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Mechanisms of Neurocognitive Dysfunction and Therapeutic Considerations in Tuberous Sclerosis Complex

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Abstract

Purpose of review—Mendelian disorders that affect cognition provide a unique opportunity to study the mechanisms of neurodevelopmental disorders through the examination of genetic defects in animals and development of hypotheses that can be tested in human subjects. Tuberous sclerosis complex (TSC) is a genetic disease that presents with epilepsy, autism and intellectual disability. Here we review recent advances in our understanding of TSC pathogenesis and signaling pathways that may be modulated to treat the neurological symptoms.

Recent findings—Accumulating evidence suggests that TSC patients have non-tuber abnormalities that contribute to the development of the neurological phenotype – in particular, disorganization of axon tracts and deficient myelination. TSC mouse models have failed to replicate the human neuropathology entirely, but have shed light on the cellular abnormalities and the neurobehavioral phenotypes. Most importantly, cell culture and animal models have identified the mTORC1 pathway as a therapeutic target in this disease.

Summary—Preclinical data strongly suggest that TSC is a disease of abnormal neuronal connectivity. The high incidence of neurodevelopmental deficits, early detection of the disease in very young ages, and availability of mTORC1 inhibitors make TSC a model for other Mendelian disorders of neurocognition and an avenue for the mechanism-based treatment trials of neurodevelopmental disorders.

Keywords

mTOR; autism; translation; DTI

Introduction

TSC is an autosomal dominant disorder characterized by hamartomas in most organ systems including the brain. Patients with TSC are frequently diagnosed with comorbid neurological disorders, including epilepsy, intellectual disability, behavioral dysregulation, sleep disorders, and autism spectrum disorders (ASD). The genes responsible for the disorder are *TSC1* and *TSC2*, which encode for the proteins TSC1 (hamartin) and TSC2 (tuberin), respectively. Together these proteins regulate the protein complex, mTORC1, constituting a key cellular pathway important for protein synthesis and cell size regulation (Figure 1)[1]. mTORC1 is directly controlled by Rheb, a small GTPase. TSC1 and TSC2 proteins together act to negatively regulate Rheb, thereby inhibiting protein synthesis. In patients with TSC,

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inactivation of either TSC1 or TSC2 leads to the overactivation of Rheb and mTORC1 with a subsequent increase in protein translation.

1. Neuroimaging correlates of TSC manifestations

To investigate the etiologies of the neurocognitive phenotypes found in TSC patients, anatomic studies have been performed, and reveal characteristic pathological abnormalities: hamartomatous tubers and subependymal nodules that may undergo neoplastic change to form subependymal giant-cell astrocytomas (SEGAs)[2]. Many studies have correlated neurological symptoms – epilepsy, intellectual disability, and ASD – with the number and location of cortical tubers. Intellectual disability has been associated with increased tuber number[3] and frontal/occipital location[4]. However, recent studies have shown that total tuber volume, not number per se, is associated with poorer cognitive outcome[5]. In addition, ASD have been associated with temporal lobe tubers or temporal lobe epileptiform discharges[6]; however, additional studies also correlate ASD with cerebellar lesions, especially with right cerebellar involvement[7,8].

Although neuropsychiatric phenotypes can be associated with tubers, many patients without significant tuber load have disabling symptoms while patients with large tuber burdens may have few neurologic symptoms, suggesting that other abnormalities are responsible for these phenotypes. Patients with TSC have in fact been found to have pathology in other brain regions implicated in neuropsychiatric disorders. Mesial temporal sclerosis and hippocampal malrotation were described in 16% of TSC patients, associated with increased tuber number and a history of febrile seizures in the first year of life[9]. Furthermore, cerebellar abnormalities were detected in approximately 30% of TSC patients (in the absence of “cerebellar” symptoms)[10]. PET studies demonstrate hyperactivation of deep cerebellar nuclei in TSC patients with ASD, consistent with cerebellar dysfunction and decreased Purkinje cell inhibitory output[11]. Combined with the fact that ASD in TSC patients correlate with cerebellar WM abnormalities[7], these data suggest that dysfunction of cerebellar connections may contribute to neuropsychiatric symptoms found in TSC. Further studies into the cerebellar contribution to neuropsychiatric dysfunction in patients with TSC is an important area of future study.

In addition, investigators have also found aberrant connectivity in patients with TSC by using diffusion tensor imaging (DTI) to study myelination and white matter (WM) integrity. With DTI, at least three parameters which reflect the integrity of white matter may be obtained: apparent diffusion coefficient (ADC) – a reflection of total diffusion, fractional anisotropy (FA) – a measure of the directionality of diffusion, and radial diffusivity (RD) – an average of diffusion perpendicular to the direction of the axonal tracts.

DTI abnormalities are reported in TSC in normal appearing (NA) WM, suggesting microstructural abnormalities beyond conventional MRI resolution[12-18]. Initially, subcortical NAWM adjacent to cortical tubers was shown to have abnormal ADC and FA values compared to the contralateral side[14]. Ictal activity may also contribute to WM disruption since NAWM and cortical tubers within epileptic zones (documented by magnetoencephalography) display decreased FA and increased RD values[12]. However, there is mounting evidence of increased ADC values in NAWM remote from areas of tubers, indicating diffuse abnormality of the white matter[13]. Reduced FA and increased RD in the corpus callosum and internal capsule have also been reported[19]. Recently, abnormalities in WM ADC values have also been found to be age and location dependent[18]. Taken together, these data support a contribution of abnormal connectivity to the neuropsychiatric phenotypes in TSC patients. However, as these studies generally involved small patient cohorts, significantly larger studies are required to validate these findings and explore their correlation with neurological symptoms.

2. Cellular genetics of TSC: interplay between haploinsufficiency vs loss of heterozygosity

TSC exhibits an autosomal dominant inheritance pattern with a high spontaneous mutation rate. About 2/3 of TSC cases are sporadic while 1/3 are familial. In agreement with the two-hit tumor-suppressor gene model, inactivation of both alleles of either *TSC1* or *TSC2* is necessary for some of TSC's clinical manifestations. Most second-hit mutations are large genomic deletions, referred to as loss of heterozygosity (LOH). LOH involving the *TSC1* or *TSC2* has been observed in angiomyolipomas, rhabdomyomas, SEGAs and lymphangioleiomyomatosis (LAM)[20]. However, LOH has not been consistently observed in cerebral cortical tubers, suggesting that tuber pathogenesis does not require inactivation of both alleles, that only some cells within a tuber have both alleles affected or that the second hit mutations do not involve LOH[21]. Recent genetic studies examining two-hit inactivation of *TSC1* or *TSC2* in tubers have been inconsistent, with one report suggesting that complete inactivation of *TSC1* or *TSC2* occurs in tuber giant cells through second hit point mutations[22]. In this study, the investigators evaluated phospho-S6 positive neurons by laser capture microscopy from tuber samples and found a somatic mutation in both alleles in 5 of 6 cases. In contrast, another group used deep sequencing to search for small mutations in *TSC1* or *TSC2* in macroscopic tuber samples. Although one TSC patient had a second allele point mutation in multiple brain samples, the remaining patients studied had no second hit mutations found[23]. Thus, questions remain regarding the precise nature and timing of the genetic events underlying tuber formation. Furthermore, if second hits occur, it is unclear whether different tubers in the same patient would carry the same somatic mutation. Unfortunately, TSC animal models failed thus far to generate cortical tubers to help answer this question.

3. TSC rodent models

To further investigate the contribution of the TSC/mTORC1 pathway to neuropsychiatric phenotypes, investigators have used a naturally occurring TSC rat strain (Eker) and have generated genetic mouse models of the disorder (Table 1). No rodent model, however, has replicated the characteristic neuroanatomical findings of TSC patients, i.e. cortical tubers, subependymal nodules, and SEGAs.

Germline homozygous *Tsc1* or *Tsc2* knockout animals are invariably lethal in early embryonic stages[27,40,41]. Heterozygous models survive and display cognitive and behavioral abnormalities. Although, $Tsc2^{+/-}$ (Eker) rat mutants have no deficits in learning and memory or anxiety[24,25], these animals have decreased novel object and social exploration[25], consistent with ASD in TSC patients. Unlike rat models, heterozygous TSC mouse models demonstrate cognitive impairments in addition to behavioral abnormalities – suggesting a background and species dependent contribution to neuropsychiatric phenotypes. Both $Tsc1^{+/-}$ and $Tsc2^{+/-}$ mice have impaired hippocampal dependent learning and memory with deficits in spatial learning and contextual fear conditioning[26,28]. $Tsc1^{+/-}$ mice also demonstrate reduced social interaction[26] while a dominant negative *Tsc2* mutant displays increased anxiety [31,32]. In addition, pups born to $Tsc2^{+/-}$ mothers display increased isolation calls, suggesting impairments in mother-pup social interaction[29]. While heterozygous TSC mouse models display no obvious neuropathological abnormalities[26], $Tsc2^{+/-}$ neurons display abnormal axon guidance *in vivo*[30], suggesting that aberrant neuronal connectivity in the *Tsc2* haploinsufficient state may underlie neurobehavioral phenotypes.

No *Tsc* heterozygous model develops seizures or pathologic abnormalities, further suggesting that neither structural abnormalities nor seizures are exclusively responsible for the neuropsychiatric phenotypes associated with TSC. Nonetheless, in $Tsc2^{+/-}$ rats, anxiety and social impairment worsen with seizure induction consistent with the association between

epilepsy and worsened outcome seen in TSC patients[5,25]. Similarly, exposure to immune activators during embryonic development may worsen social approach behavior in adult *Tsc2*$^{+/-}$ mice[42]. Etiology of this seizure- or immunity-induced worsening at the cellular and/or circuit level remains to be explored.

Conditional knockout models of TSC have also shed light on the role of TSC in specific cell types. Homozygous deletion of *Tsc1* in astroglia produces megalencephaly, neuronal death, and astroglial hypertrophy. These mice develop seizures and have reduced survival[33]. Subsequent studies revealed increased extracellular glutamate in these animals, secondary to a deficit of the glutamate transporter *Glt-1*[34,37]. In addition, these animals have decreased *Connexin43* expression and subsequent decreased gap junction coupling in astrocytes[35], likely lowering the seizure threshold in these animals. Interestingly, treatment of these mice with ceftriaxone (which increases glutamate transporter expression) prevents seizures if given prior to the onset of seizures, suggesting that abnormal glutamate transport is critical for epileptogenesis[36] and offering a potential therapy for TSC patients.

Neuronal specific models have also been generated with Cre recombinase expression driven by Synapsin (Syn) or CamKII promoters[28,38]. *Tsc1*$^{flox/-}$*SynI-Cre*$^{+}$ mice are viable perinatally, but have neuronal hypertrophy and neurofilamentous inclusions with epilepsy, tremor, hyperactivity, and mortality between 4-8 weeks postnatally[38]. Gambello and colleagues[39] also deleted *Tsc2* in radial glia, which results in ectopic neurons, megalencephaly, and astrocytosis with failure to thrive, early seizures, and mortality by 3-4 weeks postnatally. Both these models also have myelination defects, consistent with the DTI abnormalities in TSC patients. However, the severe phenotype and early mortality in all of these models have limited behavioral evaluation.

4. Mechanisms underlying abnormal neuronal connections in TSC

Mouse models have also been vital to understanding the cellular mechanisms that are deranged with loss of *Tsc1* or *Tsc2*. Over-activation of mTOR affects virtually every step of neuronal development (Figure 1). While loss of *Tsc2* does not affect the proliferation of neuronal precursors in null embryos[43], *Tsc1*$^{-/-}$ astrocytes have a growth advantage[33]. Furthermore, neuronal migration is affected in *Tsc2*-deficient cells, in the setting of abnormal neuronal orientation in the cortex[38,44], suggesting a possible contribution for abnormal neuronal polarity to abnormal migration.

Several recent observations support the hypothesis that TSC1/2 proteins play important roles in neuronal connectivity. First, TSC1/2 regulate both dendritic spine density and morphology as well as AMPAR mediated synaptic currents[45], consistent with previously known modulation of synaptic function by pathways regulating protein synthesis (Reviewed in[46]). Furthermore, TSC1/2 are found within growth cones[47]. Most CNS neurons have a single axon and multiple dendrites, and establishing this unique polarized structure is critical for proper neuronal functioning. TSC/mTORC1 pathway components are predominantly expressed in axons and regulate neuronal polarity and axon specification[44,48,49]. This particular phenotype results from the local translation of TSC/mTORC1 pathway dependent proteins in nascent axons. The full repertoire of proteins regulated by TSC/mTORC1 in neurites remains to be identified. The abnormal neuronal polarity seen in TSC mutants requires the loss of both alleles of either *Tsc1* or *Tsc2*. However, as discussed earlier biallelic inactivation occurs in only a minority of cells in the TSC patient's brain and most neurons are heterozygous for the mutation[22,23]. Thus, one would predict that the downstream networks regulated by the TSC proteins would be dysregulated in the haploinsufficent state as well. This prediction has, in fact, been confirmed by the observation that retinal neurons from *Tsc2*$^{+/-}$ mice project aberrantly to their CNS targets *in vivo*[30], secondary to an abnormal response to ephrin/Eph receptor signaling, an important

