The epileptogenic trinity

Oxidative stress, brain inflammation and iron in epilepsy

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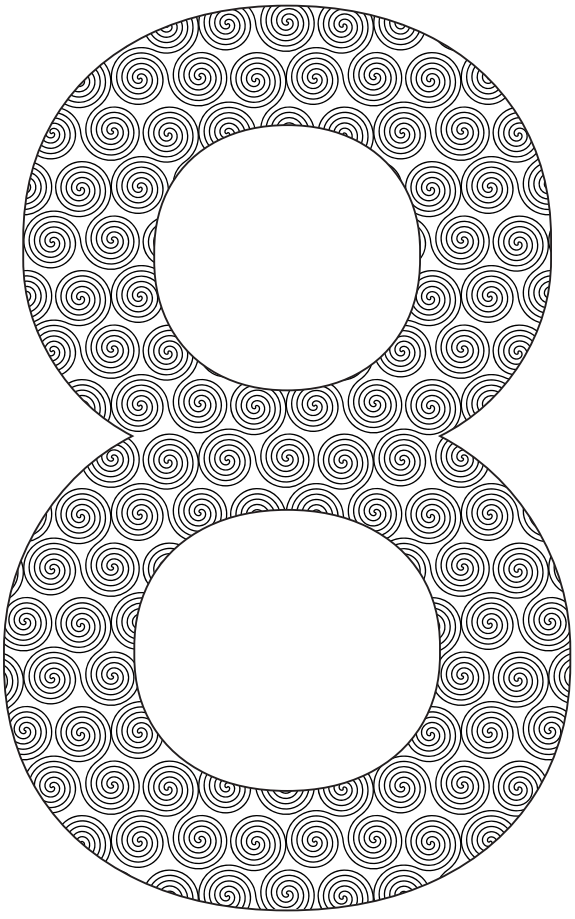

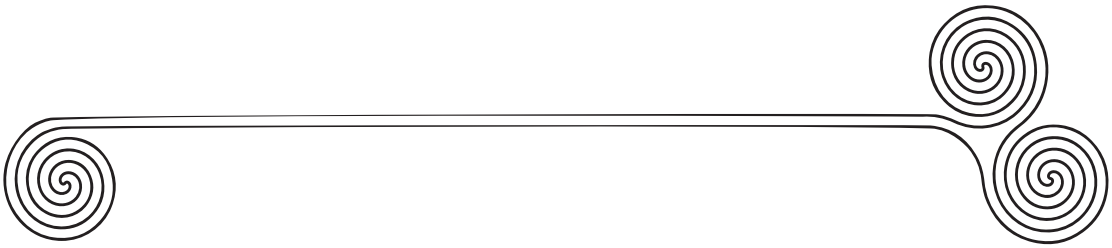

SUMMARY

Epilepsy is a neurological disease that affects approximately 65 million people worldwide. The disease is defined as "a disorder of the brain characterized by a persistent predisposition to cause seizures and the neurobiological, cognitive, psychological and social consequences of this condition". The etiology of epilepsy is diverse, ranging from genetic mutations, such as mTORopathies due to mutations in the mammalian target of rapamycin (mTOR) signaling pathway, to acquired epilepsy in response to acute brain injuries such as stroke, trauma or status epilepticus (SE). Epileptogenesis is the gradual process by which a normal brain develops epilepsy and is defined as “the development and expansion of tissue capable of causing spontaneous recurring seizures, resulting in (1) the development of an epileptic disorder, and/or (2) progression of epilepsy after it has been diagnosed”. Although anti-epileptic medication can suppress seizures in some epilepsy patients, more than 30 % of patients suffer from drug-resistant seizures and currently there are no treatments that target and modify the process of epileptogenesis that can prevent or cure epilepsy. One of the hallmark processes of epileptogenesis leading to neuronal disturbance is neuroinflammation. Additionally, the over-production of reactive oxygen species (ROS) in combination with insufficient antioxidant capacity, leads to a state of oxidative stress (OS), which is another important epileptogenic factor inducing loss of neurons. In addition, dysregulated iron metabolism in the form of iron ions (Fe^{2+}/Fe^{3+}) can enhance the potentially toxic effects of ROS, exacerbating neuronal damage. The aim of this thesis is to investigate oxidative stress, its relation to inflammation and iron metabolism, and its contribution to cellular damage and altered neuronal circuitry in epileptogenesis and epilepsy.

In **chapter 2** we investigated the expression of markers of OS (inducible nitric oxide synthase (iNOS) and solute carrier 7 A 11 (xCT)) and neuroinflammation (Toll-like receptor 4 (TLR4) and cyclooxygenase 2 (COX-2)) in mTORopathies: tuberous sclerosis complex (TSC), focal cortical dysplasia type 2 (FCD 2), and hemimegalencephaly (HME). We found strong expression of all markers in the malformed cells characteristic of the disease and a strong correlation between OS and inflammatory markers. In the same cells, we found nuclear localization of the transcription factor nuclear factor kappa-light chain-enhancer of activated B-cells (NFκB) and identified a concentration-dependent switch to an inflammatory state *in vitro* in response to OS, probably mediated by NFκB. These findings support a strong link between OS and neuroinflammation, pointing to OS as a ROS concentration-dependent cause of brain inflammation.

