Breaking the vicious cycle of epileptogenesis

Focus on brain inflammation and matrix metalloproteinases

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CHAPTER

Discussion

Epilepsy

Epilepsy is one of the most common neurological diseases, currently affecting 65 million people worldwide [1]. Despite the availability of a wide range of antiepileptic drugs (AED), around one-third of all epilepsy patients remain intractable [2-4]. Moreover, there are currently no drugs available that can stop the development or progression of epilepsy. Therefore, the need to identify novel therapeutic targets is urgent. The main aim of this thesis was to investigate several neurobiological processes that are altered in the epileptogenic brain, such as brain inflammation, blood-brain barrier (BBB) disruption and extracellular matrix (ECM) dysregulation, and to use this knowledge to discover novel treatment strategies.

Brain inflammation in epilepsy

The immune system serves to defend the host from external or internal pathogenic threats. In the central nervous system (CNS), this system can be activated in response to infectious agents or pathological events such as hypoxia, ischemia, traumatic brain injury (TBI) and (prolonged) seizure activity [5-8], all of which can lead to the development of epilepsy. The importance of the immune system in epilepsies has been increasingly recognized in the last two decades [6-9]. In chapter 2, we aimed to investigate inflammatory markers of both the innate and the adaptive immune response in human and experimental temporal lobe epilepsy (TLE). We observed that especially markers of the innate immune response were persistently upregulated in the epileptogenic hippocampus of TLE patients as well as during epileptogenesis in a rat model of TLE. The activation of the innate immune response, involving brain resident cells such as microglia and astrocytes, has previously been suggested to play a role in epilepsy [10-16]. Accordingly, the presence of reactive astrocytes and microglia has been shown in the brain of patients with TLE [13,17-19], tuberous sclerosis complex (TSC) [20-23] and malformations of cortical development (MCD) [24,25]. In these pathologies, cytokines and danger signal molecules are synthesized by activated cells of the innate immune system [18,22,24,26-29]. Looking at the adaptive immune system on the other hand, we observed that T-lymphocytes and dendritic cells were not or scarcely present in the hippocampus of TLE patients and in the post-status epilepticus (SE) rat model. Although cells of the adaptive immune system were more evident in other epilepsies, such as post-encephalitis epilepsy [8], focal cortical dysplasia (FCD) type II and TSC [8,30] as well as in several epilepsy rodent models [16,31,32], our data suggest a major proepileptogenic role of the innate immune response in TLE. However, we cannot exclude the contribution of the adaptive immune system in the development of epilepsy, since the infiltration of cells belonging to the adaptive immune system is associated with BBB dysfunction and epileptogenesis [31-33].

During the activation of the immune response, the proteasome serves important roles, being the most common intracellular degradative system. In chapter 3 and 4, we aimed to further investigate the proteasomal system in epilepsies. We found that the constitutive subunits β1 and β5 and the inducible subunits β1i and β5i were higher expressed in neurons and glia in the epileptogenic foci of patients with epilepsy secondary

to cortical malformations such as FCD and TSC and in TLE patients compared to their respective controls. Under normal physiological conditions, the constitutive form of the proteasome is ubiquitously expressed in non-immune cells where it modulates oxidative stress (OS), synaptic plasticity and gene transcription [34,35] while the catalytically more efficient immunoproteasome is limited to immune cells. Consistent with our observations, expression of the immunoproteasome is induced in non-immune cells in response to brain inflammation and accompanying inducing factors such as cytokines, danger associated molecular pattern molecules (DAMPs) [36-38], OS [39] and activation of the mammalian target of rapamycin (mTOR) signalling pathway [40]. In order to investigate the involvement of the (immuno)proteasome during the development of epilepsy, we studied the expression of constitutive and inducible subunits in the post-SE rat model for TLE. We observed higher expression as compared to controls of both the constitutive and the immunoproteasome in the acute phase of epileptogenesis (1 day after SE induction) and an even more prominent expression during the latent phase (1 week post-SE) which continued throughout the chronic phase (3 months post-SE) when the animals experienced spontaneous recurrent seizures. This pattern coincides with the inflammatory response that peaked in the latent phase and remained high during the chronic phase. Moreover, we observed that expression of constitutive and inducible subunits was correlated with seizure frequency in post-SE rats, as well as in patients with MCD. Together, this suggests the involvement of the proteasomal system in progression of epilepsy.

