BRAIN
REVIEW ARTICLE

Epileptogenesis in tuberous sclerosis complex-related developmental and epileptic encephalopathy

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Epileptogenesis in infants with tuberous sclerosis complex (TSC) is a gradual and dynamic process, leading to early onset and difficult-to-treat seizures. Several cellular, molecular and pathophysiologic mechanisms, including mammalian target of rapamycin (mTOR) dysregulation, GABAergic dysfunction and abnormal connectivity, may play a role in this epileptogenic process and may also contribute to the associated developmental encephalopathy. Disease-specific antiseizure medications or drugs targeting the mTOR pathway have proved to be effective in TSC-associated epilepsy. Pre-symptomatic administration of vigabatrin, a GABAergic drug, delays seizure onset and reduces the risk of a subsequent epileptic encephalopathy, such as infantile spasms syndrome or Lennox–Gastaut syndrome. Everolimus, a rapamycin-derived mTOR inhibitor, reduces seizure frequency, especially in younger patients. This evidence suggests that everolimus should be considered early in the course of epilepsy.

Future trials are needed to optimize the use of everolimus and determine whether earlier correction of mTOR dysregulation can prevent progression to developmental and epileptic encephalopathies or mitigate their severity in infants with TSC. Clinical trials of several other potential antiseizure drugs (cannabidiol and ganaxolone) that target contributing mechanisms are also underway.

This review provides an overview of the different biological mechanisms occurring in parallel and interacting throughout the life course, even beyond the epileptogenic process, in individuals with TSC. These complexities highlight the challenges faced in preventing and treating TSC-related developmental and epileptic encephalopathy.

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Introduction

Developmental and epileptic encephalopathies (DEE) refer to a group of disorders characterized by early onset, difficult-to-treat epileptic seizures with developmental impairment, which is mainly related to the underlying aetiology and possibly worsened by the epileptiform activity.[1–3] Many pathogenic tuberous sclerosis complex (TSC) gene variants are indicated by accumulating evidence to cause complex neurodevelopmental disorders (NDD), with the superimposed epileptic encephalopathy further affecting the developmental outcome.[4]

Tuberous sclerosis complex is a multisystem genetic disorder characterized by age-related formation of benign tumours throughout the body (including brain, kidneys, heart, skin and eyes). TSC is caused by inactivating mutations in either *TSC1* or *TSC2* genes.[5] These genes encode proteins that form an inhibitory complex for RHEB (Ras homolog enriched in brain), thus mTORC1 (mammalian target of rapamycin complex 1) hyperactivation represents a key feature of TSC.[5,6] mTOR-signalling deregulation is also observed in a large spectrum of epileptogenic developmental pathologies (termed 'mTORopathies'; mTOR pathway-related malformations of cortical development, such as hemimegalencephaly and focal cortical dysplasia, FCD type II).[7–9]

More than 2000 pathogenic *TSC1*/*TSC2* variants have been described,[10] which has challenged the correlation of genotypes with the highly variable forms of TSC that exist between patients, even within families with an inherited form of TSC.[5,11–13] Generally, the *TSC2* mutation seems to be associated with a more severe phenotype than the *TSC1* mutation, including earlier seizure onset, a lower cognitive level and a greater tuber load.[14–16] However, patients with a milder phenotype may also have *TSC2* mutations.[17–19]

Multiple genetic, epigenetic, as well as acquired and environmental, factors can influence dynamically the phenotypical outcome throughout the life course. Early seizure onset, the occurrence of epileptic spasms, neurosurgery, anti-seizures treatments and age-dependent somatic mutation rate may affect, and thus complicate, genotype–phenotype associations.[12,20–23]

Several NDD appear with epileptic seizures, or in some patients, even precede clinical seizure onset.[24] Of particular interest is the bridge between these neurological and neuropsychiatric aspects.[25] TSC offers the unique opportunity to probe the age-dependent mechanisms of mTOR pathway-related epileptogenesis, involving dynamic and complex combinations with mTOR-dependent and mTOR-independent processes.[9,26,27]

Diverse *in vitro* and *in vivo* models of TSC, more recently including human stem cell-based models, have improved the understanding of mTOR pathway deregulation on brain development and epileptogenesis. This review will focus on cellular and molecular mechanisms of TSC-associated epileptogenesis and related DEE, discussing the rationale for current therapeutic options for TSC-related epilepsy.

Search strategy

We searched PubMed for peer-reviewed publications published between 1 January 2015 and 31 August 2022, with the term 'tuberous sclerosis'. Searching for the term 'tuberous sclerosis' in PubMed returned 3449 possible articles (accessed 31 August 2022). We then refined our search terms to 'tuberous sclerosis' AND (as individual combinatory terms) 'epilepsy', 'epileptogenesis', 'diagnostic criteria', 'mTOR inhibitors', 'neurobiology', and 'treatment'. Selection criteria from full-text outputs were novelty of study findings and their relevance to neurologists, with inclusion decided collectively by all authors. Relevant historical references outside the search timeframe were also included.[28]

Cellular and molecular mechanisms of TSC associated epileptogenesis

Epileptogenesis is currently viewed as a continuum that underlies the development of spontaneous seizures and continues after epilepsy diagnosis.[29,30] The development of drug resistance as well as neurological and neuropsychiatric comorbidities are all part of epileptogenesis. This broad definition for epileptogenesis extends the therapeutic opportunities for intervention beyond the prevention of epilepsy onset to disease modification, including both antiepileptogenesis and modification of concomitant NDD.[31,32]

Although some commonalties in epileptogenic processes exist,[33] few temporal dynamics of mechanisms in acquired epilepsies apply to genetic epilepsies, in which gene-, pathway- and age-specific mechanisms (as discussed below) are likely to be involved.

Tuberous sclerosis complex affects the immature brain and so is particularly challenging, since we are often dealing with an 'immediate' epileptogenesis. Such early development of the epileptogenic network is supported by imaging, neuropathological and EEG clinical studies.[24,34–37] Foetal brain MRI shows cortical and subcortical lesions in the large majority of TSC patients evaluated, and prenatal detection of such lesions correlate with NDD and autism at 2 years.[34] Moreover, longitudinal observational studies provide evidence of early epileptiform EEG activity in infants with TSC.[37,38]

Understanding the temporal evolution of the epileptogenic process in TSC represents a key step towards the development of stage-specific therapeutic strategies. Furthermore, genetic models of TSC can be equally informative on the behavioural complexities associated with the epileptogenic network as on the temporal dynamics of mTOR-related epileptogenesis.

Insights from human pathology and genetic models

mTORC1 signalling and its roles in normal brain development

mTOR is a serine/threonine protein kinase that acts through two distinct protein complexes, mTORC1 and mTORC2.[6,39] mTOR signalling influences survival and proliferation of neural stem cells, neuronal migration and axon and dendritic formation and outgrowth. Thus, balanced spatiotemporal mTOR signalling is crucial for the proper development of human cortical structure and organization.[6,9] In particular, mTOR regulates the morphology and migration of a specific population of neural stem cells prevalent in the developing human cortex (the outer radial glial cells).[40]

Morphological and functional alterations in TSC models

Investments have been made into developing models that recapitulate or mimic the brain lesions observed in TSC patients and enable the association of specific cell types to morphological and functional alterations.

