New developments in understanding focal cortical malformations

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\textbf{Purpose of review}
Focal cortical dysplasias (FCDs) represent common cortical malformations that are frequently associated with epilepsy. They have so far not been well understood in terms of their molecular pathogenesis, and with respect to mechanisms of seizure emergence.

\textbf{Recent findings}
Several recent studies have succeeded in making significant advances in understanding the molecular genetics, in particular FCD type II. A second major advance has been the development of novel rodent models of FCDs that replicate a somatic mutation seen in humans, lead to a focal lesion, and recapitulate many phenotypic features of human FCDs. We will discuss these recent advances.

\textbf{Summary}
These advances promise significant advances in understanding the heterogeneity of FCDs at the molecular genetic level. They also promise a much better understanding of cell-intrinsic and network mechanisms underlying increased seizure susceptibility and altered cognition. Systematic studies utilizing the approaches summarized here promise to lead to specific strategies regarding when and how to treat specific subgroups of FCDs.

\textbf{Keywords}
epilepsy, focal cortical dysplasia, in-utero electroporation, mammalian target of rapamycin, seizures

\section*{INTRODUCTION}
Malformations of cortical development (MCDs) arise because of a disturbance of the normal steps that lead to the formation of a layered cortex. A wide variety of different MCDs have been described, with diverse causes. These range from MCDs because of single gene mutations that cause extensive disruption of normal cortical development to benign glioneuronal tumors such as gangliogliomas. One group of MCDs that has so far not been well understood in pathogenetic terms and with respect to mechanisms of seizure emergence are the focal cortical dysplasias (FCDs). FCDs represent a spectrum of focal malformations that are characterized by a focally disrupted normal cytoarchitecture. FCDs are common amongst cortical malformations, and have a number of special features. Firstly, they are frequently associated with epilepsy, and in fact were discovered as epilepsy-associated entities in a study by Taylor \textit{et al}. \cite{1}. Secondly, they are mostly sporadic entities, with very few familial pedigrees published.

FCDs have been subdivided into different subgroups and entities. Although distinct classification systems were applied in a parallel fashion in the past, the current International League Against Epilepsy (ILAE) classification represents a wide consensus system mainly based on histopathological features \cite{2}. Briefly, FCD type I summarizes different aberrations of cortical layer formation because of altered migration, and may display heterotopic neurons. FCD type II is characterized by dysmorphic neurons as major neuropathological hallmark. FCD type III occurs in conjunction with other brain lesions, for instance tumors. Tuberous sclerosis is a disease associated with cortical tubers, which resemble neuropathological features of, in particular, FCD type IIb in many respects, but are not included in this classification scheme.
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KEY POINTS

- Focal cortical dysplasias (FCDs) are common cortical malformations that are frequently associated with epilepsy.
- Recent studies have used molecular genetic approaches to understand the molecular pathogenesis of FCDs, resulting in a better understanding of the heterogeneity of FCDs at the molecular genetic level.
- The development of novel rodent models of FCDs that replicate human etiopathogenesis and clinicopathological feature promise a better understanding of cell-intrinsic and network mechanisms underlying increased seizure susceptibility and altered cognition.

Uncovering the genetic and, in particular, the functional mechanisms of FCDs has been difficult. The focal nature of the lesions seen in FCDs has led to the idea that somatic mosaic mutations of genes are responsible. The unequivocal detection of such mechanisms necessitates parallel molecular genetic examinations on brain tissue and other tissues, and is therefore, limited to those cases in which brain tissue is available following epilepsy surgery. Nonetheless, several recent studies have succeeded in making significant advances in understanding the molecular genetics of, in particular, FCD type II. We will discuss these recent publications and the accumulating molecular genetic evidence for involvement of specific pathways in FCDs.

A second challenge in understanding the mechanisms of FCDs is to develop conceptual models that explain how the known genetic alterations lead to cortical hyperexcitability and functional deficits. One major difficulty has, in our view, been the lack of appropriate animal models. Many models of MCDs, such as focal freeze lesions or other focal models produce circumscribed lesions [3], but fail to capture the cellular and molecular genetic heterogeneity of FCD lesions. On the other hand, many transgenic mouse models recapitulate genetic defects leading to human disorders. These, however, mostly show diffuse pathological abnormalities and lack focal lesions seen in the human disorders [4]. An ideal FCD model should consequently introduce a genetic alteration known to occur in humans, affect only a subset of neurons in the developing brain to cause a lesion that is both focal and composed of dysplastic and normal neuronal elements, recapitulate at least some of the phenotypic features of human FCDs. Recent advances in in-utero electroporation approaches as well as CRISPR-Cas9-mediated genome editing have opened new avenues to generate models of different types of FCDs. We will, therefore, also discuss the potential uses and promise of such recent approaches for uncovering functional mechanisms of FCDs.

