The unfolded protein response: a common pathomechanism in tauopathies

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Chapter 7

Summary and general discussion
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The presence of aggregates of misfolded proteins in many neurodegenerative disorders indicates that protein quality control mechanisms have been compromised or are incapable to restore protein homeostasis (Chapter 2). In this thesis we show that in neurodegenerative disorders that are classified as tauopathies activation of the unfolded protein response (UPR) is increased. This event coincides with the pathogenic accumulation of the microtubule associated protein tau. Our data indicate that UPR activation contributes to increased levels of phosphorylated tau (p-tau), a prerequisite for the formation of tau aggregates.

UPR activation: a common event in tauopathies

Previously we reported that markers of an active UPR are increased in Alzheimer’s disease (AD). These studies indicated a close association between UPR activation and increased levels of p-tau. To further elucidate the link between UPR activation and tau phosphorylation we investigated the involvement of the UPR in disorders histopathologically classified as frontotemporal lobar degeneration with tau positive inclusions (FTLD-tau). This included cases diagnosed as Pick’s disease (PiD), progressive supranuclear palsy (PSP) and familial frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). Similar to AD, in FTLD-tau we observed a strong association between UPR activation and tau phosphorylation. Markers of an active UPR were observed in neurons that contained diffusely distributed p-tau that had not (yet) formed an inclusion body. Thus, UPR activation is a common pathomechanism in tauopathies and is associated with early tau pathology (Chapter 3). Our observations from post mortem brain tissue suggest a functional relationship between UPR activation and the accumulation of p-tau.

Accumulation of insoluble protein deposits, as occurs in tauopathies, suggests an underlying disruption in cellular proteolysis (Chapter 2). We first investigated whether UPR activation is employed to enhance the activity of the cellular proteolysis systems. In vitro UPR activation led to increased activity of autophagy and had no effect on the ubiquitin proteasome system (UPS, Chapter 4). This indicates that autophagy is the major degradational pathway following UPR activation in neuronal cells. Next, we assessed the functional connection between the UPR and p-tau. Overexpression of
human tau (h-tau) did not influence the UPR in vitro. In addition, no UPR activation was found in the brains of a transgenic mouse model with high levels of phosphorylated and aggregated h-tau at different stages of tau pathology. However, in vitro UPR activation led to a mild increase in tau phosphorylation, indicating that the UPR lies upstream in the signalling cascade leading to tau phosphorylation (Chapter 5). We linked UPR activation to increased activity of the major tau kinase glycogen synthase kinase-3 (GSK-3). Our data show that UPR activation induces selective degradation of the Ser21/9 phosphorylated, inactive, GSK-3 isoform via the autophagy/lysosomal pathway (Chapter 6).

UPR activation is increased in patients with AD and FTLD-tau compared to controls (Chapter 3). The pronounced presence and relatively high number of neurons positive for UPR markers suggests that in the tauopathy brain UPR activation is not a transient event but occurs chronically. Chronic UPR activation indicates that cells are incapable of resolving the underlying disruption in ER homeostasis. The precise nature of this disruption remains unknown. Recently a polymorphism in the gene encoding PERK, EIF2AK3, was identified as a risk factor for developing PSP, one of the tauopathies we studied. In addition, a polymorphism in the gene encoding the ER chaperone protein disulfide isomerase (PDI), PDIT, was shown to be a risk factor for developing primary open angle glaucoma (POAG). These studies suggest that subtle alterations in ER stress transducers or ER protein folding factors may contribute to disease development. Furthermore, neurons rely on a continuous supply of glucose and oxygen by the blood and disruptions in glucose and oxygen metabolism have been shown to induce the UPR. Several key ER chaperones require optimal Ca\(^{2+}\) concentrations for their protein folding activity and disruptions in ER Ca\(^{2+}\) homeostasis can also lead to UPR activation. In AD, amyloid β (Aβ) may contribute to UPR activation. Aβ is generated in the ER of neuronal cells and increased intracellular Aβ production sensitizes cells for ER stress toxicity. In addition, extracellular oligomeric Aβ was readily internalized and this resulted in a moderate UPR response perhaps via induction of Ca\(^{2+}\) release from the ER.

