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Current approaches and future directions for the treatment of mTORopathies

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Abstract

The mechanistic target of rapamycin (mTOR) is a kinase at the center of an evolutionarily conserved signaling pathway that orchestrates cell growth and metabolism. mTOR responds to an array of intra and extracellular stimuli and in turn controls multiple cellular anabolic and catabolic processes. Aberrant mTOR activity is associated with numerous diseases, with particularly profound impact on the nervous system. mTOR is found in two protein complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2), which are governed by different upstream regulators and have distinct cellular actions. Mutations in genes encoding for mTOR regulators result in a collection of neurodevelopmental disorders known as mTORopathies. While these disorders can affect multiple organs, neuropsychiatric conditions such as epilepsy, intellectual disability, and autism spectrum disorder have a major impact on quality of life. The neuropsychiatric aspects of mTORopathies have been particularly challenging to treat in a clinical setting. Current therapeutic approaches center on rapamycin and its analogues, drugs that are administered systemically to inhibit mTOR activity. While these drugs show some clinical efficacy, adverse side effects, incomplete suppression of mTOR targets, and lack of specificity for mTORC1 or mTORC2 may limit their utility. An increased understanding of the neurobiology of mTOR and the underlying molecular, cellular and circuit mechanisms of mTOR-related disorders will facilitate the development of improved therapeutics. Animal models of mTORopathies have helped unravel the consequences of mTOR-pathway mutations in specific brain cell types and developmental stages, revealing an array of disease-related phenotypes. In this review we discuss current progress and potential future directions for the therapeutic treatment of mTORopathies with a focus on findings from genetic mouse models.

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Conflict of Interest Statement

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Keywords

mTORC1; mTORC2; rapamycin; Tuberous Sclerosis Complex; mTORopathy; epilepsy; neurodevelopmental disorders; Raptor; Rictor; TSC1; TSC2; PTEN

Introduction

In 1964, a scientific expedition on Easter Island led to the discovery of one of the most widely studied compounds today. This compound, named rapamycin after Rapa Nui (Easter Island), exhibited strong immunosuppressant and anti-tumor properties [1]. The target of rapamycin (TOR) was first reported in yeast in 1991 [2, 3] and a few years later followed the discovery of the mammalian TOR homologue [4, 5].

The serine/threonine-protein kinase mTOR (mechanistic target of rapamycin) was discovered just over 25 years ago [4, 5]. Initial studies on mTOR focused on its role in cancer, as it is a key regulator of cell growth and proliferation [6]. However, subsequent studies revealed broader roles for mTOR as a signaling hub, coordinating information between the intra and extracellular environment. mTOR was found to respond to various stimuli including amino acids, trophic factors and energy status and in turn regulate the balance between anabolic (i.e. protein synthesis) and catabolic (i.e. autophagy) processes [7].

mTOR's role in protein synthesis made this pathway a point of interest for neuroscientists in the early 2000s. *De novo* protein synthesis had been identified as a requirement for long-term synaptic plasticity in neurons [8, 9] and mTOR was known to control the translational regulators ribosomal protein S6 kinase beta-1 (S6K1 or p70S6K1) and 4E binding proteins (4E-BPs) [10]. Indeed, in 2002, mTOR was shown to be required for the late phase of hippocampal long-term potentiation (LTP), as LTP was inhibited by rapamycin in brain slices [11]. From then on, the list of mTOR's functions in neurons has steadily expanded. Today, mTOR signaling has been linked to fundamental neural processes such as progenitor proliferation, neuronal migration, cell survival, axon and dendrite development, membrane excitability and synaptic properties [12, 13]. Consistent with its multifaceted roles in the nervous system, deregulation of mTOR signaling is associated with numerous neurological and psychiatric disorders [14–16].

In this review we will discuss 1) how mutations in genes that encode mTOR regulators lead to disorders with shared neurological manifestations ("mTORopathies"), 2) the current status of therapeutic interventions for mTORopathies in patients and animal models, and 3) potential considerations for developing improved treatment strategies for mTOR-related disorders.

