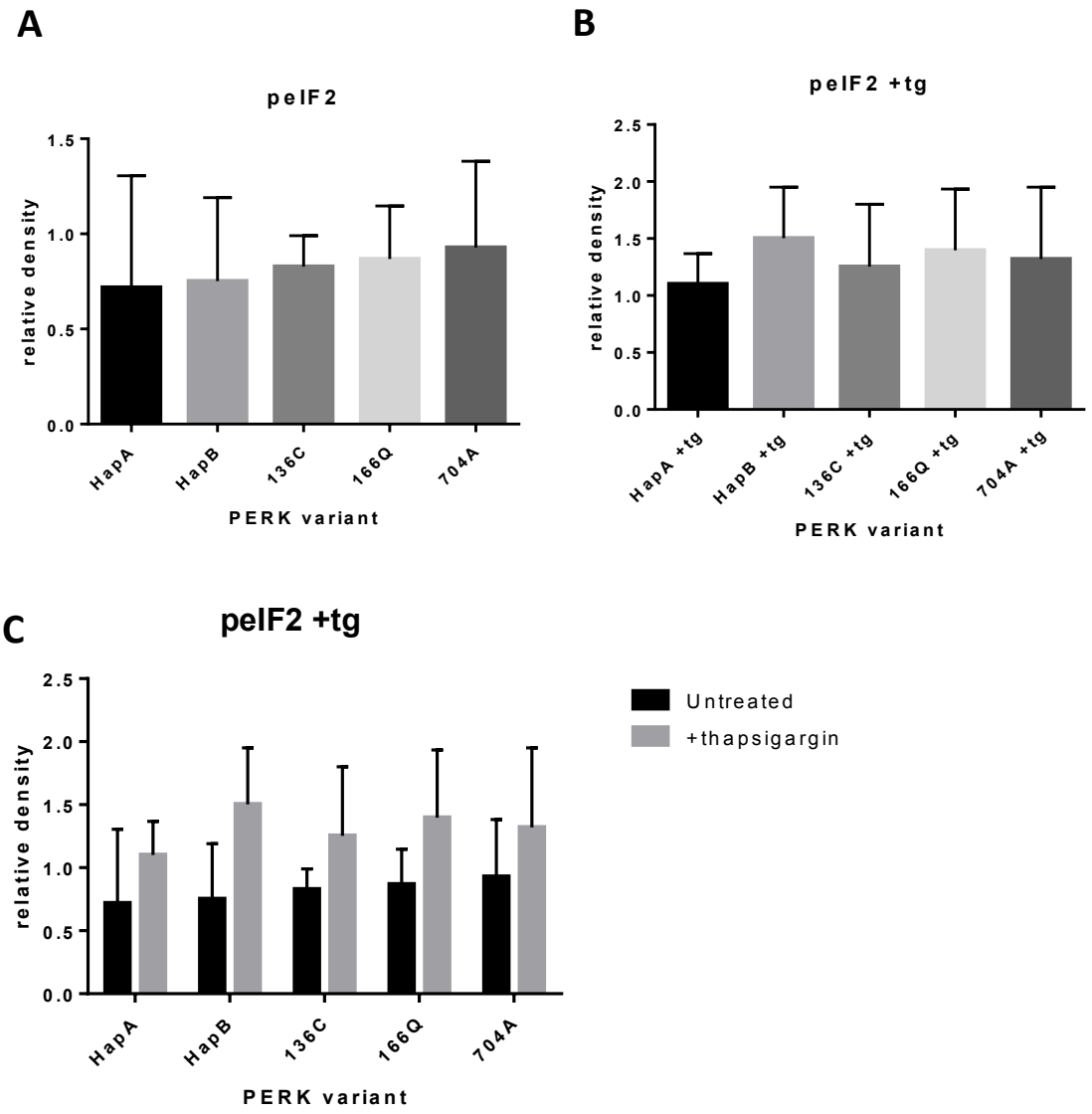


Figure 3.7 Quantification of pPERK from Western blots. A-B. Comparison of pPERK levels between constructs. P-values are comparisons between each construct and HapA. **A.** pPERK levels were not statistically different between constructs in the absence of tg treatment. **B.** pPERK levels were significantly lower in 704A relative to HapA (p=.01). **C.** Comparison of pPERK levels with and without tg treatment for each construct. P-values are comparisons within each construct with and without tg. pPERK for all constructs increased significantly with tg treatment (all p-values < 0.05). **D.** The ratio of tg treated to untreated for each construct was not significantly different from the tg treated to untreated for HapA, though tg treatment tended to have a greater magnitude of effect on HapB. Error bars represent standard deviation.