The main function of the proteasome is to degrade pathogens and misfolded and oxidized proteins, thereby protecting the cell from possible pathogenic influences. However, its involvement has also been recognized in a range of pathological diseases including epilepsy (reviewed by [41,42]). With the incorporation of the inducible subunits, the catalytic activity of the proteasome alters; an increase in trypsin- and chymotrypsin-like activity arises along with a decrease in caspase-like activity [43,44]. The cells may therefore be more capable of degrading a wider variety of disease-related misfolded and damaged proteins, which primarily could serve a beneficial cause. In relationship to epilepsy, the proteasome is known to be involved in the turnover of proteins associated with neuronal survival, neurotransmission, spine- and dendritic growth [35,45-48]. Rather than securing effective degradation, excessive proteasomal activity might also lead to uncontrolled off-target degradation. Moreover, the immunoproteasome has also been shown to activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway and to modulate proinflammatory conditions and OS responses [49-54]. Furthermore, the more diverse pool existing of pure constitutive, pure immuno- and all intermediate-type proteasomes gives the opportunity to a wide range of epitopes being presented on the cell surface [55]. Together, this might lead to the prolonged activation and further recruitment of immune cells causing the enhancement of the immune response in the epileptogenic brain. These factors might result in a prolonged decrease of seizure threshold and the persistence of epileptic seizures.

Brain inflammation and blood-brain barrier dysfunction

During brain inflammation, microglia and astrocytes are assisted by immunocompetent cells such as neurons and cells of the BBB; endothelial cells and pericytes. Clustering of pericytes with microglia has been observed in the epileptogenic brain, likely contributing to BBB disruption [56,57]. When endothelial cells become reactive, they do not only increase their expression of cytokines such as interleukin (IL)-1β and its receptor [18], but also the expression of adhesion molecules that facilitate the passage of blood-borne cells into the parenchyma [30,97]. In chapter 2, we observed increased expression of osteopontin, a protein that promotes the migration of macrophages, in the human and rat epileptogenic brain along with a positive correlation between perivascular macrophages and BBB permeability.

BBB dysfunction does not only occur as a consequence of brain inflammation, but can also lead to the activation of astrocytes and microglia, which release cytokines and chemokines [58-60] thereby inducing brain inflammation. It is known that extravasation of albumin can lead to the induction of these proinflammatory mediators [61-63]. Additionally, inflammatory regulators, including cytokines, are known to travel through the compromised BBB and enhance local inflammatory responses. We observed increased expression of the chemokine CCL2, a chemoattractant for cells of the monocyte lineage including monocytes, macrophages and microglia, that can further induce the release of proinflammatory cytokines and is associated with BBB dysfunction [10,64-68] as it also attracts blood monocytes [69,70]. Similar to CCL2, other chemokines such as CCL3, CCL4, CX3CL1, CXCL12 and their receptors expressed by activated and functionally altered glial cells and neurons are known to affect BBB function, such as by attracting blood leucocytes into the brain [31,71-74].

Blood-brain barrier dysfunction and extracellular matrix organization

BBB dysfunction is observed in several neurological diseases, including patients with drug-resistant epilepsy [11,18,75-91]. Additionally, we have shown in this thesis that BBB dysfunction was evident in cortical tubers of TSC patients. It is one of the earliest neuropathological characteristics after an insult such as SE, stroke or TBI and can last for hours to weeks and even months, implying that BBB dysfunction has a role in the development of epilepsy.

BBB dysfunction can occur as a direct result of the insult which can lead to trauma or ischemia to endothelial cells, but it can also be due to secondary events such as increased blood-pressure, hypoxia, decreased blood pH, increased metabolic activation and (as discussed in the previous paragraph) brain inflammation. The integrity of the BBB can be regulated by ECM modulatory molecules that regulate cell-cell and cell-matrix interactions. The remodelling of the ECM has been suggested to play a role in epileptogenesis [92], which is confirmed by previous studies of our group [21,93,94]. RNA sequencing studies in cortical tubers have indicated that ECM proteins and ECM remodelling are important processes in TSC patients [21,93]. Additionally, by performing an RNA microarray study, Gorter *et al.* showed that several ECM regulators were increased during epileptogenesis in a post-SE rat model,

including several matrix metalloproteinases (MMPs). Similarly, using five different models of brain injury, Kim *et al.* showed that albumin exposure leads to transcriptional changes in genes related to ECM modulation, among which several MMPs [58]. MMPs are a family of extracellular endopeptidases, responsible for the degradation of the virtually all proteins in the ECM and are thereby involved in many different processes such as synaptic plasticity and migration [95-98]. In this thesis, we have shown that gene and protein expression of MMP2, MMP3, MMP9 and MMP14 were increased both in cortical tubers of TSC patients and resected hippocampi of drug-resistant TLE patients. Interestingly, we observed increased MMP expression in regions of the brain where the BBB was disrupted, such as in cortical tubers and subependymal giant cell astrocytomas (SEGAs) of TSC patients (unpublished observations). In addition, we observed that expression of MMPs positively correlated with the presence of albumin in the hippocampus of TLE patients and patients who died after SE. This suggests that MMP dysregulation and BBB dysfunction are interrelated. MMPs are directly involved in the (dys)regulation of BBB integrity as their degradome includes several proteins of the basal membrane such as laminin, fibronectin, heparin sulphate and type IV collagen [99-101,270], and target tight junction proteins such as zona occludens-1, occludin and claudins [293]. This is supported by studies using animals models that have shown that disruption of the BBB is mediated by MMPs [267,294,295]. Furthermore, using the post-SE rat model, we observed higher MMP expression compared to controls in animals experiencing spontaneous recurrent seizures as well as prior to the occurrence of spontaneous seizures (during the acute and latent phase after SE induction), suggesting that MMPs play a role in the development and progression of epilepsy. In case of a dysfunctional BBB, infiltration of leucocytes can take place, which are also a major source for MMP9 providing a positive feedback loop for MMP-dependent proteolysis [102,103].

The activity of MMPs is governed by the four members of the endogenous tissue inhibitors of metalloproteinases (TIMPs). The relevance of TIMPs in human epilepsies has not been extensively studied, and only increased expression of TIMP2 has been documented in FCD patients [104]. In chapter 5 and 7, we examined TIMP expression in TSC and TLE patients and observed higher expression of TIMP1-4 compared to control cases [105]. Overexpression of TIMPs is also observed in animal models of epilepsy. Higher expression of *Timp1* and *Timp2* was observed in the hippocampus and/or the temporal lobe 1 day and 1 week after electrically-induced SE [94] and 15 days after kainic acid (KA)-induced SE [106]. The increased expression of TIMPs might be the result of a compensatory mechanism of the brain to overcome the increased MMP-mediated proteolysis. However, the relationship between MMPs and TIMPs goes beyond mere inhibition of activity. TIMPs have been shown to interact with the propeptide of MMPs that physically blocks the active site of the enzyme rendering it catalytically inactive. Controversially, TIMP2, an effective inhibitor of all MMPs, is also responsible for the activation of pro-MMP2 with the use of two MMP14 molecules [107,108], leading to cleavage of the propeptide. Furthermore, TIMP2, TIMP3 and TIMP4 have been

shown to interact with the propeptide of MMP2 while TIMP1 and TIMP3 are able to interact with pro-MMP9 [109]. In order to fully understand the effect of increased TIMP expression in epilepsy, further investigation about the spatial and temporal interaction with MMPs is necessary.

Matrix metalloproteinases and brain inflammation

In chapter 5, 6, and 7, we observed increased MMP expression not only in neurons, but very prominently in glia cells as well, implying that they might be involved in processes governed by these cell types. Interestingly, higher expression of MMPs is observed in TLE patients with hippocampal sclerosis (HS) compared to TLE patients without HS, a pathology that is characterized by hippocampal neuronal cell death and reactive gliosis [110]. Various stimuli, such as cytokines, growth factors, reactive oxygen species, or cell–ECM interactions are known to be responsible for the transcriptional activation of MMPs [111-114]. It is therefore tempting to speculate that the increase of MMPs in, particularly but not restricted to, glial cells such as astrocytes might be the result of the proinflammatory environment. Accordingly, we have shown that, in human foetal astrocytes and tuber-derived astrocyte-enriched cultures, MMP expression can be increased by mimicking a proinflammatory state through stimulation with IL-1β, whose signalling pathway has been shown to be prominently activated in TSC [21,22] and TLE [18,115] and in experimental models of epilepsy [18,116-121]. Transcription factors activator protein 1 (AP-1) and NF-κB, have been associated with the innate immune response, and inflammation [122,123] and are also known activators of several MMPs [124,130]. Remarkably, MMPs are not only activated in response to brain inflammation, they also contribute to the aggravation of the inflammatory response. MMPs can activate cytokines such as tumour necrosis factor (TNF)-α, IL-1β and IL-8 by cleavage of the propeptide [131-133], thereby sustaining their own activity in a proinflammatory environment.

The chicken or the egg? A vicious circle during epileptogenesis

We know that brain inflammation, BBB dysfunction and MMP dysregulation are associated with epilepsy and are likely to be involved in epileptogenesis. These processes are associated with a dysfunctional neuronal microenvironment which increases the propensity for seizures to occur. However, at this point, the chicken or the egg dilemma arises. Do these processes have a causal role in epileptogenesis or are they merely the result of the aberrant network activity present in the epileptogenic brain?

Several studies have shown that the activation of inflammatory responses can have both beneficial, neuroprotective effects as well as exacerbating effects leading to increased neuronal excitability (reviewed by [9,134]). It has been shown that activation of the immune system occurs after (induced) seizure activity [5,6,9,10,18,28,135]. Models of acquired epilepsy have indicated a rapid activation of danger signals such as HMGB-1 and associated toll-like receptors (TLRs) along with an induction of prostaglandins and molecules of the complement system [17,136]. Moreover, an eminent increase in cytokine and chemokine

production is seen in response to epileptiform activity [6,10,116-118,120,137-141]. Indeed, mRNA expression of cyclooxygenase (COX)-2, an important mediator of neuroinflammation, is rapidly upregulated (within 1 hour) after SE induction [117,142-144]. A similarly acute response is seen for cytokine expression including IL-1β and TNF-α after SE induction [117,118,143-145]. However, activation of the inflammatory system is also known to result in seizure activity, a clear example being the increased preposition to seizures that is observed in response to fever [146,147]. Furthermore, diseases in which a compromised immune system is the primary pathology, such as limbic encephalitis and several autoimmune diseases, are associated with increased susceptibility to seizures and epilepsy [148-150]. Local or systemic injection of lipopolysaccharide (LPS), a prototypical inducer of inflammation, can lead to decreased seizure threshold through the activation of TLR4 and the thereby induced release of DAMPs [27,151]. Interestingly, proinflammatory molecules such as cytokines have a dichotomous role in epilepsy showing anticonvulsive effects at low molecular concentrations. For example, daily intraventricular injections of IL-1β delayed amygdala kindling in rats and intrahippocampal administration of nanomolar amounts of TNF-α reduced seizures in mice [152-154]. However, those concentrations are greatly lower than those endogenously produced by seizures and administration of cytokines with seizure-relevant concentrations have led to a reduced seizure threshold and increased seizure duration [137,138,155-157], also mediated by uncoupling of astrocytes [158]. The effect of cytokines on seizure induction is strengthened by the fact that treatment with cytokine-inhibiting molecules has anticonvulsive effects [137,138,159]. Moreover, it has been shown that cytokines can act indirectly and directly on glutamate, NMDA, AMPA and GABA receptors leading to enhanced excitatory and diminished inhibitory transmission [160-166]. Since activated cells of the innate immune system can produce large quantities of cytokines, chronic activation of these cells can contribute to epileptogenesis.

In humans, BBB dysfunction is one of the earliest neuropathological alterations in response to brain insults such as SE, TBI or stroke [11,167-175]. BBB dysfunction is well documented in animal models of epilepsy, in which it is not only observed during the chronic phase when animals have recurrent spontaneous seizures but already during the first hours to days after SE [11,85,176-180]. It has been observed as early as 5 minutes after bicuculline-induced seizures in a guinea pig model [180]. In response to altered neuronal excitability, hypoperfusion of blood vessels can occur, leading to cellular damage and inflammation [174,181]. This indicates that BBB dysfunction can occur as a result of seizure activity [182]. However, when the BBB is opened in animals with recurrent seizures, it leads to an increase in seizure frequency [11]. This suggests that in the presence of neuropathological alterations such as brain inflammation, BBB dysfunction can play a significant role in epileptogenesis. Likewise, focal BBB damage in post-traumatic epilepsy patients is associated with abnormal EEG activity [169,170,183] and areas with a compromised BBB show focal slowing and more spikes [184,185]. Under healthy physiological conditions, artificial opening of the BBB does not typically lead to seizures [11,186-189], though acute seizures have been observed occasionally [14,188]. This underlines that the presence of a pathology is important in how the tissue reacts to BBB dysfunction [182]. The

molecular mechanisms by which BBB dysfunction can lead to epileptic activity are not fully unravelled. It is proposed that, on the membrane of astrocytes, extravasated albumin binds to transforming growth factor (TGF)-β receptors and affects potassium buffering leading to increased extracellular potassium levels. This not only lowers the action potential threshold and increases neuronal excitability [190], but also leads to proictogenic inflammation and synaptogenesis, resulting in gradual development of epilepsy [61,187,191]. It is also suggested that albumin uptake by neurons, mostly found in regions in which cell death occurs, participated in the apoptotic processes involved in epilepsy [11,85].

