Tau phosphorylation as adaptive response to metabolic dysfunction in the brain
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Discussion
Metabolic stress increases tau phosphorylation via the UPR

Metabolic disturbances are strongly associated with increased risk for tauopathies such as AD and are a potent inducer of the UPR. Previous studies show that the UPR is activated in tauopathies in close connection with early stages of tau pathology [73, 74, 76, 79]. In this thesis, we observe UPR activation and increased tau phosphorylation in torpor, which is a physiological hypometabolic response (Chapter 2). We show that metabolic stress activates the UPR causing increased tau phosphorylation. Remarkably, after removal of metabolic stress, the UPR switches off and increased tau phosphorylation disappears. Inhibition of the PERK pathway of the UPR prevents the increase in phosphorylated tau showing that metabolic stress-induced tau phosphorylation is mediated by PERK signaling. In line with this, inhibition of the PERK pathway in a mouse model of FTDP-17 shows restoration of protein synthesis, lower phosphorylated tau levels and reduced clinical signs [90]. Recent genetic data are in accordance with a direct effect of the UPR on tau phosphorylation. A polymorphism in the gene encoding PERK (EIF2AK3), which results in increased risk to develop PSP and AD, is associated with increased activity of the PERK pathway [80, 81]. Moreover, injection of TM in the rat brain results in UPR activation and increased levels of endogenous tau phosphorylation [342]. This UPR-induced tau phosphorylation may lead to tau aggregation, which in turn may contribute to prolonged UPR activation by interfering with ER associated degradation, as was recently shown to occur in a transgenic tau model with a very aggressive phenotype [190, 191]. It should be mentioned that the UPR in this study is activated by strong overexpression of mutant tau while we studied endogenous tau levels. It is possible that high levels of transgenic tau have a different effect than endogenous tau. Nevertheless, in primary rat cultures ER stress and endogenous hyperphosphorylated tau were shown to induce each other and form a vicious cycle [343]. Overall, this underlines the risk of prolonged UPR activation and the importance of balanced regulation.

It is still elusive how the UPR increases tau phosphorylation. An imbalance in kinase and phosphatase activity is the first step to increased tau phosphorylation levels. GSK3β is a major tau kinase that is associated with tau pathology. In transgenic mice overexpressing GSK3β, tau is highly phosphorylated and inhibition of GSK3β prevents this tau phosphorylation [344-347]. Interestingly, the UPR increases the activity of GSK3β [91, 92]. Therefore, UPR-induced tau phosphorylation might be regulated via GSK3β. Another kinase that is activated by the UPR is checkpoint kinase (CHK). CHK can phosphorylate tau and increase tau toxicity [348, 349]. In Drosophila, overexpression of PERK results in neurodegeneration, which is rescued by the inhibition of eIF2α phosphorylation [350]. A genetic screen showed that CHK was involved in this PERK mediated neurodegeneration in Drosophila. Moreover, the resulting recovery of protein translation inhibits CHK phosphorylation and consequently CHK activity indicating that CHK activity is regulated via the PERK pathway [351]. The fact that PERK regulates CHK activity fits with our observation that increased tau phosphorylation is regulated via PERK. However, other kinases and/or phosphatases apart from GSK3β and CHK may be involved in UPR-induced tau phosphorylation as well.
Insulin deficiency increases tau phosphorylation via PKA