A
peIF2
relative density
1.5
1.0
0.5
0.0
HapA
HapB
136C
166Q
704A
PERK variant
B
peIF2 +tg
relative density
2.5
2.0
1.5
1.0
0.5
0.0
HapA +tg
HapB +tg
136C +tg
166Q +tg
704A +tg
PERK variant
C
peIF2 +tg
relative density
2.5
2.0
1.5
1.0
0.5
0.0
HapA
HapB
136C
166Q
704A
PERK variant
Untreated
+thapsigargin

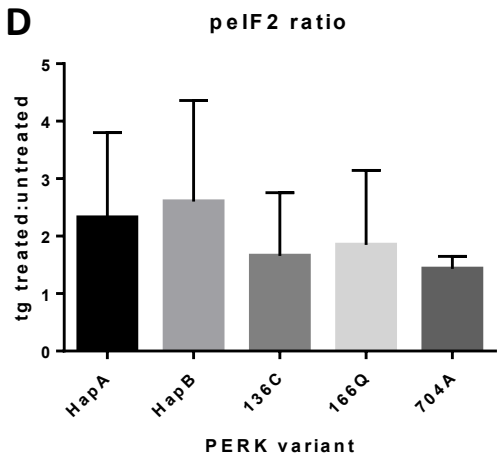
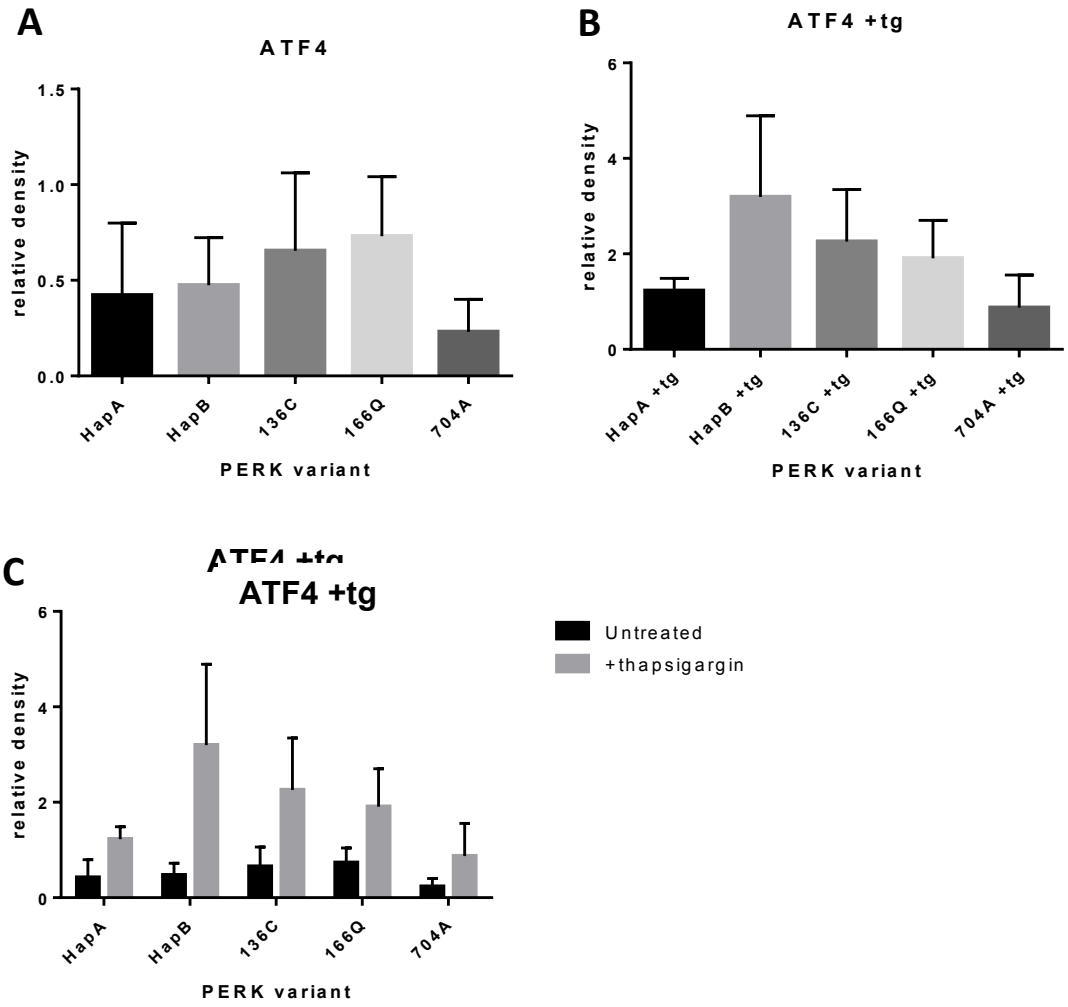


Figure 3.8 Quantification of peIF2α from Western blots. A-B. Comparison of peIF2α levels between constructs. P-values are comparisons between each construct and HapA. **A.** peIF2α levels were not statistically different between constructs with or **B.** without tg treatment. **C.** Comparison of peIF2α levels with and without tg treatment for each construct. P-values are comparisons within each construct with and without tg. peIF2α for all constructs did not increase significantly with tg treatment (all p-values > 0.05). **D.** The ratio of tg treated to untreated for each construct was not significantly different from the tg treated to untreated for HapA. Error bars represent standard deviation.


A
ATF4
relative density
1.5
1.0
0.5
0.0
HapA
HapB
136C
166Q
704A
PERK variant
B
ATF4 +tg
relative density
6
4
2
0
HapA +tg
HapB +tg
136C +tg
166Q +tg
704A +tg
PERK variant
C
ATF4 +tg
relative density
6
4
2
0
HapA
HapB
136C
166Q
704A
PERK variant
Untreated
+thapsigargin

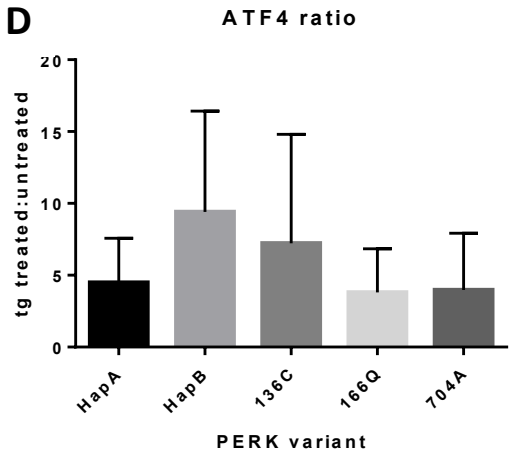


Figure 3.9 Quantification of ATF4 from Western blots. A-B. Comparison of ATF4 levels between constructs. P-values are comparisons between each construct and HapA. **A.** ATF4 levels were not statistically different between constructs with or **B.** without tg treatment. **C.** Comparison of ATF4 levels with and without tg treatment for each construct. P-values are comparisons within each construct with and without tg. ATF4 for all constructs did not increase significantly with tg treatment (all p-values > 0.05). **D.** The ratio of tg treated to untreated for each construct was not significantly different from the tg treated to untreated for HapA. Error bars represent standard deviation.

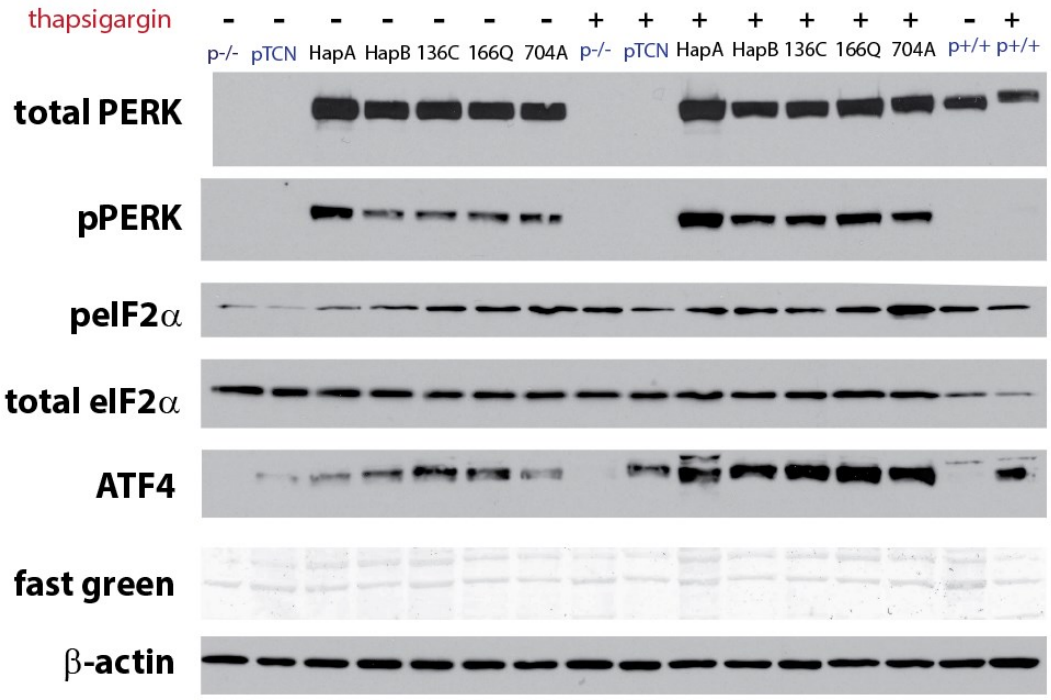


Figure 3.10. Additional Western blot included in preceding analysis. P-/-=PERK null MEFs, pTCN=empty vector. For quantification, see Figures 3.6-3.9.

CHAPTER 4: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Neurodegenerative diseases represent a major health crisis in the United States and around the world. By the year 2050, an estimated 16 million older Americans will suffer from Alzheimer's disease (AD) at a cost of 1.2 trillion dollars in direct care (Alzheimer's Association, www.alz.org). Finding treatments that halt or delay disease progression will be a major public health priority in the years to come. Because parts of AD pathology overlap with the pathology of other age-related neurodegenerative diseases, this push to discover and implement effective therapy may result in treatment options for a number of other disorders, including the tauopathy progressive supranuclear palsy (PSP). Likewise, research into the pathogenesis of PSP could have implications for treating AD and other tauopathies.

Close examination of genetic risk factors for neurodegenerative disease is one way to identify important pathogenic disease pathways and discover new inroads to potential therapies. Large-scale genome-wide association screens (GWAS) and, more recently, whole exome sequencing studies have provided researchers with a wealth of information about potential contributors to neurodegenerative disease pathogenesis (Desikan et al., 2015; Höglinger et al., 2011; Jun et al., 2015; Lambert et al., 2013; Naj et al., 2011). Risk genes identified by SNP genotyping serve as "signposts" of risk—a genome-wide significant SNP may not be the risk-conferring variant in and of itself. Rather, the "hit" SNP is likely in high linkage disequilibrium (LD) with the true genetic source of increased disease risk. The previous chapters detail and explore in-depth one

example of this: the PSP risk gene *EIF2AK3*, which codes for the PERK protein. Though the GWAS signal from *EIF2AK3* came from an intronic SNP (rs7571971), that SNP was in near complete LD with three SNPs that alter PERK protein coding (rs867529, rs13045, and rs1805165). These PERK coding variants are functionally different from one another (Liu et al., 2012) and are likely to be the source of increased PSP risk from *EIF2AK3*.

