The role of microRNAs in epileptogenesis

Modulation of brain inflammation and the extracellular matrix

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Publication date
2020

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CHAPTER 1

General introduction & outline of the thesis

Epilepsy overview

Epilepsy is one of the most common brain disorders affecting over 65 million people worldwide [1,2]. The conceptual definition of epilepsy given by the International League Against Epilepsy (ILAE) states: "Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures, and by the neurobiologic, cognitive, psychological, and social consequences of this condition" [3]. For the use in clinical practice, epilepsy has been defined as a disease of the brain, which fulfills any of the following criteria [4]:

1. At least two unprovoked (or reflex) seizures occurring more than 24 hours apart
2. One unprovoked (or reflex) seizure and a probability of further seizures of at least 60% over the next 10 years
3. Diagnosis of an epilepsy syndrome

Epilepsy is further classified based on different seizure types, epilepsy types as well as a large spectrum of associated epilepsy syndromes and co-morbidities arising from genetic and environmental factors or a combination of both [5,6]. Epilepsy usually has a complex etiology and the identified factors for the development of the disease include brain injuries (as a result of traumatic brain injury (TBI), tumors, stroke or status epilepticus (SE), as well as infections, gene mutations, metabolic and autoimmune conditions and structural abnormalities of brain development [6]. Despite considerable progress in the clinical management of epilepsy over the last 150 years, about 30% of adult patients and 15% of children with epilepsy do not remain completely seizure free for more than 1 year despite treatment with anti-epileptic drugs (AED) [7,8]. Epilepsy is diagnosed as drug-resistant when there is a failure to achieve sustained seizure freedom after adequate trials of two tolerated and appropriately chosen AED schedules [9]. The most common form of acquired focal epilepsy in adult patients is temporal lobe epilepsy (TLE), which is drug-resistant in about 30% of cases [8,10,11]. In the young patients epilepsy is diagnosed in about 85% patients with a genetic disorder tuberous sclerosis complex (TSC) and up to 75% of the cases are drug-resistant [12]. For a subgroup of patients with

drug-resistant epilepsy, resective surgery of a part of the brain may be used as a treatment. Other solutions include vagal nerve or deep brain stimulation, as well as strict dietary regimens, such as ketogenic diet [13,14]. All the listed treatments come with risks and side-effects, including adverse reaction to AEDs, post-surgical co-morbidities and death. Moreover, epilepsy is associated with a dramatic reduction in quality of life, increased risk of depression and suicide, mental disability, as well as the stigmatization of patients, all of these factors render epilepsy an enormous financial and social burden for society [15-17].

The realization that epilepsy is not a static phenomenon, but a dynamic and multifaceted process and a chronic condition, has led to a search for treatments that are able to prevent seizure development and progression, as well as identification of biomarkers that can serve as diagnostic tools for the early detection of the emerging pathology [13]. Thus, a deeper understanding of fundamental mechanisms underlying the development of epilepsy, is needed to address the current lack of diagnostic and therapeutic strategies for prevention or effective modification of epilepsy.

Histopathology of epilepsy

The examination of the post-mortem or surgically resected tissue from the brain of patients with drug-resistant epilepsy reveals various histological alterations. This tissue represents epileptogenic zones – areas identified by the analysis of seizure patterns, brain electrical activity and brain imaging. In TLE focal electrographic seizure activity can be detected as originating from the temporal lobe [18]. The most common histopathological finding in TLE is hippocampal sclerosis (HS) [10,19]. HS is characterized by the neuronal loss in the CA1, CA3 and CA4 subfields of the hippocampus, astrogliosis, dispersion of granule cell layer and sprouting of granule cell axons – mossy fibers [19,20]. However, whether HS is the consequence or cause of seizures in epilepsy has been a matter of debate. The most common histopathological finding associated with drug-resistant epilepsy in children is malformations of cortical development (MCD) [10], which is present in over 80% of TSC patients [21]. TSC is a multisystem genetic disorder, which is associated with the development of

benign tumors throughout the body, including the brain [12]. The neuropathological manifestations of TSC include three major types of lesions: cortical tubers, subependymal nodules and subependymal giant cell astrocytomas (SEGAs). The cortical tubers are considered the primary neuropathological substrate for epilepsy in TSC patients [12]. Cortical tubers represent areas of cortical dyslamination, containing a mixture of different cell types, including giant cells, dysmorphic neurons and reactive astrocytes [12,22]. However, not only the tubers itself, but also the surrounding perituberal space has been proposed to contribute to the development of epilepsy [23,24]. Despite certain common neuropathological features in both TLE-HS and TSC [12,25,26], the formation of epileptogenic lesions and mechanisms of epilepsy development are different between these pathologies, as further described in the proceeding sections.

Pathogenesis of epilepsy: epileptogenesis

The development of epilepsy after the initial precipitating injury is followed by a latent period, which precedes the onset of spontaneous recurrent seizures and can vary from days to many years between patients [27]. Furthermore, chronic epilepsy can be non-progressive or progressive, the latter meaning that the condition can further deteriorate over patient's life, which is manifested in increased seizure frequency, alterations in seizure phenotype and development of drug-resistance [28,29]. The studies performed in animal models of epilepsy, such as kindling, post-TBI and post-SE models, have provided a strong evidence that the development of epilepsy is a dynamic process occurring during the latent period and also extending further in time, contributing to the continuation and progression of epilepsy after the after the diagnosis has been established [27]. Thus, epileptogenesis is defined as the process of the development of epilepsy, and also the progression of epilepsy after spontaneous seizures first appear [27,30,31].