In **chapter 3** the contribution of the peripheral immune system to neuroinflammation in FCD 2 lesions was investigated. We wanted to specifically characterize differences between the neuropathological subtypes FCD 2a and FCD 2b, which are distinguished at the histopathological level by the presence of the FCD 2b-specific balloon cells. We found a remarkably high activation of the immune system in the cortex of

patients with FCD 2b compared to controls, especially for peripheral T lymphocyte recruitment and increased antigen presentation by malformed cells. In addition, we found that balloon cells in FCD 2b appear to attract immune cells, reducing the density of oligodendrocytes in the subcortical white matter and promoting hypomyelination. In particular, the magnitude of these effects are dependent on the density of balloon cells in the lesion. These findings imply that patients with FCD 2b could potentially benefit from additional anti-inflammatory treatment or controlled immunosuppression.

After establishing NFκB as an important transcription factor in the regulation of inflammation in chapter 2, we chose to identify novel transcriptional regulators of neuroinflammation that could be leveraged as anti-epileptogenic treatments. To this end, in **chapter 4** we used transcriptomic data from TSC tubers to identify novel transcription factors that modulate pro-inflammatory gene expression. A transcription factor enrichment analysis yielded SPI1/PU.1 as a potential candidate. We were able to validate the SPI1/PU.1 overexpression in malformed cells with mTOR activation in TSC tubers, fetal TSC tissue and FCD 2b lesions. Finally, we found *in vitro* evidence that SPI1/PU.1 RNA expression in different cell models (including tuber-derived primary cells) is ROS-dependent and not mTOR-dependent. Although we found SPI1/PU.1 RNA overexpression in different cell types, probably in response to ROS, protein expression could only be detected in cells with mTOR activation. These findings indicate that ROS and mTOR activation represent a "double hit" in malformed cells, leading to the expression of SPI1/PU.1 and subsequent enhancement of pro-inflammatory genes, establishing yet another link between OS and neuroinflammation. Targeting SPI1/PU.1, especially in malformed cells, could potentially dampen neuroinflammation.

In **chapter 5**, oxidative damage was investigated in FCD 2b and TSC, as well as the anti-oxidant nuclear factor erythroid 2 like 2 (NRF2) pathway and its modulation by the previously identified inflammation-associated microRNA (miR) miR155. We found markers for oxidative damage, but also activation of the NRF2 pathway and miR155 expression, predominately in malformed cells with mTOR activation in FCD 2b lesions, TSC tubers, and in an experimental TSC knockout model (*Tsc1*$^{GFAP-/-}$ mice). Transfection of human fetal astrocytes with miR155 *in vitro* revealed that miR155 activates several NRF2 target genes via attenuation of the expression of the NRF2 competitive transcription factor BTB domain and attenuating CNC homolog 1 (Bach-1). Long-term exposure to miR155 resulted in chronic overexpression of the NRF2 target heme-oxygenase 1 (HO-1), which in turn promoted the release of free iron and changes in the metabolic gene expression of iron metabolism-related genes. Iron metabolism dysregulation was then validated and assessed in FCD 2b lesions, TSC tubers, a TSC model and fetal TSC lesions. These findings indicate that chronic activation of antioxidant gene expression via NRF2 promotes HO-1-mediated iron

release. Since iron can potentiate ROS toxicity, these data indicate a role for iron in the pathogenesis of these mTORopathies. Moreover, these findings suggest the potential of therapeutic approaches targeting deregulation of the NRF2 signaling pathway.

Iron metabolism, oxidative damage, and antioxidant gene expression were further investigated in autopsy brain tissue from SE patients and resected brain tissue from patients with temporal lobe epilepsy and hippocampal sclerosis (TLE-HS) in **chapter 6**. In addition to iron deposition and changes in iron metabolic factors we also found oxidative damage and overexpression of antioxidant factors in the hippocampus of patients with SE or TLE-HS compared to controls. In an experimental TLE rat model, we detected iron deposition during the acute phase after electrically induced SE, but also during the chronic phase, after the development of spontaneous recurrent seizures. These changes were accompanied by changes in factors involved in iron processing and antioxidant defense. *In vitro* iron uptake in mouse brain slices is facilitated by epileptiform activity and iron exposure in these slices and pure human astrocyte cultures appears to be related to the RNA expression of pro-inflammatory factors. To gain a better understanding of whether the data from TLE-HS tissue can be extrapolated to other forms of acquired epilepsy, we characterized tissue from tumor-related epilepsy patients and found similar changes in antioxidant and iron metabolism. To investigate whether iron overload could also play a role in the pathogenesis of epilepsy, we measured iron in tissue from patients suffering from potentially epileptogenic conditions, namely stroke or traumatic brain injury. Here we have detected iron overload, similar to brain tissue from people with chronic epilepsy. In summary, in chapter 6, we show iron overload and changes in iron metabolism in epileptogenic disorders as well as in chronic epilepsy, indicating a role of iron in epileptogenesis.

The findings of this thesis are summarized and discussed in **chapter 7**. The data collected indicate that neuroinflammation and OS are involved in the development and progression of epilepsy. In addition, both processes appear to be closely related and amplify each other through different regulators including NFκB, SPI1/PU.1 and/or miR155. Finally, we identify iron overload as a new, additional pathogenic trigger that could be involved in cell loss in epilepsy. New anti-inflammatory therapies targeting NFκB or SPI1/PU.1 and therapies modulating chronic NRF2 activation in mTORopathies could be promising new therapeutic options. In addition, the control of iron overload with iron-scavenging agents or antiferroptotic agents deserves further investigation as possible antiepileptogenic therapy. However, because of the important role of iron in brain homeostasis, this potential therapeutic approach requires more thorough research.