pathway for axonal pathfinding and outgrowth (Figure 1). These experiments provide a cellular substrate for abnormal neuronal connectivity in the *Tsc2*$^{+/-}$ neurons. While this study investigated the role of TSC only in the visual system, ephrins and Eph receptors play roles in the establishment of many axon tracts in the brain, including those that connect brain regions involved in language and social cognition. Evaluation of other axonal pathways will be important in future studies.

Lastly, TSC may play a role in neuronal connectivity through its role in myelination. As demonstrated in neuroimaging studies and mutant models, CNS myelination is markedly reduced in the TSC mutant background. Importantly, there is no detectable change in oligodendrocyte number or differentiation[38]. Rather it appears that loss of *Tsc1* in neurons inhibits the induction of myelination, consistent with the crucial developmental interaction between neurons and oligodendrocytes. Abnormal myelination seen in TSC mouse models could potentially provide a mechanism underlying the radiologic differences in WM found in TSC patients. Parallel studies using DTI and histology in mouse models could help shed light on this possibility.

5. Potential Treatment Options

Recent studies not only have revealed mechanisms underlying neurological dysfunction in TSC patients but also has shed light on potential therapies. Indeed, suppressing the activity of Rheb or mTORC1 in TSC patients could result in clinical benefit. Both preclinical and clinical trials with mTORC1 inhibitors in TSC appear promising.

In rodent models, mTORC1 inhibitors can improve both anatomic (e.g. myelination) and neurological phenotypes. In mice with neuronal specific deletion of *Tsc1*, rapamycin (sirolimus) or RAD001 (everolimus) treatment normalize both survival and growth, with rescue of seizures and anatomic abnormalities[28,50]. In addition, treatment of astroglial specific *Tsc1* mutants with rapamycin prevents seizure development by increasing both Glt-1 and Connexin 43 levels[35,51], Finally, rapamycin treatment results in the reversal of spatial learning and contextual fear conditioning deficits, even when treatment is initiated in adulthood[28].

As a result of these preclinical data, clinical investigations have been initiated. Franz and colleagues[52] showed that rapamycin therapy induced regression of SEGAs. In this pilot study, five individuals with TSC were treated with rapamycin at standard immunosuppressive doses (serum levels 5-15ng/ml), and all lesions regressed. SEGA regrew in one patient whose therapy was interrupted, but regressed again when therapy was resumed. Subsequently, a larger (28 patients) open-label RAD001 treatment study confirmed efficacy of this compound in SEGAs, with 32% of patients having greater than 50% reduction in tumor volume at 6 months[53]. Based on these results, RAD001 was FDA-approved in November 2010 for treatment of SEGAs in TSC patients, who are not candidates for surgical resection.

Several questions remain: What are the long-term sequelae of mTORC1 inhibitor treatment in TSC patients? Since the SEGAs appear to grow when the treatment is stopped, how long would patients with SEGAs need to be on mTORC1 inhibitors? Can mTORC1 inhibitors improve epilepsy and prevent epileptogenesis clinically? Can they improve neurocognitive deficits and neurobehavioral difficulties? Is there a critical period during which treatment with mTORC1 inhibitors result in long-term improvement without requiring chronic treatment? Since there is growing evidence that TSC and Rheb may have mTORC1-independent functions[54], are there clinical aspects of TSC that are rapamycin insensitive?

Since rapamycin family mTOR inhibitors have significant side-effects, chronic therapy with these inhibitors may not be practical in many patients. However, perhaps using these inhibitors only during critical periods of pathogenesis or in patients refractory to other treatments. Another alternative is the intermittent use of these drugs, which has been effective in one animal model[55] and may be less toxic. Several other mTOR inhibitors recently have been developed although it remains unclear if they will have a better efficacy or side-effect profile[56-61].

Conclusions

Emerging evidence – from abnormal white matter on neuroimaging to deficits in axonal integrity in animal models – supports the hypothesis that abnormal neuronal wiring contributes to the neuropsychiatric symptoms in TSC patients. Further studies using functional imaging and electrophysiology will be important to delineate the timing and specific circuits underlying these abnormalities. While important areas of future study remain and have been highlighted throughout this review, several additional questions remain. What causes the remarkable variability of neurological phenotype in patients with the same *TSC* gene mutations? What are the genetic and non-genetic modifiers of TSC disease? Will it be possible to generate animal models that fully replicate the human neuropathology? Finally, findings from TSC may also have implications for other diseases in which the mTOR pathway is hyperactive – such as PTEN hamartoma syndrome, Fragile X Syndrome (FXS), and Neurofibromatosis 1 (NF1), all of which have been associated with ASD, behavioral dysregulation, or intellectual disability[62-66]. It will be crucial to compare and contrast the neuronal dysfunction in each of these conditions to determine whether modulation of mTORC1 function will have therapeutic or detrimental effects. Such information should shed light on the mechanisms underlying TSC and on the development of future therapeutic modalities.

Bullet points

- Accumulating evidence suggests that TSC patients have non-tuber abnormalities that contribute to the development of the neurological phenotype.
- While TSC mouse models have failed to replicate the human neuropathology in its entirety, they have shed light on the cellular abnormalities and the neurobehavioral phenotypes.
- Preclinical data strongly suggest that TSC is a disease of abnormal neuronal connectivity.
- Cell culture and animal models have identified the mTORC1 pathway as a therapeutic target in TSC, and clinical trials are in progress.
- The high incidence of neurodevelopmental deficits, early detection of the disease in very young ages, and availability of potential therapeutic targets and drugs make TSC a model for other Mendelian disorders of neurocognition.

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Annotated references in yellow highlight: Red letters indicate ••; black letters indicate •

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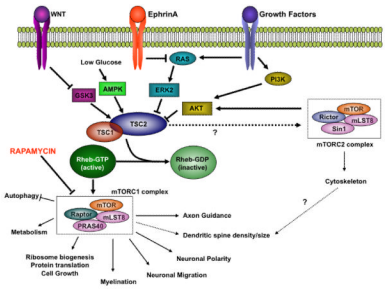

Figure 1.

TSC mediated signaling in the central nervous system. This cartoon of TSC mediated signaling has been simplified to highlight the demonstrated biologic roles for TSC mediated mTOR signaling in the nervous system. Among the upstream signaling pathways, only the growth factors and ephrins have been shown to modulate TSC-mTOR pathway in neurons (growth factors, ephrins) while others (e.g. Wnts) have been implicated, but not proven to regulate TSC signaling in the nervous system.

Table 1

Rodent Models of TSC in the Nervous System

Species	Gene	Constitutive/ Conditional (Cre promoter)	Anatomy	Neuropsychiatric phenotypes	References
Rat	Tsc2	Constitutive, heterozygous; IAP element insertion into codon 1272 (Eker Rat)	Normal	Decreased novel object and social exploration; defects exacerbated by seizures	[24,25]
Mouse	Tsc1	Constitutive, heterozygous; deletion of exons 6-8	Normal	Deficient spatial learning and contextual fear conditioning; Impaired social approach	[26]
Mouse	Tsc2	Constitutive, heterozygous; neomycin cassette inserted into exon 2, deletion of exons 2-5	Normal, except axon guidance defects	Impaired spatial learning and context discrimination; Impaired mother-pup social interaction	[27-30]
Mouse	Tsc2	Constitutive, overexpression of “dominant negative” allele	Subpial collections of granule cells	Increased anxiety	[31,32]
Mouse	Tsc1	Conditional (Glial Fibrillary Acidic Protein – Cre); deletion of exons 17-18	Astroglial hypertrophy and gliosis	FTT, seizures, early mortality	[33-37]
Mouse	Tsc1	Conditional (Synapsin – Cre); deletion of exons 17-18	Dysplastic neuronal hypertrophy, abnormal myelination	FTT, seizures, early mortality, pathological hindlimb clasping	[38]
Mouse	Tsc1	Conditional (CamKII – Cre); deletion of exons 17-18	Megalencephaly, neuronal hypertrophy, astrogliosis	FTT, early mortality, pathological hindlimb clasping	[28]
Mouse	Tsc2	Conditional (human Glial Fibrillary Acidic Protein – Cre); deletion of exons 2-4	Megalencephaly, neuronal and glial hypertrophy, abnormal migration, astrocytosis, abnormal myelination	FTT, seizures, early mortality	[39]