The studies of human epilepsy are often performed using the "end stage" of the disease in surgically resected or post-mortem brain tissue. Animal models of epileptogenesis have allowed us to gain insights into the processes accompanying epileptogenesis at the earlier stages. These models have been able to recapitulate the neuropathological features observed in human chronic

epilepsy and have revealed common processes underlying the disease development [32,33]. These common neuropathological features include morphological changes in the brain, gliosis, neuroinflammation, disruption of the blood-brain barrier (BBB), remodeling of the extracellular matrix (ECM) and neuronal death. Under normal physiological conditions glial cells, such as astrocytes, oligodendrocytes and microglia maintain a proper extracellular milieu necessary for neuronal functioning, whereas endothelial cells cooperate with astrocytes and pericytes to form the BBB, controlling the exchange of nutrients and waste products between the blood and brain [34-36]. Under pathological conditions, triggered by a brain insult or prolonged seizure activity, normal communication between these cells is disturbed, which is accompanied by morphological and functional alterations of glial cells, disruption of the BBB and concomitant brain inflammation [37,38]. These events are further associated with neuronal death and neurogenesis in the hippocampus, as well as destruction and remodeling of the ECM and rearrangement of synaptic connections, such as mossy fiber sprouting [39-41]. This may result in imbalanced neurotransmission in the affected neuronal networks, wherein the excitatory glutamatergic signaling prevails and not effectively counter-balanced by the inhibitory gamma-aminobutyric acid (GABA)-ergic neurotransmission [42]. These processes may ultimately lead to increased neuronal excitability, decreased seizure threshold and the occurrence of spontaneous recurrent seizures (Fig. 1).

Epileptogenesis in developing brain

Epileptogenesis also occurs in the immature brain, with seizure development in infancy and early childhood [43]. Of particular interest is epilepsy associated with a range of genetically-determined conditions that lead to structural abnormalities in the brain during brain cortex development. A large spectrum of epileptogenic disorders with MCDs, including TSC and focal cortical dysplasia (FCD), have been recently shown to be associated with the mutations in the mechanistic target of rapamycin (mTOR) signaling pathway [12]. TSC pathology arises from the mutations in *TSC1* or *TSC2* genes, responsible for the inhibitory control of mTOR (Fig. 2). TSC is associated with early-onset epilepsy (starting

from the first year of life) in 60-90% of individuals, as well as frequent cognitive impairment and autism spectrum disorder [12]. The cortical tubers in TSC can be detected *in utero* as early as 20-23 weeks of gestation, therefore epileptogenesis in TSC appears to have a neurodevelopmental character [44,45]. The mTOR pathway is critical for proper central nervous system development, since the dysregulation of mTOR may affect proliferation and migration of neural progenitor cells, neuronal soma size, dendritogenesis and formation of dendritic spines, axon outgrowth, astrocyte proliferation and cortical lamination [46-48] (Fig.2). Therefore, early pharmacological interventions, aimed at inhibition of mTOR, have been hypothesized to mitigate epileptogenesis and as such the various neurodevelopmental abnormalities present in TSC patients [12].

Reactive astrocytes

Astrocytes are the most abundant glial cell type in the mammalian brain [49]. Astrocytes play numerous functions providing trophic support to neurons, maintaining fluid, ion and pH homeostasis, regulating synaptic transmission and local CNS blood flow in the adult brain [36]. Accumulating evidence supports the heterogeneity of astrocytes in the adult CNS, with two major classes being the protoplasmic astrocytes, mostly found in the gray matter and fibrous astrocytes, found in the white matter [50,51]. In pathological states astrocytes can shift from homeostatic to reactive state [36,52,53] with the mode of reactivity ranging from cytotoxic to neuroprotective [54]. Reactive astrocytes are known to play a major role in the regulation of the immune/inflammatory response in various brain pathologies including epilepsy [52,55].

Reactive astrogliosis is commonly found in the epileptogenic zone [56-59] and has been hypothesized to actively participate in epileptogenesis [60]. Reactive astrocytes undergo morphological and functional changes and display impaired ability to perform their normal physiological functions: buffering of extracellular potassium and uptake of excessive extracellular glutamate, provision of glutamine supply for GABA synthesis in neurons and maintenance of the BBB integrity [58,59,61]. Moreover, astrocytes not only respond to various extracellular signals, but also become a source of a variety of factors, such as glutamate, adenosine triphosphate (ATP), D-serine, prostaglandins and

neuropeptides to alter neuronal activity and synaptic plasticity [62]. Among the factors released by astrocytes, an important role belongs to cytokines, chemokines and complement factors which can act in a paracrine and autocrine manner to promote chronic brain inflammation and neuronal damage [52,55]. Taken together, reactive astrocytes in epilepsy may lose their essential functions and secrete a multitude of epileptogenic factors, thereby contributing to neuronal damage and hyperexcitability (Fig. 1).

Reactive Microglia

Activation of microglial cells is another hallmark of the epileptogenic brain. Microglia appear in the brain during the early stages of development and perform important homeostatic functions in the healthy adult brain, such as phagocytosis of cellular debris, synaptic pruning and surveillance for dangerous signals [63]. Microglial cells are an important component of the innate immune system and under pathological conditions may undergo drastic morphological and functional changes to acquire a variety of phenotypes, both cytotoxic pro-inflammatory and reparative anti-inflammatory, depending on the microenvironmental context [63]. In epilepsy microglial activation is observed in the areas of HS in TLE patients [64,65] and in epileptogenic lesions of patients with TSC [66] and FCD [67-69]. Studies in animal models have suggested that morphological changes in microglia occur both shortly after acute seizures and in a delayed fashion as a response to neuronal injury [70]. During the acute phase following experimental SE microglial cells overexpress markers indicative of both phenotypes, however, this is not observed during the chronic phase, suggesting that microglial cells may play a role during early epileptogenesis [71]. Activated microglia release a plethora of cytokines and chemokines that may activate astrocytes, attract peripheral immune cells and modulate neuronal activity and neurogenesis, thus contributing to epileptogenesis [70,72]. Furthermore, mTOR overactivation in microglia has been shown to induce non-inflammatory changes in the hippocampus, leading to neuronal degeneration, astrocyte proliferation and development of spontaneous seizures in mice [73]. Thus, activated microglial cells are actively involved in brain inflammation and may contribute to epileptogenesis along with astrocytes (Fig. 1).

BBB dysfunction and immune cell infiltration

The BBB is a complex and highly selective barrier that separates the peripheral circulation system and the CNS and controls the movement of ions and molecules between them, as well as prevents the entrance of cells and other elements from the blood stream into the CNS [74]. The BBB is formed by brain endothelial cells (EC), interconnected by tight junctions. Astrocytic endfeet, pericytes and perivascular macrophages also contribute to the integrity of the barrier (Fig. 1). Dysfunction of the BBB is observed in several CNS pathologies, including epilepsy [37,38,75,76]. BBB breakdown is considered a major risk factor for epileptogenesis [25] and has been also shown to contribute to the disease progression as well as drug-resistance during the chronic phase [38,76].

Disruption of the BBB may lead to infiltration of immune cells and other blood components, such as albumin, into the brain where they are able to mediate pathological signaling. Under pathological conditions endothelial cells may overexpress leukocyte-recruiting cell adhesion molecules (CAMs) and chemokines, attracting immune cells to the brain [77-79]. Immune cells, such as monocytes, lymphocytes and neutrophils enter the brain parenchyma and further add to the pathology by secreting pro-inflammatory factors. In humans albumin extravasation and persistently activated monocytes/macrophages are observed in the resected hippocampal tissue from patients with TLE and TSC [66,76,80] and the duration of epilepsy positively correlates with the expression of monocyte/macrophage markers [80]. The components of the adaptive immune response, such as T-lymphocytes and dendritic cells are also occasionally detected in the brains of patients who died following SE and in patients with TLE, although their contribution to epileptogenesis is considered to be less as that of the innate immune response cells, such as monocytes/macrophages, microglia and astrocytes [65,80-82]. However, recent reports suggest that the role of the adaptive immune response in epileptogenesis may be more significant in pediatric epilepsy, associated with TSC and FCD [83,84].

Brain inflammation

The activation of immune-competent cells in the brain drives the innate immune response in the epileptogenic brain, which is responsible for both

acute and chronic inflammation in the brain (Fig. 1). These responses are evolutionarily conserved mechanisms, designed to protect the organism and limit damage to the tissue. However, a chronic unresolved inflammatory response may become an immanent self-perpetuating danger to the brain, aggravating the pathology and further extending the damage [85]. In the last decade the role of brain inflammation in epileptogenesis has become a central research focus [55,86,87]. Brain inflammation is mediated by a range of inflammatory mediators, such as cytokines, chemokines, complement factors, growth factors, proteases, as well as reactive oxygen and nitrogen species [55]. The inflammatory response after brain insults is triggered by danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) – a variety of molecules that serve as ligands for the pattern recognition receptors (PPRs) expressed by brain cells [85]. For instance, the activation of the PPR toll-like receptor (TLR) 4 through its ligand high-mobility group box-1 (HMGB1) has been demonstrated in both TLE and TSC, and shown to contribute to seizure generation in mice [88,89]. Glial cells are both producers and responders to pro-inflammatory factors through the assembly of the inflammasome and engagement of such signal transduction pathways as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1) and stress-related mitogen-activated protein kinases (MAPKs) [52,90]. The activation of pro-inflammatory pathways leads to the production and activation of cyclooxygenases (COX) and major cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α). Although lowly expressed under normal physiological conditions, IL-1β and TNF-α can be rapidly upregulated in glia, neurons and endothelial cells following pathogenic insults to the brain [72,91,92]. A multifunctional cytokine transforming growth factor β (TGF-β) is another marker of inflammation that acts through the phosphorylation of intracellular SMAD protein complexes, activating multiple MAPK pathways, which regulate a wide range of downstream signaling pathways [93]. TGF-β thus plays an important role in cell proliferation, differentiation and modulation of the ECM [94,95].

The examination of resected human TLE brain tissue revealed up-regulation of IL-1β and TNF-α [96-100], as well as TGF-β1 [68,95,99,101-104]. Moreover,

higher expression of IL-1β and TNF-α was detected in the brain of patients with TSC and FCD compared to autopsy control brain tissue [12,66,68,69,105-107]. During experimental epileptogenesis increased IL-1β and TNF-α have been shown to contribute to the acute and chronic inflammation [108-113]. The elevated expression of IL-1β persists for at least one week following an epileptogenic insult [109,112,114,115]. This increase is also observed at the chronic stage, primarily in microglia and astrocytes, in the regions of the brain involved in seizure generation and propagation [65,116]. The upregulated expression of TGF-β1 is also observed post-SE within the hippocampus at the acute and latent stages in rats [117]. Moreover, TGF-β signaling has been shown to mediate epileptogenesis via albumin uptake by astrocytes following the BBB breakdown [118,119].

Thus, there is ample evidence suggesting the involvement of these cytokines in epileptogenesis. In addition, the excessive neuronal activity, such as during epileptic seizures, may itself lead to the release of pro-inflammatory mediators [120]. Therefore, spontaneous seizure activity during the chronic phase of epilepsy may promote and perpetuate inflammation in the brain. One of the possible routes concerning how chronic inflammation may contribute to epileptogenesis is through the remodeling of the ECM, since the stimulation of cells with pro-inflammatory cytokines has been shown to regulate the transcription of many important molecules contributing to the ECM environment of brain cells.

ECM remodeling

(this section is partly based on the book "Inflammation and epilepsy: New vistas, Eds D. Janigro, N. Marchi, A. Nehlig; chapter "Perivascular inflammation and extracellular matrix alterations in blood-brain barrier dysfunction and epilepsy" by D.W.M. Broekaart, A. Korotkov, J.A. Gorter, and E.A. van Vliet, submitted)

A wide spectrum of cellular changes associated with brain lesions during epileptogenesis have a profound impact on the non-cellular component within the brain – the ECM [121]. Accounting for 10-20% of the total brain volume, the ECM represents a highly organized meshwork of proteins and glycans, occupying the parenchyma of virtually all cells [122]. The major component of the ECM is a high molecular weight glycosaminoglycan hyaluronan, which provides a structural framework for binding of the other ECM components, such as

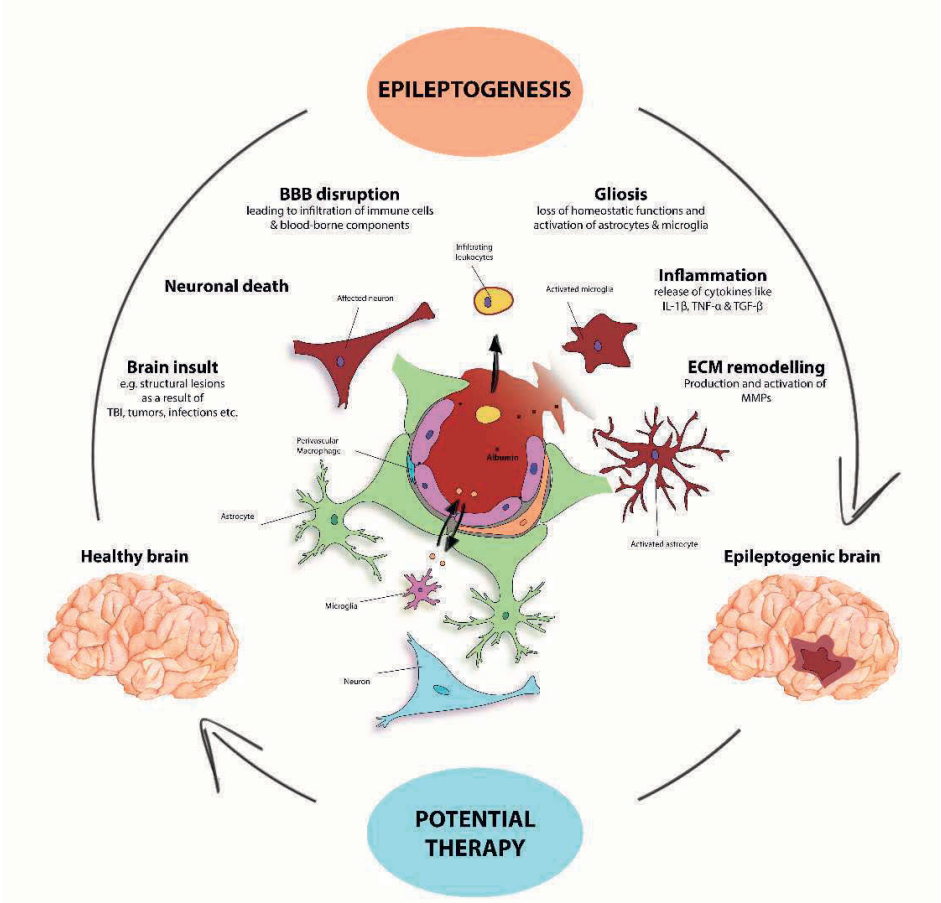

Figure 1. Schematic representation of epileptogenesis. Various brain insults may cause cell death and disruption of the BBB, which is followed by brain inflammation, mediated by major pro-inflammatory cytokines released by reactive astrocytes, microglia and infiltrating immune cells. Neuronal death, gliosis and chronic sustained inflammation are accompanied by the alterations in the composition of the ECM. The remodeling of the ECM is mediated by proteases, such as matrix metalloproteinases (MMPs). The alterations in the ECM can lead to the rewiring of synaptic connections and establishment of aberrant neuronal networks that are prone to overexcitation. This may ultimately result in the occurrence of recurrent spontaneous seizures. The potential novel therapies could be directed on prevention of epileptogenesis or modification of the disease through the targeting of key pathogenic processes.

proteoglycans, tenascins and link proteins [123]. In addition, the ECM contains a wide variety of secreted soluble factors, matricellular proteins and fragments of membrane-bound molecules, all of which may regulate cell-to-cell and cell-to-matrix interactions [40]. Therefore, the ECM does not only provide a physica scaffold for the cells, but also plays important roles in cellular growth, activity and survival [122].

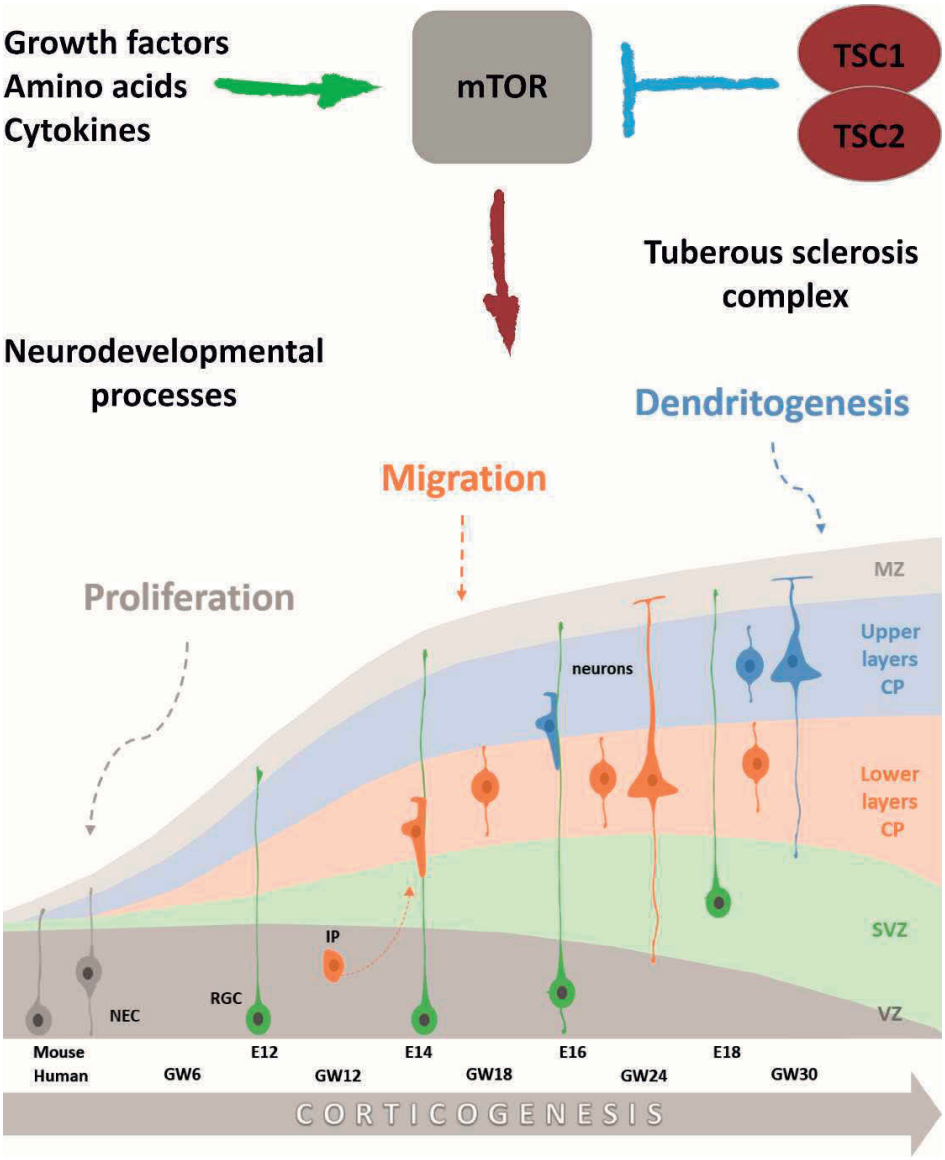

Figure 2. Overactivation of mTOR in TSC affects neural development. Growth factors, nutrients, cytokines and other environmental factors are integrated by mTOR signaling pathway to regulate cell proliferation and growth. TSC1 and TSC2 are major negative regulators of mTOR, which are mutated in TSC. As a result, mTOR is overactivated, which is associated with disturbances in crucial processes during prenatal and early post-natal brain development, including proliferation of neuroepithelial cells (NEC) in the ventricular zone (VZ), migration of intermediate progenitors (IP) along the radial glia cells (RGC) to the cortical plate (CP) and dendritogenesis during later fetal and early postnatal development; E – embryonic day, GW – gestational week. Modified from Tee et al (121).

The two specialized types of the ECM include the basement membrane (BM) and perineuronal nets (PNNs). The BM constitutes the outer ECM lining of the blood vessels in the brain and plays an important role in vascular integrity as a component of the BBB [124]. The PNNs surround somas, proximal dendrites and initial segments of axons of some neurons, predominantly interneurons in the cerebral cortex and hippocampus [40]. The PNNs can be destroyed after a brain insult and this has been hypothesized to be responsible for the death of interneurons losing their protective shield [125]. Since interneurons provide inhibitory signaling to neural networks in the brain, their loss may result in insufficient inhibition and overexcitation of the networks. Moreover, the neural ECM controls structural and functional plasticity in the brain, and with the ECM compomised, novel aberrant synaptic connections can be formed. Thus, the degradation of the ECM in the brain may be one of the key causes of epileptogenesis.

The ECM is a highly dynamic structure that undergoes continuous controlled remodeling, mediated by specific proteolytic enzymes that are responsible for the ECM degradation [126]. One of the most important players in the ECM proteolysis is a family of matrix metalloproteinases (MMPs) comprising around 25 members [126]. However, only several MMPs are expressed at a significant level in the brain (Fig. 3). MMPs can be classified based on their substrate specificity and also distinguished as bound to the plasma membrane or secreted into the ECM. Each MMP possesses a domain structure and undergoes post-translational modifications, the most significant of which is the proteolytic removal of a pro-peptide domain, required for their activation [127]. The activity of MMPs is controlled by the endogenous tissue inhibitors of metalloproteinases (TIMPs).

Under normal physiological conditions in the adult brain MMPs execute functions in routine remodeling of the ECM, angiogenesis, neurogenesis, and synaptic plasticity [128,129]. However, under pathological conditions, the expression of MMPs can be transcriptionally activated in brain cells, especially astrocytes and microglia, upon stimulation with pro-inflammatory cytokines, reactive oxygen species, hypoxia, and alterations in pH [128,130]. The following activation of MMPs is not always adequately inhibited by TIMPs and the

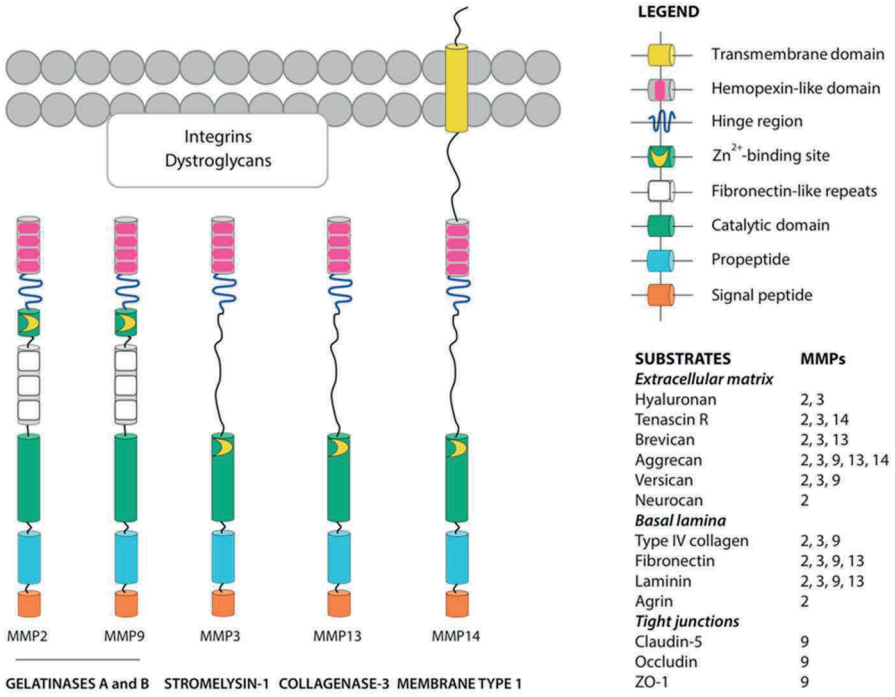

Figure 3. Schematic domain organization and targets of metalloproteinases that are studied in the epileptogenic brain. MMP2 and MMP9 belong to the class of gelatinases, MMP3 is a stromelysin, MMP13 a gelatinase and MMP14 (MT1-MMP) belongs to a type of membrane bound MMPs. MMPs consist of several domains, including a propeptide (cleavage required for MMP activation), a catalytic domain, a linker peptide or hinge region and a hemopexin domain. MMP2 and MMP9 also have three repeats of a fibronectin type II motif. The substrates of these MMPs include brain ECM components, such as hyaluronan and proteoglycans, as well as basal lamina and tight junction proteins.

resultant imbalance between the activation and inhibition of MMPs promotes the degradation of the ECM components, CAMs and receptors, which disturbs the ECM homeostasis and BBB integrity. The activation of MMPs also results in the activation of cytokines, chemokines, growth factors, complement cascade and MMPs themselves, rendering MMPs important players not only in the ECM remodeling, but also in brain inflammation, both processes involved in epileptogenesis.

In the epileptogenic brain, higher expression of MMP2, MMP3, MMP9, MMP14 as well as TIMP1 and TIMP2 have been observed in the hippocampus of TLE patients and patients who died from SE, as well as in FCD and in TSC

cortical tubers [131-135]. The studies in animal models of epileptogenesis showed that these MMPs have dynamic patterns of expression throughout different phases of epileptogenesis [136-138]. Some MMP genes, like *Mmp3*, are overexpressed not only at the chronic phase, but also during the acute and latent phases [138,139], which makes them potential targets for anti-epileptogenic therapy.

Modulation of inflammation and ECM as novel therapy for epilepsy

The growing understanding of the processes underlying epileptogenesis has extended the estimation of the "therapeutic window of intervention" for anti-epileptogenic treatments and has led to a search for strategies aimed at prevention of epileptogenesis as well as disease modification [12]. In this regard, anti-inflammatory drugs have been increasingly tested in preclinical models of epilepsy and in the clinical settings [55,87,140]. A number of clinical trials and case reports have shown efficacy of the administration of the recombinant form of human IL-1 receptor antagonist (IL-1Ra), inhibitors of caspase-1, TNF-α and COX-1/COX-2, as well as inhibitor of microglia minocycline in reducing seizure frequency in drug-resistant epilepsy [87].

Several studies in animal models have shown either neuroprotection or reduction in the frequency of spontaneous seizures following treatments with selective cyclooxygenase-2 (COX-2) inhibitors [141], inhibitors of IL-1R1/TLR4 [142] or combinations of different anti-inflammatory drugs [143,144]. Furthermore, treatments with the mTOR pathway inhibitor rapamycin led to a reduction in seizure frequency after SE [145] and inhibition of TGF-β signaling with the angiotensin II type 1 receptor antagonist losartan showed efficacy in prevention of spontaneous seizures in rats following the BBB breakdown and albumin extravasation [146]. The inhibition of MMPs has also emerged as a promising avenue for anti-epileptogenic treatment as it has been shown that broad spectrum MMP inhibitors, such as doxycycline and minocycline could prevent PNN degradation after kindling [147] or attenuate spontaneous seizures after pilocarpine-induced SE [148].

However, interventions with the existing anti-inflammatory drugs do not always show the expected anti-epileptogenic effects [149,150], furthermore

they may have serious adverse effects [151,152]. It is important to note that the inflammatory signaling and MMPs may have not only detrimental effects, but also help to resolve the brain pathology [153-155]. Therefore, the time of pharmacological intervention should be carefully chosen and the adverse effects minimized [87]. The drugs with anti-inflammatory and immunomodulatory action have arisen as a promising approach for prevention or modification of epilepsy, thus novel anti-inflammatory targets, agents and their combinations could be of great therapeutic value.

MicroRNAs and epilepsy

Transcriptomic studies over the last two decades have revealed a myriad of alterations in gene expression in the brain tissue of humans with epilepsy and in animal models during experimental epileptogenesis [156,157]. The complex intracellular regulation of gene expression also includes post-transcriptional modifications. MicroRNAs (miRNAs, miRs) are a class of small non-coding RNAs (18-22 nucleotides long) with the ability to regulate gene expression at the post-transcriptional level. miRNAs navigate the RNA-induced silencing complex (RISC) through complementary binding to the 3'-untranslated regions (UTR) of the mature mRNA. In mammals this can lead to the repression of target gene translation [158]. MiRNAs can have hundreds of direct targets within a cell and often simultaneously target several genes within a certain intracellular signaling pathway, thus regulating gene expression at a network level. Moreover, it is estimated that around 70% of all known miRNAs are expressed in the brain, many of which are specific to brain cells [159,160]. MiRNAs have been implicated in numerous pathologies of the brain [161]. A number of transcriptomic studies have shown that a great multitude of miRNAs are dysregulated in human TLE and TSC [162-166] and during experimental epileptogenesis in animals [156,157,167-170].

Several deregulated miRNAs in epilepsy have been associated with the innate immune response and inflammation [171-174]. The increased expression of inflammation-associated miRNAs miR146a, miR155 and miR132 have been shown in the hippocampus of patients with TLE [111,175,176] and miR146a, miR147b, miR155, miR21 – in TSC tubers [177,178]. Increased expression of inflammation-associated miRNAs were also observed in the rat hippocampus during epileptogenesis [167]. These miRNAs are expressed in different cells of the brain,

but their role as inflammatory modulators in epilepsy is especially intriguing in the brain cells mediating the innate immune response – astrocytes and microglial cells [175,177-179]. Experiments *in vitro* have demonstrated that such miRNAs as miR146a, miR155, miR21 can be induced by pro-inflammatory IL-1β, TNF-α or lipopolysaccharide (LPS) in astrocytes [111,177,178]. The overexpression or inhibition of these miRNAs in cell cultures may further help to gain insights into the mode of miRNA action on inflammatory signaling. Such strategies have revealed that miR146a and miR147b are potent negative regulators of inflammatory signaling [178-180] and their overexpression may be beneficial, whereas miR155 often acts as a pro-inflammatory modulator [181,182] and its inhibition may yield anti-inflammatory effects.

MiRNAs are also involved in the regulation of neurodevelopmental processes, such as cell fate determination, neurogenesis, migration of neural progenitors, neuronal polarization, dendritogenesis and synapse development [183,184]. Aberrant expression of miRNAs is associated with abnormal brain development and the pathogenesis of several neurodevelopmental diseases [185]. This may have implications for the pathogenesis of TSC. A large-scale transcriptomic analysis in TSC tubers identified many dysregulated miRNAs [166]. The expression of the miR34 family (miR34a, miR34b and miR34c) was found to be among the most significantly overexpressed miRNAs in epileptogenic tubers [186] and miR34b has been shown to modulate neurite outgrowth in *vitro* [166]. However, the evaluation of miR34 expression during prenatal and early post-natal development in TSC has not been performed.

MicroRNA-based therapy for epilepsy

Discovery of miRNA dysregulation in epilepsy and the fact that miRNAs act as potent modulators of pathological pathways involved in epileptogenesis have led to the idea that miRNAs could be used to develop new treatments of acquired epilepsy and associated co-morbidities. MiRNAs can be detected not only in the tissue associated with pathology, but also in biofluids, such as blood and cerebrospinal fluid (CSF), where they remain relatively stable and protected from RNase activity [187,188]. It has been hypothesized that these circulating miRNAs may serve as diagnostic tools, or biomarkers, that could be objectively

measured to identify the development, presence, severity, progression, or localization of an epileptogenic abnormality [189].

Several attempts have been made to modulate epileptogenesis in TLE models using miRNA-based interventions [190,191]. Inhibition of a neuronal miR-134 by intracerebroventricular injection of its antagomiR was shown to decrease seizure frequency and exert neuroprotective effects in mice during the first 2 weeks post-SE [190]. Inhibition of another neuronal miRNA miR135a has been shown to be effective in reducing seizure frequency during the chronic stage post-SE in mice [192]. Increasing the levels of anti-inflammatory or decreasing the pro-inflammatory miRNAs in glial cells may also be used to modify or prevent epileptogenesis and to alleviate associated neuropathological consequences. As an example, intracerebroventricular administration of the miR146a mimic reduced the frequency of spontaneous seizures post-SE in mice [144]. In another study, intranasal administration of miR146a mimic prior to a pilocarpine-induced SE extended the latency to generalized convulsions and reduced seizure severity in mice [193]. This improvement in disease outcome was associated with a reduction in expression of the inflammatory mediators TNF-α, IL-1β, and IL-6 [193]. Silencing miR155 in the brain may also have a beneficial effect; inhibition of miR155 could reduce seizure frequency after pilocarpine-induced SE in rats [194] and mice [195], as well as protect the integrity of the BBB and improve neurological recovery after TBI [196,197]. Moreover, treatments based on non-coding RNAs for genetic drug-resistant epilepsy in Dravet syndrome are currently considered for clinical trials in humans (https://dravetsyndromenews.com/cur-1916/), and miRNAs may soon follow its lead.

Thesis overview

The aim of this thesis was to investigate the involvement of miRNAs in epileptogenesis through the regulation of brain inflammation, alterations in ECM and corticogenesis, in order to find novel therapeutic targets and miRNA-based approaches in the treatment of acquired forms of epilepsy. We employed molecular, histological and bioinformatical techniques to examine miRNA expression and regulation in various human epileptogenic pathologies, such as

TLE, TBI and TSC, as well as in animal models of epileptogenesis, such as electrically-induced post-SE and post-TBI rat models.

Although a large amount of data has been accumulated in regard to the expression of miRNAs during epileptogenesis, the results of these efforts have been largely discordant. Therefore, in **chapter 2**, we performed a meta-analysis and review across the published large-scale profiling studies of hippocampal miRNA expression in animal post-SE models of epileptogenesis and human TLE-HS. We created a database of differentially expressed miRNAs and reviewed various parameters, such as the animal model used, time-point following SE and subregion of the hippocampus. We also sought to identify consistently differentially expressed miRNAs across different studies, as well as biological pathways associated with altered expression of these miRNAs.

One of the most consistently up-regulated miRNAs in epileptogenesis, as identified by the meta-analysis, was miR132, therefore in **chapter 3** we further investigated its expression in the human TLE-HS hippocampus and in the rat post-SE model of TLE. Our preliminary data had suggested that miR132 was expressed not only in neurons, but also in reactive glial cells, however the functions of miR132 in glia were not known. To address this question we characterized the cell-type specific expression of miR132 in glia in TLE-HS and studied the potential involvement of miR132 in the regulation of the inflammation-associated pathways IL-1β and TGF-β in human astrocytes.

We further studied the potential of inflammation-associated miRNAs to regulate the ECM. In **chapter 4**, we investigated whether miR155 and miR146a could modulate the expression of MMPs associated with epileptogenesis, such as MMP2, MMP3, MMP9 and MMP14.

In **chapter 5** we extended the analysis of miR155, as well as studied another miRNA identified by the meta-analysis, miR142, in the perilesional cortex in a rat post-TBI model. The potential of these miRNAs to serve as biomarkers was assessed in the rat plasma. Furthermore, the cell-type specific expression (including neurons, glia and immune cells) of these miRNAs was studied in the human cortex post-TBI and the potential to mediate brain inflammation through astrocyte activation was assessed *in vitro*.

Finally, in **chapter 6**, we investigated the expression of miR34a and miR34b in the cohort of resected human TSC tuber samples with the focus on fetal and early postnatal periods. The effect of miR34a and miR34b overexpression on neuronal migration and apoptosis during embryonic development was investigated in mice. We further investigated the expression of the target of these miRNAs, contactin-3, in TSC brain specimens and studied its regulation by these miRNAs in a neuronal cell line *in vitro*.

In **chapter 7**, we discuss the content of the thesis, give a future outlook and provide the conclusions.

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