Epidemiological studies show that DM, a severe metabolic disease, is a risk factor for AD [141]. In animal models, DM induces or exacerbates the phosphorylation of tau, suggesting that DM may influence the risk at AD by directly facilitating tau phosphorylation. In chapter 4 and 5, we investigated abnormal tau phosphorylation in the brain of diabetic rats. Increased tau phosphorylation is observed in the STZ model, which mimics T1DM, but not in rats on a fcHFHS diet that mimics the early phase of T2DM. Since increased tau phosphorylation was not observed in both the STZ model and fcHFHS diet model, insulin deficiency and insulin resistance may have a differential effect on tau phosphorylation. Unlike T1DM, T2DM slowly develops with initially normal glucose levels and high insulin levels to compensate for insulin resistance [352]. As T2DM progresses hyperglycemia increases before an extreme hyperglycemic state arises due to a relative or absolute decrease in pancreatic insulin secretion. This final stage of T2DM is difficult to mimic in animal models. High-caloric diets result in slight hyperinsulinemia and therefore these models represent the prediabetic state wherein insulin resistance already exists but insulin production is still intact. For that reason, the final state of T2DM in animal models is often mimicked by a STZ treatment at the end of the high-caloric diet. Such combined models show increased tau phosphorylation [273, 353], indicating that the prediabetic phase with insulin resistance and slight hyperglycemia is not sufficient to increase tau phosphorylation. In other words, not the prediabetic state, but only the end stage of T2DM is probably involved in diabetes-induced tau phosphorylation.

We demonstrate in the diabetic brain that neither energy deficiency as observed in torpor (Chapter 5) nor neuroinflammation (Chapter 4) is the trigger for increased tau phosphorylation. However, insulin deficiency is sufficient to induce tau phosphorylation (Chapter 5). Concomitantly with increased tau phosphorylation upon insulin deficiency, we observed upregulation of tau kinase PKA. The increase in PKA activity upon insulin deficiency is not surprising, since PKA and the downstream signaling target cAMP response element binding protein (CREB) are known to play an important role in neuronal survival during stress stimuli [354]. Remarkably, diabetes-induced tau phosphorylation is reversed upon insulin treatment in vivo. This indicates that both PKA and tau phosphorylation are part of a physiological response rather than a pathological defect. Indeed, stimulation of PKA activity in a transgenic tau mouse model was shown to prevent cognitive dysfunction early in disease [63]. PKA phosphorylates the proteasome resulting in increased proteasome activity and consequently in decreased tau aggregation levels. Possibly PKA activates a dual adaptive response by increasing tau phosphorylation and decreasing tau aggregation which consequently results in better cognitive performance in cellular stress situations like insulin deficiency.
**Tau phosphorylation as adaptive response to various stressors**

Both metabolic stress and insulin deficiency can result in increased tau phosphorylation via different signaling pathways (Figure 1). Hence, other stressors might also increase tau phosphorylation. In fact, hyperphosphorylated tau is also observed in traumatic brain injury (TBI, [355]). The trigger for this increased tau phosphorylation in TBI is still elusive, but decreased oxygen supply due to disruption of blood vessels could be involved in TBI-induced tau phosphorylation. Tau might be a general stress protein that can be hyperphosphorylated during different stress situations. In line with this, the many phosphorylation sites of tau, which can be phosphorylated by various kinases and phosphatases, makes tau accessible for regulation by different stress pathways [356]. For instance, GSKβ kinase activity is increased upon UPR activation whereas diabetes-induced tau phosphorylation is not regulated via GSKβ. In contrast, the inactive form of GSKβ is increased during insulin deprivation. Instead, insulin deficiency increases PKA activity resulting in increased tau phosphorylation (Chapter 5). Interestingly, 2DG-induced UPR activation increases PKA activity in vitro (Chapter 5, Figure 6). PKA activity is also increased during starvation in cancer cells to promote survival [357]. In cancer cells, PKA attenuates the UPR suggesting a close interaction between PKA and the UPR. Both metabolic stress and insulin deficiency eventually converge at increased tau phosphorylation. It would be interesting to further investigate which kinases are essential for the increased tau phosphorylation in different stress situations. For instance, is the observed increase in PKA activity upon metabolic stress sufficient to result in hyperphosphorylated tau?