UPR activation is essentially a protective response aimed at restoring ER homeostasis. However, if homeostasis can not be resolved, UPR activation leads to caspase activation and apoptosis. Chronic UPR activation in tauopathies might be insufficient to induce apoptosis. A strong increase in immunoreactivity for pPERK, pIRE1 and the downstream UPR targets pelF2α and GRP-78/BiP was observed in AD brain. We also observed a
strong increase in reactivity against phosphorylated (p) PERK and pIRE1 in FTLD-tau (Chapter 3, peIF2 and BiP were not assessed). This indicates that at least two of the three UPR signalling routes are highly active in the tauopathy brain. Even though signs of apoptotic cell death are not very pronounced in the AD brain, rapidly occurring apoptosis may not be readily identified. Alternatively, the UPR might be modulated in such a manner that favours the pro-survival pathways. This is in line with findings that mature neurons, which need to survive for a lifetime, have downregulated their apoptotic pathways.

In our model system UPR activation lies upstream of tau phosphorylation. We and others only observed a moderate increase in tau phosphorylation upon UPR activation in vitro (Chapter 5). This is in line with the difficulties to reproduce aggregates of hyperphosphorylated tau in cell models by increasing levels of p-tau. Also, in animal models the expression of high levels of mutant h-tau containing FTDP-17 associated mutations is necessary to produce disease-like tau pathology. This implies that an additional event is necessary to cause the accumulation and subsequent aggregation of p-tau. Mounting evidence suggests that a disruption in lysosomal metabolism is involved in AD disease pathogenesis. Abnormalities in the lysosomal system have been identified in neurons of the AD brain in both familial (fAD) and sporadic cases (sAD). This included disruptions in the endocytic pathway at an early disease stage and a progressive build-up of incompletely digested substrates in autophagosomal vesicles. Both the endocytic and the autophagy route converge at the level of the lysosomes and accumulation of endocytic and autophagic vesicles is indicative of a disruption in lysosomal proteolysis. Presenilin-1 (PS1) was shown to be essential in lysosomal acidification and PS1 mutations causing early-onset fAD show the accumulation of undegraded autophagosomal vesicles in patient fibroblasts. It remains unknown whether in sAD and in fAD not associated with PS1 mutations, lysosomal degradation is impaired because of a functional defect in the pathway or whether the increased demand for lysosomal metabolism disrupts autophagosomal flux. Our group demonstrated that oligomeric, but not fibrillar, Aβ is internalized into SK-N-SH neuroblastoma cells via the endocytic pathway and is transported to the lysosomes. Other studies have indicated that Aβ, poorly degraded by lysosomes and that its accumulation in lysosomes causes leakage of lysosomal enzymes, referred to as lysosomal membrane permeabilization (LMP). Aβ peptides of varying size and with varying posttranslational modifications are found in the human brain. One of these variants is pyroglutamate modified Aβ (Aβ,pE-42), which is more
stable and has an increased aggregation propensity and toxicity compared to unmodified Aβ.\textsuperscript{476,491} Data generated in our group indicate that oligomeric Aβ\textsubscript{1\textendash}42\textsuperscript{(pE)} induces LMP more potently than oligomeric Aβ\textsubscript{1\textendash}42.\textsuperscript{492,493} Thus, in AD Aβ\textsubscript{1\textendash}42 may contribute to the defect in the lysosomes and hamper lysosomal degradation of endocytic and autophagosomal substrates. Disruptions in lysosomal metabolism have been most extensively studied in AD. In this thesis we show evidence that lysosomal dysfunction also occurs in other tauopathies. In our study we observed markers of UPR activation in granules that morphologically resembled granulovacuolar degeneration (GVD) in AD and FTLD-tau (Chapter 3). Furthermore, we observed high levels of the autophagy protein LC-3 in neurons that had activated the UPR in AD (Chapter 4). In addition, the inactive form of GSK-3, which is targeted for autophagy/lysosomal degradation by the UPR, was found to accumulate in lysosomal structures in FTLD-tau (Chapter 6).

