The epileptogenic trinity

Oxidative stress, brain inflammation and iron in epilepsy

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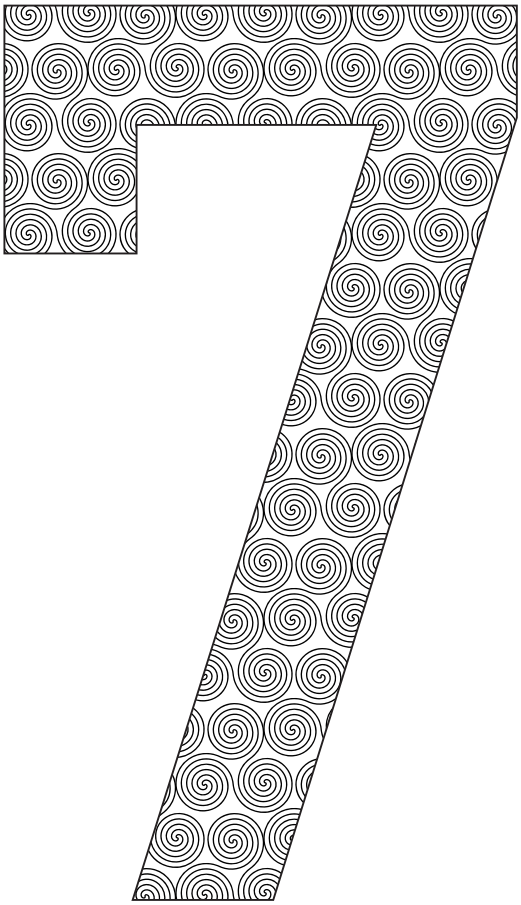
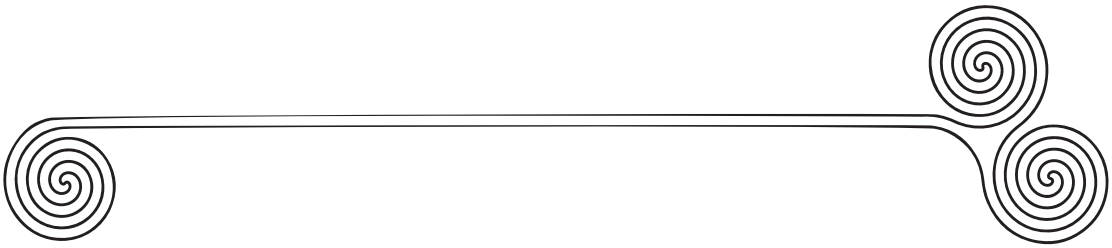




GENERAL DISCUSSION

The origin and evolution of life and its indispensable dependence on O_2 are tightly interlaced (1). Though, utilizing O_2 in oxidative phosphorylation allowed the emergence and maintenance of ever more complex multicellular life it came at the cost of requiring explicit regulation due to the inevitable by-production of reactive oxygen species (ROS) (2). Failure of antioxidant regulatory mechanisms fosters an imbalance in favor of ROS generation over elimination, thereby provoking oxidative stress (OS) (2). Since the brain is explicitly vulnerable to OS, many neurological disorders manifest with OS, including epilepsy. Since epilepsy therapy currently depends on purely symptomatic treatment and lacks disease modifying effects, elucidating if OS plays a role in epileptogenesis could yield novel avenues in the quest for anti-epileptogenic remedies. Though it is widely accepted that neuronal function can be compromised by ROS in processes like excitotoxicity, our current understanding of OS in patients suffering from epilepsy of varying etiology is sparse. Moreover, its role in epileptogenesis and its interaction with other pathogenic mechanisms such as neuroinflammation remain limited.

Balloon and giant cells are drivers of oxidative stress, inflammation and immune system activation in FCD 2b lesions and TSC tubers

Neuroinflammatory processes are an integral feature of resected brain tissue from epilepsy patients and tissue from experimental epilepsy models (3, 4). Moreover, inflammatory pathways such as i.a. the interleukin (IL)-1 receptor/toll-like receptor (TLR), cyclooxygenase (COX)-2 or transforming growth factor (TGF) β signaling are upregulated in response to pro-epileptogenic insults and were shown to alter neuronal excitability and promote ictogenesis, thus suggesting a crucial role in epileptogenesis (5, 6). In turn, these inflammatory processes can trigger the production of ROS via NADPH oxidases (NOX) by innate immune cells, including neuroglia (7-10). In addition, there is also ample evidence for ROS modulating a wide array of inflammatory processes (10, 11). Hence, a better understanding of the crosstalk and interdependence between ROS and inflammation in epileptogenesis could greatly benefit in the development of novel drug candidates that rapidly terminate these processes after an initial pro-epileptogenic event. To this end a strong correlation between the OS response markers inducible nitric oxide synthase (iNOS), cysteine-glutamate antiporter (xCT) and the pro-inflammatory markers TLR4 and COX-2 in brain tissue from hemimegalencephaly (HME), focal cortical dysplasia (FCD) 2 and tuberous sclerosis complex (TSC) patients was identified in chapter 2. Importantly, cell responses were cell-type specific, namely we found stronger overall expression and correlation in dysmorphic neurons and balloon/giant cells with intrinsic mammalian target of rapamycin (mTOR) activation, particularly in FCD 2b and TSC. In addition, glia also displayed higher expression of genes linked to brain inflammation. The link between OS and brain inflammation and the consequent pro-oxidant and pro-inflammatory environment could be nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) dependent. Moreover, we identified a

concentration-dependent switch via ROS to an inflammatory state mediated by NFκB *in vitro*. Interestingly, in lesional tissue, nuclear NFκB translocation was found primarily in balloon/giant cells, indicating that this switch predominantly occurs in these cells. Combined with the evaluation of iNOS, xCT, TLR4 and COX-2 expression, balloon/giant cells seem to promote ROS production and OS, but also respond with NFκB-dependent pro-inflammatory gene expression, promoting a pro-inflammatory environment. Additionally, ROS production by these cells was shown to create a positive selection pressure in favor of themselves by having a higher antioxidant capacity mediated by glutamate cysteine ligase (GCLC) overexpression, making them more resistant to OS (12). Of note, expression of these markers was also higher in dysmorphic neurons in FCD 2b and TSC compared to HME and FCD 2a. Considering that the functional propensity for this expression pattern in dysmorphic neurons is similar in all mTORopathies, the trigger for expression of inflammatory factors likely originates from balloon/giant cells. Finally, overexpression of iNOS and COX-2 could also be detected in activated glia in FCD 2b and TSC, indicating an additional effect on resident innate immune cells. From the data presented in chapter 2 it can be suggested that balloon/giant cells express pro-epileptogenic factors such as the TLR4-NFκB axis (13-16) or COX-2 (17). Considering the anabolic effect of mTOR activation, one has to keep in mind that the expression of e.g. functional TLR4 is a complex process, including post-translational modification, proper intracellular shuttling, membrane insertion and association with downstream transducers and, thus, might not function appropriately. Nonetheless, IL-1β as downstream product of NFκB activation and potentially ictogenic cytokine could be detected in balloon cells in FCD (18) and giant cells in TSC (19), indicating operative NFκB activation.

The investigation of balloon cells in FCD 2 was expanded in chapter 3 by comparing activation of the brains' innate and the peripheral immune system in FCD 2a and FCD 2b tissue. Here, FCD 2b lesions presented with higher immune system activation supporting the findings from chapter 2. While chapter 2 examined brain intrinsic responses, evidence for involvement of peripheral immune cells in reinforcing neuroinflammation was identified in chapter 3. By comparing the transcriptome from FCD 2a and FCD 2b lesions, genes involved in adaptive immunity, innate immunity and cytokine signaling were overexpressed in FCD 2b compared to FCD 2a. Protein expression of immune cell markers and pro-inflammatory factors in lesions confirmed transcriptomic data and revealed that parenchymal T-lymphocyte infiltration is a characteristic feature of FCD 2b lesions. Moreover, this was coupled with excessive expression of human leukocyte antigen (HLA) I and HLA II, implying elevated antigen presentation via major histocompatibility complex (MHC) I and MHC II. Analysis of cytokine expression provided a clue as to why T-lymphocytes might infiltrate FCD 2b lesions, as indicated by higher expression of the lymphocyte homing factors C-C motif ligand (CCL) 2 and CCL19 in balloon cells in FCD 2b. Additionally, we found that complement factor expression was enriched around balloon cell-rich regions and that balloon cell density correlated positively with the number of T-lymphocytes.

On the other hand, balloon cell density correlated negatively with oligodendrocyte number and myelin content, indicating subcortical white matter damage and hypomyelination. Taken together, balloon cells seem to be highly immunogenic and can recruit peripheral immune cells to the brain, particularly T-lymphocytes and macrophages, and amplify neuroinflammation. While dysmorphic neurons in FCD 2a also displayed expression of pro-inflammatory factors, these lesions probably suffer less from the effects of peripheral immune cells. Furthermore, based on chapter 2 and chapter 3 we suggest that neuroinflammation and immune system activation play a more pronounced role in FCD 2b than in FCD 2a, facilitated by balloon cells. In our studies, the patients with FCD 2b did not suffer higher epileptogenicity in terms of seizure frequency or duration compared to FCD 2a. Hence, while somatic mTOR mutations in neural progenitor cells cause focal epileptogenic lesions, the developmental emergence of balloon cells in FCD 2b might alter the trajectory of lesional evolution towards an inflammatory milieu. During the course of these investigations, FCD 2a lesions generally presented with much higher densities of dysmorphic neurons, while FCD 2b lesions contained balloon cells, less dysmorphic neurons, and presented with severe white matter alterations. Therefore, one might conclude that the epileptogenicity in FCD 2a lesions might be primarily neurogenic, mediated by aberrant circuitry, while the epileptogenicity of FCD 2b lesions might be promoted by neuroinflammation, immune system activation and white matter alterations. These mechanisms probably do not represent absolute cause, but rather a relative polarity towards one of the two epileptogenic mechanism (i.e. inflammation vs. epileptogenic network development) discussed here.

Though, we emphasized a stronger immune system activation in FCD 2b, pro-inflammatory signaling certainly plays a role in the epileptogenicity of FCD 2a (16, 20-23). Importantly, inflammation could not only contribute to epileptogenesis (5) but also might promote mTOR activation (24, 25), which could impact aberrant neurogenesis of dysmorphic neurons (26). Similarly, ROS and OS were demonstrated to modulate mTOR activity (27-30). Consequently, OS and brain inflammation could affect the maldevelopment of focal FCD lesions and tubers. Since it was demonstrated that giant cells appear very early in TSC, even before the appearance of dysmorphic neurons (31), OS and brain inflammatory processes could impact the preliminary stages of neurogenesis and cortical development in TSC. Assuming a similar time-course for genesis of balloon cells in FCD 2b, these mechanisms could also impact the development of FCD 2b lesions. While HME lesions display occasional balloon cells and are considered “hemispheric” FCD 2a/b lesions (32), we could not find evidence for extensive neuroinflammation as in FCD 2b foci. This finding, together with the observation that white matter damage positively correlates with balloon cell density, indicates that balloon cell load of the lesion and the concentration of immunogenic factors released by these cells might play a decisive role in establishing the neuroinflammatory environment observed.

Identification of novel transcriptional regulators of FCD 2b lesions and TSC tubers