Due to the strong interactions with both brain inflammation and BBB dysfunction, it is not unreasonable that also MMPs are readily increased in response to seizure activity. Indeed, several animal studies support the induction of multiple members of the MMP family acutely after an initial insult such as an SE [94,192-199]. For example, increased MMP2 and MMP9 activity has been observed as early as 8 hours after KA-induced in SE [197]. Furthermore, in TBI patients, after which acute seizures or even epilepsy can develop, the largest increase in MMP9 concentrations in the pericontusional cortex was found during the first 24 h post-injury [200]. On the other hand, upregulation of MMPs can also result in seizure activity as shown by genetic manipulation in rodent models. Overexpression of *Mmp9* leads to higher susceptibility to kindling-induced epileptogenesis [201]. Likewise, *Mmp9*-overexpressing mice had more seizures in response to TBI [202]. Besides the aggravation of BBB dysfunction and neuroinflammation, MMPs are able to directly affect neuronal excitability because of their wide range of substrates. Due to their capabilities to degrade the ECM proteins of perineuronal nets (PNNs), a structure of specialized ECM, MMPs can cause dysregulation of the inhibitory interneurons surrounded by PNNs [203-205]. This leads to reduced inhibitory transmission which is a known pathological factor in epilepsy and brain injury [58,206]. Furthermore, MMPs can target the propeptides of several growth factors, such as the brain-derived growth factor (BDNF), resulting in cell proliferation [207-210]. By activating BDNF, MMP9 might be one of the driving forces behind mossy fibre sprouting and the thereby caused synchronized firing seen in the epileptogenic brain [211-213]. MMPs are also involved in spine formation and remodelling, due to their abilities to cleave membrane-associated adhesion molecules and via integrin-signalling pathways [214,215]. Similar to cytokines, MMPs have also been shown to directly act on AMPA [216] and NMDA receptors [214], indicating that they play an important role in the generation of seizures and development of epilepsy [217-219].

Overall, it seems that although brain inflammation, BBB disruption and ECM dysregulation have important and essential functions under normal physiological conditions, the persistent activation of these processes can have deleterious effects especially in an already compromised environment such as the epileptogenic brain. As they can occur as a consequence of (prolonged) seizure activity, but more importantly, can also cause a reduced seizure threshold, their abberant activation can lead to the aggrevation of pathology and progression of epilepsy (Fig. 1). Therefore, interfering with this vicious cycle provides an interesting therapeutic target in order to inhibit or block epileptogenesis.

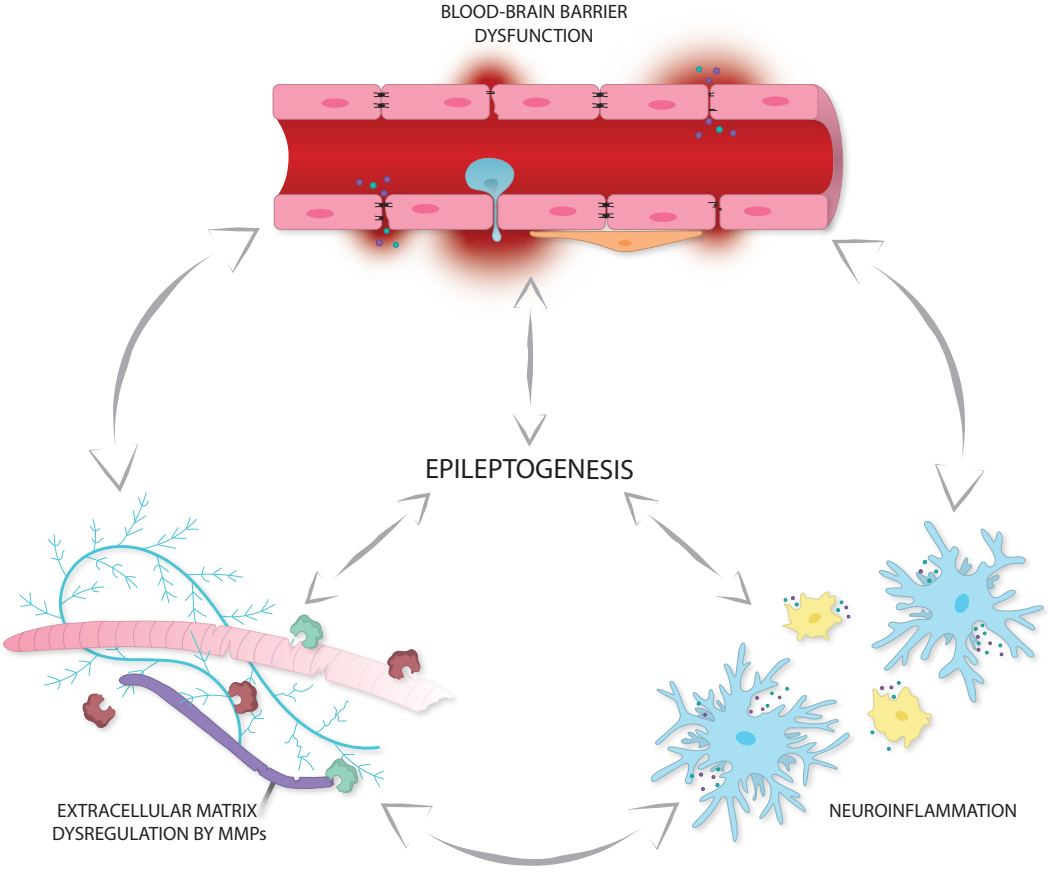

Figure 1. Schematic representation of the vicious circle consisting of blood-brain barrier dysfunction, neuroinflammation and extracellular matrix dysregulation by MMPs in the epileptogenic brain.
Blood-brain barrier (BBB) dysfunction: the BBB consists of several cells such as endothelial cells (pink) and pericytes (orange) that control the influx and efflux of molecules between the brain parenchyma and blood. Upon BBB dysfunction, infiltration of cells (blue) and permeability for blood-borne molecules (green/purple) increases. Neuroinflammation: among the many alterations during a proinflammatory state, reactive astrocytes (blue) and microglia (yellow) synthesize and secrete high number of inflammatory factors (green/purple) such as cytokines, chemokines and danger-associated molecules. Extracellular matrix dysregulation by MMPs: persistently activated matrix metalloproteinases (MMPs; green/red) can lead to dysregulation of extracellular matrix (ECM) proteins such as collagen (pink), fibronectin (purple) and proteoglycans (blue). As previously explained in detail, BBB dysfunction, neuroinflammation, ECM dysregulation by MMPs are interconnected and reciprocally aggravate each other. These pathological alterations can be enhanced by seizure activity, but most importantly can also lead to seizures and the development of epilepsy.

Therapeutic strategies targeting inflammatory processes and matrix metalloproteinases

The data presented in this thesis in combination with the available literature indicates that proinflammatory conditions, BBB dysfunction, and ECM dysregulation by MMPs are intertwined and are of great importance in the development and progression of epilepsy. The fact that increased expression and activation of markers of inflammation, BBB disruption and ECM remodelling are already evident before spontaneous seizures occur in animal models of epilepsy, indicates that there is a therapeutic time window. Some of the possible treatments targeting the aforementioned processes will be discussed in the following paragraphs.

Anti-inflammatory/immune-suppressive therapy

Anti-inflammatory/immune-suppressive therapy has been shown to be successful in several epilepsy cases. For example, treatment with the corticosteroid dexamethasone resulted in relief of seizure load, improved EEG and cognitive function in almost half of the included patients with refractory epileptic encephalopathy with continuous spike-and-wave during sleep [220]. In another study, dexamethasone reduced the seizure frequency in 5/13 children with drug-resistant epilepsy [221]. In animals, dexamethasone reduced BBB leakage, ictogenesis and epileptogenesis after pilocarpine-induced SE in rats [15]. However, the severe side effects of steroids have prevented long-term or widespread use of these drugs [222]. Moreover, anti-inflammatory treatment in animal models of epilepsy has proven challenging as different inflammatory mediators are induced at different time points. An initial wave of inflammation, including the induction of cytokines and its receptors is observed rapidly after experimental SE, followed by subsequent wave with COX-2 and prostaglandin induction as well as the upregulation of molecules of the complement system [5,9,116,223,224]. Therefore, great consideration has to be taken in terms of timing when targeting the inflammatory system as treatment for epilepsy. Several therapies targeting proinflammatory modulators, such as cytokines and chemokines, are reviewed in detail elsewhere [10,225].

Potential novel therapeutic targets

The immunoproteasome

Enhanced activity of the proteasomal system might lead to uncontrolled off-target degradation, prolonged proinflammatory conditions and further recruitment of immune cells sustaining BBB dysfunction. Using hippocampal slices, Engel *et al.* (2017) investigated the effects of inhibition of the immunoproteasome and observed that KA- or glutamate-induced cell death was rescued with pretreatment of two different inhibitors, epoxomicin and MG132 [226]. Also *in vivo*, intracerebroventrical pretreatment with epoxomicin resulted in less neurodegeneration 24 hours following KA-induced SE in mice, however without having an anticonvulsant effect. Specific inhibition of subunit β5 or β5i was investigated by Mishto *et al.* (2015) using brain slices from pilocarpine-induced SE rats with chronic epilepsy.

Pretreatment with ONX-0914, a specific inhibitor of β5i, but not with PR825, a specific inhibitor of β5, resulted in a delay or prevention of AP-4-induced seizure-like events [38]. Unfortunately, these inhibitors have bad bioavailability and poor capacities to cross the BBB suggesting that different target techniques are needed.

Considering the potential of the mTOR signalling pathway in activating the immunoproteasome and the overactivation of this pathway in many epilepsies such as FCD and TSC, we investigated whether rapamycin, an inhibitor of the mTOR signalling pathway able to pass the BBB, could attenuate overexpression of the (immuno)proteasome. Indeed, treatment of FCD-derived astrocytes and human foetal astrocytes with rapamycin reduced IL-1β-induced increase of (immuno)proteasome subunits. Moreover, rapamycin treatment after electrically-stimulated SE in rats did not only result in a decreased seizure frequency, but also in decreased neuronal expression of subunits β5 and β5i. The beneficial effect of rapamycin has already been evident from studies including rodent models of epilepsy [76,227-232]. It has shown to suppress seizures in post-SE models and other models of acquired epilepsy, as well as attenuate mossy fibre sprouting, cell death and neurogenesis [229,233-237]. Furthermore, in response to rapamycin treatment, anti-inflammatory effects [238-241] and improvement of BBB integrity [77,229,242,243] have been documented. A rapamycin derivative, everolimus, has been approved by the FDA for treating TSC patients with SEGA [244,245] and as adjunctive treatment for adults and paediatric patients (>2 years of age) with TSC-associated partial-onset seizures as it leads to attenuation of the seizure burden [246,247]. Further studies are needed to unravel the precise mechanism by which rapamycin affects proteasome activity and proteasome-dependent inflammation and epileptogenesis.

microRNAs

The first study about the relevance of microRNAs (miRNAs) in epilepsy arose almost a decade ago [248] and from that time on, multiple studies investigated the identification and expression patterns of miRNAs in epilepsy patients and in several rodent models of epilepsy. Because of the widespread range of miRNA targets, research has focused more and more on the therapeutic potential of miRNAs as either a target or therapeutic agent itself. A recent meta-analysis evaluated pathways associated with experimental and human TLE and showed that among the most-enriched pathways is the regulation of the ECM [249]. Targeting these miRNAs might therefore be beneficial in patients in which an altered ECM milieu has a causative role or is contributing to the pathology. Evaluating the therapeutic potential of proinflammatory miR155, we used IL-1β-stimulated human foetal astrocytes and observed that while overexpression of miR155 resulted in increased MMP3 expression, silencing of miR155 using an antagomiR, that inhibits the miRNA to bind to its target mRNA, reduced the IL-1β-induced increase of MMP3. The anti-inflammatory miRNAs 146a and 147b were studied in relation to TSC, where they are highly expressed in dysmorphic cells [250]. In cortical tuber-derived cells, overexpression of miR146a or miR147b successfully attenuated the IL-1β-induced increase in MMP3. Furthermore, TIMP expression that was decreased

after IL-1β stimulation could be attenuated by miR146a or miR147b overexpression. Together, this suggests that inhibition of proinflammatory miRNAs and overexpression of anti-inflammatory miRNAs could restore the MMP/TIMP imbalance and resulting ECM alterations that are seen in the brain of patients with epilepsy(-related) disorders such as TLE and TSC.

The antiepileptogenic potential of miRNA silencing or overexpression has been investigated in a limited number of animal studies. Iori and colleagues observed reduced neuronal excitability in hippocampal slice cultures of mice injected with a miR146a synthetic mimic. Furthermore, in the KA-induced SE mouse model, they observed that intracerebroventricular injection of miR146a mimic delayed the onset, frequency and duration of acute seizures as well as the occurrence of spontaneous seizures [251]. In another study it was shown that delivery of miR155 antagomiR prior to pilocarpine-induced SE resulted in less behavioural seizure severity and a trend towards better survival rates [252]. Other miRNAs with antiepileptogenic potential supported by *in vivo* studies, are miR134a and miR135a. miR134, which is involved in activity-dependent dendritogenesis [253], is shown to be upregulated in human [254,255] and experimental TLE (reviewed by [256]). Silencing of this miRNA resulted in reduced spontaneous seizures after SE and increased delay in seizure onset [254,255,257,258]. Inhibition of miR135a, which is upregulated specifically in neurons in the epileptogenic brain, has antiseizure effects in the chronic stage of intrahippocampal KA mouse model, most likely due to reduced expression of *Mef2a*, a key regulator of excitatory synapse density [259].

Besides directly affecting disease-relevant pathways, miRNA modulation can also be regarded as an add-on to current drug treatment. Interestingly, a recent study showed that miR298 binds to the P-glycoprotein gene, reducing its ability to pump AEDs out of the brain [260]. Thus far, preclinical studies have not led to clinical trials that aim at treating brain diseases by modification of miRNA expression. However, miR155 modification is being investigated in a phase II trial for patients with T-cell lymphoma and phase II trials are planned for a long non-coding RNA-based treatment for Dravet syndrome [261]. Challenges that need to be considered when aiming for miRNA modulation in disease are adverse inflammatory effects, charge-related toxicity, targeting the diseased brain area and routes of administration [262]. For the latter, promising results have been obtained in experimental models of epilepsy using intranasal delivery [263,264]. With regard to epilepsy, more research is needed about the medical utility of miRNAs, though miR155, miR146a and miR147b are interesting candidates and deserve further investigation.

Metalloproteinases

As described previously, prolonged expression and increased activity of MMPs can be devastating for brain homeostasis and can influence cellular physiology and brain circuitry. Inhibition of excessive MMP activity might therefore be an interesting therapy in several

neuropathologies such as epilepsy. Genetic manipulation in experimental models of epilepsy have already shown the effectiveness of MMP activity management, showing it has both positive effects on seizures or epilepsy development [201,202,211] as well as on attenuation of BBB dysfunction [102,265-267].

Also natural products, such as tetracycline –produced by Streptomyces– and its derivatives are considered to inhibit MMPs, though the exact mechanisms of function are unknown. Treatment with doxycycline and minocycline has proven to restore BBB dysfunction [268-273], although the effects on epileptogenesis have been moderate and model- and dose-dependent. Unfortunately, treatment with tetracycline derivatives can lead to severe side effects [274-279]. A case report of a patient with astrocytoma and drug-resistant epilepsy shows that treatment with 50 mg minocycline twice daily greatly decreased seizures but resulted in severe skin irritations after one month [280]. In chapter 7, we treated rats with a sub-toxic dose (45 mg/kg) of minocycline daily during rapid kindling and did not observe decreased seizure severity compared to vehicle-treated animals. Importantly, MMP9 activity did not change, indicating that this dose might not be sufficient to inhibit MMP9 activity in the epileptogenic brain.

Nowadays, crystallography has provided more insight into the several pockets of the MMP enzymes, which can contribute to drug design methods [281,282]. It is now thought that the previously seen side effects of the broad-spectrum MMP inhibitors were due to the compounds' lack of specificity. Broad-spectrum targeting of MMPs resulted in inhibition of non-pathological MMPs as well as non-MMP inhibition of members of the a disintegrin and metalloproteinase (ADAM) and ADAM with thrombospondin motifs (ADAMTS) families [283]. For this reason, next generation inhibitors were developed to more specifically bind a selection of MMPs. For example, a thiol-based inhibitor SB-3CT was developed that specifically binds MMP9 and MMP2. Preclinical studies have shown that SB-3CT has neuroprotective effects after ischemia [284,285], and reduces astro- and microgliosis, hippocampal cell loss and BBB permeability after TBI [286,288]. DP-b99, a MMP9-inhibitor, delays the onset and severity of pentylenetetrazole (PTZ)-induced seizures in mice [289] and shows neuroprotective effects in cultures [290]. However, despite encouraging preclinical and phase II clinical trial data, DP-b99 did not show efficacy in ischemic stroke patients [291].

The newly-developed MMP inhibitor IPR-179 has high bioavailability, moderate water solubility and is BBB permeable [292] making it an interesting replacement for earlier mentioned MMP-inhibitors. Its inhibiting capacities for MMP2 and MMP9 are in nanomolar range while IC50 values for other MMPs and non-MMP proteases are >10 µM. Administration of IPR-179 had anticonvulsive effects on acute PTZ-induced seizures in rats [292]. In this thesis, we administered IPR-179 in two rodent models of epilepsy; the rapid kindling rat model and the intrahippocampal KA mouse model. In the rapid kindling rat model, IPR-179-treated rats showed less severe seizures in response to electrical stimulation compared to vehicle-treated animals. In the absence of the drug, these animals still had less

severe behavioural electrically-induced seizures. In the intrahippocampal KA mouse model, IPR-179-treated animals showed less epileptiform epochs, together with decreased seizure duration and overall decreased amount of seizures compared to vehicle-treated animals, which lasted for 7 weeks after IPR-179 was discontinued. Seizure-induced cognitive deficits such as impaired spatial navigation and novel object recognition, were attenuated with IPR-179 treatment. Together, these data show that IPR-179 decreased MMP9 activity effectively in rodent models and has disease-modifying effects without adverse effects. This renders IPR-179 extremely interesting in the search for novel therapeutics for drug-resistant epilepsy patients. Due to the promising results of MMP inhibition by this drug, the company that developed IPR-179 is currently aiming at improving the oral bioavailability of the compound while preserving the compound's BBB permeability, low cytotoxicity and high stability in order to bring the drug into clinical trials.

Conclusion

Taken together, this thesis has provided evidence that brain inflammation, BBB dysfunction and ECM dysregulation by MMPs play important roles in epileptogenic brain. Reciprocally interacting with each other, a vicious circle is formed which leads to the aggravation of pathology and progression of epilepsy. Therefore, alleviating brain inflammation, BBB dysfunction and ECM dysregulation could be a novel therapeutic strategy to inhibit or block epileptogenesis. We have shown that rapamycin, inflammation-related miRNAs and MMP inhibitor IPR-179 have disease-modifying effects in experimental models of epilepsy. In this respect, inhibiting the (immuno)proteasome, restoring a dysfunctional BBB and inhibiting MMPs are promising new approaches, which should be further investigated. Hopefully, the research presented in this thesis has helped to shorten the path towards an effective treatment for drug-resistant epilepsy patients and will aid in the development of a drug that ideally prevents and cures epilepsy.

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