The mTOR complexes

The regulatory-associated protein of mTOR (RAPTOR) was the first mTOR binding partner to be discovered and was identified as an obligatory component of mTOR complex 1 (mTORC1) [17, 18]. A few years later, a second mTOR complex was found (mTOR

complex 2, mTORC2), after the discovery of a protein that bound to mTOR independently of RAPTOR, the rapamycin-insensitive companion of mTOR (RICTOR) [19]. mTORC1 and mTORC2 share some of the same protein components, while others are unique to each complex (shown in Fig. 1A). Shared proteins include the mTOR kinase itself, mTOR-associated protein, LST8 homolog (mLST8 also known as GβL), DEP domain-containing mTOR interacting protein (DEPTOR), TELO 2 interacting protein 1 (TTI1) and telomere maintenance 2 (TELO2) [20–22]. Specific to mTORC1 are RAPTOR and proline-rich AKT1 substrate 1 (AKT1S1 or PRAS40) [23]. Besides RICTOR, mTORC2 also contains proline-rich protein 5 or 5-like (PRR5/5L or PROTOR1/2) and target of rapamycin complex 2 subunit MAPKAP1 (MAPKAP1 or mSIN1) [24, 25] (shown in Fig. 1A).

Both complexes work as integrators of extra and intracellular signals to orchestrate cellular responses [12, 26] (shown in Fig. 1A). mTORC1 is regulated by various inputs including nutrients, growth factors, neuropeptides, and neurotransmitters [12]. In response to these stimuli, mTORC1 controls fundamental cellular processes such as protein synthesis, metabolism and autophagy. In neurons, mTORC1 controls differentiation, migration, cell morphology, physiology and synaptic properties [12, 13]. Our knowledge of the functions of mTORC2 in the central nervous system is still limited. Several studies, however, have shown that mTORC2 responds to growth factors, hormones and neurotransmitters to regulate cytoskeletal organization and thus can impact neuronal morphology and physiology [12].

mTORopathies

The critical importance of balanced mTOR signaling is underscored by the fact that mutations in genes encoding mTOR regulators cause neurodevelopmental disorders collectively termed mTORopathies (Table 1) [27, 28]. Some of the most well characterized genes associated with mTORopathies are *TSC1*, *TSC2*, *PTEN*, *AKT*, *STRADA*, and *DEPDC5* [29]. While mutations in these genes can affect multiple organ systems, for this review we will focus on their shared neurologic and psychiatric manifestations, which can include cortical malformations, intellectual disability, epilepsy, and autism spectrum disorder (ASD).

Tuberous Sclerosis Complex (TSC)

TSC is a multisystem developmental disorder with varying symptom severity caused by mutations in either the *TSC1* or *TSC2* genes that encode for the proteins tuberin and hamartin, respectively [30, 31]. TSC1, TSC2 and TBC1 domain family member 7 (TBC1D7) form a protein complex that acts as an essential negative regulator of mTORC1 [32–34] (shown in Fig. 1B). Loss of either TSC1 or TSC2 results in destabilization of the complex, leading to loss of its GTPase activating protein (GAP) activity towards the GTP-binding protein Rheb (RHEB), a direct activator of mTORC1 [35]. Loss-of-function (LoF) mutations in *TSC1* or *TSC2* therefore lead to constitutive mTORC1 activity.

TSC neuropathology includes focal malformations called tubers that contain enlarged and dysplastic neurons, astrocytes, and so called “giant” or “balloon” cells [36]. Tubers form during embryonic development and are primarily found in the cortex and occasionally in other regions such as the cerebellum [37, 38]. Tubers, or the peri-tuberal cortex can

become seizure foci [39, 40] and may be surgically resected as a treatment for intractable epilepsy in TSC [41]. Approximately 80% of individuals with TSC develop benign growths emanating from the ventricular walls called subependymal nodules (SENs) [42]. In 5–15% of TSC patients, SENs can progress to benign glioneuronal tumors called subependymal giant cell astrocytomas (SEGAs) [42]. A prevailing model is that TSC-associated brain lesions including SEGAs and tubers are caused by somatic second-hit mutations [29, 43]. According to this model, individuals with TSC have a germline heterozygous LoF mutation in *TSC1* or *TSC2* and during brain development a small number of neural progenitor cells acquire a somatic mutation that disrupts the expression of the functional allele, causing bi-allelic inactivation. In this scenario, progenitor cells with a second-hit mutation give rise to abnormal, dysplastic tuber cells that are surrounded by cells with normal-appearing morphology, which are derived from heterozygous progenitors. This model, in which the secondary mutation occurs stochastically, is consistent with the variable number, size and location of cortical tubers observed in individuals with TSC. Second-hit mutations have been consistently observed in resected SEGAs and TSC-associated tumors (called hamartomas) in peripheral organs [44–46]. However, they have only been identified in a subset of cortical tubers, resulting in some debate over the origins of cortical tuber cells [47–50].