PERK activation in neurodegenerative disease

Chapter 2 of this dissertation provides biological evidence that PERK is associated with tau pathology in the brain. In a comparison of post-mortem brain tissue from PSP, AD and normal controls, the greatest number of phosphorylated PERK-positive cells were in brain regions highly affected in each disease. pPERK and peiF2α were highest in the brainstem in PSP and in the hippocampus in AD. This puts PERK "at the scene of the crime" not only in PSP, but also in AD and in some elderly controls (Stutzbach et al., 2013). This also confirms and expands upon similar findings from other groups (J J M Hoozemans et al., 2007; Jeroen J M Hoozemans et al., 2009; D. a T. Nijholt et al., 2011). pPERK postitive cells also tended to be positive for htau, especially diffuse htau. This suggests that PERK activation precedes formation of neurofibrillary tangles (NFTs) and thus may be an early event in disease pathogenesis. This, too, reinforces findings from other groups (Jeroen J M Hoozemans et al., 2009).

Intriguingly, some elderly controls exhibited moderate pPERK staining in the hippocampus. A correlation analysis demonstrated that PERK activation increased with both age and tau pathology. This novel finding suggests that PERK activation and tau both tend to increase in the aging brain. Whether PERK and tau directly influence one another is a question for future study. PERK may affect tau pathology in one or more

steps of the pathogenic process: 1.) PERK could influence whether a cell forms aggregates, either at the point of protein delivery to the cytoplasm or at the point of aggregation and/or 2.) PERK could influence downstream effects of tau aggregation. The UPR and tau intersect in several protein degradation pathways, including the proteasome system (Yen, 2011), which is the target of the ERAD pathway (Travers et al., 2000), and the endosome to lysosome pathway (Guo & Lee, 2011).

Another finding that could account for the connection between PERK variation and tauopathy is the association of tau with the rough endoplasmic reticulum (Iqbal et al., 2009). Recent work demonstrated that abnormally phosphorylated tau partially colocalized with several subcellular compartments, including the ER (Tang et al., 2015). This colocalization would bring tau aggregates and the UPR machinery in close proximity, providing an opportunity for these two processes to interact more directly with one another.

One cell culture model that could be useful to investigating this question was established by the Lee group at University of Pennsylvania (Guo & Lee, 2011). Guo and Lee (2011) transduced pre-formed tau fibrils (pffs) into QBI-293 cells expressing exogenous tau. This transduction resulted in formation of NFT-like tau aggregates within a matter of hours. Strikingly, incubating these same tau-expressing cells in media containing tau pffs resulted in spontaneous endocytosis-mediated uptake of these protein seeds and subsequent tangle formation, even in the absence of a protein delivery reagent. This model is thus useful for studying intracellular tau aggregation, abnormal tau degradation, and/or tangle-induced toxicity in cell culture and also could provide insight into cellular processes that affect uptake of abnormal tau and delivery to

the cytoplasm. Other potentially useful cell culture models of tau aggregation use fluorescently tagged tau to monitor aggregation in real time (reviewed in Lim et al. 2014) and may bypass some of the experimental drawbacks of post-fixation examination of tau aggregation.

Using one of these cell culture models of tau aggregation, future work could examine 1) whether/when PERK is activated during the uptake, seeding, or aggregation process and 2) whether perturbing PERK affects tau uptake, seeding, or aggregation. PERK could be manipulated in several ways: 1) Gross pharmacological induction of the UPR via thapsigargin (Rogers et al., 1995) or tunicamycin (Elbein, 1987) 2) specific pharmacological prolongation of eIF2α phosphorylation via salubrinal (Boyce et al., 2005) 3) expression and activation of a stress-independent, drug-inducible PERK construct (Fv2E-PERK; Lu et al., 2004) 4) PERK knockdown *via* RNAi 5) PERK inhibition *via* treatment with GSK2656157 (Atkins et al., 2013) or 6) expression of the PERK variant constructs detailed in Chapter 3 of this dissertation.