Loss of *Tsc1* or *Tsc2* in germline knockout-mouse models is associated with embryonic lethality, whereas monoallelic loss of TSC genes fails to result in cortical tuber-like formation in rodent brains. Nevertheless, $Tsc2^{+/-}$ rats and mice, as well as $Tsc1^{+/-}$ mice, display aberrations in neuronal function, resulting in impaired learning and social behaviour, even in the absence of apparent cerebral

pathology and spontaneous seizures.[41–43] Hence, haploinsufficiency for the TSC genes can lead to cognitive deficits independently from seizure activity, at least in these models.

Cognitive deficits in *Tsc2*$^{+/-}$ mutant mice could be rescued by rapamycin treatment.[43] Sato *et al.*[44] reported that impaired social interaction of both *Tsc1*$^{+/-}$ and *Tsc2*$^{+/-}$ mice lessened with rapamycin treatment. However, in the *Tsc2-hGFAP* mouse model (*Tsc2* removal in embryonic neural progenitor cells)—which is characterized by cortical abnormalities, seizures and cognitive deficits—differential effects on neurodevelopmental defects of rapamycin treatment were reported depending on prenatal and/or postnatal administration.[45]

Recently, developmental status epilepticus was induced in *Tsc2*$^{+/-}$ (Eker) and wild-type rats by 12 days after birth.[46] In this study, both *Tsc2*$^{+/-}$ mutations and developmental status epilepticus may have caused social behaviour deficits and epileptiform EEG abnormalities; however, the mTOR inhibitor everolimus improved only the autistic-like behaviours related to *Tsc2* haploinsufficiency.[46] These observations suggest that seizures during early postnatal development can lead in later life to autism spectrum disorder (ASD) symptoms that cannot be ameliorated by mTOR inhibitors.

Forebrain-specific *Tsc1* deletion in mice (*Tsc1*$^{flox/flox}$; CaMKIIα-cre) causes both epilepsy and autism-like behaviours.[47] With this model, McMahon *et al.*[47] show that epileptiform activity spreads to the brainstem, resulting in seizure-dependent hyperactivation of mTOR in serotonergic neurons—indicating the role of the serotonergic system dysfunction in TSC. Moreover, mTOR hyperactivation targeted to serotonergic neurons (*Tsc1*$^{flox/flox}$; Slc6a4-cre mice) produced autism-like behaviours only, which rapamycin treatment reversed.[47]

Wu *et al.*[48] have recently generated a *Tsc1* conditional knock-out mouse model in which *Tsc1* inactivation in late-embryonic radial glia produces cytomegalic pyramidal neurons with development of both spontaneous seizures and social/cognitive impairment. To mimic persistent mTOR activation at different level of activity, a constitutively active Rheb (RhebCA; the canonical activator of mTORC1) was expressed in cortical neurons of mouse embryos using *in utero* electroporation (IUE) with dose ranging. Intriguingly, mTOR-hyperactivity levels correlated with the severity of epilepsy and associated neuropathology in this model.[49]

To study mTORC1-driven epileptogenesis, a brain-specific inducible *Tsc1* mouse model has been developed (*Tsc1*$^{fl/fl}$-Camk^{2a}-Cre^{ERT2}). Deletion of *Tsc1* resulted in strong activation of the mTORC1 pathway in this model, and both epileptogenesis and lethality could be prevented by inhibitors of the mTOR pathway.[50] Time-dependent changes were observed in the transcriptome and neuronal excitability, with an increase in the excitation-to-inhibition ratio in the hippocampus (but not in the cortex) later in epileptogenesis.[51]

The high co-occurrence and link between early onset epilepsy, especially infantile spasms, and neurodevelopmental outcomes in TSC has long been emphasized.[37,52] However, despite the development of multiple animal models of TSC and epilepsy, few models have reported spasm-like seizures or age-dependent seizure patterns that mimic infantile spasms.[53,54] Gataullina *et al.*[53] described age-dependent electrographic discharge patterns (including early onset 'spike clusters', 'spasm-like' and 'tonic–clonic like' patterns) with *Tsc1*$^{+/-}$ mouse pups, reporting a sequence similar to TSC patients. Although the *Tsc1*^{GFAP}CKO mouse model does not have spontaneous infantile spasms, it has been reported to have increased NMDA-induced spasms (with a reduced threshold for these), supporting the concept of a 'two-hit' model for infantile spasms that may explain their development in a subset of TSC patients.[54] One hypothesis is a requirement for an inflammatory insult.[55]

Zebrafish models reproduce several human-like disease features and have proven useful for transcriptomic and pharmacologic analyses—exploring the mechanisms of drugs [i.e. mTOR inhibitors or cannabidiol (CBD)] and their effects on TSC-related phenotypes, including behavioural effects.[56,57] In-depth analysis of changes in brain connectivity of the *TSC*^{2vu242} mutant zebrafish during development has implicated TrkB signalling (now a potential therapeutic target) in the complex TSC pathology, providing a mechanistic link between brain anatomy and human NDD.[58]

Other recent models include patient-derived induced pluripotent stem cells (iPSC) and cortical organoids. These models have the advantage of studying the earliest stages of neural development and so may discern the contribution of specific cell populations and cell type-specific dysfunction to the disease network in TSC and related mTORopathies.[40,59–62] Notably, a recent study in iPSC-derived neurons from TSC patients suggested that mTOR-independent processes for impairing axon extension or guidance could contribute.[63] Concordantly, the neural network connectivity abnormalities of these TSC patients cannot be ameliorated by mTOR inhibitors.[63]

Morphological and functional alterations in human brain

mTOR signalling pathway hyperactivation represents a shared pathogenic mechanism for a group of developmental malformations with similar histopathological abnormalities.[9,64] Brain lesions with evidence of mTORC1 activation can be detected prenatally in TSC patients.[36,65] Second-hit loss of either *TSC1* or *TSC2*, inducing mTORC1 activation, has been identified in various TSC neoplastic lesions, such as subependymal giant cell astrocytoma (SEGA), whereas TSC inactivation in cortical tubers is less frequently detected.[22,66] Still debated is whether only a small, poorly detectable, portion of cells is affected for tuber development or whether a causal complex is required between mono-allelic mutation and additional molecular mechanisms (dependent or independent of mTOR).[22]

Cortical tubers are focal developmental malformations and represent the neuropathological basis of the epileptogenic cellular network in patients with TSC. The availability of surgical specimens has provided a unique opportunity to dissect the cellular contributors to seizure development and progression and associated comorbidities.[9,24]