TSC is also associated with several neuropsychiatric conditions, collectively termed “TAND” (TSC-Associated Neuropsychiatric Disorders) [51]. Approximately 80–90% of TSC patients develop seizures that can begin in infancy (infantile spasms) [52]. Earlier onset and increased severity of TSC-related seizures is correlated with greater risk of intellectual disability (ID), ASD, and attention deficit hyperactivity disorder (ADHD) [52–56]. Impaired and disordered myelination has also been observed in individuals with TSC using brain imaging techniques [57, 58]. Interestingly, while mutations in *TBC1D7* have not been reported in TSC patients, they have been identified in individuals with macrocephaly/megalencephaly and ID [59, 60], presentations that are shared among several mTORopathies.

Phosphatase and tensin homolog (PTEN) hamartoma tumor syndrome (PHTS)

PHTS is a spectrum of multi-system disorders caused by LoF germline mutations in *PTEN* [61]. PTEN is a phosphatase that negatively regulates the PI3K/AKT/mTOR pathway by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the cell membrane. LoF mutations in *PTEN* cause elevated PIP3 that recruits several proteins including 3-phosphoinositide-dependent protein kinase-1 (PDK1) and AKT family members. PDK1 and subsequently mTORC2 phosphorylate and fully activate AKT, which in turn phosphorylates and inhibits TSC2 leading to increased mTORC1 activity (shown in Fig. 1C) [62].

PHTS disorders include Cowden syndrome (CS), Bannayan–Riley–Ruvalcaba syndrome and adult Lhermitte–Duclos disease [61]. These disorders are associated with increased cancer and tumor risk [61]. Neuropsychiatric manifestations associated with LoF mutations in *PTEN* include macrocephaly, developmental delay, ASD, and intellectual disability [61]. Some studies have also identified patients carrying *PTEN* mutations who present with cortical malformations and seizures [63–65]. In addition, *PTEN* mutations have been linked to ASD and *PTEN* is one of the most prominent risk genes in idiopathic ASD [66, 67].

Malformations of cortical development (MCD)

MCD is a group of disorders characterized by abnormal development of the cerebral cortex, such as focal cortical dysplasia (FCD), megalencephaly (ME), and hemimegalencephaly (HME) [68]. FCD is defined by focal regions of the cortex that contain enlarged, dysplastic, and mislaminated neurons and glia, which can be observable by MRI and vary in size and location [69]. ME is defined by increased head circumference two standard deviations above the age-related mean, which is caused by increased growth of brain structures. HME is the enlargement of an individual hemisphere of the cerebral cortex [70, 71]. MCDs are common causes of pediatric epilepsy, ID and neurological deficits [72]. Epilepsy in MCD patients is often intractable, occasionally life-threatening, and can require surgical resection [73, 74]. Studies of resected tissue from FCD and HME patients have shown increased phosphorylation of the mTORC1 downstream target, ribosomal protein S6 [75–78]. More recent sequencing studies revealed that somatic brain mutations in mTOR regulators including *mTOR* itself, *PIK3CA*, *RHEB*, *AKT3*, *TSC1* and *TSC2* [79–82] can result in focal cortical dysplasia (Table 1), which share several features with syndromic disorders including cortical malformations and epilepsy [81, 83]. In addition, mutations in *DEPDC5*, *NPRL2* and *NPRL3*, which are components of the mTORC1 inhibitor GATOR complex 1 [84], have been linked to FCD, infantile spasms, focal epilepsy and sudden unexpected death in epilepsy (SUDEP) [85–87].