Strikingly, tau phosphorylation is reversible upon removal of both metabolic stress and insulin deficiency. In tauopathies, UPR activation, PKA activity and consequently increased tau phosphorylation could be part of an adaptive response. In line with this, tau KO mice show no obvious neuronal phenotype indicating that tau might be especially important in stressful situations [39]. It would be interesting to expose tau KO mice to stressors like metabolic deprivation and insulin deficiency and investigate whether this results in neuronal defects. How tau exactly contributes to the restoration of neuronal homeostasis is still elusive. It is also unclear whether this adaptive tau response differs for different stress inducers. Tau is mainly known for its function in axonal transport. Increase in tau phosphorylation results in destabilization of the microtubules and consequently in decreased axonal transport. In case of metabolic stress, this might be a mechanism to temporally decrease energy-costly axonal transport in an environment with low glucose availability. Interestingly, the PERK pathway also decreases protein translation, which not only diminishes the protein load in the ER, but also decreases ATP utilization by protein synthesis [358]. Therefore, upon metabolic stress the PERK pathway could contribute to decreased ATP expenditure by reducing both protein synthesis and axonal transport. It is likely that it is important in other stress situations as well to reduce energy costly processes to dedicate energy sources to solving the initial problem.
Figure 1. Tau phosphorylation as adaptive response
A) Insulin deficiency results in activation of PKA. PKA activity increases tau phosphorylation as part of a physiological adaptive response to restore homeostasis. Hyperphosphorylated tau relocates to the synapses and decreases axonal transport. Glucose deprivation activates the UPR, which also phosphorylates tau via affecting the activities of as yet unknown kinases or phosphatases. Overall this results in decreased axonal transport, protein synthesis and metabolism to restore homeostasis. However, if homeostasis is not restored tau becomes pathological and eventually tau inclusion bodies will form. Additional factors like Aβ, age and neuroinflammation may contribute to the transition from physiological to pathological tau.

B) Therapeutic intervention should focus on enhancing the adaptive response (1), preventing neuronal stress (2) and blocking the transition to pathological tau.
In chapter 3, we studied the direct effects of the UPR on glucose metabolism. The UPR results in decreased glucose metabolism via the IRE1 pathway. The decrease in glycolysis and mitochondrial respiration is not accompanied by apoptosis or damage indicating that the change in metabolic rate is a normal non-apoptotic response. This reduction of the metabolic rate could be a mechanism to anticipate on the low glucose resources. Interestingly, decreasing mitochondrial respiration in a neuronal cell line reduced the toxicity of neuronal toxins like Aβ [230]. This suggests that in tauopathy patients the UPR-induced decrease in glucose metabolism might be a protective mechanism against neurotoxins. Multiple studies show that increased tau phosphorylation also affects mitochondrial function [359]. Increased tau phosphorylation results in decreased mitochondrial respiration. The effects of the UPR and tau on mitochondria are probably regulated via independent pathways, since UPR-induced tau phosphorylation is increased via the PERK pathway and the effect of the UPR on mitochondria is regulated via the IRE1 pathway. Interestingly, PKA also localizes to the mitochondrial matrix where it has a positive effect on neuronal survival [360]. It is unknown whether this beneficial effect of PKA on mitochondria is regulated via tau. The UPR, PKA activity and tau phosphorylation all result in decreased mitochondrial respiration indicating that mitochondria are involved in a general strategy for neuronal survival [361].

A striking gain of function of highly phosphorylated tau is the relocalization of tau to the synapses where it can interfere with synaptic function [56]. Indeed, a mouse model of tauopathy with moderate tau accumulation shows reduced neuronal firing prior to cell death [362]. Additionally, the UPR affects synaptic function by diminishing synaptic proteins due to the overall translational block of the UPR [363]. Interestingly, PKA is also associated with synaptic function. For instance, PKA interacts with the NMDA receptor and enhance NMDA receptor currents [4, 364]. Moreover, increase of PKA activity reduces neuronal membrane excitability [365]. These effects on synaptic function could be a survival mechanism to reduce energy utilization or to protect neurons from hyperexcitability. Overall, it is interesting to observe that although initiated by different stressors like glucose or insulin deficiency, the UPR, PKA activity and tau phosphorylation all affect similar cellular processes like mitochondrial metabolism and synaptic function to restore homeostasis.

**Transition from adaptive response to pathology**

In this thesis, we demonstrate that different stressors can induce reversible tau phosphorylation. The question remains what determines the conversion from reversible phosphorylated tau into irreversible pathological tau. In our studies, increased phosphorylated tau is diffuse and comparable with the phosphorylated tau early in pathology. Later in pathology, dense tau aggregates are observed that form the end stage tau inclusion bodies. These end stage tau aggregates are difficult to degrade and therefore are probably irreversible [366]. A possibility is that just prolonged stress and consequently prolonged tau phosphorylation eventually result in irreversible tau aggregates. This suggests that if the torpor or diabetic state in animals is prolonged that eventually dense tau aggregates will form. Indeed, black bears have the longest torpor period and the
densest form of phospho-tau from all hibernating species [132]. However, this torpor-induced phospho-tau in black bears is still reversible, therefore it would be interesting to even further prolong stress to observe whether eventually irreversible tau aggregates will form. Prolonged stress and consequently tau aggregates might also become pathological by negative side effects. For instance, the initial protective effect of decreased axonal transport might eventually lead to starvation of synapses and permanent synaptic damage [293, 367]. Moreover, prolonged PERK activation leads to synaptic loss and neurodegeneration in animal models for prion disease and Aβ pathology [89, 368, 369]. PERK activity results in decreased synthesis of proteins including synaptic proteins, which could be a mechanism of how chronic UPR activation facilitates neurodegeneration [363]. Overall, the activated processes that are initially dedicated to solving the problem might eventually become pathological when homeostasis is not restored.

Alternatively, an additional problem might be necessary to switch from normal tau phosphorylation to tau pathology. For instance, the tau mutations in patients with FTDP-17 may affect the outcome of the adaptive response [7]. These mutations promote self-aggregation of tau, which may result in irreversible tau aggregates upon stress whereas in individuals with normal tau, tau phosphorylation may still be reversible after restoration of the homeostasis. In addition, these mutations may result in dysfunctional tau that cannot contribute to the adaptive response resulting in prolonged and extreme tau phosphorylation. Moreover, a polymorphism in the gene encoding PERK is associated with increased risk to develop PSP and AD [80, 81]. In these cases, the UPR might not function properly thereby reducing the capacity to restore homeostasis. In other words, similar levels of stress may lead to a pathological state due to a defect in the adaptive stress response.

Besides malfunction of the adaptive response, environmental factors may also contribute to the transition from physiological tau to pathological tau. For instance, Aβ in AD may influence the negative side effects of tau, since the toxic effect of Aβ is mediated by the relocalization of phosphorylated tau to the synapses [370]. Therefore, Aβ might be crucial for permanent synaptic dysfunction resulting in irreversible damage. Although neuroinflammation is not a prerequisite for diabetes-induced tau phosphorylation (Chapter 4), neuroinflammation could be involved in the progression of tau pathology. In tau transgenic models, genes involved in neuroinflammation are upregulated after 18 months [371]. Moreover, neuroinflammation is observed before tau-related neuropathological changes in humans [372, 373]. Activation of the immune system may create a situation in which tau hyperphosphorylation is not reversible resulting in pathological tau as observed in tauopathies. Finally, aging is the most important risk factor for neurodegenerative disease. Aging is defined as the progressive accumulation of unrepaired changes. The hallmarks of aging are among others mitochondrial dysfunction, increased insulin resistance, decreased proteasome activity and a chronic state of inflammation [374-376]. These changes during aging may result in inefficiency of the adaptive response and may push the neuron to a pathological state. For instance, on a background of mitochondrial dysfunction the UPR might not adequately restore ER homeostasis resulting in prolonged UPR activation with detrimental effects.
Implications for therapeutic intervention in tauopathies