Our data indicate that induction of autophagy/lysosomal degradation by the UPR is an important event in the pathomechanism of tauopathies. The autophagy/lysosomal system is activated in response to an increased demand for proteolytic degradation in the ER or cellular compartments connected to the ER. Markers of an active UPR are observed as GVD granules in both AD and FTLD-tau, consistent with the notion that upon UPR activation parts of the ER are targeted for degradation by the autophagy/lysosomal system. The ER is an extensive network localized throughout the neuron and the selective degradation of certain parts of the ER, the part that for example senses and signals the ER stress, may more quickly return it to its homeostatic state. In addition, the autophagy/lysosomal pathway regulates the sequestration and degradation of inactive, Ser21/9 phosphorylated, GSK-3 and in this manner enhances tau phosphorylation. On a background of disrupted lysosomal clearance ER homeostasis might not be adequately restored by the UPR and prolonged UPR activation may lead to detrimental effects.

Continued activation of the UPR will further increase GSK-3 activity by sequestering it into lysosomal structures (Chapter 6). It is expected that even though these structures are not degraded, inactive GSK-3 is still sequestered and this will still lead to increased overall GSK-3 activity and tau phosphorylation. Phosphorylated tau is highly aggregation prone and quickly forms aggregates that can not be efficiently cleared if the autophagy/lysosomal system is disrupted. Diminished activity of the tau protein phosphatase PP2A has been reported in AD brain \textsuperscript{97,494,495} and reduced activity of PP2A, and possibly other protein phosphatases, may contribute to the increase in levels of p-tau. Little is
Figure 1: The mechanisms of the UPR in tauopathies. UPR activation leads to increased activity of the autophagy/lysosomal pathway. This increases the cell’s proteolytic capacity and enhances tau phosphorylation via the selective degradation of inactive GSK-3. These events are aimed at restoring homeostasis to the ER. However, if homeostasis can not be restored, UPR activation is prolonged and becomes a chronic event. On a background of already impaired autophagy/lysosomal degradation, as is observed in tauopathies, this leads to the accumulation of p-tau and neurodegeneration. Therapeutical approaches (orange boxes) are aimed at decreasing levels of p-tau and its aggregation and different players in the signalling route may be targeted. Inhibition of GSK-3 activity by lithium decreases levels of p-tau. The same may be achieved by enhancing activity of protein phosphatases, e.g. PP2A. Enhancing activity of PP2A may have dual effects as it directly targets tau and the Ser 21/9 residue on GSK-3. Furthermore, levels of p-tau may be lowered by enhancing activity of the proteasome or by tau vaccination protocols. Tau aggregation inhibitors may prevent tau oligomerization and keep it available for proteasomal degradation. Upstream in the signalling cascade, lysosomal replacement therapy may enhance efficiency of the autophagy/lysosomal system, favouring the rapid degradation of p-tau. Modulation of the UPR by salubrinal, valproate or small molecule modulators may permit activation of UPR protective routes but inhibit detrimental routes.
known concerning the dynamics of tau mediated regulation of the microtubule network. However, tau hyperphosphorylation is thought to disrupt transport of cargo into the axons and towards the synapse. Strikingly, we observed diminished UPR activation in cells that had formed a highly aggregated inclusion body, e.g. neurofibrillary tangles and Pick bodies in AD and PiD, respectively. This might imply that storing aggregated proteins in an inclusion body, sequestered away from essential components, is beneficial. Removing these proteins from the immediate load of targets destined to be degraded might restore the balance between proteolytic demand and capacity and diminish accumulation in the autophagy/lysosomal pathway. If cells fail to form an inclusion body prolonged UPR activation may eventually lead to apoptosis and cell loss. Alternatively, cells that form an inclusion body may become senescent and can therefore no longer maintain the UPR or other cellular functions. This is in accordance with ghost tangles that can be observed in the AD brain. Here the surrounding neuron has been ‘cleared’ possibly via degradation by microglia and the tangle remains. This thesis demonstrates that the UPR is an early event in tauopathies that in conjunction with the autophagy/lysosomal system enhances levels of p-tau leading to neurodegeneration. A model describing this pathway is depicted in Figure 1.

**Therapeutic options in tauopathies**

Directly targeting the formation of p-tau either by inhibition of phosphorylation or stimulation of dephosphorylation are therapeutic options for tauopathies. Lithium, an aselctive inhibitor of GSK-3 activity, is capable of lowering p-tau levels both in vitro and in vivo. Lithium was shown to decrease levels of p-tau in rat brain and was shown to arrest tangle formation in a transgenic mouse model with advanced tau pathology. In addition, lithium treatment prevented tau hyperphosphorylation and tangle formation in FTDP-17 h-tau and GSK-3 overexpressing mice. However, in this model already formed tangles were not cleared. Interestingly, lithium is a drug that is already widely used in the clinic for the treatment of bipolar disorder and is being tested for its efficacy in AD and mild cognitive impairment (MCI). In a recent study lithium treatment was associated with a significant decrease in cerebrospinal fluid concentrations of p-tau and with better performance of cognitive tasks in patients with MCI. An earlier study using patients diagnosed with mild AD failed to show an effect of lithium on levels of p-tau in the CSF or on other AD markers. However, in this study
patients were treated with lithium for 10 weeks, compared to 12 months in the earlier described study. Long term treatment with lithium may be necessary to observe effects on GSK-3 activity and levels of p-tau and is expected to be most beneficial in early stages of the disease. Treatment with lithium gives many side effects and currently more specific GSK-3 inhibitors are being developed including competitive inhibitors that bind to the GSK-3 substrate binding site. Furthermore, animal studies have shown that lithium treatment does not reduce already formed tangles, indicating that another -for example an autophagy/lysosomal degradation stimulating factor- is necessary to clear inclusion bodies. An alternative to decreasing activity of tau kinases is enhancing the activity of tau phosphatases. The small molecule FTY720 (fingolimod, Gilenya) that was recently (2011) approved by the European Medicines Agency for use in multiple sclerosis enhances activity of PP2A, the major tau phosphatase. This drug might also be applicable for lowering levels of p-tau in AD and other tauopathies. In addition, synthetic RVxF-peptides have been shown to activate PP1 by binding to its regulatory site.

Other strategies that target the tau protein are aimed at preventing its aggregation or at clearing p-tau via immunization strategies. Tau aggregation inhibitors aim at preventing the formation of tau oligomers and fibrils. One of the first compounds indentified that blocked tau oligomerization was methylene blue, which was shown to disrupt the structure of isolated PHFs and inhibited tau multimerization. A more recent study demonstrated that methylene blue attenuated tau pathology in a transgenic mouse model via the induction of autophagy. In a library screen of over 200 000 compounds, members of the family of the anthraquinones, organic compounds that contain an aromatic ring, were shown to prevent paired helical filament (PHF) formation and dissolve preformed PHFs. Historically, in AD vaccination techniques focused on Aβ and the clearance of Aβ from the brain. However, these studies have shown limited efficacy and removal of Aβ did not halt disease progression. More recently, tau vaccination techniques have been explored to reduce levels of p-tau in several transgenic animal models. In these studies transgenic animals were vaccinated with p-tau peptides and this significantly lowered levels of p-tau and prevented the formation of NFTs. In an AD mouse model expressing all 6 h-tau isoforms and a presenillin mutation, tau vaccination prevented cognitive decline and cleared abnormal tau from the brain. However, in these studies animals were vaccinated before the onset of NFTs and this did not address whether pre-existing tangles also dissipate with vaccination.
In several mouse models for neurodegeneration, including Huntington’s disease, Parkinson’s disease (PD) and also AD, induction of autophagy by the mTOR inhibitor rapamycin has proven beneficial in degrading already formed inclusion bodies.\textsuperscript{514-516,361} However, in human tauopathies we do not expect rapamycin to be beneficial as it only enhances autophagosome formation on a background of defective or impaired lysosomal degradation. Alternatively, lysosomal enzyme replacement therapy (ERT)\textsuperscript{517} and restoration of the function of the lysosomes, could be used to enhance or restore autophagic flux. ERT was initially developed to treat lysosomal storage disorders. These disorders are caused by a hereditary defect in a lysosomal enzyme, or in its targeting to the lysosomes, that leads to lysosomal accumulation of complex macromolecules, e.g. sphingolipids and glycoproteins.\textsuperscript{518} The prototypical LSD that is successfully treated by ERT is Gaucher’s disease ty pe 1. Here, systemic administration of the lysosomal enzyme β-glucosidase restores lysosomal degradation of the lipid glucosylceramide.

Enhancing lysosomal capacity in affected cells in the tauopathy brain is expected to contribute to restoring ER homeostasis but also to clearing already formed small aggregates and inclusion bodies. Lysosomal ERT could be a viable treatment option for patients with FTDP-17. This disorder arises between the 3\textsuperscript{rd} and 5\textsuperscript{th} decade and p-tau pathology is thought to accumulate even earlier. This indicates that the autophagy/lysosomal system, efficiency of which decreases with age, is quickly overwhelmed and is incapable of degrading accumulated p-tau. An underlying difficulty with using ERT for neurodegenerative disorders is crossing of the blood brain barrier (BBB). However, several studies have addressed this problem in animal models and novel techniques, e.g. reversible osmotic barrier opening and use of BBB receptor mediated transport, are being developed. In the case of fAD with PS1 mutations lysosomal acidification is impaired and therefore in these disorders ERT is not a viable option. In this case an alternative would be enhancement of the activity of the UPS, strategies for which are described in Chapter 2. Degrading monomeric, phosphorylated or newly translated tau, would prevent further accumulation into inclusion bodies.

The UPR as a therapeutical target in tauopathies

As the UPR is activated early in tauopathies and precedes increased tau phosphorylation and aggregation it poses an interesting therapeutical target. Attenuation of the UPR, by for example, designing small molecules that either prevent ER stress sensor activation or that
inhibit their downstream signalling, could prevent chronic UPR activation. Full inhibition of the UPR is not expected to be beneficial as cells can no longer adequately control ER homeostasis. Alternatively, modulating different pathways of the UPR might convey a protective effect. Several drugs have already been shown to act on the UPR. Salubrinal, an inhibitor of eIF2α dephosphorylation, was shown to protect against ER stress induced cell death and was protective in a PD cell model. In addition, the chemical valproate was shown to protect against ER stress induced toxicity by increasing intracellular levels of GRP-78/BiP and thus enhancing the capacity of the ER to cope with disrupted homeostasis. Furthermore, valproate preserved oligodendrocytes and axons in a model for spinal cord injury by inhibiting the UPR pro-apoptotic protein CHOP. In addition to a direct effect on the UPR, valproate was recently shown to reduce tau phosphorylation via the inhibition of CDK-5 and GSK-3β in a cell and animal model. Thus, valproate could be an interesting treatment option with dual action in tauopathies.
Conclusion and future studies

In this thesis we demonstrate that the UPR is an early event in tauopathies that lies upstream of tau pathology. Therefore, \textit{in vivo} models of tauopathies, in which mutant tau is overexpressed, bypass this important early step of UPR activation. It is thus pivotal to identify the initial inducer of ER stress in the tauopathy brain. Interestingly, diabetes is a risk factor for developing AD, the major tauopathy.\textsuperscript{523} Glucose deprivation is a known inducer of the UPR \textsuperscript{524} and a disruption in insulin signalling and glucose metabolism in the brain may trigger ER stress. Regional reductions in brain glucose metabolism are apparent in MCI \textsuperscript{525} and in AD \textsuperscript{526}, thus linking reduced brain metabolism to pathology. Furthermore, ischemia and traumatic brain have been shown to activate the UPR in animal models. In tauopathies it is unknown whether ER stress is caused by a transient stress inducer, after which neurons are inadequate at restoring ER homeostasis and the UPR is not switched off, or whether ER stress is caused by a continuous disruption of ER homeostasis.

Much is known about the signalling of the separate arms of the UPR, however little is known about the timing of their activation or whether cell specific modulation of the UPR occurs. It is important to further dissect the involvement of selective pathways of the UPR, to determine whether a specific signalling route of the UPR is responsible for tau phosphorylation. This may be achieved with drugs like Salubrinal or constructs that express active UPR components and transcription factors. This will facilitate the development of selective UPR modulating drugs that may be implemented for the treatment of tauopathies.