PERK variation and its effect on PERK function

Chapter 2 also presents data supporting the hypothesis that a three amino acid coding variant haplotype of the PERK protein, HapB, contributes to PSP risk (Stutzbach et al., 2013). HapB PERK induces higher levels of eIF2α phosphorylation in response to thapsigargin treatment than does HapA PERK (Liu et al., 2012). Because the higher activity HapB PERK is also higher risk for PSP, it follows that this increased activity is not adaptive and may actually be pathogenic. One explanation for this could be that prolonged phosphorylation of eIF2α damages the neuron *via* extended translational inhibition (Moreno et al., 2012). Continual synthesis of new proteins and neurotransmitters are essential to neuronal function in general and memory formation in

particular. Long-term potentiation (LTP), an important component of memory formation at the cellular level, is inhibited by eIF2α phosphorylation (Ma et al., 2013), and an over-active eIF2α kinase could disrupt this important process.

Thus, though PERK activation in the short-term and in moderation may be adaptive, extended PERK activity may irreparably damage a neuron. Even a modest increase in PERK activity might bias a cell toward dysfunction, especially as pathological protein aggregates accumulate in the cytoplasm and challenge homeostasis in myriad ways. It could be that as we age, our neurons are more vulnerable to these stressors as more pathological proteins accumulate, increasing the likelihood of cell death in response to a challenge.

Chapter 3 explores the origin of this differential eIF2α kinase activity between HapA and HapB. HapB is composed of the following three amino acid variations: Ser136Cys, Arg166Gln, and Ser704Ala. Amino acids 136 and 166 are located on the luminal, dimerization end of the PERK protein; amino acid 704 is on the cytoplasmic, kinase-domain side of the protein. Though all three coding variants are usually inherited together (Liu et al., 2012; Stutzbach et al., 2013), it is possible that only one of the changes is responsible for the difference in activity between the two versions of the PERK protein.

This work explores this possibility in two ways. First, data from Chapter 3 replicated the finding from Liu et al. (2012) that HapB PERK phosphorylates more eIF2α in response to thapsigargin treatment than does HapA. Seemingly in contrast, HapB β-lymphocytes also showed relatively less PERK phosphorylation in response to this same drug treatment. If this effect is not due to a difference in pPERK antibody affinity

between the haplotypes, it could indicate that HapB is actually less likely to transautophosphorylate and more likely to phosphorylate eIF2α. Future work should explore this possibility.

Expressing artificial PERK variants with these individual amino acid changes in PERK null mouse embryonic fibroblasts (MEFs) did not yield significant differences in PERK activity. However, these results suggest a trend toward HapB-expressing cells having a stronger response to tg treatment. HapB expressing cells showed a bigger difference between baseline levels of pPERK, peIF2α, and ATF4 and these same levels after tg treatment. This difference in the magnitude of response to thapsigargin between HapA and HapB echoes the findings in β-lymphocytes discussed above. Interestingly, MEFs expressing the single amino-acid variant PERK constructs may have an "intermediate" effect on thapsigargin response, though the current data are only preliminary given the lack of statistical significance.

Further study into functional variations of PERK should also examine how post-translational modifications affect differences in activity between HapA and HapB. HapA contains two serines (S136 and S704) not present in HapB. Phosphorylation at either of these sites could potentially affect activity. Thus, future studies could incorporate PERK constructs pseudophosphorylated at these sites (136E and 704E). Conversely, this work could also examine activity of PERK that is unphosphorylatable and incapable of disulfide bonding at residue 136 (136A). Another way to approach this question would be to determine by mass spectrometry whether either of these serines are phosphorylated on endogenously expressed HapA PERK, either at baseline or in response to stress.

It may be that PERK -/- MEFs are not an ideal model to study PERK variation and its contribution to disease pathogenesis. Because PERK plays a role in neurodegeneration, future work could benefit from using neuronal cultures to explore this question. The specialized functions of neurons and their heavy reliance on protein translation may make them especially vulnerable to sustained PERK activation. Though there are, as of yet, no neuronal cell culture models of tau aggregation (Guo & Lee, 2014), this type of system would be ideal for studying the interaction between PERK variation and tau pathogenesis.

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