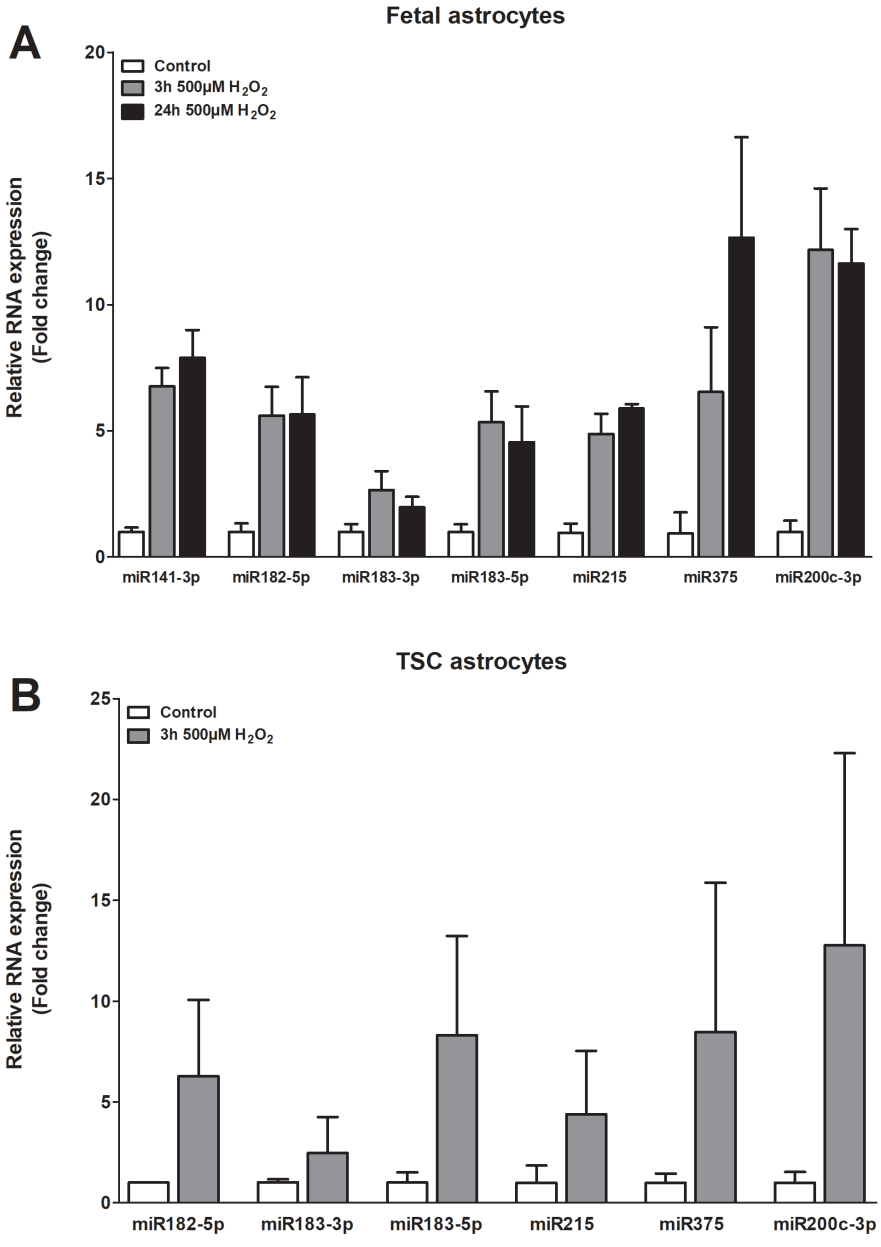
In chapter 2 and 3 we found strong evidence for OS, immune system activation and expression of pro-inflammatory mediators in mTORopathies, particularly in tubers and FCD 2b lesions. In doing so, mainly the TLR4-NFκB axis and its downstream factors were investigated. In this context, selectively modifying signaling pathways, and in particular transcriptional regulators such as transcription factors that are improperly regulated pose attractive therapeutic options in various disease contexts including epilepsy (33, 34). Additionally, several studies have identified altered gene regulation via epigenetic mechanisms and its long-lasting effects on intercellular communication, network organization and, ultimately, epileptogenesis due to aberrant neuronal activity (34, 35).

Since modulating deviant gene networks by targeting the activity of a single or several regulators in unison could potentially deliver disease modifying effects, the aim in chapter 4 was to identify novel transcriptional regulators of the pathological transcriptional landscape in TSC by utilizing transcriptomic data from resected tubers. By analyzing the promotor region of the differentially overexpressed genes and comparing these motifs with transcription factors that are predicted to bind to these regions (transcription factor enrichment), SPI1/PU.1 was identified as novel transcriptional regulator of the tuber microenvironment. Here, SPI1/PU.1 was predicted to target a plethora of genes involved in immune system activation and inflammation. SPI1/PU.1 is a well-known transcription factor essential in the differentiation of microglia. Since SPI1/PU.1 in microglia was shown to be required for their viability and activation in neurodegenerative conditions (36-38), finding SPI1/PU.1 in malformed cells suggests that mTOR hyperactivity promotes an immunogenic transcriptional profile, supporting the data from chapter 2 and 3. Moreover, the finding that SPI1/PU.1 expression is highly inducible by ROS *in vitro* suggests yet again a strong link between OS and inflammation, as was also reported for peripheral macrophages (39). Interestingly, using *in situ* hybridization to SPI1 RNA we found strong transcriptional activation of SPI1/PU.1 in tubers and also in an experimental TSC model in various cell-types, not restricted solely to microglia. Since we detected elevated OS in these tissues previously, induction of SPI1 likely resulted from OS-dependent transcriptional activation. Moreover, this suggested that translation of SPI1 is aberrantly turned on only in malformed cells and that mTOR hyperactivation in conjunction with other pathogenic mechanisms, in this case OS, can activate pro-inflammatory gene networks.

The successful *in silico* identification of transcriptional master regulators and potential drug targets utilizing large-scale transcriptomic data was recently demonstrated for acquired epilepsy (40). In the context of chapter 4, SPI1/PU.1 could indeed be exploited as drug target in TSC as previously demonstrated for leukemia (41).

However, compared to high-grade cancer, tubers grow slowly and are composed of a mixture of normal-appearing, supposedly healthy cells, and mutation-carrying cells with mTOR hyperactivation. Hence, targeting SPI1/PU.1 could interfere with microglia development and, consequently, also their vital function in neurodevelopment (42-45). Whilst this could have an especially pronounced effect on the pediatric TSC population, it could, on the contrary, also dampen microglia activation. In addition to SPI1/PU.1, numerous changes in DNA methylation and expression of post-transcriptional modifiers such as miRNAs have been demonstrated in tubers (46, 47), indicating that solely targeting SPI1/PU.1 is probably not sufficient. Finally, while we found higher expression of SPI1/PU.1 in malformed cells in tubers, its expression was heterogeneous, with some cells expressing much more than others, potentially reflecting the redox state of the microenvironment or translational dysfunction of individual cells. Nonetheless, the findings in chapter 4 consolidate the findings from chapter 2 and 3 in that OS and brain inflammation are tightly intertwined and that mTOR hyperactivation in conjunction with secondary pathogenic mechanisms promotes immunogenic gene expression. Moreover, we found a similar distribution of SPI1/PU.1 in FCD 2b lesions, implying that these findings translate also to this patient population. Finally, single-cell RNA sequencing could represent a better approach for the cell-specific identification of transcriptional regulators, which might be undetectable in bulk tissue RNAseq. Nevertheless, transcription factor enrichment analysis yielded SPI1/PU.1, in addition to NFκB, as novel transcription factor regulating pro-inflammatory gene expression in TSC tubers and FCD 2b lesions. In addition to transcriptional regulators, post-transcriptional modulators represent attractive targets to better understand and potentially modify gene regulation. Consequently, previous pursuits trying to identify regulators of the TSC transcriptome focused on identifying micro RNAs (miRNA) (46, 48, 49). MiRNAs are short (18-22 nucleotide long), non-coding RNAs that can bind and modulate the stability of mRNA and therefore can facilitate the repression of target translation (50). Since there is strong evidence for OS as pathogenic stimulus in the tuber microenvironment, we opted to identify novel miRNAs induced by OS. To this end, primary human fetal astrocytes were stimulated with H_2O_2 followed by small RNA sequencing. Two time points were selected to discriminate between miRNAs upregulated acutely (3 h) and those potentially having long-lasting effects (24 h). Seven miRNAs were differentially expressed that exhibited strong upregulation after 3 h and 24 h (Fig. 1, unpublished data). Importantly, since previous data from this stimulation scheme indicated that astrocytes were able to detoxify H_2O_2 approximately after 6-8 h, upregulation after 24 h indicated that these miRNAs could potentially play a role in chronic OS and exert long-lasting effects. Subsequently, these targets were validated to be upregulated in TSC tuber-derived astrocytes 3 h after H_2O_2 stimulation. Overexpression experiments *in vitro* could help unravel what downstream pathways are modulated by these miRNAs and if these processes also play a role in tubers in response to OS. Moreover, this could help shed light on whether these miRNAs play a role only in astrocytes or

