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MECHANISMS OF EPILEPTOGENESIS IN TUBEROUS SCLEROSIS COMPLEX AND RELATED MALFORMATIONS OF CORTICAL DEVELOPMENT WITH ABNORMAL GLIONEURONAL PROLIFERATION

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Summary

Malformations of cortical development (MCDs) are increasingly recognized as causes of medically-intractable epilepsy. In order to develop more effective, rational therapies for refractory epilepsy related to MCDs, it is important to achieve a better understanding of the underlying mechanisms of epileptogenesis, but this is complicated by the wide variety of different radiographic, histopathological, and molecular features of these disorders. A subset of MCDs share a number of characteristic cellular and molecular abnormalities due to early defects in neuronal and glial proliferation and differentiation and have a particularly high incidence of epilepsy, suggesting that this category of MCDs with abnormal glioneuronal proliferation may also share a common set of primary mechanisms of epileptogenesis. This review critically analyses both clinical and basic science evidence for overlapping mechanisms of epileptogenesis in this group of disorders, focusing on tuberous sclerosis complex, focal cortical dysplasia with balloon cells, and gangliogliomas. Specifically, the role of lesional vs. peri-lesional regions, circuit vs. cellular/molecular defects, and non-neuronal factors, such as astrocytes, in contributing to epileptogenesis in these MCDs is examined. An improved understanding of these various factors involved in epileptogenesis has direct clinical implications for optimizing current treatments or developing novel therapeutic approaches for epilepsy in these disorders.

Keywords

epilepsy; seizures; tuberous sclerosis complex; focal cortical dysplasia; ganglioglioma

Introduction

Malformations of cortical development (MCDs) are increasingly recognized as causes of epilepsy. The clinical significance and impact of MCDs is especially high, because MCDs are frequently associated with pharmacoresistant epilepsy that is refractory to available seizure medications. While a subset of epilepsy patients with MCDs may be candidates for epilepsy surgery, a better understanding of underlying mechanisms of epileptogenesis in this group of developmental brain disorders could lead to more effective therapeutic strategies, including anti-epileptogenic treatments to prevent epilepsy in at-risk patients. However, one problem with studying epileptogenesis in MCDs is that MCDs consist of a wide spectrum of disorders, involving a variety of clinical, etiologic, radiographic, and pathological properties

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and likely encompassing equally diverse mechanisms of epileptogenesis. For example, on one extreme, lissencephaly has widespread cortical involvement and severe neurological symptoms usually including global developmental delay in addition to epilepsy. On the other extreme, milder MCDs may be much more localized, such as in focal cortical dysplasia or limited heterotopic neurons in white matter, and involve isolated epilepsy or be clinically silent. Despite this large diversity of MCDs, many MCDs share a number of similar molecular and histopathological, as well as clinical, features, suggesting that a common set of primary mechanisms of epileptogenesis could predominate in a subset of MCDs. In particular, many MCDs exhibit focal "dysplastic" histological and cellular features of both neurons and glia, including dysmorphic or cytomegalic neurons, glial proliferation, and distinctive undifferentiated cells with mixed neuronal and glial features, all of which are likely due to abnormalities in glioneuronal proliferation during embryonic cortical development. Tuberous sclerosis complex (TSC) is often identified as a "model disease" that exemplifies these histopathological abnormalities and has a particularly high incidence of intractable epilepsy. Focal cortical dysplasia (FCD) with balloon cells and low-grade gangliogliomas (GG) also share similar clinical, pathological, and molecular features, involving a mixture of neuronal and glial abnormalities.

In this review, I will first discuss the evidence supporting the existence of this subgroup of MCDs characterized primarily by similar defects in glioneuronal proliferation and differentiation, which are highly associated with epilepsy and may share common mechanisms of epileptogenesis. Then, I will critically analyze available data related to three key questions about epileptogenesis in these disorders: 1) Do seizures start within the lesion or the perilesional region? 2) Is epileptogenesis primarily a result of circuit abnormalities or cellular/molecular defects? 3) Do non-neuronal factors, such as glial cells, contribute to epileptogenesis? I will review relevant data from both human studies and animal models, focusing primarily on TSC as a model system, but also drawing comparisons from other related disorders, such as FCD and GG. Finally, the potential clinical implications of these findings will be discussed, related to optimizing current treatments or developing novel therapies for epilepsy.

MCDs with Abnormalities of Glioneuronal Proliferation and Differentiation: A Group of Related Disorders with Shared Mechanisms of Epileptogenesis?

Over the past decade, with advances in neuroimaging, histological and molecular genetic techniques, the number and characterization of MCDs have become increasingly large and complex, often leading to confusion in the literature about terminology and classification of MCDs. A variety of different types of MCDs have been identified, differing in radiographic, pathological, and etiological/genetic features. Several different classification schemes have been proposed for MCDs, with varying emphasis on molecular genetic, pathological, or radiographic criteria (Mischel et al., 1995; Sarnat, 2000; Palmini et al., 2004; Barkovich et al., 2005). One comprehensive classification scheme has attempted to incorporate several of these features, based conceptually on the specific stage of embryonic brain development at which the initial developmental defect occurs, including cellular proliferation and differentiation, neuronal migration, and cortical organization (Barkovich et al., 1996, 2001, 2005). During normal cortical development, immature cortical neurons and glia are primarily generated in the germinal ventricular zone (proliferative stage), although some GABAergic cortical interneurons have also recently been shown to originate from the subcortical ganglionic eminences (Parnavelas, 2000). While the different stages of cortical development overlap temporally, neurons generated during the proliferative stage proceed to migrate either radially or tangentially to their final location in the cortex (neuronal migration

stage) and then develop mature dendrites and axons and form synaptic connections (cortical organization stage).

Although many MCDs may involve abnormalities in more than one of these developmental stages, a subgroup of MCDs have been identified with cellular, molecular, and histopathological features indicating a primary genetic defect or environmental insult in the early proliferative stage of cortical development (Barkovich et al., 2005). These disorders involving abnormal glioneuronal proliferation include both non-neoplastic and low-grade neoplastic processes and have an especially high association with epilepsy, such as TSC, FCD with balloon cells, hemimegalencephaly, and GG. While this group of disorders may have a variety of properties, they do share a number of basic abnormalities of both neurons and glia on the histopathological, cellular and molecular level, as well as common clinical features, suggesting that they could also have common mechanisms of epileptogenesis (Table 1).