Mutations in *STRADA* and *NF1*, which encode negative regulators of mTORC1 [88, 89] have been identified in syndromes that present with MCD, epilepsy and cognitive deficits. Specifically, LoF mutations in *STRADA* cause polyhydramnios, megalencephaly, and symptomatic epilepsy syndrome (PMSE) [90] and LoF mutations in *NF1* cause neurofibromatosis type 1 (NF1) syndrome [91]. Polyhydramnios refers to excess amniotic fluid during pregnancy, which is a hallmark of PMSE. Aside from mTORopathies, aberrant mTOR signaling has been detected in several neurodevelopmental disorders such as Fragile X syndrome, Down syndrome and idiopathic ASD [92–94]. In addition, mTOR dysregulation has been observed in several psychiatric disorders and neurodegenerative diseases [12, 14, 15, 95]. However, a direct causal link between mTOR and these disorders has yet to be defined. In particular, given that mTOR signaling is highly responsive to neuronal activity [96], it is possible that altered mTOR signaling may occur as a secondary phenotype to changes in network activity in many of these diseases.

Current treatment strategies for mTORopathies

Several clinical studies have demonstrated the utility of rapamycin and its analogues (rapalogs), such as sirolimus and everolimus, as treatments for TSC and other mTORopathies including PHTS, NF1 and PMSE [97–99]. Rapamycin is an allosteric mTOR inhibitor that binds to FK506-binding protein 12 (FKBP12). The rapamycin-FKBP12 complex then binds to the FKBP-rapamycin binding domain on mTOR and blocks its catalytic domain [4, 100]. The first clinical evidence of rapalog efficacy for neurological manifestations came from Franz et al. in 2006 who showed SEGA regression in five TSC patients following rapamycin treatment [101]. Several larger follow-up studies corroborated the effectiveness of everolimus treatment in TSC patients with SEGAs [102–104]. Clinical

studies have also shown that rapalogs can be useful treatments for other TSC-related phenotypes including seizures [104–106], with one study demonstrating 40% reduction of seizure severity in 40% of patients in a trial of over 300 patients spanning a broad age range [105]. However, not all studies have reported successful outcomes with rapalogs in individuals with TSC-related seizures [107]. Recent clinical studies such as "Stopping TSC Onset and Progression 2: Epilepsy Prevention in TSC Infants (STOP2) (https://clinicaltrials.gov/ct2/show/NCT04595513)" have focused on early treatment or prevention of seizures in infants and children with TSC, as it may be difficult to control seizures with rapalogs or other drugs after prolonged occurrence [108, 109]. Notably, other antiseizure medications, including vigabatrin for infantile spasms, have been successfully used in TSC patients [110].

A limited number of studies have explored the effect of rapalogs on the cognitive and psychiatric conditions associated with TSC. In 2017, Krueger et al. found that a 6-month long everolimus treatment did not significantly improve neurocognitive function or behavioral abnormalities in children with TSC [111]. Similarly, in 2019, Overwater et al, showed that 12-month everolimus treatment in children with TSC did not improve intelligence quotient (IQ) or ASD symptoms [112]. However, a study involving 35 Japanese patients showed promising results of everolimus treatment on both TSC-related ASD behavioral symptoms and seizures [113]. The mixed results of these studies suggest that while rapalogs can be effective at treating neuropsychiatric aspects of TSC in some individuals, further research and development of therapeutic approaches is warranted.

While rapalogs are extensively used in clinical trials as therapeutic interventions for mTORopathies, there are limitations to their usage. For instance, rapamycin does not equally block the phosphorylation of all mTORC1 substrates [114]. Specifically, while rapamycin abolishes phosphorylation of ribosomal protein S6, inhibition of 4E-BP1 phosphorylation is incomplete [115], suggesting that phenotypes arising due to 4E-BP1 deregulation may be largely rapalog-resistant. In addition, while the phenotypes of mTORopathies are generally thought to be due to mTORC1 hyperactivity (although see further discussion below), it is known that chronic rapamycin treatment inhibits both mTORC1 and mTORC2, likely via sequestering mTOR kinase and prohibiting mTORC2 formation [116]. This non-selective inhibition, in addition to on-target off-tissue effects, has been shown to contribute to the adverse effects associated with rapalog treatment such as glucose intolerance, insulin resistance and new-onset diabetes [117–119]. These side-effects are an important consideration given that long-term treatment with rapalogs may be required as the beneficial results can be reversible. For example, upon cessation of rapalog treatment, seizures can resume and SEGAs can regrow [101]. However, studies in mice have shown that intermittent, low-level dosing of rapamycin can maintain the mTOR inhibiting effects while minimizing unwanted systemic side effects [120]. Consistent with this, it was shown that the antiepileptic properties of rapamycin in a mouse model of TSC could be maintained with as long as 24 day inter-treatment intervals [121].

Development of novel drug-based therapeutics

Currently, much research is geared towards the development of new drugs, including novel rapalogs, with more favorable pharmacokinetic characteristics, greater selectivity towards mTORC1, and fewer or less severe side effects. A recently developed rapalog, DL001, showed higher selectivity for mTORC1 when compared to rapamycin and had substantially fewer side effects when tested in mice [122]. In addition to rapalogs, a second generation of ATP-competitive mTOR kinase inhibitors have been under development [123]. These inhibitors target the catalytic site of mTOR and while all downstream mTORC1 targets are equally affected, these inhibitors exhibit no selectivity between the two mTOR complexes, similar to extended rapamycin treatment. Recent studies have attempted to improve selectivity by targeting mTOR regulators. For example, the small molecule NR1 potently binds to Rheb and selectively inhibits mTORC1 activity in mice [124]. More recently, another small molecule, EN6, inhibits mTORC1 by binding to an ATP-proton pump that normally aids mTORC1 recruitment onto the lysosome, which is necessary for its activation [125]. *In vitro*, EN6 was shown to suppress phosphorylation of both S6 and 4E-BP1, increase autophagy, and did not affect the mTORC2 target AKT [125].

The off-tissue effects of systemically administered small molecules are often a challenge in drug development. The ability to selectively target aberrant mTOR signaling in the brain would be transformative to long-term management of patient care. One recently developed strategy is a dual-molecule approach aiming to restrict mTOR inhibition to the brain. Specifically, Shokat et al. used RapaLink-1, a brain permeable mTOR inhibitor, along with RapaBlock, a brain impermeable FKB12 ligand. RapaBlock inhibits RapaLink-1 function outside the brain and thus prevents mTOR inhibition in peripheral tissues [126]. This approach has the potential to overcome the problem of systemic side effects.

While small molecule mTOR inhibitors are an active area of research, the question of whether we fully understand the functions of the mTOR complexes and the consequences of manipulating these functions remain. The variability of the clinical data underscores the importance of further exploring the mechanisms underlying neuropsychiatric presentations in mTORopathies. In addition, treatment strategies may need to be optimized and tailored toward specific phenotypes. For example, when targeting cells in tumors, complete suppression of mTORC1 activity may be beneficial to inhibit cell growth and eventually induce apoptosis. However, balancing mTOR activity rather than completely inhibiting it, may be a more desirable outcome for treating neuropsychiatric conditions. Thus, it will be important to examine alternative treatment strategies and utilize robust models to study these disorders.

Animal models of mTORopathies

Various animal models have been generated to enable investigations into the underlying mechanisms of mTORopathies. In early studies, drosophila and rat models of TSC were used to uncover the roles of *Tsc1* and *Tsc2* in the cell cycle, proliferation and cancer [127–131]. Subsequently, numerous genetic mouse models have been developed harboring mutations in genes that encode for mTOR regulators including *Strada* [132, 133], *Nf1*

[134] and *Depc5* [135] (Table 2). In this review, we will focus on several of the most well-studied mouse models with mutations in the *Tsc1*, *Tsc2* or *Pten* genes (Table 2). Germline homozygous loss of these genes is embryonically lethal [136–139]. While mice with heterozygous LoF mutations in *Tsc1*, *Tsc2* or *Pten* can exhibit some synaptic, cellular and behavioral phenotypes [138, 140–147], they often do not fully recapitulate the spectrum and severity of human disease phenotypes. In particular, mice with heterozygous *Tsc1* or *Tsc2* mutations do not exhibit robust mTORC1 hyperactivity, cortical malformations, or significant spontaneous seizures.