also in other cell types. Taken together, in addition to redox sensitive SPI1/PU.1, a novel set of miRNAs that are overexpressed by human fetal astrocytes in response to ROS stimulation, could play a role in TSC and other epileptogenic pathologies characterized by OS.



Chronic activation of antioxidant pathways in FCD 2b and TSC links OS and iron metabolism

Seizures and epilepsy certainly contribute to ROS generation, brain OS and neuroinflammation as outlined previously. The importance of a rapid but appropriate response by the cellular antioxidant machinery with subsequent deregulation back to homeostatic redox balance is exemplified in chapter 5. FCD 2b lesions and TSC tubers were characterized by oxidative damage markers and activation of the Nrf-2 signaling cascade. Malformed cells revealed a strong activation of nuclear factor erythroid 2 like 2 (Nrf-2) and its downstream target heme oxygenase (HO) 1, as well as markers of oxidative damage in the very same cells and similar changes were also detected in *Tsc1*$^{GFAP-/-}$ mice. Several upregulated miRNAs were found in TSC tubers, and initial screening revealed that the pro-inflammatory miR155 (48, 51) could mediate chronic Nrf-2 activation. Transfection of cells *in vitro* revealed that miR155 i.a. targeted Bach-1, a competitive, negative regulator of Nrf-2 (52-54). Indeed, miR155 overexpression coincided with higher HO-1 expression as result of Bach-1 inhibition and subsequent Nrf-2 activation. Though, the Nrf-2-mediated antioxidant response by cells is generally considered beneficial and assumed to increase cell survival, these results indicate that prolonged Nrf-2 stimulation could foster oxidative damage and OS. One possible mediator of the pro-oxidant effect could be HO-1, which degrades heme into CO, biliverdin and Fe^{2+}. Induction of HO-1 by ROS and a variety of xenobiotics mediates antioxidant effects via CO and bilirubin, the reduced form of biliverdin mediated by biliverdin reductase (55). The induction of HO-1 is generally followed by rapid inactivation by degradation of the enzyme (56). While rapid induction and termination of HO-1 seem essential for cytoprotection, prolonged HO-1 activation has been demonstrated to promote cell damage/death by accumulation of Fe^{2+} and iron-catalyzed ROS (57-60). Indeed, higher expression of the iron binding protein ferritin and the iron exporter ferroportin was induced in cells chronically exposed to miR155 and HO-1 activity, indicating iron accumulation. Subsequently, strong expression of ferritin was found in FCD 2b lesions and TSC tubers, a surrogate marker of iron accumulation (61) and also iron (Fig. 2, unpublished data) could be detected. Moreover, dysregulation of other iron-responsive genes in epileptogenic lesions and *Tsc1*$^{GFAP-/-}$ mice were detected. In conclusion, in chapter 5 we identified a link between sustained Nrf-2 activation, chronic HO-1 activity and iron accumulation, which could potentially exacerbate oxidative damage via the Fenton reaction. Interestingly, while we showed in chapters 2-4 that OS can stimulate the expression of pro-inflammatory genes, the experiments presented in chapter 5 revealed that miR155 can promote OS. Since miR155 is regulated via

Figure 1: Validation of differentially expressed miRNAs identified from small RNA sequencing of primary fetal astrocytes *in vitro* stimulated with H_2O_2 for 3 h and 24 h revealed long-lasting upregulation of miR141-3p, miR182-5p, miR183-3p, miR183-5p, miR215, miR375 and miR200c-3p (A). Stimulation of TSC tuber-derived astrocytes confirmed induction of these miRNAs, except miR141-3p, 3 h after H_2O_2 stimulation (B). n = 3 independent cultures.

NFκB (62), inflammatory stimuli could activate Nrf-2 indirectly via miR155. In fact, HO-1 was also shown to directly respond to NFκB (63, 64), which itself can also be activated by ROS (65). In chapter 5 we identified an essential third component to the interplay between OS and inflammation, i.e. iron metabolism. While iron responsive genes are tightly regulated by intracellular iron concentrations, predominantly on the post-transcriptional level, (66), their transcription is also sensitive to OS (67) and