MCDs related to abnormal glioneuronal proliferation often feature a number of characteristic cellular and histological abnormalities (Becker et al., 2006). First, dysmorphic or cytomegalic neurons with aberrant and disoriented processes associated with a loss of normal cortical lamination are frequently seen in these MCDs. Proliferation of normal-appearing glia or abnormal reactive astrocytes, which overexpress specific intermediate filaments and other immature molecular markers, usually accompany the neuronal abnormalities. Furthermore, large, poorly-differentiated cells that have features of both neurons and glia, such as the balloon cells of FCD and giant cells of TSC, are often the distinctive hallmarks of these types of MCDs and strongly implicate primary defects in cellular proliferation and differentiation. Overall, cortical tubers of TSC and FCD with balloon cells can be virtually indistinguishable in their histological appearance, involving loss of cortical lamination and characteristic dysmorphic or cytomegalic neurons, glial proliferation, and large, undifferentiated (balloon or giant) cells described above, suggesting overlapping pathophysiological mechanisms common to TSC and FCD. Parenthetically, it should be noted that different histological subtypes of FCD have been identified. Per a recent pathological classification of FCD (Palmini et al., 2004), FCD Type IIB contains balloon cells and is most directly related to TSC and other MCDs with abnormal glioneuronal proliferation (thus, the analysis of FCD in the remainder of this review refers specifically to FCD with balloon cells or FCD Type IIB). In addition to TSC and FCD, other MCDs have their own distinct pathological properties, but also share some histological features with TSC and FCD with balloon cells. For example, while GGs usually lack cytomegalic neurons and balloon/giant cells, GGs typically contain poorly-differentiated cells, dysmorphic neurons, and glial proliferation, similar to TSC and FCD. Although hemimegalencephaly has the most histologically-disparate properties of this group and may involve unique pathophysiologically mechanisms (Salamon et al., 2006), the prototypical balloon cells are often found in hemimegalencephaly, suggesting a similarity with TSC and FCD.

In addition to shared histopathological properties, many of the aforementioned MCDs also display significant overlap in molecular characteristics. TSC is very well-characterized on a molecular genetic level, being caused by single-gene mutations in either the *TSC1* or *TSC2* genes on chromosomes 9 and 16, respectively (European Chromosome 16 Tuberous Sclerosis Consortium, 1993; van Slegtenhorst et al, 1997). The *TSC* genes, and their protein products, hamartin and tuberin, regulate cell growth and proliferation and serve as “tumor-suppressors”. Thus, mutation of these genes in TSC may lead to histopathological features of increased cell size, immature or poorly-differentiated cell types, cellular proliferation, and tumor formation in various organ systems. In the brain, loss of hamartin or tuberin function in glioneuronal progenitors may result in formation of cortical hamartomas (“tubers”), with

characteristic giant cells and glial proliferation, as well as subependymal giant cell astrocytomas (SEGAs). Recent work has revealed exciting insights into the molecular mechanisms mediating the effects of the *TSC* genes on cell growth and proliferation, as described in more detail in other reviews (Kwiatkowski, 2003; Holmes et al., 2007). Briefly, hamartin and tuberin normally work together to inhibit the phosphatidylinositol 3-kinase (PI3)/insulin-activated signaling pathway, involving the mammalian target of rapamycin (mTOR) and a cascade of other downstream kinases and translational factors that stimulate protein translation, cell growth and proliferation, such as ribosomal protein S6 (S6), S6 kinase (S6K), eukaryotic translation initiation factor 4 (eIF4), and eukaryotic initation factor 4E binding protein 1 (4EBP1). Thus, mutation of hamartin or tuberin in TSC leads to hyperactivation of the downstream mTOR pathway and the associated kinase signaling cascades and translational factors, resulting in increased cell growth and proliferation.

To test for similarities on the molecular level among different MCDs with abnormal glioneuronal proliferation, a number of studies have looked for evidence of TSC-related signaling abnormalities in other, histopathologically-related MCDs. Interestingly, mutational analysis detected significant polymorphisms in the *TSC1* gene, but not the *TSC2* gene, in balloon cells in almost 50% (11 of 24 cases) of FCDs resected from patients with intractable epilepsy, compared to 200 control cases (Becker et al., 2002). Similarly, significant polymorphisms in the *TSC1* or *TSC2* genes were found in 75% of 20 cases of GGs resected due to intractable epilepsy (Becker et al., 2001). The pathogenic relevance of the *TSC* gene polymorphisms in these cases of non-TSC-related MCDs is not clearly established, as definitive “loss of function” mutations have not been demonstrated. However, abnormalities in components of *TSC*-regulated signaling pathways have also been found in these other MCDs. Similar to giant cells in TSC, balloon cells and dysmorphic neurons in FCD exhibit hyperactivation of the signaling molecules, p-S6 and p-eIF4, which can regulate cell growth and proliferation (Baybis et al., 2004; Miyata et al., 2004; Ljungberg et al., 2006). In contrast, other upstream and downstream components involved in the *TS*C-mTOR pathways, such as p-Akt and p-S6K, are differentially activated in balloon cells from FCD compared to TSC samples, suggesting some differences in regulation of these signaling mechanisms between TSC and FCD (Baybis et al., 2004; Schick et al., 2007). By comparison, similar molecular analyses of epileptogenic GGs demonstrated increased activation of both p-S6 and p-S6K, further demonstrating the remarkable overlap in signaling pathways between GG, FCD, and TSC (Samadani et al., 2007). Furthermore, other molecular markers, such as the stem cell epitope CD34, are present in GGs, FCDs, and TSC specimens from epilepsy patients, indicating a potential common cellular origin in these disorders early in glioneuronal differentiation (Fauser et al., 2004; Deb et al., 2006).

Thus, a multitude of studies point to numerous similarities on the histopathological and molecular levels between different MCDs involving abnormal glioneuronal proliferation and differentiation. Although the specific relevance of these shared cellular and molecular properties to epilepsy remains less clear, the clinical and pathophysiological features of epilepsy in these disorders do also exhibit some important parallels. The incidence of epilepsy is especially high in these MCDs with abnormal glioneuronal proliferation. In some series from specialized centers, epilepsy is estimated to occur in about 90% of TSC patients (Sparagana et al., 2003), although other studies with less referral bias report numbers closer to 60% (Webb et al., 1991). As minimally symptomatic or asymptomatic patients with mild MCDs may go undiagnosed, it is difficult to estimate an accurate incidence of epilepsy in some of these disorders, but FCD and GG have also been noted to have an especially high association with epilepsy, especially medically-refractory epilepsy (Aronica et al., 2001a; Tassi et al., 2002). In addition, epilepsy in this group of disorders is of particular clinical relevance, because they exhibit such a high rate of medical intractability and low rate of spontaneous remission. For instance, in one study, seizure remission was achieved in only

14% of TSC patients (Sparagana et al., 2003). Furthermore, FCD and GG were among the most common causes of intractable epilepsy identified in pediatric patients undergoing epilepsy surgery (Wyllie et al., 1998). Pathophysiologically, patients with these types of MCDs usually all have partial seizures that are believed to originate from within or close to the focal region of cortical abnormality (or in some cases of TSC, multifocal seizures coming from multiple tubers). The clinical relevance of the lesional versus peri-lesional origin of seizures in these disorders (see below) is especially applicable to surgical treatment of intractable epilepsy, as removal of the lesion is often a very effective therapy for these patients. Although the degree of surgical success varies depending on the study and type of MCD, most series report a comparable seizure-free rate of ~50–75% following lesionectomy in TSC, FCD, and GG (Aronica et al., 2001a; Tassi et al., 2002; Weiner et al., 2006).

While a number of pathological, cellular, and molecular similarities have been observed among different MCDs with abnormal glioneuronal proliferation, it should be emphasized that significant differences still exist between TSC, FCD, and GG. Thus, one must consider the alternative hypothesis that these different disorders only overlap or converge superficially in some phenotypic features, but ultimately have completely distinct pathophysiological and etiologic origins. For example, as mentioned above, the pathogenic significance of *TSC* gene polymorphisms documented in FCD and GG is currently unknown, give that true “loss of function” mutations have not been definitely demonstrated. Furthermore, only some, but not other, components of the mTOR pathway that are abnormally activated in TSC have been shown to be dysregulated in FCD and GG. Thus, it remains to be determined whether the similarities observed between these different MCDs have meaningful pathophysiological relevance and clinical applications.

Despite these caveats, overall a variety of data suggest that a subgroup of MCDs shares a remarkable number of clinical, histological, cellular, and molecular features that may ultimately relate to abnormal glial and neuronal proliferation (Table 1) and thus probably do have significant pathophysiological relevance. As these lesions are all highly associated with epilepsy, it is likely that this group of MCDs may also share common mechanisms of epileptogenesis. In the remainder of this review, I will critically analyze the evidence related to several key questions about epileptogenesis in these disorders.

Do seizures start within the lesion or the perilesional region?

A frequently discussed, but incompletely resolved, question is whether seizures in MCDs, such as TSC or FCD, start within the lesions themselves, or in adjacent “perilesional” regions. This question has direct pathophysiological relevance for epileptogenesis and important clinical implications for surgical approaches to epilepsy in these disorders. Based on the focal, circumscribed nature of the lesions in most of these MCDs, it would seem to make intrinsic sense that seizures originate within the lesions. The high success rate of “lesionectomy” during epilepsy surgery, with many studies reporting over a 60–75% seizure-free rate, also supports the idea that the lesions directly produce seizures (Aronica et al., 2001a; Tassi et al., 2002; Weiner et al., 2006). However, this still leaves a substantial minority of patients that continue to have seizures following lesionectomy, suggesting that the epileptogenic zone was not contained within the lesion in those cases. Furthermore, the success of lesionectomy in eliminating seizures may have other interpretations: The margins of resection typically contain some “normal” perilesional tissue, which may actually be the primary source of the seizures. Alternatively, perilesional cortex, immediately adjacent to or even distant from the lesion, may generate the seizures, but may be somehow dependent on the lesion for epileptogenesis; removal of the lesion eliminates this driving force for seizure-generation within the remaining cortex. Finally, studies correlating radiographic and pathological data increasingly indicate that areas of “normal”-appearing cortex on magnetic

resonance imaging (MRI) in patients with other discrete regions of MCD often contain subtle histopathological abnormalities (Porter et al., 2003). Thus, the debate about the actual source of seizures in the group of MCDs with abnormal glioneuronal proliferation is of high clinical and pathophysiological relevance and warrants more detailed examination.

In TSC, a variety of clinical studies, including electrophysiological and radiographic investigations, suggest that cortical tubers are the primary site of epileptogenesis. Electroencephalography (EEG) often identifies both interictal epileptiform abnormalities and seizures originating from the immediate region of a putative epileptogenic tuber on MRI (Cusmai et al., 1990; Koh et al., 2000). Several recent studies suggest that magnetoencephalography (MEG) may be even more accurate than traditional EEG in identifying epileptogenic tubers (Iida et al., 2005; Jansen et al., 2006; Wu et al., 2006). Furthermore, nuclear medicine radiographic studies, such as positron emission tomography (PET) and single photon emission-computed tomography (SPECT), often point to specific tubers as being the source of seizures (Chugani et al., 1998; Koh et al., 2000). Finally, surgical approaches for epilepsy specifically targeting tubers often result in seizure-freedom in at least 75% of patients (Koh et al., 2000; Weiner et al., 2006), strongly supporting the idea that tubers are the source of the seizures in these cases.

Despite the abundant clinical evidence implicating tubers as the epileptogenic foci in TSC, a number of limitations reduce the certainty of this conclusion. In many cases, radiographic and electroencephalographic data do not have a high enough spatial resolution to clearly distinguish an epileptogenic source from within a tuber itself versus the adjacent perituberal region. As mentioned earlier, most surgical resections also include at least some margin of normal-appearing cortex surrounding the tuber, making it difficult to rule out the perituberal region as the source of seizures and the reason for success with surgery. Furthermore, some patients continue to have seizures despite an appropriately targeted tuberectomy. Finally, much of the clinical data supporting the importance of seizures derives from series of TSC patients that underwent epilepsy surgery, which likely represents a biased, pre-selected group. Other TSC patients, who were not deemed to be good surgical candidates, as well as patients who failed epilepsy surgery, may have other mechanisms of epileptogenesis that are not as tightly linked to tubers.

Although these limitations are difficult to fully resolve, especially in human studies, some clinical evidence, as well as data from animal models of TSC, suggest that the non-tuber regions of cortex could also be a source of epileptogenesis. First, quantitative MRI studies indicate that TSC patients have diffusely decreased cortical grey matter volume, not specifically related to cortical tubers (Ridler et al., 2001; Chandra et al., 2007). Second, although pathological studies of non-tuber cortex from TSC patients are rare, diffuse cellular abnormalities have been reported in some cases of TSC brains independent of tubers, such as atypical poorly-differentiated cells (Roske et al., 2003) and decreased neuronal counts (Chandra et al., 2007). Although the relevance of these more diffuse radiographic and histological abnormalities to epilepsy is not established, they at least raise the possibility that non-tuber cortex is abnormal and may be capable of generating seizures. In addition, more direct clinical evidence for the role of non-tuber cortex in causing seizures is seen in rare reports of TSC patients with intractable epilepsy who become seizure-free following surgical resection of normal-appearing, tuber-free brain tissue (Wang et al., 2007).

While clinical data may sometimes be ambiguous or difficult to interpret with respect to the source of seizures in TSC, animal models provide additional evidence that tubers may not be necessary for seizure generation. Mice involving conditional inactivation of the *Tsc1* gene primarily in glial cells (*Tsc1*^{GFAP}CKO mice) develop severe, progressive seizures (Uhlmann et al., 2002; Erbayat-Altay et al., 2007). While the brains of *Tsc1*^{GFAP}CKO mice do exhibit

diffuse histological abnormalities, including glial proliferation and neuronal disorganization, there is no evidence of focal abnormalities resembling tubers, indicating that *Tsc1* deletion-induced cellular and molecular abnormalities are sufficient to cause seizures independent of tubers. Similarly, knock-out mice involving inactivation of *Tsc1* in neurons (*Tsc1*^{synapsin}CKO mice) exhibit neuronal hyperexcitability and seizure activity, despite having no evidence of focal histological abnormalities or tubers (Meikle et al., 2007; Wang et al., 2007). More limited information relevant to seizures can also be derived from the Eker rat, which has a spontaneous mutation in the *Tsc2* gene and, with very rare exceptions (Mizuguchi et al., 2000), also displays minimal evidence of cortical abnormalities or tubers. Although Eker rats do not appear to have spontaneous epilepsy or altered seizure susceptibility (Waltereit et al., 2006; Tschuulun et al., 2007), these rats do have a moderately enhanced seizure response to chemical kindling (Waltereit et al., 2006). Overall, while clinical studies strongly implicate tubers as being epileptogenic, evidence from multiple animal models of TSC, in addition to limited clinical data, also suggests that seizures can potentially arise from non-tuber cortex as well.

Similar conclusions can be made regarding the source of seizures in FCD with balloon cells, which again often has a histopathological appearance that is virtually indistinguishable from tubers in TSC. Clinical evaluations, including EEG, MEG, and various imaging methods, frequently provide evidence that seizures originate intrinsically from within the FCD evident on MRI (Palmini et al., 1995; Bast et al., 2004). Surgical resection of FCD results in seizure freedom in at least 50% of patients (Wyllie et al., 1998; Tassi et al., 2002), again supporting the concept of the epileptogenic foci being contained within the FCD. However, this still leaves a significant proportion of patients with persistent seizures, despite apparent resection of the FCD. Even more than in TSC, there is substantial clinicopathological evidence that seizures can also arise from regions beyond the FCD, or at least outside the area of the FCD that is grossly evident on MRI. A likely explanation for surgical failures in patients with FCD is that the true area of FCD may extend on the microscopic level beyond the region of obvious abnormality apparent on MRI (Gomez-Anson et al., 2000; Tassi et al., 2002). In fact, a retrospective diagnosis of FCD is often made by pathological analysis of brain tissue resected from patients with intractable epilepsy that had no evidence of FCD on preoperative MRI (Bautista et al., 2003; Porter et al., 2003). In this respect, the question of whether seizures originate in the “lesion” or perilesional regions in FCD may be more of a semantic issue, limited by the resolution of imaging technology – as the resolution of imaging methods continues to improve, brain regions that appear “normal” by current techniques may eventually be identified as “lesional”. Consistent with this idea, advanced quantitative MRI techniques have found abnormalities in gray matter volume beyond the regions of FCD identified by conventional visual inspection (Bonilha et al., 2006). Finally, while there are few relevant animal models of FCD per se, animal models of other types of MCD, such as polymicrogyria and heterotopia, suggest that epileptogenesis may occur in normal-appearing cortex adjacent to the obvious lesions (Jacobs et al., 1999; Chen et al., 2000). Thus, there is abundant evidence that the perilesional regions may be just as important as the lesion itself in causing seizures in FCD.

Finally, perilesional mechanisms of epileptogenesis are also strongly implicated in cases of GG. Clinical data, including epileptiform discharge patterns emanating from GG on electrocorticography (Ferrier et al., 2006) and the success of lesionectomy in eliminating seizures (Aronica et al., 2001a), suggest the possibility that seizures could directly start within the GG itself. However, the limited spatial resolution of this type of clinical data, as similarly discussed above for TSC, makes it difficult to rule out that peritumoral mechanisms actually account for these clinical observations. Furthermore, although more differentiated cells in other types of gliomas have more of a neuronal phenotype and may be capable of generating action potentials (Patt et al., 1996; Bordey and Sontheimer, 1998),

there are limited electrophysiological data documenting whether cells in GG are electrically-excitable. Thus, many studies have focused on secondary effects of the tumor on peritumoral regions as the basis for epileptogenesis in GG. In fact, electrophysiological data from both human tissue and animal models indicate that the regions adjacent to or at the border of gliomas have the highest potential to generate epileptiform activity (Patt et al., 2000; Kohling et al., 2006).

In summary, studies of different MCDs with abnormal glioneuronal proliferation, such as TSC, FCD, and GG, share some interesting trends regarding the site of origin for seizures. Both the lesion and the perilesional region have been implicated in causing epileptogenesis in these disorders, but, somewhat paradoxically, an accumulating amount of evidence demonstrates the importance of perilesional cortex in producing seizures. It is likely that the relative contribution of perilesional versus lesional mechanisms varies between different types of MCDs and different patients with the same type of MCD. Recent studies suggest that the "perilesional" region may have subtle structural and cellular abnormalities. With further advances in mechanistic studies and improved resolution of imaging techniques, future research should reveal more detailed information about the perilesional region and its relationship to the lesion. As the clinically-practical definition of "lesion" is currently limited to the anatomical resolution of imaging methods, future advances will likely result in continual expansion of the definition and extent of the epileptogenic "lesion" from the anatomical to the cellular and molecular levels, so that the present distinction between "lesion" and "perilesional" regions may become obsolete.

Is epileptogenesis primarily a result of circuit abnormalities or cellular/molecular defects?

Independent of the question of the lesional versus perilesional origin of seizures, another controversial issue is whether epileptogenesis in MCDs is primarily due to circuit abnormalities or cellular and molecular defects. Seizures clearly consist of synchronous electrical activity reverberating through complex neuronal networks and thus ultimately must always include abnormalities on the circuit level. However, from a pathophysiological standpoint, mechanisms of epileptogenesis could involve either primary changes in circuit organization or initial cellular and molecular defects that secondarily translate to the network level. On the extremes, circuit abnormalities ("the epileptic circuit") might consist of aberrant connectivity of neurons that are otherwise completely normal in function, whereas cellular/molecular mechanisms ("the epileptic neuron") would involve a defect involving intrinsic neuronal function in the context of normally-wired and fully-operational circuits. Ultimately, both network and cellular/molecular abnormalities will stimulate epileptogenesis by upsetting the normal physiological balance between excitation and inhibition in the brain. Based on mechanistic studies of other epilepsy models, examples of circuit abnormalities might include selective loss of inhibitory neurons within a network ("disinhibition"), development of excessive or aberrant excitatory connections within a network, or non-specific "mass effect" of a lesion causing destruction or disruption of all connections in a network, but ultimately resulting in excitatory effects on adjacent intact networks. Classic examples of cellular/molecular defects promoting epileptogenesis would include increased expression and function of excitatory neurotransmitter receptors or channels (e.g. glutamate receptors, calcium channels) or decreased expression and function of inhibitory molecules (e.g. GABA receptors, potassium channels).

In most MCDs, the presence of focal, structural lesions automatically indicates disruptions in normal cortical organization and circuitry. However, this does not necessarily prove that these network abnormalities are the primary cause of epileptogenesis. It is also possible, especially in perilesional regions, that neuronal circuits are intact, but instead a cellular or

molecular defect is primarily responsible for promoting neuronal hyperexcitability and seizures. Thus, distinguishing between network and cellular/molecular mechanisms is important, not only for understanding basic concepts governing neuronal excitability in the brain, but also for designing therapies for epilepsy with rational mechanistic targets.

In TSC, surprisingly little is known about neuronal circuit organization and function within and between tubers and perituberal cortex. Few data have been published about physiological properties of neurons, such as giant cells or dysmorphic neurons, or anatomical connectivity of neuronal elements within tubers or surrounding tubers in human TSC to determine whether primary circuit abnormalities could be the cause of seizures. A recent study reported some limited immunohistochemical evidence for anomalous GABAergic inhibitory circuits within cortical tubers (Valencia et al., 2006), but this study did not address issues of synaptic connectivity or physiological function of such putative networks. In one report involving intracellular recordings of normal-appearing neurons in non-tuber tissue resected from a TSC patient, there was evidence of neuronal hyperexcitibility but no impairment of synaptic inhibition (Wang et al., 2007). Similarly, in the *Tsc1*$^{\text{synapsin}}$CKO mouse model, which lacks tubers, normal-appearing cortical pyramidal neurons exhibited exaggerated excitatory synaptic currents, but normal intrinsic membrane properties, suggesting a potentiation of excitatory synaptic circuitry (Wang et al., 2007). In the Eker rat model of TSC, synaptic plasticity, such as long-term potentiation, is impaired, indicating abnormalities in network function, but the relationship of this defect to epileptogenesis or a primary circuit mechanism is unclear (von der Brelie et al., 2006). Thus, overall, animal models suggest the possibility of primary network abnormalities in TSC, but this conclusion is not definitive due to limitations of the animal models (e.g. absence of tubers) and the relative paucity of data in human TSC.

On the cellular level, a popular hypothesis about seizure-generation in TSC is that an abnormal cell type, in particular the giant cell in tubers, could serve as an intrinsic “pacemaker” that initiates and drives epileptiform activity and seizures. Although the amount of data is limited, intracellular recordings from giant cells in tubers have been reported from a few cases of TSC (Cepeda et al., 2003, 2005b). Contrary to the “pacemaker” hypothesis, giant cells (referred to as “balloon cells” in the study) from TSC patients were actually found to be electrically inexcitable, with no evidence of voltage-activated sodium or calcium currents. Cytomegalic neurons from TSC specimens were capable of generating action potentials, including repetitive calcium spikes in response to stimulation, and thus have potential for contributing to epileptic discharges, but showed no evidence of intrinsic pacemaker properties. Thus, the available physiological data do not support the concept that giant cells or other dysmorphic neurons within tubers are, by themselves, the primary generators of epileptiform activity in TSC.

The most evidence for potential abnormalities promoting epileptogenesis in TSC arguably exists on the molecular level, largely derived from single cell polymerase chain reaction (PCR) and microarray analysis of cells from tubers resected from TSC patients with intractable epilepsy. Molecular characterization revealed increased mRNA expression of specific glutamate NMDA receptor subunits and a decrease in specific $GABA_A$ receptor subunits in giant cells and dysplastic neurons (White et al., 2001). These specific changes in neurotransmitter expression within tubers could have obvious effects in promoting hyperexcitability and seizures, although the physiological significance of these molecular changes is uncertain given the electrophysiological data on these cell types described above. Other molecules related more to inflammatory cytokine signaling pathways have also been found to be activated in tubers, such as cell adhesion molecules, tumor necrosis factor, and mitogen activated protein kinase (Maldonado et al., 2003). Although the possible role of these signaling molecules in generating seizures is less clear and direct, cytokine activation

has been implicated in other models of epileptogenesis (Vezzani et al., 2004). In addition, in the *Tsc1*^{GFAP}CKO mouse model of TSC, abnormalities in molecules functioning primarily in astrocytes, such as glutamate transporters and potassium channels, have been implicated in promoting epileptogenesis in these mice (see below; Wong et al., 2003; Jansen et al., 2005). Overall, there is some evidence supporting both network and molecular mechanisms of epileptogenesis in TSC, but more data are needed to determine which mechanisms are the most critical.

Compared to TSC, significantly more data exists in FCD examining potential mechanisms of epileptogenesis on the circuit, cellular, and molecular levels, focusing primarily on the interplay between glutamatergic and GABAergic forces. On the circuit level, both immunohistochemical and electrophysiological studies indicate that abnormal, potentially hyperexcitable networks exist within FCD tissue specimens resected from patients with intractable epilepsy. Several studies demonstrate a decrease or abnormal organization of GABAergic interneurons within FCD, as assayed immunocytochemically by markers, parvalbumin, calbindin, or glutamic acid decarboxylase (Spreafico et al., 1998; Alonso-Nanclares et al., 2005; Calcagnotto et al., 2005). Intracellular recordings from pyramidal neurons in neocortical slices from human dysplastic cortex demonstrated physiological evidence of decreased GABA-mediated synaptic inhibition (Calcagnotto et al., 2005). Although there are essentially no animal models that precisely mimic the histological features of FCD, animal models with more widespread “dysplasia” and heterotopias also exhibit a reduction in GABAergic interneurons (Roper et al., 1999; Zhu and Roper, 2000). While there is strong evidence for impaired GABAergic circuits in FCD, which could clearly promote hyperexcitability and seizures, less is known about the organization of circuits within and around FCD that actually generate the seizures. Balloon cells do not appear to receive synaptic contacts (Alonso-Nanclares et al., 2005). Furthermore, similar to giant cells in TSC, recordings from balloon cells and cytomegalic pyramidal neurons from FCD have revealed minimal signs of hyperexcitability and thus these cells likely do not directly generate seizures (Cepeda et al., 2003, 2005b). However, a recent study has described a small subpopulation of cytomegalic interneurons in FCD that exhibit intrinsic bursting behavior (Andre et al., 2007). Furthermore, basket-like clusters of GABAergic interneurons often surround cytomegalic neurons and could serve to synchronize epileptiform activity (Spreafico et al., 1998; Alonso-Nanclares et al., 2005). Thus, it is conceivable that a primary impairment of GABAergic circuits could lead to disinhibition, synchronization and hyperexcitability of otherwise normal-appearing cortical pyramidal neurons within and surrounding the regions of FCD (Calcagnotto et al., 2005).

In addition to higher-level network abnormalities, there is abundant evidence for a number of molecular abnormalities on the single cell level in FCD that could directly influence neuronal excitability and promote epileptogenesis. Dysplastic and heterotopic pyramidal neurons microdissected from human FCD specimens exhibit decreased expression of specific $GABA_A$ receptor subunits (Crino et al., 2001), which, in addition to the loss and disorganization of GABAergic interneurons on the circuit level, might contribute to a further decrease in GABAergic tone on the single cell level. Complementing the changes in GABAergic inhibition, there is also strong evidence that glutamate receptors may be upregulated in FCD. Multiple studies involving immunocytochemical and single-cell mRNA amplification techniques have shown that specific subunits of NMDA, AMPA, and metabotropic glutamate receptors are altered, typically increased, in dysplastic neurons from FCD (Ying et al., 1998; Najm et al., 2000; Crino et al., 2001; Aronica et al., 2003). Physiologically, NMDA receptors of pyramidal neurons from FCD display decreased sensitivity to magnesium inhibition, which could promote increased neuronal excitability (Andre et al., 2004) Thus, a number of molecular changes in neurotransmitter receptors

seem to shift the balance of cellular excitability and favor glutamatergic excitation over GABAergic inhibition.

While glutamate and GABA receptors tend to receive the most attention in mechanisms of epileptogenesis, a number of other molecular players can also influence excitability in FCD. Directly related to the underlying concept that FCD and similar MCDs result from abnormal proliferation and differentiation of glioneuronal progenitor cells, some evidence indicates that epileptic tissue from FCD may recapitulate or maintain immature properties. The immature brain tends to have a decreased seizure threshold, which may, in part, be due to a paradoxical excitation due to GABA during early brain development. While GABA causes hyperpolarization and inhibition of neurons in adulthood, a relatively elevated intracellular chloride concentration in immature neurons leads to depolarization and excitation by GABA in neonatal rodent and human cortex (Dzhala et al., 2005). Developmental regulation of specific ion transporters dictates changes in the chloride gradient, such that strong expression of the sodium-potassium-chloride transporter, NKCC1, accounts for the higher intracellular chloride concentrations and depolarizing action of GABA in early development. By analogy, depolarizing epileptiform potentials paradoxically mediated by GABA have been documented in human slices of FCD exposed to convulsant drugs (Avoli et al., 1999; D'Antuono et al., 2004). Furthermore, recent studies have found increased expression of NKCC1 in cortical neurons and glia from FCD, again suggesting that FCD mimics properties of an immature developmental state (Aronica et al., 2007a; Sen et al., 2007). This upregulation of NKCC1 may contribute to hyperexcitability and seizure development in FCD. Finally, other molecular and histological features also support the general concept that FCD may maintain an immature, hyperexcitable developmental state (Cepeda et al., 2006). Furthermore, developmental differences may arise with age between pediatric and adult cases of FCD in mechanisms of epileptogenesis (Cepeda et al., 2005a).

Less is known about the effects of GG on circuit properties and network excitability, although, as discussed earlier, it may be neuronal networks at the border or perilesional regions of GG that are the most epileptogenic (Patt et al., 2000; Kohling et al., 2006). One recent study has found evidence for decreased GABAergic interneurons in perilesional epileptic networks adjacent to GGs (Aronica et al., 2007b). On the cellular and molecular level, a number of abnormalities in glutamate and GABA receptors have been documented in GG that are analogous to TSC or FCD, again suggesting that these MCDs share common pathophysiological origins and mechanisms of epileptogenesis. Immunocytochemical studies indicate that neuronal components of GGs highly express NMDA and AMPA receptors (Aronica et al., 2001b). Single cell mRNA analysis detected changes primarily in metabotropic glutamate receptors in GGs, but also demonstrated reduced expression in $GABA_A$ receptor subunits (Samadani et al., 2007). In addition, similar to FCD, upregulation of the transporter NKCC1 also occurs in GG (Aronica et al., 2007a).

Thus, overall there is some evidence for both network and cellular/molecular defects that may contribute to epileptogenesis in different MCDs with abnormal glioneuronal proliferation. There is especially strong data on the cellular and molecular level, demonstrating abnormalities in glutamate and GABA receptors, as well as other molecules directly affecting neuronal excitability, that are remarkably similar among TSC, FD, and GG. These molecular similarities support the concept of a common pathophysiological origin and indicate common mechanisms of epileptogenesis among these MCDs. Although the molecular data are very consistent and convincing, the search for a "pace-maker" cell that might directly translate such molecular defects into cellular and ultimately network hyperexcitability has thus far been unsuccessful. At the same time, while a decrease in GABAergic networks is consistently reported in different MCDs, more data are needed, especially in TSC and GG, to better define primary abnormalities in circuit organization

within and surrounding lesions in these disorders. Thus, presently it is difficult to determine whether primary circuit or cellular/molecular defects are more important in driving epileptogenesis. It is likely that both network and cellular/molecular mechanisms are involved to some, varying degree in different cases. Future research will hopefully better define the relationship and interaction between different mechanistic levels of epileptogenesis in these MCDs.

Do non-neuronal factors, such as glial cells, contribute to epileptogenesis?

Most mechanisms of epileptogenesis, such as those discussed above related to MCDs, as well as for other models of epilepsy, have predictably focused on the primary role of neurons in causing seizures. However, accumulating evidence over the past decade indicates that non-neuronal factors could be equally important in promoting epileptogenesis. In particular, glial cells, which traditionally have been viewed as simply passive, supporting cells of the brain, have been increasingly recognized as more active in regulating neuronal excitability. Astrocytes control the extracellular levels of excitatory ions and neurotransmitters, such as potassium and glutamate, which may directly affect neuronal excitability. In addition, astrocytes recently have been shown to release glutamate and other substances as intrinsic “gliotransmitters”, which can directly stimulate neurons and participate in synaptic signaling in the so-called tripartite synapse (Haydon, 2003). In addition to regulating normal neuronal excitability, glia have been implicated in causing or contributing to epileptogenesis in a number of epilepsy models (Binder and Steinhauser, 2006). Given the prominent role of mature glial cells and glioneuronal progenitors cells in the MCDs with abnormal glioneuronal proliferation, it would be logical to hypothesize that glial abnormalities might also contribute to epileptogenesis in these MCDs, such as TSC and FCD.

Histological abnormalities in glia in tubers, such as the presence of poorly-differentiated giant cells with mixed glial-neuronal properties and astrocyte proliferation, suggest a possible role of glia in epileptogenesis in TSC. More direct evidence linking glial abnormalities with seizures in TSC comes from the *Tsc1*^{GFAP}CKO mouse model, which involves Cre-mediated inactivation of the *Tsc1* gene in GFAP-expressing cells during embryonic brain development. Although there is some neuronal disorganization in these mice, especially in hippocampus, which could be related to direct neuronal involvement of the *Tsc1* gene in early GFAP-positive neuronal progenitor cells, by far the most obvious histological abnormality involves massive glial proliferation leading to increased brain size (Uhlmann et al., 2002). Furthermore, a number of cellular molecular abnormalities related to cell signaling, growth, and proliferation have been observed in cultured astrocytes from these mice, as well as in vivo (Ess et al., 2004; Uhlmann et al., 2004). Of more direct relevance to epilepsy, specific astrocyte neurotransmitter transporters and ion channels are abnormal in *Tsc1*^{GFAP}CKO mice, identifying mechanisms by which astrocyte dysfunction might promote the development of seizures that are observed in these mice. For example, astrocytes from *Tsc1*^{GFAP}CKO exhibit decreased glutamate transporter expression and function, which may cause elevated extracellular glutamate levels, excessive synaptic activation, and neuronal death, all of which might promote epileptogenesis (Wong et al., 2003). In parallel, *Tsc1*^{GFAP}CKO mice have reduced expression of inwardly rectifying potassium channels of astrocytes, which could lead to impaired potassium buffering and neuronal excitability, as demonstrated by an increased propensity of slices from the KO mice to generate epileptiform activity in response to elevated potassium (Jansen et al., 2005). Analogous to this work in animal models, preliminary studies involving recordings from tubers resected from TSC patients suggest that similar astrocyte dysfunction, such as in glutamate transport, occurs in human TSC (Wu et al., 2005). Similar defects in glutamate and potassium transport in reactive astrocytes have also been reported in other animal

models and human epilepsy, suggesting that this represents a common response in abnormally differentiated or reactive astrocytes that could generally promote epileptogenesis (Binder and Steinhauser, 2006).

There are fewer data related to astrocytic regulation of glutamate and potassium in FCD and GG, although the similarities in histological features of glia in these MCDs compared with TSC makes it likely that analogous astrocytic abnormalities occur in all these disorders. In addition, activated microglia have been demonstrated in FCD, GG and TSC specimens from patients with epilepsy (Maldonado et al., 2003; Aronica et al., 2005; Boer et al., 2006). Although it is possible that microglia activation could occur as a secondary response to seizure activity, accumulating evidence supports the possibility that inflammatory signaling pathways, such as the cytokines, triggered by microglia can promote epileptogenesis (Vezzani et al., 2004; Ravizza et al., 2006).

Thus, overall there is substantial evidence that non-neuronal factors could be involved in epileptogenesis in MCDs with abnormal glioneuronal proliferation. The importance of astrocytes and other glial cells in directly controlling neuronal excitability has received increasing attention in neuroscience in general. In addition, there are accumulating data identifying relevant abnormalities in non-neuronal cells in MCDs and other epilepsy models. While many of the glial defects found in these disorders should intuitively increase neuronal excitability and promote epileptogenesis, at this point many of the data are circumstantial and correlative. Additional mechanistic studies, ideally involving selective modulators or antagonists of glial versus neuronal function, are required to prove a causal role for these non-neuronal mechanisms in producing seizures.

Conclusions and Clinical Implications

Although clearly more data are needed to better define mechanisms of epileptogenesis in MCDs, a number of conclusions can be drawn from available studies to date. As an overall theme, there does appear to be a subset of MCDs, including TSC, FCD, GG, and other associated disorders, that share numerous histological, cellular, and molecular features and that likely all originate from related defects in proliferation and differentiation of glioneuronal progenitors during early stages of cortical development. Accordingly, these MCDs with abnormal glioneuronal proliferation also exhibit a number of similar, overlapping abnormalities in circuit, cellular, and molecular entities that can directly affect neuronal excitability and thus may represent shared mechanisms of epileptogenesis (summarized in Fig. 1). In terms of specific properties of epileptogenesis in these disorders, the existing data allow some, tentative conclusions to be made and identify areas where additional studies should lead to further refinement of these conclusions. First, while clinical studies and the results of surgical resection suggest that seizures originate from within radiographically-visible lesions, especially in TSC and FCD, accumulating evidence from a variety of human and animal models also increasingly implicate the perilesional region as a source for seizures. Perilesional regions that appear normal by current radiographic techniques may contain cellular and molecular abnormalities that can promote epileptogenesis and, as the resolution of imaging and other diagnostic methods continue to improve, the extent of the identifiable lesion will expand. Second, mechanisms of epileptogenesis may occur both on the primary circuit level and the cellular/molecular level, but future research is needed to determine the relative importance of each in individual disorders. In particular, while single-cell molecular analysis has revealed a number of molecular defects that could promote hyperexcitability on the cellular level, physiological studies have yet to find definitive evidence of a “pace-maker” cell driving epileptiform activity. More information about neuronal circuit organization and function within and between the lesion and perilesional regions are definitely needed to integrate the cellular and

molecular findings back to the network level. Finally, a number of abnormalities in glial cells have been documented in these MCDs that would intuitively be predicted to increase neuronal excitability and risk for seizures, but more definitive mechanistic studies are required to prove a causal role of glia in epileptogenesis in these disorders.

Despite the limitations, these conclusions have some definite clinical implications and again point out future areas of research that are needed to address the most therapeutically-relevant issues. First, the potential importance of the perilesional regions in generating seizures has the strongest implications for surgical approaches to intractable epilepsy patients with MCDs. Although many patients become seizure free with a standard “lesionectomy”, a significant proportion of patients continue to have seizures despite lesionectomy and might benefit from a more extensive surgical resection involving broader perilesional regions. Future advances in imaging techniques and molecular markers might be able to more accurately identify the true extent of the epileptogenic zone that needs to be resected to achieve seizure freedom. Second, identifying specific mechanisms of epileptogenesis, whether localized to the circuit, cellular, or molecular level or involving neuronal or glial elements, will obviously help identify rational targets for novel drug development. At this stage, current antiepileptic drugs are only moderately specific, usually having multiple mechanisms of action. While having multiple mechanisms may improve efficacy, this also increases the risk of developing side effects. Identifying the most critical participants in epileptogenesis at the circuit, cellular, and molecular levels may allow tailoring drugs to more specific targets that could minimize side effects and correspondingly increase efficacy by allowing the use of higher drug doses. By a similar logic, establishment of critical glial mechanisms of epileptogenesis should trigger search for drugs that can selectively modulate glia. For example, a large-scale screening of currently available drugs have identified a number of drugs that can upregulate astrocyte glutamate transporters, which may counteract the detrimental effects of impaired astrocyte glutamate transport seen in epilepsy and other neurological disorders (Rothstein et al., 2005). Glial-selective drugs may also have the benefit of fewer neurological and cognitive side effects than one would expect with a comparable drug affecting neurons. Finally, returning to the probable developmental defects in glioneuronal proliferation in the relevant MCDs, classes of drugs are already available that can inhibit cell proliferation. In particular, the recent discovery of mTOR pathway dysregulation in TSC, as well as FCD and GG, offers a remarkably direct “bench-to-bedside” strategy for treating these MCDs. Rapamycin will specifically inhibit the mTOR pathway, leading to decreased cellular proliferation (Kwiatkowski, 2003), and has already been tested in clinical trials for tumor growth in TSC (Franz et al., 2006). Since it is likely that these same upstream signaling mechanisms that control proliferation may also directly or indirectly affect other downstream mechanisms involved in epileptogenesis, investigation of the novel use of these drugs for epilepsy seems warranted, at least in TSC and possibly the other related MCDs. In addition to pharmacological interventions, future therapeutic gene replacement or gene manipulation approaches may eventually allow a more definitive correction of the molecular genetic defects in these diseases. Thus, as more is discovered about the specific mechanisms of epileptogenesis in these disorders, the variety of different upstream and downstream targets for antiepileptic drug development will continue to grow.

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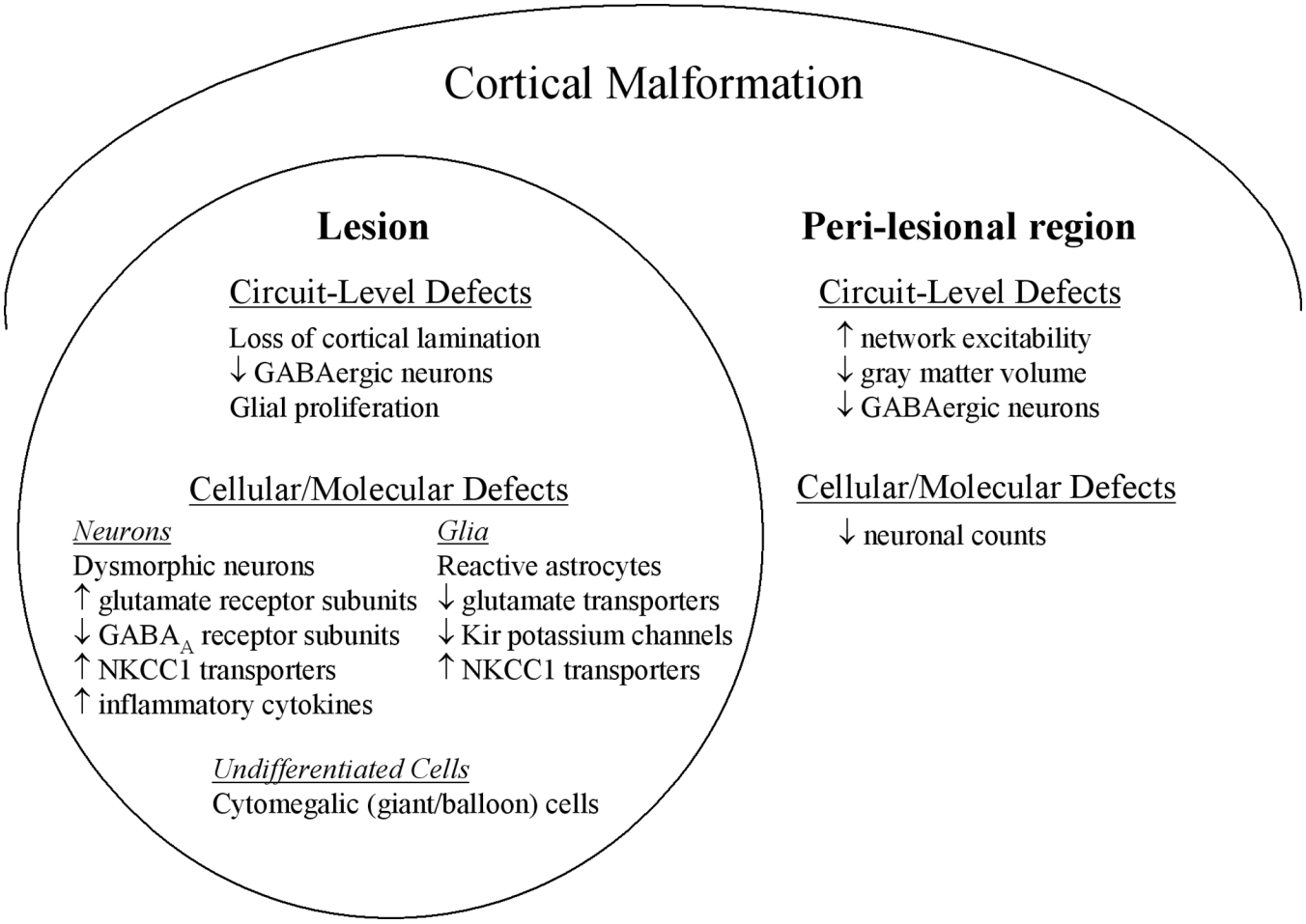

Figure 1.

Circuit, cellular, and molecular abnormalities potentially contributing to epileptogenesis in MCDs with abnormal glioneuronal proliferation and differentiation. On the circuit level, both the lesion and the peri-lesional regions have been implicated in promoting epileptogenesis. Although cellular and molecular data on peri-lesional regions have been limited by availability of clinical tissue, accumulating evidence implicates the importance of the peri-lesional networks on the circuit level. A loss of GABAergic interneurons has been consistently identified as a potential network mechanism of epileptogenesis in both the lesion and peri-lesional regions. On the cellular level, abnormal cell types, such as dysmorphic neurons, reactive astrocytes, and undifferentiated cytomegalic cells, are distinctive hallmarks, especially within the lesion of these MCDs. The potential role of glial cells in epileptogenesis is receiving increasing attention. On the molecular level, alterations in specific glutamate and GABA receptor subunits, as well as other transporters and ion channels, may predispose to increased neuronal excitability and seizures.

Table 1

Comparison of Clinical, Histopathological, and Molecular Features in MCDs with Abnormal Glioneuronal Proliferation and Differentiation

	Tuberous Sclerosis Complex	**Focal Cortical Dysplasia With Balloon Cells**	**Ganglioglioma**
Clinical Features			
Intractable Epilepsy	Frequent	Frequent	Frequent
Epilepsy helped by surgical resection	Sometimes	Frequent	Frequent
Cognitive deficits & Systemic manifestations	Frequent	Rare	Rare
Histopathological Features			
Loss of cortical lamination	Yes	Yes	Yes
Dysmorphic neurons	Yes	Yes	Yes
Glial proliferation	Yes	Yes	Yes
Distinctive cytomegalic undifferentiated cells	Giant cells	Balloon cells	No
Molecular Features Related to Glioneuronal Proliferation			
TSC1/TSC2 mutations/polymorphisms	*TSC1/TSC2* mutations	*TSC1* polymorphisms	*TSC1/TSC2* polymorphisms
Dysregulation of mTOR signaling pathways	↑ p-S6, p-S6K ↑ p-eIF4, p-4EBP1	↑ p-S6 ↑ p-eIF4	↑ p-S6, p-S6K
Stem cell epitope expression	CD34+	CD34+	CD34+

Abbreviations: *TSC1/TSC2 – Tuberous Sclerosis Complex 1* and *2* genes; p-S6 – phospho-ribosomal protein S6; p-S6K – phospho-ribosomal protein S6 kinase; p-eIF4 – phospho-eukaryotic translation initiation factor 4; p-4EBP1 – phospho-eukaryotic initation factor 4E binding protein 1; CD34 – CD34 antigen.