The use of Cre-dependent conditional knock-out mice avoids the confound of embryonic lethality and enables the generation of models with cell type- and developmental stage-specific mutations. Several mouse models with deletion of either *Tsc1, Tsc2* or *Pten* in specific cell types have been generated, which recapitulate various disease phenotypes including dysmorphic neurons, astrogliosis, hypomyelination, mislamination, seizures and behavioral alterations (as reviewed previously [12, 148]). In these models, the onset and severity of phenotypes depend on the developmental stage of the genetic perturbation. For example, studies of *Tsc1* conditional knock-out mice (*Tsc1*-cKO) have shown that Cre expression driven by the *Emx1* or *Syn1* promoters, which lead to loss of *Tsc1* from neurons during mid-embryogenesis, causes spontaneous seizures first observable at two weeks of age with premature mortality and a median survival of ~18–35 days [149, 150]. Loss of *Tsc1* or *Pten* from postmitotic neurons between 2–4 weeks of age using *Camk2a*-Cre mice results in seizures that begin later, between 5–10 weeks of age, with a median survival of approximately 50 days [151–153]. This demonstrates that seizures can occur independent of early developmental alterations. A study by Zou et al. using a tamoxifen-inducible system showed that *GFAP*-Cre-driven *Tsc1* loss primarily from astrocytes at 2 weeks of age, but not at 6 weeks, was sufficient to cause seizures, signifying that the timing of the perturbation affects the phenotypic severity [154]. These findings are important to consider when interpreting the results of therapeutic interventions in these models. For example, later-onset seizures may arise from distinct cellular or circuit mechanisms and may therefore respond differently to treatment compared to early developmental seizures. In addition, studies that provide treatment prior to or at the onset of seizures (i.e. prevention) may have a better outcome than those in which treatment occurs after seizures have already begun.

Cell type-specific disruption of mTOR regulators has revealed brain regions and cell types that may be key drivers of cognitive and behavioral deficits in TSC and PHTS. For instance, LoF mutations in *Tsc1*, *Tsc2* or *Pten* in Purkinje cells (PC) of the cerebellum induce ASD-like phenotypes including altered sociability and cognitive inflexibility [155–157]. Selective loss of *Tsc1* from dopaminergic (DA) neurons using *Slc6a3*(DAT)-Cre mice leads to cognitive inflexibility in a reversal learning task [158], while DA-neuron specific loss of *Pten* impairs social preference and novelty [143]. Selective loss of *Tsc1* from serotonergic neurons using *Slc6a4*(SERT)-Cre mice was also sufficient to cause autism-like behaviors including social behavior deficits and increased repetitive behaviors [159]. Deletion of *Tsc1* from one of the primary targets of dopamine neurons, striatal projection neurons (SPNs), results in enhanced motor routine learning, which only occurs when Tsc1 is lost from direct pathway, but not indirect pathway, SPNs [160]. In addition, deletion of *Tsc1* from thalamic neurons during mid-embryonic development leads to repetitive grooming [161].

Notably, loss of Tsc1 from cerebellar, dopaminergic, serotonergic or striatal neurons can induce behavioral alternations in the absence of seizures. Therefore, different cell types and circuits may be responsible for the distinct neuropsychiatric manifestations of TSC and related disorders.

Novel approaches have emerged to capture the focal nature of mTOR-driven cortical malformations using *in utero* electroporation in mice to induce mutations in a small population of cortical cells. In one study, Cre was electroporated at ~E15 into the cortex of $Tsc1^{flox/-}$ mouse embryos to induce a second-hit mutation. This led to the formation of cortical heterotopic nodules with neuronal hypertrophy and abnormal migration together with decreased seizure threshold [162]. Similarly, Lim et al. used *in utero* electroporation to induce CRISPR/Cas9-based gene disruption of *Tsc1* or *Tsc2*. These mice exhibited spontaneous seizures, neuronal hypertrophy and cortical mislamination [82]. Focal, constitutive activation of Rheb in the developing mouse cortex, which results in robust mTORC1 hyperactivity, has also been shown to result in neuronal hypertrophy, misplacement and spontaneous seizures [163].

As a complement to these animal models, human stem cell-based models of TSC and other mTORopathies have emerged over recent years due to advances in human stem cell and genome engineering [164–168]. These systems can capture human or patient-specific aspects of cell biology, genetics and brain development and thus provide an important complement to mouse models. Together, animal and human cellular models of mTORopathies provide a platform from which investigate key outstanding questions, in particular: 1) what is the neurodevelopmental impact of mutations in mTOR regulators? 2) what are the molecular, cellular and circuit mechanisms that drive pathophysiology? 3) which brain regions and cell types are responsible for different disease manifestations? and 4) what is the best therapeutic approach to maximize improvement and minimize side effects?

