Molecular alterations in epilepsy-associated malformations of cortical development
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General discussion
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GENERAL DISCUSSION

Focal malformations of cortical development (MCDs) are highly associated with medically pharmacoresistant epilepsy [69, 78, 205], which has deleterious effects on the central nervous system, especially in the case of a developing child, and has a significant emotional and psychological impact on the child and family. Despite the development of new antiepileptic drugs (AEDs) and polytherapy programs, a substantial number of patients continues to have seizures and focal MCDs are frequently encountered in the epilepsy surgery programs [9-11, 396, 510]. The access to this surgically removed epileptogenic tissue combined with gene expression profiling and immunocytochemical techniques provides the opportunity to study the underlying molecular alterations as described in this thesis. Here, the observed pathogenetic and epileptogenetic alterations and their clinical relevance will be discussed.

PI3K-MTOR SIGNALING IN FOCAL MCDS

The phosphatidylinositol-3 kinase - mammalian target of rapamycin (PI3K-mTOR) signaling pathway is a potential candidate pathway in the molecular pathogenesis of focal MCDs. The hyperactivation of mTOR and its downstream proteins, including the ribosomal S6 protein, has been described in the cortical tubers in patients with the tuberous sclerosis complex (TSC) and in focal cortical dysplasia (FCD type IIb), which likely explains the abnormal enlargement of the dysmorphic neurons and giant/balloon cells [58, 62, 63]. In chapter 2, we demonstrated that activated components of this signaling pathway are present in the neuronal component of ganglioglioma (GG) and not in dysembryoplastic neuroepithelial tumor (DNT), which emphasizes the suggested pathogenetic relationship of GG with FCDIIB and TSC. Nevertheless, this signaling pathway is differentially regulated in the different malformations (chapter 2; [64, 65]) and a remaining question is: what are the underlying regulatory mechanisms? As stated in chapter 2, variations in the secretion of growth factors and/or other neurotrophic factors in the microenvironment [56] or different regulation of signaling pathways associated with the TSC1-TSC2 complex [154, 155, 157] in the different MCDs provide possible explanations. The robust expression of AMOG in GG (chapter 2), which activates mTOR and S6 independently of PI3K and the TSC1-TSC2 complex [141], represents an additional mechanism of activation. Moreover, mTOR signaling can also be activated via stimulation of the group I metabotropic glutamate receptors (mGluRs; [470, 471]), which are prominently expressed in the neuronal component of TSC (chapter 9 of this thesis), FCDIIB [113] and GG [115]. Despite these associations with the PI3K-mTOR signaling pathway, the different regulatory mechanisms in the different MCDs are still incompletely resolved. Another question is whether modulation of this signaling pathway represents a potential target for treatment therapies in patients with focal MCDs. Aberrant mTOR signaling is involved in many processes, including tumor growth and innate and adaptive immune responses, and mTOR inhibitors, as for example rapamycin, has been used as effective immunosuppressants and anticancer agents [511, 512]. Rapamycin, also known as sirolimus, is a macrolide antibiotic that was discovered as a product of the bacterium Streptomyces hygroscopicus in the early 1970s from a soil sample obtained at Easter Island [513]. The Polynesian name of Easter Island, ‘Rapa Nui’, explains its name and rapamycin was...
Molecular alterations in epilepsy-associated malformations of cortical development

initially identified as potential antifungal agent. However, due to its immunosuppressive and anti-proliferative properties, rapamycin was abandoned as antifungal agent and developed as anticancer agent [514]. Despite its relatively high molecular weight, rapamycin can cross the BBB due to a high lipophilic index [515], which is important for systemic administration in the treatment of brain tumors or epilepsy. Moreover, leakage of the BBB under epileptic conditions facilitates the entry of large molecules into the brain. In TSC patients, rapamycin has been shown to reduce the volume of TSC-associated renal angiomyolipomas [516, 517] and subependymal giant cell tumors (SGCTs or SEGA; [518]). Nevertheless, compared to these hamartomas, cortical tubers are considered as non-neoplastic lesions with structural abnormalities arising during fetal development and might, therefore, need in utero approaches to prevent their development. Despite the fact that mTOR inhibitors are unlikely to fully reverse cortical tuber formation, they might suppress seizures or even prevent the development of epilepsy as demonstrated in a mouse model of TSC [519] or cortical dysplasia [520]. Similar results are recently reported in the kainate model of epilepsy, in which epileptogenesis is triggered by a status epilepticus induced with the chemoconvulsant kainate [521], indicating a general role of mTOR signaling in epileptogenesis. The mechanism of seizure control induced by mTOR inhibition is unknown, but given the fact that mTOR is also known to mediate both the innate and adaptive immune response [315, 411, 417], mTOR inhibition could possibly prevent chronic inflammation in the epileptic brain and thereby suppressing seizures. These findings indicate potential clinical applications of mTOR inhibitors for patients with MCDs, which is supported by the recent observed reduction in seizure frequency in a young girl with TSC treated with rapamycin [522]. Nevertheless, the activation of components downstream of mTOR by PDK1 in GG and FCDIIB (chapter 2; [64, 65]), and the unknown involvement of possible other signaling pathways makes it speculative to what extent mTOR inhibitors are effective in these lesions. Given the fact that mTOR signaling is also involved in synaptic transmission [523] and plasticity [524], cognitive side effects can be expected; though significant deterioration of memory and executive skills was not reported in an interim-report after one year of treatment with rapamycin [517]. Thus, promising results are reported for the use of mTOR inhibitors to suppress seizures; however, further research to elucidate both the short- and long-term consequences of mTOR inhibition is clearly required.

IMMUNE AND INFLAMMATORY REACTIONS IN FOCAL MCDs

The original consideration of the brain as an ‘immune privileged’ site has changed and it has become clear that the resident cells of the brain, especially microglia and astrocytes, produce inflammatory mediators which are involved in many CNS disorders including epilepsy [79, 80, 82, 410]. Cytokine production is rapidly induced by seizures and observations in various animal models of epilepsy demonstrated that inflammatory reactions contribute to seizure generation (Fig. 1, [82, 410]). Cytokines have also been shown to modulate blood-brain barrier (BBB) integrity [83, 415] which contributes to seizure generation by extravasation of serum albumin promoting chronic neuronal hyperexcitability [327, 328] or by facilitating the entry of leukocytes enhancing the inflammatory response [81]. Noteworthy, BBB leakage positively correlates with the frequency of spontaneous seizures in animal models of epilepsy [182, 217].
Evidence for immune and inflammatory reactions in focal MCDs is provided in several chapters in this thesis (chapter 3-7). The growth factor VEGF, prominently expressed in FCDIIIB (chapter 3), is recently described as an inflammatory mediator and may increase the BBB permeability [182, 195, 211, 216]. The exact mechanisms of modulation of the BBB permeability by inflammatory mediators is not fully elucidated, however, increased production of selectins, adhesion molecules and cytokine receptors contribute to this phenomenon [82, 416]. Increased expression of inflammatory mediators and cell adhesion molecules was detected with microarray analysis in GG and TSC (chapter 6 and 7), and we demonstrated leukocyte infiltration and albumin extravasation in TSC (chapter 5), all supporting a sustained inflammatory response. Furthermore, the decoy receptor IL-1RII and the naturally occurring antagonist of IL-1β, IL-1Ra, are relatively low expressed in MCDs (chapter 4), suggesting that the brain lacks an efficient mechanism to terminate the inflammatory effects of IL-1β. Activated microglia are prominently present in focal MCDs [108, 109], which is supported and extended by our findings in chapters 5-7. The density of activated microglia correlates with both the duration of epilepsy and the seizure frequency of these patients [108, 109] demonstrating their role in epileptogenetic mechanisms. The role of activated microglia in the development of behavioral and/or cognitive dysfunctions [320], awaits further investigation.
Despite evidence of activation of immune and inflammatory reactions in focal MCDs, the development of therapies is complicated since, in addition to the direct detrimental effects, inflammatory mediators have beneficial neuroprotective effects in the long-term repair and recovery [79, 180, 219, 270, 271]. Possible treatment strategies might include administration of inhibitors of the produced inflammatory mediators presented in this thesis, such as IL-1Ra and complement inhibitors [79]. Promising opportunities are given by IL-1Ra, since IL-1Ra is well tolerated in humans [525] and beneficial effects of IL-1Ra have been demonstrated in acute brain disorders such as stroke [79, 526]. Caution is needed by the use of complement inhibitors in the developing brain as the complement factors C1q and C3 have also non-immune functions and are critically involved in synapse elimination during development [527]. Failure of synapse elimination during development results in enhanced excitatory synaptic connectivity which generates spontaneous seizures in C1q knock-out mice [528] indicating that C1q is necessary to stabilize the synaptic network. It has been suggested that this mechanism of synapse elimination is reactivated by reactive astrocytes in brain injury [527] and possibly also in focal epilepsies as reactive astrocytes and C1q and C3 were observed in temporal lobe epilepsy associated with hippocampal sclerosis [189] and focal MCDs (chapters 5-7). Synapse elimination may play a role in synaptic loss and establishment of an aberrant network contributing to epileptogenesis, indicating that in the mature brain complement inhibitors can have beneficial effects. Disruption of the leukocyte-endothelial interactions at the BBB represents an additional therapeutic target with promising results in an animal model of epilepsy [418]. Nevertheless, the dual or multiple effects of inflammatory mediators and their non-immune functions in the CNS have to be explored carefully and taken into account before successful antiepileptic therapeutic strategies can be developed.

GABA AND GLUTAMATE SIGNALING IN MCDS

Both the neurotransmitters GABA and glutamate regulate neuronal excitability and various antiepileptic drugs (AEDs) are based on either enhancing inhibitory GABAergic effects or reducing excitatory glutamatergic effects [529]. During development, however, GABA has an excitatory effect. Activation of GABA$_A$ receptors in immature neurons triggers depolarization of the cell membrane due to a high intracellular chloride (Cl$^-$) content [438, 530]. GABAergic actions become inhibitory by lowering the intracellular chloride content via increased K$^+$-Cl$^-$-cotransporter (KCC2) and decreased Na$^+$-K$^+$-2Cl$^-$-cotransporter (NKCC1) expression during the first year of life [442, 449]. Focal MCDs show an immature expression pattern of NKCC1 and KCC2 (chapter 8), suggesting that GABA agonists triggers depolarization and cannot reduce seizures. Functional effects cannot be concluded from this immunocytochemical study, however, an NKCC1 inhibitor, like the diuretic bumetanide, may have more potential therapeutic effects in focal MCDs than GABA agonists. Accordingly, blocking NKCC1 with bumetanide has been proposed as anticonvulsant strategy to treat neonatal seizures [530], though some caution is needed as diverse effects of bumetanide on epileptic activity has been reported in different experimental models of epilepsy [531, 532].

Neuronal group I metabotropic glutamate receptors (mGluR1 and -5) generally mediate postsynaptic excitation, while group II (mGluR2 and -3) and III (mGluR4, -6, -7, and -8) receptors have presynaptic inhibitory effects [465, 466]. Therefore, the prominent neuronal expression of group I metabotropic glutamate receptors in focal MCDs (chapter 9, [27, 113,
together with the relatively low expression of group II and III mGluRs represent a critical factor in the epileptogenicity of these lesions. Several studies indicate a role for these receptors in epileptogenesis and suggest that mGluR subtypes are significant molecular targets for treatment of epilepsy [466, 468]. In particular activation of group II and III mGluRs has been shown to have anticonvulsant effects, as opposed to the convulsant action of group I mGluRs reported in a variety of experimental models [468]. In addition to epileptogenesis, group I mGluRs also mediate several processes in brain development (synaptogenesis, and proliferation, differentiation and survival of neural precursors) and aberrant signaling via particularly mGluR5 have been implicated in neurodevelopmental disorders [469]. An example is given by the Fragile X syndrome (FRAX) in which enhanced mGluR5 signaling induces synaptic dysfunction which account for the diverse neurological and psychiatric symptoms (including mental retardation, autism and seizures; [533]). Negative modulators of mGluR5 have been shown to reverse multiple phenotypes in both mouse and drosophila models of FRAX [497, 534] and has already resulted in the first clinical trial with the mGluR5 antagonist fenobam in 12 FRAX patients [535]. A single dose of fenobam had promising results with no significant adverse effects, which set the stage for more extensive clinical trials and further research to therapeutic implications of group I mGluR antagonists.

To summarize, focal MCDs caused by abnormal proliferation of neural precursor cells share similar molecular alterations potentially contributing to their epileptogenesis (Fig. 2). Additionally, alterations at the circuit level in the focal lesion themselves, the surrounding perilesional regions or even throughout the whole brain can contribute to initiation and propagation of seizures associated with focal MCD.

Figure 2. Alterations at both the cellular/molecular level and the circuit level in the focal lesion, the perilesional region and the global network throughout the brain potentially contribute to their associated epileptic activity.
CONCLUSIONS AND FURTHER PERSPECTIVES

Studying the molecular alterations in focal MCDs has revealed novel insights in both the pathogenetic and epileptogenetic mechanisms in these developmental lesions associated with intractable epilepsy. A better understanding of these mechanisms is critical in the development of more effective treatment strategies; nevertheless, functional studies in either in vivo or in vitro models are clearly necessary to support our findings and to further elucidate the clinical relevance of these findings. The surgical specimens used in our experiments are generally obtained at advanced or even final stages of the development of epilepsy and for obvious reasons the preceding period cannot be studied in human material. Though, the understanding of these mechanisms involved in the development of chronic epilepsy have high priority, as early intervention prevents further progression of epilepsy and may prevent surgery. The currently available animal models of epilepsy contribute significantly to our understanding of cortical development and epileptogenesis. None of them fully reproduce the characteristic features of focal MCDs and the development of animal models that do reproduce the human pathology and epileptogenicity will help to further elucidate critical mechanism in focal MCDs and possibly link in vitro observations to therapeutic implications. In addition to studying the molecular alterations involved in the pathogenesis and epileptogenesis of focal MCDs, it is also worthwhile to use or improve current imaging techniques to recognize early circuitry alterations associated with epileptogenesis.

In the epilepsy surgery programs, the removed tissue is in general sequentially fixed in formalin, embedded in paraffin and used for histological and immunocytochemical reactions to set the diagnosis. Unfortunately, this fixed material is unsuitable for electrophysiological or gene expression studies, as fresh or freshly frozen material is needed. Nevertheless, the removed material is very valuable and can be used to study the pathogenetic and epileptogenetic mechanisms with various research techniques (Fig. 3). Therefore, improved communication and collaboration between different clinical disciplines and basic researchers will provide novel opportunities to shed new light on developmental disorders associated with epilepsy, improving their clinical/pathological classification and, more important, recognizing new potential therapeutic options.
Figure 3. Workflow to study underlying pathogenetic and epileptogenic mechanisms in focal MCDs.