The reversibility of increased tau phosphorylation provides an opportunity for therapeutic intervention, because treatment directed to tau could be successful in removal of hyperphosphorylated tau. This is especially exciting because it indicates that patients with early stage tau pathology may be completely cured. However, because tau phosphorylation appears to be an important player in adaptive stress responses, it is necessary to be cautious with treatments that reduce tau phosphorylation. The intervention can reduce the initial beneficial effects of tau and may even result in faster progression of the disease. Therefore, in early disease stages medication should probably focus on enhancing the adaptive response to facilitate the restoration of homeostasis. In case of metabolic stress, the UPR could be an interesting target for intervention. IRE1 could be stimulated for its effect on mitochondria while PERK could be inhibited to prevent tau phosphorylation and diminished protein synthesis. The activation of the IRE1 arm of the UPR may suffice to restore the energy balance without the detrimental effects of prolonged PERK activation. Inhibition of the PERK pathway could be a successful approach in particular for patients with the polymorphism in the PERK gene associated with increased PERK activity and increased risk to develop PSP and AD [80, 81]. Boosting PKA activity also could be a good therapeutic intervention since it stimulates tau phosphorylation and increases proteasome activity, which consequently decreases the formation of tau aggregates [63]. Since mutant tau aggregation result in proteasome dysfunction, increasing PKA activity might be a good therapy for tauopathy patients in general [577].

Interfering in the transition from reversible to irreversible tau is another possibility for intervention. If prolonged activation of the adaptive response can result in a pathological state than this has obvious implications for the timing of potential therapeutics. When additional factors like Aβ, aging and neuroinflammation are required for the irreversible pathological state than inhibition or removal of these factors could be successful strategies. Therefore, better understanding of the factors that determine the transition to the pathological state are important to develop successful therapies.

Finally, removal of the neuronal stressor that activates the adaptive response could be part of a potential therapeutic intervention. We demonstrate that insulin treatment in case of insulin deficiency and return to a normal metabolic state during hypometabolism both restore homeostasis. The fact that different stressors result in increased tau phosphorylation via different signaling pathways complicates prevention of neuronal stress. It implies that different stressors cause tau pathology in patients, which would require a personalized medicine approach. For instance, sporadic AD appears to be a multifactorial disease in which insulin resistance but also hypometabolism are reported [104, 289, 297, 378]. For therapeutic intervention, it is essential to precisely diagnose individual patients to better understand the underlying problem for the increased tau phosphorylation. DM can be diagnosed, but also brain insulin dysfunction can be determined in the CSF of tauopathy patients [331, 379]. In addition, positron emission tomography (PET) can be used to measure hypometabolism in the brain [380, 381]. Interestingly, more methods become available to diagnose
tauopathy patients ante-mortem. For instance, CSF of tauopathy patients shows reliable phosphorylated tau levels and a PET scan can be used to visualize tau aggregates in the brain of tauopathy patients [382-384]. Less invasive approaches are investigated like blood-based biomarkers, but also more specific biomarkers besides phosphorylated tau in CSF are explored [385]. For instance, a biomarker to measure UPR activation in the brain of tauopathy patients is not yet available, but would have the potential to identify patients that would benefit from UPR intervention. The development of more specific biomarkers is essential to have personalized therapies.

In conclusion, tauopathies are complex diseases for which there is currently no treatment. Here, we show that various insults lead to increased tau phosphorylation via different mechanisms (Figure 1). This increased tau phosphorylation initially contributes to an adaptive response and protects neurons, but eventually could result in tau inclusion bodies as observed in tauopathies. Future perspectives may include highly personalized therapy dependent on the time of intervention. Successful therapeutics should consist of (a combination of) removal of the neuronal stressor, enhancing the adaptive response and preventing the transition to a pathological state.