RECENT FINDINGS IN MOLECULAR GENETICS OF FOCAL CORTICAL DYSPLASIAS

The past 2–3 years have seen a number of studies reporting on somatic mutations in FCDs using parallel sampling from brain and other tissue specimens. Lim et al. [5] have used deep whole-exome sequencing in both blood and brain biopsy specimens from FCD type II patients to examine brain somatic mutations. This study has – as some other recent studies – focused on the mammalian target of rapamycin (mTOR) pathway. Lim et al. [5] found somatic mutations in the mTOR gene in a significant portion of patients with FCD type II (12 of 77). Similarly, somatic mTOR mutations were identified in a further study using blood–brain paired samples in FCD type IIb (6/13 individuals). A follow-up study of similar design investigating five further genes involved in the mTOR pathway (PIK3CA, PIK3R2, AKT3, TSC1, and TSC2) used paired brain and saliva samples from FCD type II patients that did not have mTOR mutations. Five of 40 patients had somatic brain mutations in TSC1 and TSC2 [6]. Remarkably, all identified mutations induced hyperactivation of the mTOR pathway, either as a direct effect on mTOR or by disrupting the formation or function of the TSC1–TSC2 complex. Likewise, somatic duplications in the 1q chromosomal region that affect the AKT3 gene have been associated with FCD type Ib [7,8].

The simple view, however, of a single somatic mutation causing FCDs is very likely not the whole picture. In addition to somatic mutations, there are also germline mutations that have been associated with the occurrence of FCD. For instance, patients with familial DEPDC5 mutations in some cases, display FCDs [9,10]. In addition, germline mutations in the gene encoding nitrogen permease regulator-like 3 (NPRL3), which are predicted to lead to mTOR hyperactivation have been associated with FCD type IIa [11]. An additional recent study reported a new TSC1 germline mutation in patients with FCD type IIb [12]. How germline mutations can give rise to focal lesions is still puzzling, the most straightforward explanation being that a somatic second hit, that is, a different somatic mutation at a different developmental timepoint, may cause the focal lesion. This is similar to the second hit hypothesis in TSC that could explain how cortical tubers arise in the presence of a germline TSC1 or TSC2 mutation.
mutation [13]. One problem with the detection of such mutations could be that they are present only in few cells. Therefore, identification of such mutations may require extremely sensitive detection techniques (i.e. high coverage next generation sequencing), ideally of brain tissue in the center of the lesion. Single cell RNAseq approaches may provide some advances, but may pose similar problems of sensitivity.

**NEW MODELS OF CORTICAL MALFORMATIONS**

Two advances are particularly promising in generating pathogenetically meaningful models of FCDs. Firstly, refinement of in-utero electroporation techniques allow improved control over the extent to which genetic material is introduced into cortical precursors. This approach has been used very effectively in combination either with genetic silencing techniques [14,15], or by inducing expression of mutant protein in cortical precursors [5] to induce cortical malformation in rodents. Advances in electroporation techniques have, for instance, been improved targeting. Novel three-electrode approaches allow to direct the electroporation with much better accuracy to specific neurogenic areas, and therefore, to achieve more focal lesions [16].

The second approach that may be extremely useful whenever combined with the advantages of in-utero electroporation is CRISPR-Cas9-mediated genome editing. This approach will afford us with the opportunity to mimic specific genetic changes, not limited to a single gene, found in human FCD. This approach has, for instance, been used by Lim et al. [6], in which CRISPR-Cas9-mediated genome editing of the Tsc1 or Tsc2 genes resulted in generation of cytomegalic neurons, cortical lamination defects and the development of spontaneous behavioral seizures. This study shows that in-utero application of the CRISPR-Cas9 system is useful to generate both etiologically and as phenomenotypically plausible disease models of neurodevelopmental disorders arising from somatic brain mutations.

Thus, the instantiations of such models, so far, indicate that they may replicate many specific features of FCD disease, as well as the functional changes in excitability including seizures. In particular whenever considering second-hit hypotheses, useful extensions of such models may include in-utero CRISPR-Cas9 editing on the background of germline mutations. Many of the relevant mutant rodent models with germline mutations in human patients are already available (i.e. Depdc5 knockout rats [17]). A systematic application of such approaches on different backgrounds may provide the clearest evidence on how somatic and germline mutations in different genes might interact to cause FCDs.

**THEORIES OF HYPEREXCITABILITY DEVELOPMENT AND COGNITIVE IMPAIRMENT**

The novel models available also afford us with new opportunities to understand the development of hyperexcitability and cognitive dysfunction. Firstly, the generation of a pathological cortex constituting dysplastic and abnormally positioned neurons will allow to bring to bear optogenetic and classical physiological approaches to dissect the connectivity matrix of these different network elements in malformed cortex.