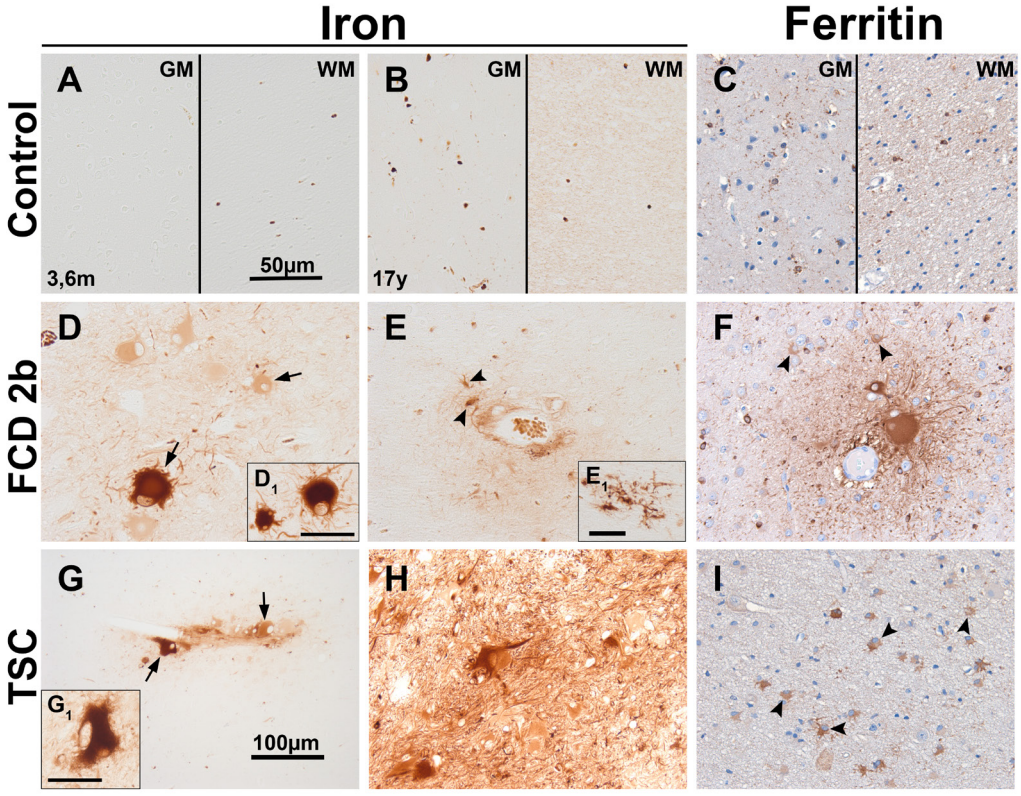


Figure 2: In the epileptogenic pathologies FCD 2b and TSC iron accumulation is elevated in dysmorphic cells, perivascular astrocytes and in microglia. Iron accumulation in autoptic control is age dependent. While iron could only be detected in the white matter of a 3.6 months-old patient, iron accumulated in the neuropil of the white matter, as well as oligodendrocytes in a 17-year-old autopsy case (A, B). Ferritin expression was detected only in microglia in cortical tissue of autopsy control (C). In FCD 2b, dysmorphic cells displayed very strong iron accumulation, especially in balloon cells with laterally displaced nuclei (D_1, arrows). Additionally, peri-vascular iron accumulation in processes and cells with glial morphologies could be detected (arrowheads). Occasionally, clusters of iron laden microglia could be found as well (E_1) (D, E). Ferritin expression in FCD 2b lesions was found in balloon cells as well as astrocytes (F, arrowheads). Similar to FCD 2b tissue, iron accumulation in TSC tubers could be found in giant cells (G_1, arrows) and sometimes cell clusters around vasculature. Iron accumulation in the tuber of an older subject (22 years) displayed massive iron load of the tissue (G, H). Ferritin expression in TSC tissue was found in cells with astrocyte morphology in tuber, but also peri-tuber tissue (I). Sections C, F, I were counterstained with hematoxylin. Scale bar = 100 µm in G, 50 µm in A (representative for A-D, E, F, H, I) and D_1, 25 µm in E_1 and G_1; arrowheads = glia, arrows = balloon/giant cells.

inflammation (68). Although, iron concentrations could increase due to the catabolic activity of HO-1, brain iron accumulation could also be facilitated by seizure-mediated blood-brain-barrier (BBB) damage and an influx of iron-containing blood components such as heme or transferrin-bound iron (69-71). Thus, there appears to be a strong interconnection between OS, inflammation and iron in mTORopathies, primarily FCD 2b and TSC. Why cells with mTOR activation display higher expression of e.g. ferritin could be explained again by overexpression conferring a survival advantage for malformed cells in conditions of iron accumulation and OS. Additionally, since iron is a vital micronutrient involved in e.g. iron-sulfur cluster assembly and thus, cellular respiration, its metabolism is not surprisingly also directly regulated by mTOR (72, 73).

Anti-inflammatory/anti-oxidant therapies for mTORopathies?

What novel therapies could help control the pathogenic mechanisms investigated in chapter 2-5? Clearly, neuroinflammation is involved in the pathogenic mechanisms in mTORopathies, above all in FCD 2b and TSC, and its decrease could benefit the disease course. Here, not only epilepsy but also the frequently reported neurological co-morbidities of patients suffering from FCD or TSC, such as autism spectrum disorder or intellectual disability could be targeted (74). One clear complication of developmental malformations of cortical development in mTORopathies is the genetic etiology, a process that leads to irreversible brain maldevelopment due to excessive mTOR activation already *in utero*. Though, aberrant corticogenesis produces a highly epileptogenic brain substrate as indicated by the high incidence of epilepsy, not all patients suffer from recurrent seizures and epilepsy manifests itself only in the first years of life, not immediately after birth. Considering the high plasticity of the brain in these early phases of postnatal brain development, immediate drug intervention with anti-inflammatory drugs might be of benefit for epileptogenesis and the development of cognitive abilities since chronic neuroinflammation could represent an additional, secondary pathogenic hit. Moreover, brain development in TSC patients is delayed by seizures, suggesting that seizure-mediated processes such as OS/inflammation delay cognitive abilities and worsen the overall prognosis for FCD 2 and TSC patients (75-78). Based on our data, two potential pro-inflammatory pathways that could be targeted are the NFκB or the SPI1/PU.1 axis. However, considering the potential side-effects of general anti-inflammatory treatment in the vulnerable pediatric population of FCD and TSC patients this likely requires a more tailored approach towards malformed cells. For example, as mentioned previously, non-specific SPI1/PU.1 inhibition could inhibit microglia activation in response to extrinsic immune challenges, an aspect of particular importance in the epileptogenic brain with BBB dysfunction.

As for increased ROS production and OS in mTORopathies, the therapeutic implications are more ambivalent. While it is possible to interfere with specific pro-

oxidant pathways, investigation of the Nrf-2 pathway in FCD 2b and TSC revealed chronic overactivation in malformed cells. Since this pathway is of crucial importance to normal neural cells in lesions, downregulating it would probably result in higher OS in these cells. The resistance of malformed cells to OS coupled to their high ROS production due to i.a. iron accumulation makes these cells particularly toxic to their environment. Although it cannot be completely excluded, based on the data of chapter 2, overall less production of pro-oxidant versus pro-inflammatory factors was observed in normal appearing glia. This result could pose giant/balloon cell-targeted therapy regarding pro-oxidant pathways in mTORopathies intriguingly effective. In this context, exploiting the overdependence of malformed cells on endogenous antioxidants for their higher antioxidant capacity could hypothetically be exploited by decreasing e.g. GSH synthesis as suggested previously (12). Together with the apparent iron accumulation and ROS susceptibility in malformed cells, decreasing antioxidant capacity could decrease survival rate and induce specific cell death. This approach has been effective in the therapy of some cancers using small molecules inhibiting xCT as rate limiting importer for GSH synthesis, thereby inducing ferroptosis (an iron-mediated form of regulated necrosis) (79, 80). Alternatively, one might consider targeting metabolic pathways that promote the production of ROS. For example, reducing ROS generation during oxidative phosphorylation via a ketogenic diet (KD) is a highly effective intervention in epileptic children (81, 82). Alternatively, decreasing overall metabolic rate by repurposing drugs such as e.g. metformin (83, 84) could indirectly affect ROS production and reduce associated neuroinflammation in mTORopathies, though this hypothesis awaits more thorough investigation.

An obvious, major hurdle in exploiting the abovementioned therapeutic strategies are variables like timing (probably early), dosing (probably low, but chronic), mixed pathological/normal-appearing cell substrate (targeting malformed cells) as well as maintaining an activation/inhibition ratio for physiological processes to commence. Here, mTOR inhibitors possibly provide, after all, the best therapeutic option for mTORopathies, a hypothesis supported by clinical trials in TSC (85-89). Importantly, mTOR suppression would effectively target epilepsy but could also act on neuroinflammation and OS as primary or secondary consequence of mTOR activation. Consequently, a careful examination of surgical material from patients with TSC that were treated with rapamycin or its analogues but still suffered seizures could give valuable insights into the actual effect of mTOR inhibition on neuroinflammation and OS. More importantly, it could give insights into changes in the cellular architecture e.g. if there is a lower burden of dysmorphic neurons versus giant cells or dysplastic glia. Taking our findings from chapter 3 into account, this could also help identify the individual contribution of particular cellular elements or pathogenic mechanisms to seizure generation. Besides TSC, mounting evidence accumulates that somatic mutations in the mTOR pathway cause FCD 2 (32, 90-

93). Thus, it is very probably that mTOR inhibitors would benefit these patients as well. In the context of this thesis, trials with mTOR inhibitors could also elucidate the role of inflammation/OS in epilepsy and epileptogenesis in FCD 2a versus 2b as presented in chapter 3. Finally, adjunctive treatment to mTOR inhibition regarding OS/inflammation could potentially decrease the disease burden further by decreasing the aforementioned second-hit required for the progression of epilepsy and associated co-morbidities.

Oxidative stress and iron metabolism in acquired epilepsy

The changes in redox state and its close connection to iron metabolism in mTORopathies were intriguing so these findings were extrapolated to acquired epilepsy, in particular temporal lobe epilepsy with hippocampal sclerosis (TLE-HS) in chapter 6. While etiologically distinct, we hypothesized that ROS, OS and iron could play an important role in epileptogenesis. Here, particularly the process of OS and if its exacerbation via iron catalysis could be involved in the loss of hippocampal neurons were of interest. Due to its potentially detrimental catalytic function intracerebral iron concentrations are tightly regulated, mainly by endothelial cells and astrocytes at the BBB (94). Since BBB dysfunction is a hallmark of epilepsy (71, 95), it was hypothesized that extravasation of iron-rich blood components like transferrin-bound iron or heme from hemoglobin could influence brain iron concentration and metabolism. Additionally, seizure-mediated release of protein-bound iron or heme could represent another source of potentially toxic iron-catalyzed OS (96). Finally, acute epileptogenic insults such as status epilepticus (SE), stroke and traumatic brain injury (TBI) were included in this study to infer if acute insults leading to large increases in intracerebral iron concentrations (97) could also play a role in neuronal damage during the initiation of epileptogenesis.

Investigation of surgically resected hippocampi from TLE-HS patients and hippocampi of acute *ante mortem* SE tissue from autopsy revealed that 4-hydroxynonenal (4-HNE) as end-product of lipid peroxidation, accumulates in neurons in the CA1 and that these cells also display higher expression of HO-1, indicative of OS. Moreover, 4-HNE adducts were also found in the cerebrospinal fluid from TLE-HS patients and factors involved in the synthesis of glutathione (GSH) were upregulated. Many of the GSH synthesis factors were controlled by Nrf-2, indicating that antioxidant genes are activated in hippocampi of TLE-HS patients. On the other hand, iron accumulation could be detected in neuronal nuclei, microglia and astrocytes in SE and TLE-HS tissue. In contrast, ferritin expression was restricted to microglia in control tissue, whereas astrocytes in SE and TLE-HS specifically displayed higher ferritin expression. Interestingly, neurons did not display ferritin expression indicating a low capacity for storing iron. Measurement of iron metabolism genes in the hippocampus of TLE-HS patients revealed an overall lower iron uptake and retention but higher iron export. To better resolve the time-course of changes in iron metabolism we investigated

hippocampal tissue of electrically stimulated SE rats. Similar to human tissue we found strong accumulation of iron in glia and higher expression of HO-1 in neurons and glia cells at 1 day post-SE and after the development of spontaneous recurrent seizures. Quantification of total tissue iron in the hippocampus recapitulated these changes indicating iron accumulation in the acute and chronic stage. In contrast to human tissue, ferritin expression could solely be detected in microglia. While these findings clearly implicate iron accumulation and OS acutely after an epileptogenic insult and in chronic epilepsy, the higher expression of antioxidant genes and the pattern of changes in iron metabolic genes we detected in human tissue were not recapitulated after SE in rat brain tissue. Moreover, the very specific expression of ferritin in astrocytes in human tissue could not be found in rat hippocampi after SE, indicating that either iron sequestration is specific to human astrocytes or only persistent iron exposure in chronic epilepsy promotes this function. However, human brain tissue after stroke or TBI also revealed higher ferritin expression in astrocytes, supporting the notion that astrocytes are iron "sponges" especially in chronic conditions of iron overload. In support of this, we detected iron accumulation in fetal astrocytes exposed to iron and hemin *in vitro*, indicating that astrocytes are competent to take up iron, potentially protecting more vulnerable neurons from iron-mediated toxicity.

Since previous studies showed that intracortical injections of low concentrations of iron salts could promote acute seizures and epileptogenesis (98, 99), the pro-ictogenic effect of iron was investigated *in vitro*. Our analyses showed that iron *per se* does not cause seizure-like activity in acute hippocampal slice preparations. There are several reasons for this discrepancy. For example, the iron donor and consequent bioavailability, ionization of the available iron, slice preparation versus whole brain or total iron concentration could all contribute to the pro-ictogenic effects. However, we found that neuronal activity could facilitate iron entry into tissue and in particular neuronal nuclei. In addition, we found that iron exposure promotes the expression of pro-inflammatory mediators in hippocampal slices and cell cultures and that stimulation of iron coupled to OS could promote high mobility group box (HMGB) 1 release, linking dysregulated iron metabolism to inflammation. Although the interconnection of iron with OS was the focus of chapter 6, a strong and evolutionary conserved link between iron and immune functions exist (68), which adds another layer of complexity considering that immune cell infiltration in TLE-HS hippocampi has been demonstrated previously (100). Moreover, astrocyte specific ferritin expression as marker of iron dysregulation was also detected in mTORopathies indicating a common function of astrocytes in sequestering iron (Figure 2).

Although TLE-HS and mTORopathies are etiologically very distinct, they are united in their high epileptogenicity. In this context, it would be of interest for future studies

to assess if the commonalities we detected here could be due to seizure activity or common mechanisms such as BBB dysfunction. To this end, analysis of tissues from neurological or neurodegenerative diseases presenting with similar dysfunction of e.g. the BBB but absence of seizures could give insights into the conservation of these changes specifically in epilepsy versus neuropathology in general. It is worth mentioning that the electrically induced SE epilepsy model investigated here presented with similar processes as human TLE-HS such as iron accumulation in response to acute and spontaneous recurrent seizures. On the other hand, ferritin expression as indicator for iron binding was detected solely in microglia but not astrocytes, exemplifying the limitations of animal models and the importance of investigating human tissue to understand the individual players in pathogenic processes such as dysregulated iron metabolism.

Anti-oxidant/iron chelation therapies for TLE-HS?

While historically associated with a variety of pathological conditions and generally regarded pathogenic, more recent studies appraised ROS and OS more critically. Within this framework, the concept of hormesis plays an essential role since low ROS levels are crucial signals for sensing the cells metabolic state and can mediate activation of signaling cascades. On the other hand, excessive ROS generation induce cytotoxicity (101). The results of chapter 6 point towards the latter in chronic epilepsy, probably by i.a. ROS production by NOX (9, 102) or mitochondrial ROS generation during seizures (103). Stimulation of the whole GSH synthesis machinery suggests also an overall higher antioxidant demand but also a higher adaptive capacity. Moreover, elevated parenchymal iron and the alterations in iron cycling genes suggest less retention and more export and could represent an adaptation to prevent iron-amplified ROS toxicity. While the electrically induced SE model of acquired epilepsy and brain tissue from drug-resistant epilepsy patients with recurrent seizures point towards OS during chronic epilepsy, pro-epileptogenic brain injuries like stroke, SE and TBI all presented with oxidative damage shortly after the injury and iron overload as well. This implies a role for OS and iron in cell damage prior to or at the onset of epileptogenesis, but also in the progression and pathophysiology of epilepsy.

An important question in this context is if the observed elevation in antioxidant capacity and reduced iron retention are sufficient to protect cells and in particular vulnerable neurons. The fact that 4-HNE accumulation in chapter 6 was detected in neurons implies insufficient ROS detoxification in neurons. Indeed, antioxidant therapy in experimental models was demonstrated to have long-lasting disease modifying effects. For example, supplementation with the GSH precursor N-acetylcysteine (NAC) together with the Nrf-2 stimulant sulforaphane in an electrically-induced SE model delayed epilepsy onset and progression as well as frequency of spontaneous seizures (104). Similarly, indirect stimulation of Nrf-2 via kelch like ECH associated

protein (KEAP) 1 inhibition alone or combined with NOX inhibition were shown to reduce epilepsy development and seizure frequency (105, 106). Both approaches clearly show that antioxidant treatment has a beneficial disease modifying effect in experimental models. While these results are promising it is worth considering the aforementioned physiological role of ROS in cells. Disregarding this function and treating epilepsy patients chronically with antioxidants could therefore result in unwanted side-effects. Rather, a timely administration (i.e. acutely post-ictal) to reduce ROS-mediated damage to neurons might be a more suitable approach. Moreover, the failure of a multitude of clinical trials to treat a variety of diseases with dietary antioxidants (e.g. vitamin C/E) exemplifies the strict regulation of antioxidants in the body and the importance of the type of antioxidant therapy (107). Here, providing precursors of endogenous antioxidants (e.g. NAC) or stimulation of endogenous antioxidant pathways (e.g. weak pro-oxidants, Nrf-2 stimulation, KEAP1 inhibition) may represent promising approaches for chronic epilepsy (107). Unfortunately, the Nrf-2 target xCT was shown to be overexpressed in high-grade glioma and serve as biomarker for epileptic seizures at diagnosis (108, 109). xCT imports cystine in exchange for glutamate which is crucial to sustain tumor growth as tumor cells are characterized by intrinsic OS due to their drastically increased metabolic activity (110-112). As a consequence, the build-up of extracellular glutamate was concluded to be ictogenic and cause excitotoxicity in healthy brain tissue surrounding the tumor. While this ictogenic effect of xCT casts doubt on Nrf-2 targeted therapies in epilepsy, the properties of glioma versus epileptic tissue likely differ greatly in the propensity to excrete/metabolize glutamate and the results of antioxidant therapy in epilepsy models contradict this finding. Nevertheless, these results exemplify that antioxidant drugs, e.g. Nrf-2 stimulants, have to be developed with care. Another promising line of research with potential antioxidant effects is the KD. Its efficacy in epilepsy (113) suggests a strong connection between seizure activity and (lipid)metabolism, which are themselves intricately linked to ROS and OS (114-116).

Common therapeutic approaches to restrict iron overload are based on iron chelation. Several genetic disorders collectively termed “neurodegeneration with iron accumulation” (NBIA) as well as Alzheimer’s or Parkinson’s disease are characterized by iron accumulation and iron-mediated toxicity. Moreover, iron reduction in experimental models revealed beneficial effects. Consequently, iron chelation therapy has already reached clinical stages (117). In the context of epilepsy, several lines of evidence along with the results of chapter 6 point towards a potentially beneficial role of iron chelation in preventing cellular dysfunction, damage and cell loss via ferroptosis. First, ferroptosis susceptibility has been demonstrated in interneurons (118, 119), a neuronal subpopulation rapidly lost in chronic epilepsy, and epilepsy models display features of ferroptosis (120, 121). Secondly, ferroptosis inhibitors were shown to improve the disease course in epilepsy models (120, 122-

124). However, these studies mostly relied on the ferroptosis-preventing specificity of the used inhibitor but did not convincingly demonstrate that iron-mediated cell death is implicated in the respective experimental model. In contrast, chapter 6 provides strong evidence that lipid peroxidation and iron accumulation, both characteristic features of ferroptosis, are present in neurons of epilepsy patients and animal models. Though, it is tempting to speculate that iron chelation benefits epilepsy patients, systemic administration was shown to evoke side-effects such as gastrointestinal but also auditory and visual problems (125). Moreover, in agreement with the high iron concentrations in oligodendrocytes in chapter 6, myelination was demonstrated to critically depend on iron (126-129), making iron chelation especially in the developing brains of the pediatric epilepsy population problematic. Nevertheless, acute epileptogenic brain injuries such as stroke, TBI or SE characterized by an acute increase in intracerebral iron could potentially benefit from iron chelator therapy by preventing acute toxicity and, consequently, dramatically reducing the probability for epileptogenesis (130). Importantly, indications for ferroptotic cell death in TBI have been demonstrated (131). However, while hypothetically very intriguing, side-effects and efficacy of the first clinical studies in stroke cast some doubt on the immediate benefit (132). On the contrary, this trial did not evaluate long-term outcome in terms of epilepsy. One essential variable to consider for the design of iron chelation therapy is certainly the magnitude of iron overload which differs significantly when comparing acute trauma versus chronic epilepsy.

Since the results from chapter 6 suggest iron overload as a result of BBB leakage, iron toxicity in epilepsy could alternatively be targeted by inhibiting BBB breakdown, an approach that already demonstrated beneficial effects in experimental models (133). Moreover, as mentioned for antioxidant therapy, the KD also indirectly targets ferroptosis. Since ferroptosis critically relies on iron-catalyzed lipid ROS generation, modification of metabolic ROS generation (ketosis>glycolysis) and altered lipid composition due to the KD might prevent ferroptosis in vulnerable neuronal subpopulations such as interneurons. Importantly, ketogenic dieting suppresses seizures in experimental models acutely (134, 135), thereby likely having additional acute effects. On the other hand, long-term preservation of physiological neuronal networks and attenuation of epileptogenesis by preventing ferroptotic cell death could explain some of the anti-epileptogenic effects observed (136). To test this hypothesis, investigating volumetric changes of hippocampal subfield utilizing advanced high-resolution imaging techniques such as magnetic resonance imaging at 7 (137) or prospectively 10.5 Tesla in epilepsy patients on the KD could give insights into the rate of cell loss and progression of epilepsy. Another tempting line of research in the context of stroke found protective effects of selenium supplementation against ferroptosis, but also excitotoxicity and endoplasmic reticulum stress, apparently by activating transcription of protective factors (138). Although this study injected selenium directly into the brain, it is tempting to speculate that these findings could potentially also translate to dietary modifications in epilepsy.

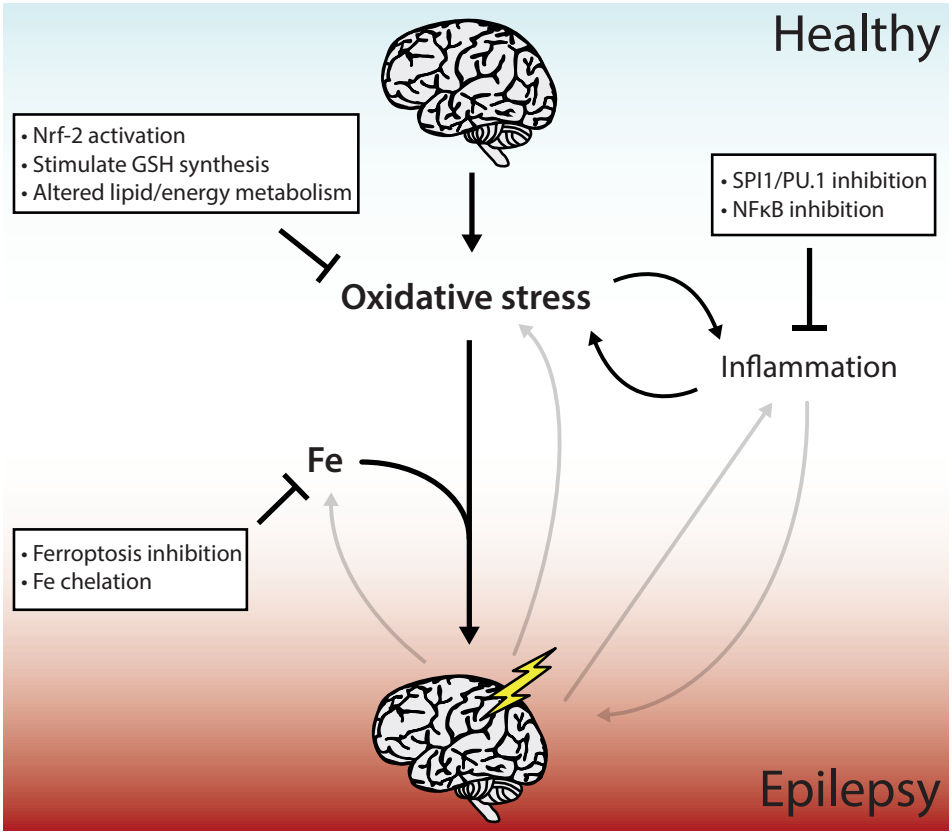


Figure 3: Epileptogenesis is driven by OS, inflammation and iron. OS in conjunction with iron-catalyzed ROS potentiation induces cellular dysfunction, damage and potentially ferroptosis. In addition, OS is tightly associated with neuroinflammation, both of which reinforce each other. While not the focus here, inflammatory mediators also contribute to epileptogenesis directly. All these processes are reinforced in the epileptic brain by spontaneous recurrent seizure activity and the associated changes in metabolism, BBB permeability etc. Potential therapeutics could target OS by stimulating intrinsic antioxidant mechanisms such as Nrf-2 dependent gene expression, GSH synthesis or altering brain and lipid metabolism to reduce ROS susceptibility. Inflammation, specifically in mTORopathies, could be targeted by suppression of NFκB or SPI1/PU.1 activation. Lastly, iron overload in epilepsy could be targeted by modifying altered iron concentration via iron chelation, especially acutely after seizures, or by targeting ferroptosis trying to rescue vulnerable neuronal populations.

Conclusion

This thesis aimed 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. We found convincing evidence for OS in genetic mTORopathies, but also acquired epilepsy mainly targeted at neurons, leading to pro-epileptogenic neuronal dysfunction and death. Furthermore, we discovered an intricate interconnection between brain inflammation, immune system activation and excessive ROS production in OS. Here, we identified the novel ROS-sensitive transcriptional regulator SPI1/PU.1 and a set of miRNAs which require further functional assessment (Figure 3). In the course of these investigations, we discovered the double-edged nature of antioxidant defense mechanisms in mTORopathies, which when activated chronically can potentiate ROS toxicity by iron-catalysis via HO-1. Moreover, in acquired epilepsy we discovered iron overload, likely due to BBB dysfunction, leading to OS and neuronal damage. These findings implicate ferroptosis as novel cell death mechanism in epilepsy. Therapeutic approaches taking the findings of this thesis concerning OS, the associated neuroinflammation and iron into consideration might aid in the search for novel, neuroprotective drugs that possess anti-epileptogenic potential. Furthermore, the close relationship between ROS generation and cellular respiration points towards potential effectiveness of therapies affecting metabolism such as modified diets.

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