Neural circuit dysfunctions and altered neurotransmission

Functional studies indicate that focal seizures and interictal epileptiform discharges in TSC arise within the epileptogenic tubers and may propagate in the perituberal cortex and other epileptogenic tubers.[67] Electrophysiological recording in *ex vivo* slices of surgical specimens has demonstrated that the neurons display hyperexcitable intrinsic membrane properties that may contribute to the mechanisms of epileptogenesis in different mTORopathies.[68,69] Several studies in both models and human tissues provide evidence of neuronal network alterations resulting in a disrupted excitatory-inhibitory balance as the underlying mechanism of epileptogenesis (Fig. 1).[9,48,60]

Alterations in the expression of ionotropic glutamate receptor subunits (iGluRs; such as NMDA, GluN and AMPA, GluA receptors)

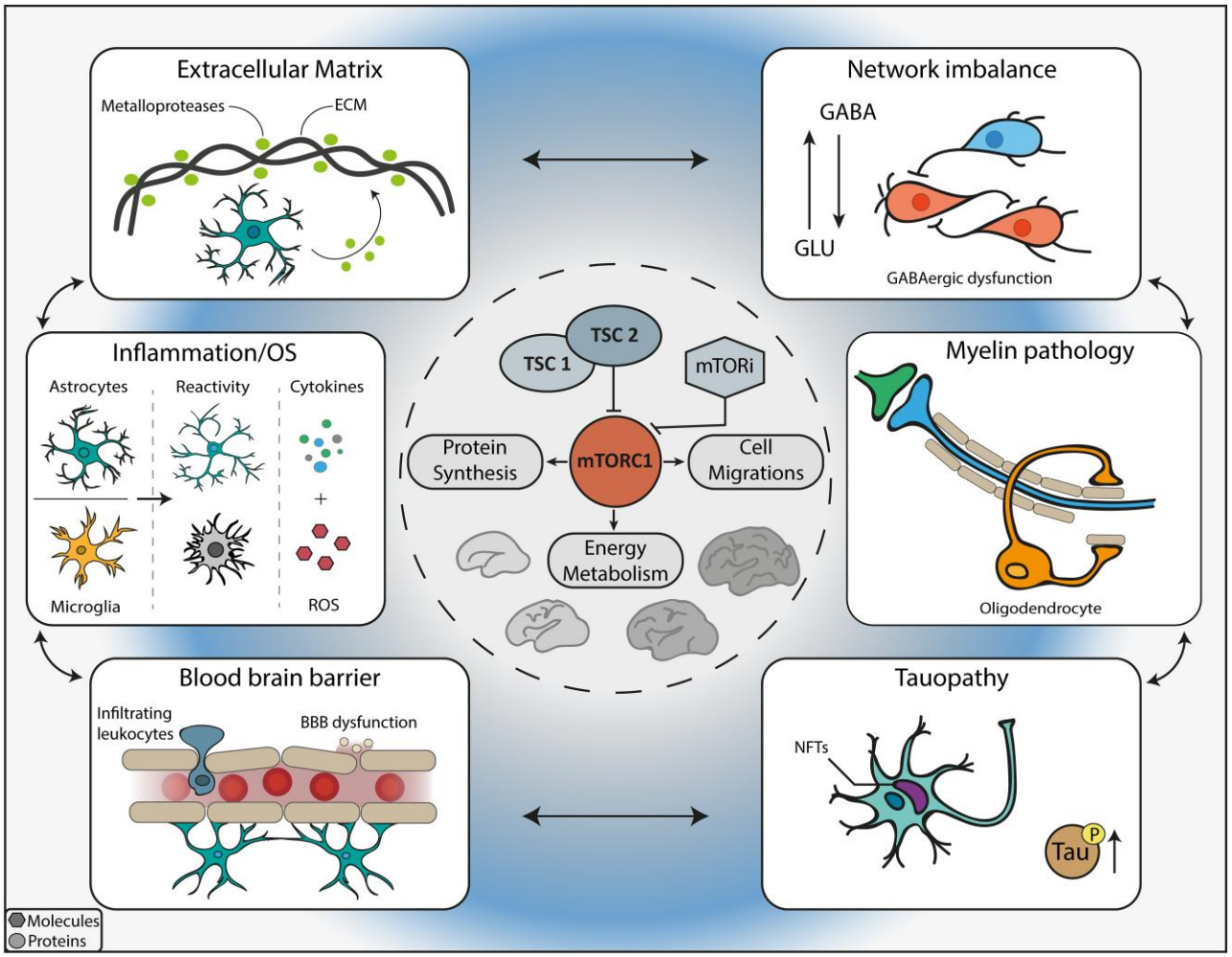

Figure 1 Cellular and molecular mechanisms underlying mTOR-related DEE in TSC. Overview of the complex cellular and molecular mechanisms contributing to the epileptogenic process and developmental disabilities in TSC. mTORC1 signalling plays a central role during brain development, regulating many basic cellular functions, such as energy metabolism and protein synthesis to regulate cell growth, proliferation and migration. mTORC1 is inhibited by the multiprotein complex consisting of TSC1 and TSC2. Loss-of-function mutations in either *TSC1* or *TSC2* result in prenatal mTOR overactivation; mTOR inhibitors (mTORi; such as everolimus and sirolimus) are powerful inhibitors of mTORC1 activity (for a more detailed illustration of the mTOR pathway, see Curatolo *et al.*[26]). Knowledge about the complex cellular and molecular consequences of mTORC1 overactivation, affecting different cell types, during brain development is rapidly increasing. Early and sustained oxidative stress and inflammation (with enhanced pro-inflammatory cytokines production and recruitment of the peripheral immune cells), accompanied by blood–brain barrier (BBB) dysfunction/leakage and alterations in the composition of the ECM are observed in TSC brain. Crosstalk among these multiple cellular and molecular can contribute to network dysfunction during brain development. Dysregulation of mTOR signalling itself results in developmental alterations of the balance between excitation and inhibition (i.e. GABAergic system dysfunction). Moreover, mTOR hyperactivity affects the oligodendroglial turnover (with failure to produce proper myelin), further contributing to abnormal cell-signalling and premature activation of mechanisms of neurodegeneration. Glu = glutamate; NFTs = neurofibrillary tangles; ROS = reactive oxygen species.

have been reported in TSC and related mTORopathies. These alterations involve an increase in the GluA1-to-GluA2 subunit ratio and in GluN2B-containing GluN receptor expression, which may contribute to increased network excitability.[26,70–73] The *Tsc1*$^{+/-}$-mouse model exhibited an mTOR-dependent increase in GluN-mediated excitatory activity due to an upregulation of the GluN2C subunit, and these findings were also seen in human surgical TSC resection samples.[74,75] Also reported in relation to both epilepsy and co-occurring NDD in TSC is abnormal synaptic transmission through alterations of expression and function of group I metabotropic glutamate receptors (i.e. mGluR5).[76,77]

GABAergic deficit/imbalance is also implicated in the dysfunctional neural circuitry underlying the epileptogenesis in a large variety of NDD characterized by marked genetic and phenotypic heterogeneity (see the 'Age-dependent pathophysiological mechanisms and GABA signalling' section).[78,79]

Non-neural mechanisms

Astrocytes may contribute to epileptogenesis due to either impairment of their homeostatic function or gain of aberrant properties.[80] In TSC models (e.g. *Tsc1*^{GFAP}cKO mouse), increased astroglial proliferation has been observed at the time of onset of spontaneous seizures, and both astrocyte-mediated glutamate and K^+ reuptake were impaired.[81,82] Interestingly, postnatal reduction of TSC1 (*Tsc1*$^{GFAP\text{-}CreER}$ mouse) was sufficient to cause both astrogliosis and spontaneous seizures.[83] Astroglial morphological and functional changes concordant with the intrinsic epileptogenicity of

Table 1 Risk factors for epilepsy and TSC-related DEE

	Risk factor for epilepsy	Risk factor for co-occurring NDDs	References
TSC2 mutation	X	X	Farach *et al.*,[15,19] Mongrain *et al.*,[16] Ogorek *et al.*[14]
Structural abnormalities: tubers and microlesions	X	X	Curatolo *et al.*,[24] Catlett *et al.*,[63] Hulshof *et al.*,[34,100] Pagani *et al.*[101]
Myelin pathology		X	Scholl *et al.*,[102] Prohl *et al.*,[103] Peters *et al.*,[104] Sato *et al.*[105]
Neural circuit dysfunctions and altered neurotransmission: glutamatergic transmission	X	X	Wu *et al.*,[48] Catlett *et al.*,[63] Cepeda *et al.*,[68] Talos *et al.*,[72] Lozovaya *et al.*,[74] Gataullina *et al.*,[75] Catania *et al.*[76]
Neural circuit dysfunctions and altered neurotransmission: GABAergic deficit/ imbalance	X	X	Eichmuller *et al.*,[62] Ruffolo *et al.*,[73] Katsarou *et al.*,[78] Amegandjin *et al.*,[106] van Andel *et al.*[107]
Non-neural mechanisms: astrocytes	X	X	Zou *et al.*,[83] Sosunov *et al.*,[84] Zimmer *et al.*[27]
Non-neural mechanisms: microglia	X	X	Zhang *et al.*,[87,88] Koike-Kumagai *et al.*,[108] Zimmer *et al.*[27]
Inflammation, oxidative stress and BBB dysfuntion	X	X	Eichmuller *et al.*,[62] Boer *et al.*,[85] Arena *et al.*,[89] Zimmer *et al.*,[90,95] Mills *et al.*,[91] van Scheppingen *et al.*,[93] Gorter *et al.*[96]
ECM remodelling	X	X	Mills *et al.*,[91] Long *et al.*,[97] Bongaarts *et al.*,[98] Broekaart *et al.*,[99] Lewis *et al.*[109]
Neurodegeneration progression: tauopathy	–	X	Iyer *et al.*,[110] Kovacs *et al.*,[111] Sarnat *et al.*,[112] Hwang *et al.*,[113] Liu *et al.*[114,115]

Most of the cellular, molecular and clinical events play a significant role in determining a high risk for both epilepsy and co-occurring NDDs.

the tuber (including decreased homeostatic function related to ion homeostasis and neurotransmitter metabolism) are evident in resected TSC cortical tissue.[27,84]

Increased density and activation of microglia cells are also observed in both TSC models and resected tubers, suggesting supportive roles of these cells in the pathogenesis of seizures in TSC.[27,85–88] Particularly interesting is the crosstalk between microglia and astrocytes to maintain a pro-inflammatory environment with the induction of pro-epileptogenic inflammatory pathways in TSC brain (Fig. 1).[27,87]

Immunohistochemical and large-scale transcriptomic studies in brain tissue from TSC patients showed induction of various inflammatory pathways, some of which (i.e. interleukin-1 receptor/ toll-like receptor and complement pathways) may contribute to epileptogenesis and associated comorbidities.[27,89–92] Notably, prenatal activation was evident for inflammatory pathways,[36] as were transcription factors (such as SPI1/PU.1) involved in the pro-inflammatory gene expression observed even in developing TSC brain lesions,[90] supporting the hypothesized role of immune–inflammatory responses to early epileptogenic processes. Specific small non-coding RNAs—and in particular microRNAs, such as miR-146a, miR147b and miR155—have been shown to contribute to the regulation of the astrocytic inflammatory phenotype in TSC.[93–95]

Astrocytes release cytokines that activate receptors on endothelial cells and pericytes of micro-vessels. In this way, perivascular astrocytes may contribute to blood–brain barrier (BBB) dysfunction, with increased BBB permeability and facilitated leukocyte diapedesis.[96] Brain extravasation of serum albumin and its uptake into astrocytes has been reported in brain tissue from TSC patients (Fig. 1).[85] Reactive astrocytes represent also an import source of the metalloproeinases (MMPs) upregulated in TSC brain tissue, the release of which contributes to extracellular matrix (ECM) remodelling and the pathological network underlying epilepsy and/or co-occurring NDDs in TSC (Fig. 1 and Table 1).[91,97–99]

Strong interdependence between inflammation and oxidative stress in TSC has been revealed recently.[27,89] Involving NF-κB signalling, extent of oxidative stress is suggested to predict the neuroinflammatory state of the brain.[27,89] Moreover, oxidative stress is closely linked to iron metabolism and may act synergistically to exacerbate cell dysfunction or death.[95,116]

The activation of adaptive immune responses with recruitment of the peripheral immune system is another feature of TSC pathology that further contributes to the sustained inflammation and related pro-epileptogenic mechanisms.[85,86,91,117] The presence of T cells has recently been correlated with myelin pathology, suggesting an involvement of the adaptive immune response in the pathogenesis of hypomyelination (even beyond the white matter) that has previously been linked to cognitive dysfunction in TSC patients (Fig. 1).[118]

mTORC1 is essential for the differentiation of oligodendrocytes (myelin-producing cells).[119] Thus, the maturation of oligodendrocytes and production of a proper myelin sheath is also impaired as a result of mTOR pathway disturbance in TSC.[120] Evidence of specific interactions between oligodendrocytes and inhibitory interneurons has been reported,[121] which raises an interesting hypothesis for impaired bi-directional communication that results in a pathological network.[122] This hypothesis deserves further investigation in the context of epileptogenesis in TSC-related DEE. Most non-neuronal mechanisms mentioned above also play a role in other epilepsy syndromes, suggesting that TSC may also be targeted with non-mTOR specific antiseizure drugs as mentioned later.

Age-dependent pathophysiological mechanisms and GABA signalling

Increasing evidence supports the concept of GABAergic dysfunction as a unifying mechanism underlying the variety of DEE.[79,106,107,123–125] Several studies support the link between

mTOR dysregulation and the development of GABA signalling. Experimental studies indicate that mTOR dysregulation affects the maturation and function of the GABAergic system, even beyond postnatal neurodevelopment.[106,126–129] Conditional knock-out mice with selective deletion of the *Tsc1* gene in GABAergic interneuron progenitor cells show alterations in interneuron development and function, along with a concomitantly decreased seizure threshold.[126] A key role of mTORC1 signalling in the development of parvalbumin interneurons is supported by Amegandjin *et al.*,[106] using conditional *TSC1*-mutant mice and single-cell genetics in cortical organotypic cultures. This study also identified a critical developmental period during which deficits in both parvalbumin interneuron-connectivity and social behaviour of mice can still be rescued by rapamycin.[106]

Using the human cerebral organoid model of TSC, a recent study has identified a specific neural stem cell type, caudal late interneuron progenitor (CLIP) cells, suggesting that dysregulation of specific interneuron generation may plausibly be a mechanism underlying vulnerability to pathology in TSC.[62] Evaluation of neuronal networks derived from ASD-patient iPSCs with a *TSC2* mutation showed abnormal network connectivity, resulting from an excitatory/inhibitory imbalance due to increased GABA-signalling at inhibitory synapses.[129]

The link between the GABAergic system and mTOR dysregulation is further supported by studies indicating a delay (or lasting impairment) of the physiological maturation of GABAergic signalling in TSC. Expression of $GABA_A$-receptor subunits and cation-chloride cotransporters (NKCC1 and KCC2) are altered, leading to alterations in excitatory/inhibitory (E/I) balance at the network level.[73,130,131] The concept of GABAergic 'immaturity' may represent another common mechanism underlying mTOR-related epileptogenesis and NDD in TSC. Interestingly, CBD at low doses acts as positive allosteric modulator on $GABA_A$ receptors.[132]

Biomarkers for TSC-related developmental and epileptic encephalopathies

Clinically relevant biomarkers for TSC-related DEE would greatly facilitate appropriate patient selection for combination drug therapy and/or epilepsy surgery (treatment personalization) and, ultimately, improve quality of life. Particularly important is the identification of individuals at risk of developing neurological and neuropsychiatric comorbidities.

Several types of biomarkers (genetic, imaging, EEG, molecular and behavioural) are established and could be implemented in early diagnostic protocols. Early assessment of gene variants can inform on the risk of seizure development and co-occurring NDDs. Therefore, TSC infants and young children can potentially benefit the most from early assessment and more timely, appropriate pharmacological and/or behavioural intervention.[14]

Structural and functional MRI may improve prognostication of co-occurring NDDs in patients with TSC.[133,134] Early MRI characteristics are predictive of neurologic manifestations and neurodevelopmental outcome at 2 years.[34,100] Several clinical studies have had promising results in support of the value of EEG-based biomarkers of epileptogenesis and co-occurring NDDs.[38,135–141]

Serum-based biomarkers, such as circulating microRNAs (miRNAs) and their isoforms (isomiRs), have recently shown potential to aid standard clinical testing in the early risk assessment of ASD and intellectual disability development in TSC patients.[142] Future studies are needed to elucidate further potential applications of circulating miRNAs to predict and monitor treatment efficacy.[143]

Early behavioural biomarkers also have the potential to be utilized in several aspects of clinical care in children, targeting the earliest symptoms of abnormal neurodevelopment.[144–146] Future studies of integrative biomarker research, using machine learning techniques, could be important to further understanding the relationships among different biomarkers and to establish and provide superior prognostic information in individuals with TSC-related DEE.

Mechanisms of developmental encephalopathy

mTOR dysregulation has been observed as a possible mechanism in idiopathic ASD.[147] Furthermore, evidence of a pathogenetic role for hyperactive mTOR signalling in TSC-associated ASD has been reported, in addition to the reversal of impaired social interaction with rapamycin in a mouse model of TSC (see the 'Cellular and molecular mechanisms of TSC associated epileptogenesis' section).[44,147]

As discussed above (see the 'Age-dependent pathophysiological mechanisms and GABA signalling' section), dysfunction of cortical GABA interneurons are hypothesized to contribute to the large variety of NDDs and further investigations are required in TSC-related DEE.[148,149] Figure 1 and Table 1 provide an overview of the convergent cellular and molecular mechanisms contributing to both the epileptogenic process and co-occurring NDDs in TSC, some of which are highlighted below.

Contributing mechanisms in TSC

Inflammation

The evidence of early inflammation and its long-term effects on brain development and function could provide a means by which multiple mechanisms associated with epilepsy may lead to co-occurring NDD.[150–152]

Extracellular matrix and cell adhesion

ECM remodelling and dysfunctional cell adhesion have been implicated in the pathogenesis of NDD.[97,153] ECM/cell adhesion could also contribute to the pathological network underlying TSC and co-occurring NDD. In concordance, changes in the expression of genes associated with cell adhesion have been observed in cortical tubers.[91] Notably, lower expression of the cell-adhesion molecule contactin-3 in TSC brain during the early postnatal period is a hypothetical pathophysiological mechanism.[109]

Myelin pathology

Myelin pathology represents a major feature of TSC brain pathology, linked to the hyperactivation of the mTOR pathway ('Cellular and molecular mechanisms of TSC associated epileptogenesis' section).[9,26] Several imaging studies have further emphasized hypomyelination in TSC, supporting its contribution to behavioural and cognitive dysfunctions in TSC patients.[154–157] Dysfunctional white matter, responsible for clinical manifestations of TSC, including co-occurring NDD, has been investigated in a plethora of studies, supporting this as one mechanism underpinning a network disorder.[102–105,118,120]

Early neurodegeneration progress: tauopathy

The link between neurodevelopmental and neurodegenerative mechanisms is well supported, with developmental disorders showing evidence of premature neurodegeneration associated with deregulation of the mTOR pathway, including TSC.[9,110–113] Both apoptotic cell death and ferroptosis-mediated cell death could also contribute.[95,110] Particularly interesting are studies that point to accelerated (early) neurodegeneration with tau dysregulation in TSC.

Tau is microtubule-associated protein involved in a group of neurodegenerative diseases (called 'tauopathies'), including infantile disorders with enhanced levels of phosphorylated tau (phosphor-Tau immunoreactivity).[158] Enhanced levels of phosphorylated tau have also been reported in different mTORopathies, such as hemimegalencephaly, FCD type II and TSC.[110,112,159] Clinical evidence points to the overlap between TSC and frontotemporal dementias.[115] Moreover, adult patients with TSC have recently been reported to have elevated aggregation of phosphorylated tau isoforms (3R/4R tau);[114] hence, co-occurring NDD could represent a novel 3R/4R tauopathy, independent of amyloid plaque formation, linked to the hyperactivation of the mTOR pathway and accelerated (early) neurodegeneration.[114] A recent study has provided additional evidence for a specific pattern of post-translational modifications in TSC (with differences between TSC1 and TSC2 mutation carriers), suggesting that individuals with TSC may have increased risk for tauopathy in mid-life.[113]

Untangling the complex interplay between genotype and resulting phenotype in a dynamic disease network is crucial to the characterization and subtyping of TSC phenotypes. Figure 2A–C illustrates the complexities within genotype–phenotype associations and the hypothetical temporal dynamics of disease progression. Table 1 provides an overview of the risk factors (convergent cellular and molecular mechanisms) for epilepsy and/or co-occurring NDDs in TSC.

Abnormal functional connectivity

Networks of abnormal functional connectivity are increasingly supported as underlying the comorbidity between TSC and ASD. Identifying the pathological brain connectivity patterns in TSC individuals with ASD may yield neurophysiological markers, facilitating early intervention.[101,105,160] In particular, the study by Sato *et al.*[105] suggests that white-matter microstructural integrity is associated with connectivity dysfunction, underlying co-occurring NDD. Evidence that large-scale network aberrations are associated with both ASD and mTOR-related connectopathy (characterized by fronto-cortico-striatal hyperconnectivity and rescued by inhibition of mTOR) has recently been reported using resting-state fMRI, electrophysiology and *in silico* modelling in *Tsc2* haplo-insufficient mice.[101]

Sleep disorders are a common neurological symptom and a cause of decreased quality of life in TSC patients.[161] Exploration of the abnormal functional connectivity in TSC may provide a link to the novel mechanisms for sleep dysfunction recently reported in experimental models.[162]

Mechanism of action of drugs and targeted therapeutic options

Mechanisms of drug resistance

Inflammatory mediators and release of glutamate by perivascular astrocytes may contribute to up-regulation of multidrug transport proteins on BBB endothelial cells in TSC.[163,164] Multidrug transport proteins (notably p-glycoprotein) are generally overexpressed at the luminal side of endothelial cells and astrocytic endfeet but are further upregulated in TSC brain lesions.[163,165] p-Glycoprotein overexpression may limit the access of several antiseizure medications to the intended brain targets, reducing their therapeutic effects and contributing to drug resistance.[163,164] For mTOR inhibitors, several additional molecular mechanisms of resistance have been explored previously within the field of oncology.[166] Interestingly, other compounds interacting with the mTOR pathway have been tested for the treatment of TSC-associated AML, SEGA and epileptic seizures, such as metformin,[167] however, it deserves further investigation.

Table 2 summarizes how approved therapeutic options for the treatment of TSC-associated epilepsy are currently being used.

Vigabatrin

Vigabatrin (VGB) can be considered as a neuromodulator agent: its major effect is to influence the activity of neurons. VGB is an irreversible inhibitor of GABA-transaminase (GABA-T),[174] an enzyme that degrades GABA, resulting in elevated brain GABA levels.[175] Higher concentrations of GABA terminate seizure activity. Additionally, vigabatrin may facilitate the synaptic release of GABA and prevent its neuronal uptake.[176] It may also inhibit glial uptake of GABA.[177] Beyond GABA mechanisms, VGB may reduce glutamate/glutamine cycling between astrocytes and neurons, and the antiseizure effects may also be related to this glutamatergic effect.[178]

VGB is effective when treating patients with TSC with both focal seizures and infantile spasms. VGB inhibits mTOR pathway activity,[179] which could represent a further mechanism of action that may contribute to the distinctive efficacy of VGB in TSC. VGB also showed an inhibitory effect on glial proliferation, at least in hippocampus,[179] and reduced astrocyte numbers in the neocortex in the mouse model. Effects of VGB on glial proliferation can likely be attributed to mTOR pathway inhibition.

In practice, a minimal effective dose should be considered to limit the risk of serious adverse events, including MRI changes and visual field abnormalities.[169]

Cannabidiol

CBD has broad spectrum anticonvulsive efficacy in animal model of seizures and epilepsy (including acute pentylenetetrazol and maximal electroshock for generalized seizures, acute pilocarpine and penicillin for focal seizures, audiogenic model of genetic epilepsy, chronic lithium-pilocarpine for generalized epilepsy and *Scn1a*$^{+/-}$ for developmental and epileptic encephalopathy).[180–182] Additionally, CBD has improved survival and behavioural comorbidity outcomes in a mouse model of Dravet syndrome[181] and in a rat model of temporal lobe epilepsy.[183]

The mechanism of action of CBD remains to be fully elucidated. CBD has very poor affinity for CB1 receptors and lacks euphoric side effects, and no direct effects seem to be mediated via cannabinoid receptors.[180,184,185] CBD is hypothesized to act at a synaptic level by reducing excitatory neurotransmission through at least three possible mechanisms: blocking GPR55 receptors, desensitization of TRPV1 channels or inhibition of ENT1 adenosine reuptake pumps.[186,187]

Other potential mechanisms for CBD in epilepsy include the reduction of signalling in the mTOR pathway observed in zebrafish.[188] Different effects of CBD on signalling within the mTOR pathway

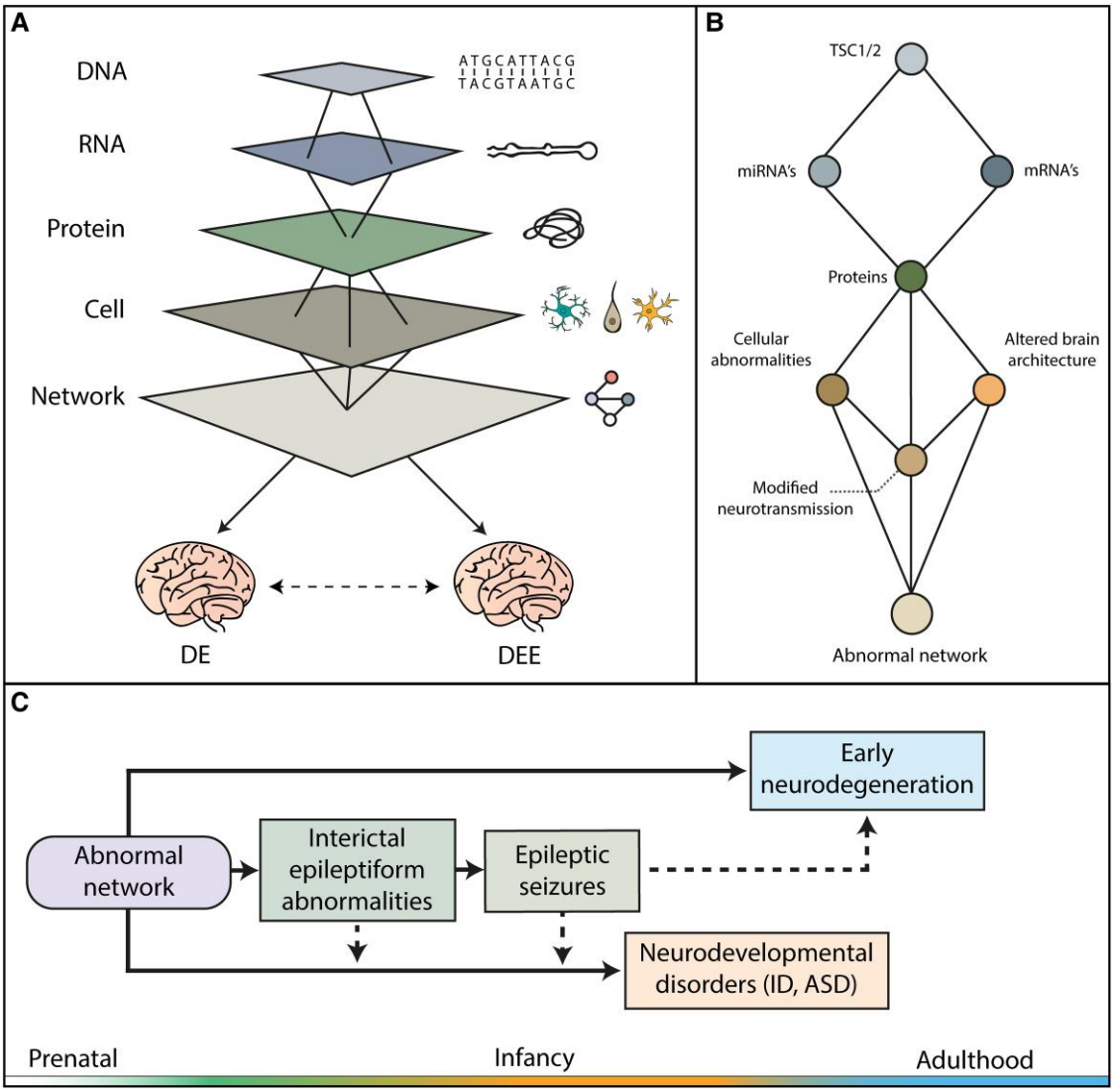

Figure 2 The early development of a disease network in TSC. (**A**) Schematic illustration of the levels of complexity from DNA to RNA and proteins, to cells, network and phenotype. The black lines show the relationships between levels from genotype (*top*) to phenotype (*bottom*) with emergence of developmental encephalopathy (DE) or DEE. (**B**) The convergent and divergent relationships between genetic defects (*TSC1* and *TSC2* mutations) and the abnormal network with hidden levels of complexity at the level of the genetic information (protein-coding or non-coding genes) and structural/cellular and functional abnormalities influencing the phenotype. (**C**) Hypothetical temporal dynamics of disease progression in TSC (early and late epileptogenesis), highlighting the establishment of an mTOR-related epileptogenesis associated with co-occurring NDDs and accelerated (early) neurodegeneration. ID = intellectual disability.

have been reported across diverse experimental models. This may suggest a potential pro-homeostatic mechanism for CBD, whereby it counteracts the disease-associated perturbation in mTOR signalling.[189]

Everolimus

Everolimus, a derivative of rapamycin (sirolimus), is a potent mTOR inhibitor. Its major effects are related to immunosuppression and antiangiogenic properties, and it was first approved as an immunosuppressant to prevent the rejection of organ transplants.[190]

Everolimus acts only on the mTORC1 protein and not on the mTORC2 protein.[191] It binds with high affinity to the FK506 binding protein-12 (FKBP-12), forming a drug complex that can inhibit the activation of mTOR,[192] resulting in inhibition of T-lymphocyte activation and proliferation.[193]

Reduction of neuronal excitability by everolimus (the antiepileptic activity) is mediated by a prolonged opening of Ca^{2+} and K^+ channels, via an increased expression of $K_v1.1$ in cortical and hippocampal neurons and reduced expression of AMPA receptors.[69,194,195] Everolimus also has some neuroprotective activities exerted via the modulation of synaptic plasticity, regulation of neuronal death and regulation of neurogenesis.[196,197] Chronic treatment leads to changes in synaptic membranes, with reduced excitability and increased GABA-mediated synaptic activity.[69]

Prevention of epilepsy has been suggested in a mouse model of TSC. Early treatment with rapamycin itself (age postnatal Day 14) showed prevention of seizures and premature death, whereas late treatment (age 6 weeks) only induced suppression of seizures and prolonged survival in a *Tsc1*-GFAP CKO mouse model.[198] Different TSC mouse models have shown concordant results for antiepileptogenic effects; however, rapamycin has also been

Table 2 Current and emerging treatment options: mechanism of action and level of evidence

Drug	Mechanism of action	Level of evidence	FDA/EMA approval	Studies in TSC	Minimal effective dose	Time to response	References
Vigabatrin	Irreversible inhibitor of GABA-T, it reduces glutamate/glutamine cycling between astrocytes and neurons, inhibits mTOR pathway	1b	FDA: Aug 2009 EMA: Oct 1999 (epilepsy)	Randomized trial comparing VGB and hydrocortisone in infantile spasms due to TSC	50–80 mg/kg/day	1 week	Chiron *et al.*,[168] Ounissi,[169] Rodrigues *et al.*[170]
Cannabidiol	Block of GPR55 receptors, desensitization of TRPV1 channels, inhibition of ENT1 adenosine reuptake pumps, reduction of mTOR pathway signalling	1b	FDA: Aug 2020 EMA: Jan 2021 (TSC)	Placebo-controlled phase 3 trial GWPCARE6 (NCT02544763)	25 mg/kg/day	4 weeks	Thiele *et al.*[171]
Everolimus	Potent mTOR (mechanistic target of rapamycin) inhibitor	1b	FDA Apr 2018 EMA Jul 2020 (epilepsy in TSC)	EXIST-3 (NCT01713946), randomized, double-blind, multicentre trial	3–7 ng/ml	8 weeks	French *et al.*,[172] Franz *et al.*[173]
Ganaxolone	Positive allosteric modulator of $GABA_A$ receptors synthetic analog of allopregnanolone	1b	FDA Mar 2022 (epilepsy in CDD)	Phase 2 CALM study (NCT0485346) Phase 3 TRUSTTSC study (NCT05323734)	Study ongoing in TSC	Study ongoing in TSC	NCT0485346

1b = individual randomized controlled trials (with narrowconfidence intervals); EMA = European Medicines Agency; FDA = Food and Drug Administration.

shown to restore cell size and myelination, indicating a possible disease-modifying effect.[199–202] Recently, rapamycin has been shown to relieve seizures and neuropsychiatric symptoms, except for ASD, and this effect seems to be related to the regulation of microglia polarity in the TSC mouse model.[108]

Everolimus improves white matter microstructural integrity, particularly in younger patients during a period of a rapid white matter maturation.[203] Furthermore, due to the targeted mechanism of action, everolimus is also effective in reducing several tumour manifestations associated with TSC (including subependymal giant cell astrocytoma and renal angiomyolipomas); hence, may represent a systemic treatment for this devasting condition.[204]

In the randomized controlled trial EXIST-3, both low and high exposure to adjunctive everolimus treatment in patients with drug resistant epilepsy showed a clinically meaningful reduction of seizure frequency in comparison with the placebo arm at the end of the core phase.[172] This response remained sustained in the post-extension phase. Furthermore, in a *post hoc* analysis, the response was particularly high in the younger subgroup (children <6 years).[205] The long-term safety of everolimus was assessed in an interventional post authorization safety study of 179 patients who received everolimus for the licensed indications in the European Union.[206] The most frequent treatment-related adverse events were stomatitis (6.7%) and mouth ulcers (5.6%). Serious adverse events, such as grade 2–3 pneumonia, were reported in 3% of patients, reflecting the safety and tolerability of everolimus in the management of TSC in real-world routine clinical practice.

Interactions with CBD have been reported, with an increase of everolimus blood levels.[207] Detection and further evaluation of potential drug interactions in patients using the combination of everolimus with CBD should be considered to increase patient safety.[208]

Ganaxolone

Ganaxolone (GNX) received the first approval in March 2022 within the USA for the treatment of seizures associated with CDKL5 deficiency disorder (CDD).[209] A phase III study named MARIGOLD (NCT03572933) documented its efficacy in patients with CDD. A phase III study (TRUSTTSC) is ongoing in patients with TSC. GNX is a synthetic analog of allopregnanolone, a metabolite of progesterone, with a methyl substitution at the 3β position that prevents back-conversion to any active intermediates.[210] GNX also acts as a positive allosteric modulator of $GABA_A$ receptors in the CNS via receptor binding at different sites, all of them different from the benzodiazepine binding sites.[211]

Discussion

Epileptogenesis in TSC is a multi-layered and dynamic process, and the epileptogenic network evolves over time (Fig. 2A and B). Overactivation of mTOR signalling is associated with cellular effects, and altered excitatory/inhibitory balance may be an important mechanism promoting epilepsy.[212] Mechanisms manifesting epilepsy, such as altered interneuron development, may also co-manifest in autism-like features.[148,149,212–215]

Recently, altered expression of mTOR and MAPK pathways—both key regulators of synaptogenesis and protein synthesis—were identified in children affected by idiopathic autism.[147] Progress in understanding the molecular basis of DEE and discerning the pathogenetic mechanisms that trigger both the epileptic and developmental encephalopathy components may help to find the most appropriate treatment.

Close EEG monitoring may allow early identification of pre-symptomatic EEG patterns and thus allow immediate commencement of treatment against seizures. This early seizure recognition and treatment is crucial to minimizing the risk of a poor neurological outcome.[216] Pre-symptomatic diagnosis of TSC may allow early identification of patients at high risk of developing drug-resistant epilepsy and DEE. EEG, MRI and genetic biomarkers have all been used successfully to identify infants at high risk of developing epilepsy and autism.[13–15,34,37,144] There is an optimum time window for planning and implementing therapeutic intervention; this should include not only the use of specific antiseizure medications (such as vigabatrin or CBD) but also targeted medications such as mTOR inhibitors.

Early abnormalities in developmental trajectories up to 6–12 months of age also predict a higher risk for autism.[144] Despite a solid biological rationalization based on animal models and preclinical data for the improvement of learning disabilities[43,217] and autism,[44,46,218] clinical trials of everolimus targeting mTOR overactivation have not yet yielded unequivocal positive results in TSC-associated intellectual disability and autism; however, some improvements have been seen in the 3–6-year-old age group.[172,219] The timing of therapeutic intervention may be crucial in reshaping brain development and normalizing its function.[214,220]

Genetic and acquired risk factors during critical and sensitive periods of synaptic plasticity and circuit development may have a significant impact on developmental trajectories.[221] The study of developmental trajectories in TSC infants may enable the discovery of biomarkers that have the potential to help in identifying infants at high risk of ASD before the onset of the first behavioural abnormality and consequently help to find targeted therapies for DEE.[222]

Epilepsy in young $Tsc1^{+/-}$ mice exhibits age-dependent expression that mimics that of human TSC.[53] Animal models show that mTOR inhibitors are potentially effective not only in reducing seizure frequency but also in improving cognitive function. These outcomes have not, however, been reflected by clinical experience, where the age-dependent profile and appropriate dose need consideration.[223] Future clinical studies need to include more patients aged under 2 years; for example, rapamycin has been tested as a preventative treatment in TSC patients.[224]

Evidence is growing to support that co-occurring NDD is not only a consequence of epilepsy and epileptiform abnormalities but may also reflect a common branched effect of *TSC1* and *TSC2* genetic variants. Medically reducing seizures still has only minimal impact on cognitive and behavioural symptoms.

The concept of EEG monitoring with pre-symptomatic treatment has changed clinical practice within the past few years. In a recent study, the epilepsy rate in a subgroup of patients receiving preventative treatment was much lower when compared with the conventional treatment approach.[225] Despite the use of preventative treatments, even when the delay between seizure onset and vigabatrin initiation is short, a reduction in the risk of epileptic encephalopathy is by no means a certainty; the effect of seizures may be minimized, but not the effect had on developmental encephalopathy.

Different biological mechanisms should be considered to explain these findings. Firstly, the current treatment approach is able to modify the shape of neurons but has no effect on the dyslamination that starts prenatally. Again, time dependency of treatment is critical, even if the results are equivocal with effectiveness of mTOR inhibition in ASD remaining controversial.[44,101]

Selection of the children who are likely to benefit from mTOR therapy requires predictive biomarkers. Yet, no interventional

trials of mTOR inhibitors in patients with epilepsy and autism exist that would enable us to evaluate both the effects on seizure frequency as well as the symptoms behind ASD. Further studies are needed to optimize mTOR inhibitor use, the safety and efficacy with long term use of rapalogs, and to determine whether early drug therapy combined with behavioural cognitive intervention can prevent progression to DEE or mitigate DEE severity.

Interestingly, increased EEG connectivity has been shown to precede the onset of epileptic spasm in TSC infants, indicating the establishment of a progressive pathological network synchronization.[139]

Despite getting closer, targeted treatment is still not available. In TSC, the outcome with respect to seizures, cognitive dysfunction and comorbid conditions is highly variable, even within the same family. Considering the great heterogeneity of TSC, an integrative approach is essential, and clinical trials are needed on biologically homogeneous subgroups.

The advances made in our understanding of the mechanisms underlying epileptogenesis in infants with TSC have led to the emergence of new concepts in the management of TSC-related epilepsy. A predictive and preventive approach can help both to delay seizure onset and improve seizure response, offering new avenues for targeted medicine. Novel treatment options should be explored that target the cellular and molecular pathway alterations which contribute to epileptogenesis.

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Competing interests

E.A. has received speaker honoraria from Novartis, Nutricia and UCB; has served as an investigator for UCB and Nutricia; and has served on scientific advisory boards for Novartis and UCB. N.S. has served on scientific advisory boards for GW Pharma, BioMarin, Arvelle, Marinus and Takeda; has received speaker honoraria from Eisai, Biomarin, Livanova, Sanofi; and has served as an investigator for Zogenix, Marinus, Biomarin, UCB and Roche. P.C. has served on scientific advisory boards for Novartis. M.L. reports no competing interests.

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