Secondly, we believe that combining such models with the currently available techniques to record neuronal activity at the population level with cellular resolution [18] will yield novel insights into the mechanisms underlying the generation of abnormal activity. In particular, 2-photon in-vivo imaging approaches appear extremely promising because they allow both identification of genetically abnormal neurons, and detection of neuronal activity with a spectrally separated genetically encoded calcium indicator such as GCaMP6. Using new techniques for deep imaging [19], it will likely also be possible to detect activity patterns in aberrantly positioned subcortical neurons that have not achieved migration to their proper cortical position. Thirdly, the focal nature of these models now permits us to address how seizures might spread from a focally abnormal area of the cortex to more remote brain areas. Equally important, focal models can also be used to study mechanisms of functional disturbance in remote brain areas. Such a remote impairment has been hypothesized, primarily because focal lesions may show a wide range of neuropsychiatric problems, many of which are not easily reconciled with a focal functional disturbance within a limited lesion. One study has addressed this important issue, showing that mice exhibiting focal heterotopias mainly in the somatosensory cortex, exhibited cognitive deficits commonly associated with impaired function of the medial prefrontal cortex (mPFC). Both an analysis of immediate early gene expression and local field potential measurements suggested altered activity in the mPFC in animals with heterotopia. Importantly, chemogenetically manipulating the activity of abnormal neurons in heterotopic animals improved some of the cognitive defects [20]. It will be important to follow up on this intriguing finding to determine the specific network mechanisms leading to remote disturbance, the variability of remote disturbance.
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disturbances between models, and the response of remote disturbances to treatment.

The novel emerging models also have an additional advantage, namely that vectors can be used that allow to induce, or reverse genetic modifications at specific timepoints. This may be very instructive to narrow down the spectrum of abnormalities most importantly for a functional phenotype such as seizures. An example for this approach is a recent publication by Hsieh et al. [21**], in which mTOR activity was increased by in-utero electroporation of a plasmid encoding a constitutively active form of Rheb (RhebCA), the canonical activator of mTORC1. The electroporation approach preferentially targeted layer 2/3 cells of frontal cortex, and caused a neuropathology with hallmarks of FCD type II. Activating the RhebCA expression only after birth leads to a similar phenotype, but lacking the dyslamination and white matter heterotopia. Importantly, these mice also exhibited seizures, indicating that migration defects are not required for the generation of spontaneous seizures [21**]. Interestingly, a congruent phenomenon could be observed whenever all FCD-related defects, including neuronal misplacement and dysmorphogenesis, were prevented by early rapamycin treatment. In this case, seizures recurred after rapamycin withdrawal even in the absence of migration defects [21**]. Aside from informing on causative aspects, these experiments are, therefore, also highly relevant for treatment considerations, because they indicate that continuous treatment with inhibitors of the mTOR cascade may be required to maintain seizure freedom. However, chronic mTOR antagonist treatment is associated with adverse drug effects in substantial number of patients [22]. Therefore, more targeted symptomatic as well as gene therapy approaches may in the future become increasingly relevant for the clinical management of neurosurgically intractable FCDs.

In the context of symptomatic treatments, it may be fruitful to target individual, functionally relevant consequences of mTOR hyperactivation. For instance, heterozygotic TSC1+/− mice display a functional upregulation of cortical GluN2C-containing N-methyl-D-aspartate receptors (NMDARs) in an mTOR-dependent way. These mice lack malformative lesions, but suffer from recurrent, unprovoked seizures during early postnatal life (<P19 [23]). Specific GluN2C/D− antagonists suppressed seizure activity in TSC1+/− mice. Correspondingly, GluN2C expression was augmented in TSC human brain biopsy tissue and a GluN2C/D antagonist-reduced episodic hyperexcitability [23].

Finally, in-utero electroporation models of circumscribed neurodevelopmental lesions may be useful to study and determine strong candidates for gene therapy. A seminal study has demonstrated in an in-utero RNAi rat model of subcortical band heterotopia that re-expression of the Dcx gene in aberrantly positioned neurons after the time of birth again initiated their migration and increased the previously reduced seizure threshold [15]. With respect to tuber-related as well as FCD type II-related dysplastic neurons, we observed that expression of TSC1 stop mutants frequent in TSC patients in an TSC1 knockout background resulted in a substantial increase in size and aberrant shape of emerging neuronal cells and mTOR pathway activation, whereas co-electroporation of only a minimal gene dosage of functional TSC1 was sufficient to rescue the phenotype [24].

**PERSPECTIVES**

Understanding the cellular mechanisms of FCDs has led to several strategies to develop treatments. Firstly, the discovery that mTOR pathways are hyperactive in many etiological subgroups of FCDs, similar to tuberous sclerosis, has triggered clinical studies testing the efficacy of mTOR inhibitors in these disorders. Secondly, the discovery of mechanisms leading to hyperexcitability in FCD lesions, such as the upregulation of specific NMDA receptor subunits, may be leveraged for treatment. Finally, the translation of innovative gene therapy strategies into clinical settings will require to overcome numerous obstacles including precise diagnostics ideally at an preepileptogenic stage as well as entirely safe vector systems for gene therapy. Nevertheless, the outlined rodent models provide powerful tools to improve our understanding of the functional consequences of increasingly recognized molecular genetic alterations in FCDs, the cellular complexity of emerging brain lesions as well as the characterization of critical dynamics of neuronal network development in the future.

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**Conflicts of interest**

H.B. has received honoraria from UCB and BIAL, and has performed contract research for UCB and BIAL. A.J.B. has no conflicts of interest.
REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest