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Invited review

Targeting the unfolded protein response in neurodegeneration: A new approach to therapy

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ABSTRACT

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and the rarer prion diseases, share a number of key similarities, including aggregation of disease-specific proteins in the brain and neuronal loss. The focus of research in these disorders has centred on pathogenesis caused by individual proteins and their build up in their specific diseases, but there are also likely to be more generic pathways that are active in neurodegeneration across the spectrum of these disorders. The unfolded protein response (UPR) has recently emerged as one such pathway. The UPR is normally a protective cellular response that protects against endoplasmic reticulum (ER) stress, which occurs with the build up of misfolded proteins. Recent evidence indicates that in neurodegenerative disease this pathway becomes constitutively activated, preventing protein translation. UPR activation is found in post mortem brains in a variety of diseases, including AD, PD and prion diseases, and has also been found to be activated in mouse models of neurodegeneration and in various *in vitro* models. We propose that modulation of the UPR in neurodegeneration is therefore a promising target for future therapeutic treatments.

This article is part of the Special Issue entitled 'The Synaptic Basis of Neurodegenerative Disorders'.

1. Introduction

Neurodegenerative diseases are one of the greatest challenges facing society and medicine at present. Due to an ageing population, they are expected to increase in prevalence and are predicted to become the second most common cause of morbidity in the developed world by 2040. Alzheimer's disease alone is estimated to affect 25 million individuals worldwide (Wimo et al., 2003). Since Alois Alzheimer first described a specific form of dementia that would later bare his name, a great amount of research has been directed at deciphering the molecular and biochemical mechanisms that lead to neurodegeneration. Despite having distinct clinical, pathological and biochemical signatures, neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and the rarer prion diseases, all share remarkable similarities: protein aggregation in the brain and fatal neuronal loss. The build up of misfolded proteins is the major common feature; this group of diseases is often referred to as protein misfolding disorders. Each disorder exhibits a build up of disease specific misfolded proteins, amyloid-β (Aβ) in AD, α-synuclein in PD, huntingtin in HD or the prion protein (PrP) in prion disease. Much effort has been directed into elucidating how the build up of these specific misfolded proteins contributes to the pathology of their respective diseases. Aβ is known to be toxic to synapses, reducing synaptic transmission as well as the number of dendritic spines (Yu and Lu, 2012). The accumulation of α-synuclein can damage mitochondria, leading to cell death in the substantia nigra (Cookson, 2009). Expanded huntingtin can form inclusion bodies that interfere with normal cellular processes and induce the misfolding of proteins (Hatters, 2008). But as well as these disease-specific toxic mechanisms, are there more general similarities between these neurodegenerative diseases? Neurodegeneration starts with synaptic dysfunction, which leads to the loss of dendritic spines and the postsynaptic density, and ultimately to the failure of neuronal networks and neuronal cell death. Cellular processes such as protein recycling (Rubinsztein, 2006) and mitochondrial dysfunction (Lin and Beal, 2006) have already begun to explain some of the common footprints of neurodegeneration. Recently, the UPR has emerged as a central player in the pathology of prion disease (Moreno et al., 2012), and importantly, it is likely to be a shared common feature in neurodegeneration.

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1.1. The unfolded protein response

The unfolded protein response (UPR) is a protective cellular mechanism that is induced during periods of cellular and endoplasmic reticulum (ER) stress. Secreted and transmembrane proteins enter the ER as unfolded proteins to be properly assembled, or to be targeted for degradation. The UPR maintains the protein-folding homoeostasis within the ER, ensuring the proper functioning of the produced proteins, and therefore the cell. A variety of conditions can interfere with this process and cause ER stress, including amino acid deprivation, viral replication and, as the name suggests, the presence of unfolded proteins (Ron and Walter, 2007). This activates the UPR, which seeks to restore the normal functioning of the ER, using multiple strategies that act individually and in synergy. Chaperone proteins are produced to prevent protein aggregation and facilitate correct protein folding (Sitia and Braakman, 2003). Protein translation is temporarily reduced to lower the amount of proteins present in the ER (Zhao and Ackerman, 2006). Lipid synthesis is also stimulated to increase ER volume, and the degradation of unfolded proteins is induced by activating the endoplasmic reticulum-associated protein degradation (ERAD) pathway (Meusser et al., 2005).

1.2. The three arms of the UPR

When misfolded proteins accumulate within the ER, GRP78/BiP dissociates from three proteins that mediate the UPR stress response: protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) (Lai et al., 2007). Dissociation of GRP78/BiP from PERK, IRE1 and ATF6 allows the activation of these factors resulting in the induction of three UPR-related pathways (Fig. 1).

Activation of PERK leads to a reduction in global protein synthesis via the phosphorylation of eukaryotic initiation factor 2α (eIF2α) (Harding et al., 1999). This phosphorylation causes an eIF2α-mediated translational repression, which halts protein synthesis, helping to alleviate the overload of unfolded proteins inside the ER. There are also three other kinases that can phosphorylate eIF2α, each of which is activated by a different cellular stress: the double-stranded RNA-activated protein kinase (PKR) responds to viral infection (Clemens, 2004), general control non-derepressible-2 (GCN2) is activated during amino-acid starvation (Deng et al., 2002), and the heme-regulated inhibitor kinase (HRI) represses protein synthesis in heme-deficient erythroid cells (Han et al., 2001). Once the ER stress has been resolved and any unfolded proteins have been removed, the translational repression is reversed by dephosphorylation of eIF2α by the phosphatase GADD34 (Novoa et al., 2001). Although the phosphorylation of eIF2α causes the reduction in the synthesis of most proteins, some are upregulated, like activating transcription factor 4 (ATF4) (Blais et al., 2004). ATF4 is a key transcription factor involved in the regulation of genes related to protein folding, amino acid metabolism and redox control (Ma and Hendershot, 2003). Important targets of ATF4 include NRF2, which regulates the functions of a variety of antioxidant genes (He et al., 2001), and CHOP, which conversely is key in the activation of apoptotic pathways and cell death (Han et al., 2013).

There are two paralogs of IRE1: IRE1α and IRE1β (Wang et al., 1998). IRE1α is a kinase and endoribonuclease, that when activated, catalyses the splicing of the mRNA encoding the transcription factor X box-binding protein 1 (XBP1), removing a 26 base-pair intron (Calfon et al., 2002). This splicing changes the reading frame of the XBP1 mRNA, resulting in a potent transcription factor that regulates a subset of UPR targets genes involved in ER protein synthesis and folding, ERAD, autophagy and redox metabolism (Acosta-Alvear et al., 2007). IRE1β controls the site-specific cleavage of 28S rRNA, which contributes to translational repression (Iwawaki et al., 2001).

ATF6 has a CREB/ATF bZIP transcription factor domain at the amino terminus. Upon the accumulation of unfolded proteins in the ER, ATF6 is released from Grp78/BiP, and is trafficked to the Golgi apparatus where it is cleaved by site 1 and site 2 proteases at the

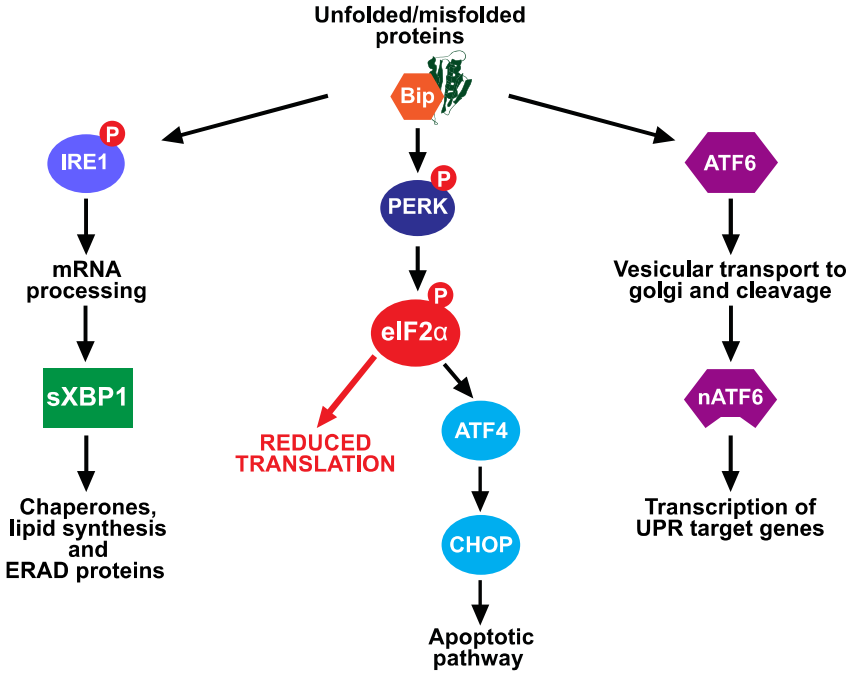

Fig. 1. Schematic of the unfolded protein response. After the detection of unfolded proteins by GRP78/BiP, the three arms of the UPR (PERK, IRE1 and ATF6) are activated. The PERK arm causes a reduction in global protein synthesis via the phosphorylation of eIF2α. Activation of IRE1 leads to XBP1 splicing (sXBP1) and the transcription of chaperones and ERAD proteins. ATF6 is cleaved to nATF6, which leads to the expression of a variety of UPR target genes.

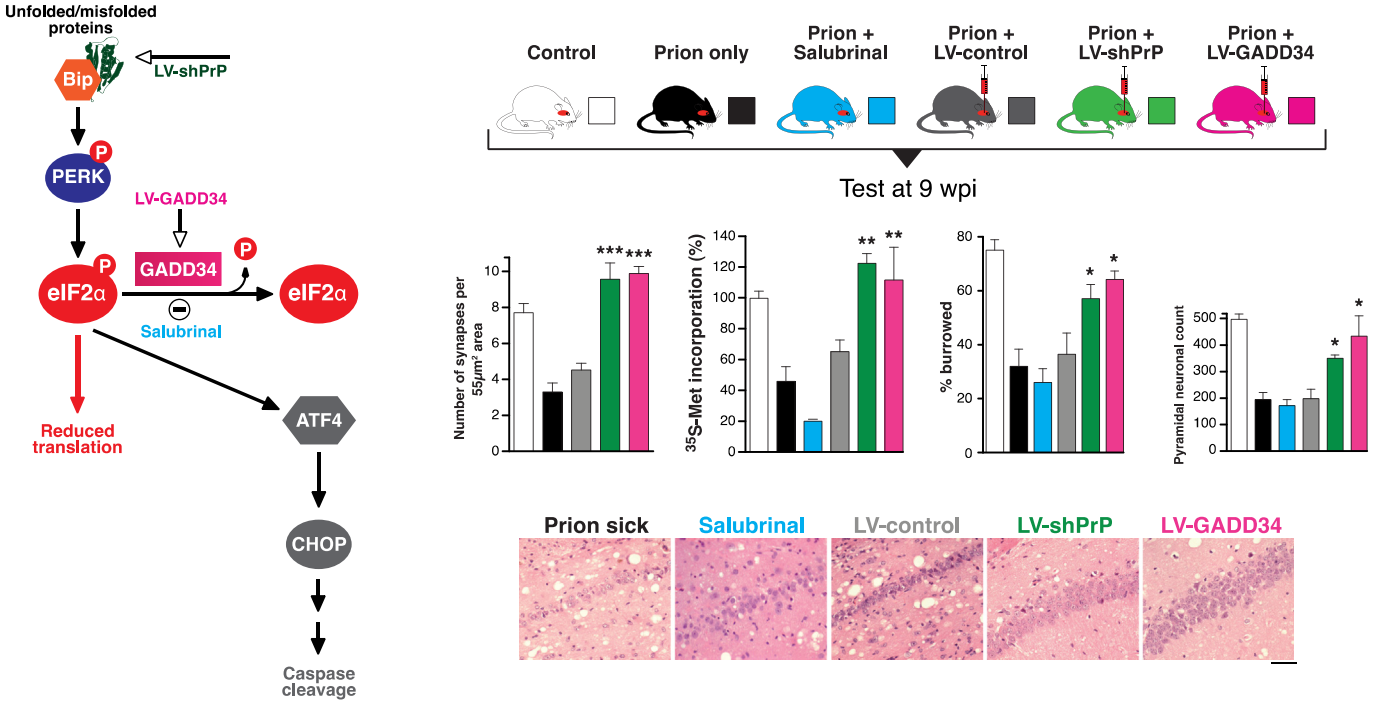

Fig. 2. Manipulation of the UPR changes the progression of prion disease. RNAi against PrP (green), or overexpressing GADD34 (pink) restored synaptic protein levels, synaptic transmission, burrowing behaviour, synapse number and neuronal pathology in prion disease when compared to untreated prion diseased mice (black) or empty vector controls (grey). Salubrinal (blue) had a detrimental effect in the same experiments.

transmembrane site, yielding a cytosolic fragment known as ATF6 p50 (or nATF6), which migrates to the nucleus to activate UPR gene expression (Haze et al., 1999). The chaperones GRP78/BiP and GRP94, the transcription factors CHOP and XBP1 as well as other proteins such as p58IPK/DNAJC3 and SERCA are all induced by ATF6 (Bravo et al., 2013). ATF6 also plays a role in regulating ER volume increases and stimulates cellular adaptation to chronic ER stress (Ron and Walter, 2007).

2. Targeting the UPR in prion disease

Recently, we have shown that the unfolded protein response is a potential therapeutic target in prion disease (Moreno et al., 2012). Mice that are infected with prions follow a stereotypical disease progression, which begins with a reduction in the number of synapses. Later, behavioural signs such as decreased burrowing activity and loss of object recognition memory, as well as a reduction in hippocampal synaptic transmission and the first neuropathological changes are all established. Extensive neuronal degeneration follows, with the animals becoming clinically ill several weeks later, the timing depending on the strain of both the infectious prions and that of the recipient mice. In particular, the incubation period and onset to death is inversely related to levels of PrP in the infected mice (Bueler et al., 1993; Manson et al., 1994).

We studied Tg37 mice that over-express PrP at around 3 fold wild type levels, and succumb to Rocky Mountain laboratory (RML) prion infection in around 12 weeks (Mallucci et al., 2002). Our first key observation biochemically was the finding of abrupt reduction in the number of synaptic proteins at 9 weeks post infection (wpi). This correlated with increasing accumulation of the misfolded PrP, a further decrease in synaptic number and a critical decline in neurotransmission (Moreno et al., 2012). This reduction in synaptic protein levels could result from increased degradation, or decreased synthesis. The ubiquitin proteasome pathway is known to be inhibited in prion disease, causing a reduction, not an increase, in protein degradation (Andre and Tabrizi, 2012). We therefore asked if protein synthesis was reduced through translational control mechanisms. Given that the level of misfolded PrP rises during disease, and that PrP is synthesised in the ER, we hypothesised that translational repression by the UPR was the cause of the decrease in proteins. We found that there was a progressive increase in phosphorylated PERK (PERK-P) and eIF2α (eIF2α-P) as the disease progressed. GADD34 levels did not change, despite the rising eIF2α-P levels, suggesting that there was insufficient GADD34 to dephosphorylate the increased amounts of eIF2α-P. This shows that the PERK/eIF2α arm of the UPR is activated in prion disease, inhibiting protein translation and leading to a reduction in the levels of synaptic proteins.

To confirm this hypothesis, we measured total protein synthesis rates in the hippocampus via the uptake of radioactive methionine into hippocampal slices. A 50% decline in [^{35}S] methionine was observed. We also measured overall translation of messenger RNA (mRNA) by polysome profiling, and found a simultaneous reduction in the overall number of actively translating ribosomes at 9 wpi, as well as a reduction in active translation of specific transcripts of SNAP-25 and β-actin mRNA. In contrast ATF4 mRNA, which escapes eIF2α-P mediated inhibition of translation, due to the structure of its 5′ untranslated region (UTR), showed increased active translation (see for review, Spriggs et al., 2010). PrP mRNA did not show reduced translation, likely due to the presence of similar translational control elements within the 5′ UTR of the PrP gene. As total mRNA levels remained unchanged, the reduction in protein synthesis in prion disease is controlled at the translational, not the transcriptional, level (Harding et al., 2000).

While transient eIF2α phosphorylation is beneficial to cells experiencing ER stress due to misfolded proteins, persistently high levels of eIF2α-P are likely to be detrimental. To test if eIF2α-P is directly involved in prion neurodegeneration *in vivo*, we asked if reducing the levels of eIF2α-P in prion disease would be neuroprotective. To do this we overexpressed GADD34 using a lentiviral

vector, to reduce eIF2α-P levels directly, and in parallel we used targeted RNA interference (RNAi) of PrP to remove the source of UPR activation and prevent eIF2α-P formation. We also asked if increased levels of eIF2α-P exacerbate prion neurotoxicity by using salubrinal, an inhibitor of eIF2α-P dephosphorylation.

At 9 wpi, mice injected with a lentivirus expressing GADD34 showed a similar level of PERK-P as prion infected mice treated both with empty virus and with no treatment, demonstrating that the UPR was still being activated, but eIF2α-P levels were reduced. RNAi against PrP prevented the PrP-induced rise in PERK-P and eIF2α-P seen in untreated animals, confirming prevention of UPR activation. Both GADD34 overexpression and PrP knockdown restored global translation rates at 9 wpi. As a result, synaptic protein levels, synaptic transmission and synapse number in prion-diseased mice treated with GADD34 or PrP knockdown were protected and equivalent to levels in uninfected control mice. Burrowing deficits were prevented and there was extensive neuronal protection in the hippocampus, with no neuronal loss and markedly reduced spongiform change (Fig. 2). Importantly, targeted expression of GADD34 and focal PrP knockdown had a modest, but highly significant, effect on survival.

Critically, treatment with salubrinal had the opposite effect, by preventing dephosphorylation of eIF2α-P. Thus, eIF2α-P levels were markedly higher at 9 wpi than in prion-only controls, causing further repression of global translation. Salubrinal treatment resulted in earlier severe neuronal loss, and significantly accelerated disease compared with untreated prion-infected mice.

These data demonstrate that manipulation of the UPR represents a novel target for treatment in prion disease. Genetic manipulation of this pathway was successful in our model of prion disease (Fig. 2), but there are many barriers to translating genetic approaches for therapy in humans, including potential immune reactions and the possibility of insertional mutagenesis (Pauwels et al., 2009). Small molecule inhibitors provide a much more attractive target for therapy. We propose the inhibition of PERK as a promising target for drug discovery, as it would prevent the phosphorylation of eIF2α, and the downstream pathological translational repression, while allowing chaperone protein expression via the IRE1 and ATF6 arms of the UPR. Importantly, compounds for pharmacological inhibition of PERK have recently been developed for use as anti-tumour agents (Atkins et al., 2013; Axten et al., 2012). It is possible that these, or related compounds optimised for penetration of the blood brain barrier, would be potential therapeutic agents, or allow for the development of new compounds for treatment of the over-activation of the PERK branch of the UPR.

3. Wider relevance of UPR activation in neurodegeneration

There are currently many animal models available to researchers that aim to recapitulate the symptoms and pathology of neurodegenerative disease. Most rely on the introduction of known mutations that have been shown to increase the risk of disease, or the overexpression of the disease related misfolding protein. Although these models have proved to be extremely useful in understanding of neurodegeneration, they often do not produce neuronal cell death, the major pathological event in diseases such as AD, PD and HD (Jucker, 2010). Prion infected mice do develop stereotypical prion disease with the resultant neuronal cell death (Telling, 2008), but care needs to be taken before translating results into humans. With this in mind, what evidence is there from human studies, and other evidence from animal work, to suggest targeting the UPR would be a viable therapeutic option? As discussed below, there is also much evidence to suggest that UPR activation is a common phenomenon in neurodegenerative disease, suggesting the prospect of a treatment aimed at common pathways of disease that are independent of disease-specific proteins.

3.1. Prion disease

Upregulation of several chaperones and ER stress proteins such as GRP78/BiP, GRP94 and GRP58/ERp57 is observed in patients with Creutzfeldt–Jakob disease (CJD), the most common form of prion neurodegeneration in humans, as well as in some mouse models (Hetz et al., 2003; Yoo et al., 2002). This suggests ER stress and abnormal homoeostasis is a feature of prion disease.

One of the ER's most important roles is in calcium homoeostasis and signalling, and the ER contains the largest intracellular store of calcium in the cell. Disruption of calcium homoeostasis, and the resulting ER stress, has emerged as another component of the development of prion disease. Exposing N2A cells to purified PrP^{Sc} from the brain of scrapie-infected mice, induces the release of calcium from the ER stores as well as ER stress. This is associated with the upregulation of several chaperones that are involved in the UPR, that are also found in the brains of CJD patients, such as GRP78/BiP, GRP94 and GRP58/ERp57 (Torres et al., 2010). Cells chronically infected with prions are more susceptible to ER stress mediated cell death, linked with a stronger UPR activation after exposure to ER stress-inducing agents such as tunicamycin and thapsigargin (Torres et al., 2010).

ER stress can also facilitate the generation of intermediary misfolded forms of the prion protein, increasing its vulnerability to conversion into the misfolded PrP^{Sc} form *in vitro* (Orsi et al., 2006). PrP^{Sc} has also been shown to result in the accumulation of proteins in the ER, which can lead to ER stress induced apoptosis (Wang et al., 2011).

3.2. Alzheimer's disease

There have been multiple reports of UPR activation in the brains of Alzheimer's patients (Hamos et al., 1991; Hoozemans et al., 2009, 2005). Importantly, PERK-P and eIF2α-P are also widely reported to be associated with AD post mortem brains (Chang et al., 2002; Nijholt et al., 2011; O'Connor et al., 2008). eIF2α-P levels correlate with elevated BACE1 (an enzyme that cleaves the amyloid precursor protein into Aβ) levels in transgenic mice as well as AD patient brains (O'Connor et al., 2008). Levels of GRP78/BiP, the ER stress sensor and UPR activator, are increased in the temporal cortex and the hippocampus of AD cases compared to non-demented control cases (Hoozemans et al., 2005). A comparison of the expression of GRP78/BiP in the different Braak stages of AD suggests that UPR activation occurs early in AD.

Treatment of cells with Aβ peptides leads to the activation of ER specific caspases, that correlates with the induction of apoptotic cell death (Nakagawa et al., 2000). Exposing cells to Aβ oligomers or fibrils in different experimental models can also trigger ER stress, which has been shown to lead to the phosphorylation of eIF2α, PERK and other indicators of UPR activation (Katayama et al., 2004).

UPR activation is also associated with hyperphosphorylated Tau. PERK-P has been observed in neurons and glia that exhibit tau pathology (Nijholt et al., 2011). IRE1 and PERK phosphorylation have also been observed in patients affected with AD, as well as a wide range of frontotemporal dementias that exhibit tau pathology (Nijholt et al., 2012). *In vitro* studies suggest that the induction of ER stress by the exposure of cells to Aβ oligomers correlates with the induction of Tau phosphorylation, suggesting a link between ER stress, Aβ mediated neurotoxicity and Tau hyperphosphorylation (Resende et al., 2008). Importantly, induction of UPR signalling has been shown to induce Tau phosphorylation, possibly via the activation of glycogen synthase kinase 3β (GSK-3β) (Sakagami et al.,

2013), demonstrating a direct link between UPR activation and neurodegenerative processes. Neurons displaying PERK-P coexpress active GSK-3β in AD brains, suggesting a possible mechanism (Hoozemans et al., 2009). ERAD has been shown to be blocked by tau accumulation, leading to UPR activation in the tg4510 mouse model of tau pathology (Abisambra et al., 2013), demonstrating a novel mechanism of tau toxicity via the disruption of normal proteostasis.

3.3. Parkinson's disease

The UPR has been shown to be activated in dopaminergic neurons of the substantia nigra bearing α-synuclein inclusions in the brain of patients affected by Parkinson's disease (PD), suggesting that the UPR may be involved in dopamine neuron degeneration (Hoozemans et al., 2007). α-Synuclein has also been shown to accumulate within the ER, directly activating the PERK arm of the UPR by binding to GRP78/BiP (Bellucci et al., 2011). Additionally, the accumulation of α-synuclein in dopaminergic cells increased the expression of GRP78/BiP and induced the expression of the UPR-related transcription factor ATF4. The authors also suggested that activation of the UPR pathway in cells by α-synuclein, coincided with pro-apoptotic changes (Bellucci et al., 2011).

The A53T missense mutations in the gene coding for α-synuclein causes dominant familial PD. This mutation is associated with UPR activation, as observed by an increase in CHOP and GRP78/BiP expression, and increased phosphorylation of eIF2α, suggesting the UPR is active in these cells (Smith et al., 2005). ER stress leads to mitochondrial dysfunction, but inhibition of caspase-12, a downstream caspase of UPR activation (Nakagawa et al., 2000), protected the A53T α-synuclein-overexpressing cells from cell death, suggesting that the activated UPR was inducing apoptosis (Smith et al., 2005).

LRRK2 mutations also cause dominant familial PD, by impairing protein degradation pathways in an age dependent manner. This leads to the build up of α-synuclein and ubiquitinated proteins, impairment of autophagy, and increased apoptosis, which is likely to lead to the build up of unfolded proteins (Tong et al., 2010).

Mutations in Parkin result in an impairment of the ubiquitin proteasome pathway, which can result in the accumulation of misfolded proteins within neurons and may underpin the development of PD in people with this mutation (Imai et al., 2000). Parkin has been shown to be up-regulated via ATF4, following ER stress and this event is associated with neuroprotection. It was also found that CHOP could down-regulate Parkin expression. These findings suggest wild-type Parkin plays a protective role following ER stress by preventing stress induced mitochondrial damage, and the loss of function of Parkin due to mutation can be a factor in the development of PD.

4. Conclusions

The UPR is emerging as a promising target in neurodegeneration. UPR activation is associated with a number of neurodegenerative diseases in post mortem human brains as well as a number of animal and cell based models. Manipulation of the UPR via genetic methods has also demonstrated that UPR activation can directly contribute to neurodegeneration. This raises the tantalising prospect of a general treatment for neurodegeneration, independent of any disease-specific mechanisms.

Targeting the UPR may well prove beneficial in several of these disorders, especially by inhibiting the formation of eIF2α-P. However, there is conflicting evidence as to whether inhibiting or activating eIF2α-P, and consequently protein translation, is the prudent approach to take when modulating the UPR. Salubrinal inhibits the dephosphorylation of eIF2α-P, and has been shown to be protective in cells exposed to ER stress (Boyce et al., 2005). Phosphorylated eIF2α was shown to be protective in cells exposed to tunicamycin by preventing oxidative stress that can lead to apoptosis (Han et al., 2013), and eIF2α-P can also induce ATF4 and consequently the NRF2 cell survival pathway (Lee et al., 2003).

How can the conundrum of the conflicting reports on the beneficial or detrimental effects of phosphorylated eIF2α be reconciled? UPR activation is undoubtedly an advantageous response to ER stress and unfolded proteins. The answer may lie in the nature of the ER stress, and more importantly, in its duration. Attenuation of protein synthesis is commonly protective in cellular based models, where ER stress is acute, and strongly induced. In animal models, especially those that model the unfolded proteins found in neurodegenerative disease, the ER stress gradually builds up until it is chronically induced. Here the UPR is constitutively active, and the translational repression that is beneficial during acute insults becomes detrimental, as essential proteins aren't produced. It is likely that a certain amount of fine-tuning will be needed to observe the largest therapeutic benefit if the UPR is to become a valuable drug target. It is likely that such approaches will ultimately form part of a combined approach to inhibiting translation, including manipulation of the elongation step of translation via eIF4E/4E-BP (cap-dependent translation) (Merrick, 2004), in addition to the UPR-mediated effects on the initiation step of protein synthesis. Indeed, inhibition of cap-dependent translation through mTOR inhibition has been shown to be protective in neurodegeneration (see for review, Bove et al., 2011). Ultimately, the ideality is to achieve a balance between restoring global translation and manipulation of target-specific translation, for maximal protection of neuronal function, while preventing the pathological effects of dysregulation of protein synthesis.

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