Drug-based therapies in animal models

There is a large literature examining the effects of rapalogs in animal models of mTOR-related disorders. These studies have revealed a range of possible outcomes including full reversal of neuropsychiatric phenotypes even after they have been established, prevention of phenotypes by pre-symptomatic mTOR inhibition, rescue within certain critical treatment windows, or a lack of response to rapalog treatment. One of the first studies to show efficacy of rapalogs in treating brain phenotypes was in a conditional mouse model of TSC. Meikle et al. showed that by administering rapamycin every other day starting at P7-P9 in *Syn1*-Cre*;Tsc1*-cKO mice they could shift median survival from 33 to over 100 days and reverse neuronal hypertrophy and demyelination phenotypes [169]. In subsequent work, Carson et al began rapamycin treatment at P13 in *Emx1*-Cre*;Tsc1*-cKO mice and were able to prevent premature mortality and largely reverse glial pathology. However, abnormal neuronal lamination was rapamycin-resistant [150]. Lin et al. showed that mislamination in a mouse model of hyperactive mTORC1 could, in fact, be prevented through administration of a constitutively active mutant 4E-BP1 if this occurred at the same time as the manipulation that caused mTORC1 hyperactivity [170]. Similarly, a study

examining *Pten* loss from dentate gyrus neurons showed that rapamycin could prevent but not reverse abnormal migration [171]. Together, these studies show that while seizures may be partly treatable with postnatal rapamycin, abnormal lamination and neuronal migration are early developmental phenotypes that may be preventable by mTORC1 suppression but cannot be reversed at later stages of development. For phenotypes such as these it will be key to determine how much they contribute to disease manifestations and whether functional improvement can be achieved in the absence of complete rescue of these abnormalities.

The idea of critical periods for treatment has gained attention in recent studies. Two studies led by Tsai et al. showed that mice which lack *Tsc1* from cerebellar Purkinje cell's exhibit social deficits and repetitive behaviors [155, 172]. The authors demonstrated that treatment starting within the first week of life could rescue both phenotypes [155]. However, treatment starting at 6 weeks of age, after the phenotypes were already established, could only reverse social behavior aberrations, but not behavioral inflexibility or repetitive behaviors [172]. Cox et al. characterized the effects of rapamycin on dendritic arborization and spine deficits caused by embryonic *Tsc1* deletion from cortical neurons at two different time points. In the early treatment group (P1-P7) they could rescue abnormal arborization and with later treatment (P15-P27) they could reverse abnormal spine maturation [173]. Critical periods might also exist for treatment of seizures in mTORopathies. Studies reporting the most success have either treated animals prior to symptom onset or have shown rescue in models in which the mutation occurs later in life [148]. It is possible that early on, potentially during epileptogenesis, seizures are sensitive to rapamycin [174] but once epilepsy is established, seizures are less responsive to mTOR inhibition. Together, these data suggest that it is important to identify critical periods for disease phenotypes in order to determine the optimal timing of treatment. Treatment given past the critical window may be responsible for some of the differential drug effectiveness observed in clinical studies.

Rapalogs have been the main pharmacological approach used in animal and cell models to inhibit mTORC1 in the context of genetic mTORopathies. However, as discussed above, chronic rapamycin inhibits both mTOR complexes and may not be effective at targeting all cell types in the brain, for example dopamine neurons [158]. In addition, a study assessing the effect of prenatal rapamycin treatment in wild-type animals showed that a single dose at embryonic day 16 resulted in adverse effects including motor abnormalities and increased anxiety that persisted in adulthood [175]. Thus, strong and non-specific suppression of mTOR activity, particularly in the developing brain, may not be an optimal therapeutic approach. Further work is needed to understand the basic biology of the two mTOR complexes, especially in neurons, the contribution of each complex to specific disease endophenotypes, and whether selective targeting of one complex will yield improved treatment with fewer side effects.

Genetic approaches to manipulating mTORC1 and mTORC2 in disease models

Several studies have attempted to selectively manipulate mTORC1 or mTORC2 in the mouse brain by disrupting their specific components, Raptor or Rictor, respectively. Such

studies have revealed that while the two complexes share some common functions such as regulation of somatodendritic morphology [176], they also have distinct functions and differentially impact a range of processes from development to synaptic transmission to behavior [177–179]. The distinct contributions of the two mTOR complexes to neural development and function suggest that therapeutic approaches for modifying disease phenotypes may benefit from an ability to control each complex independently and in a temporally precise manner.

Recent studies have begun disentangling the contribution of mTORC1 and mTORC2 to disease phenotypes in genetic mouse models of mTORopathies. Huang et al. crossed germline *Pten*$^{+/-}$ mice with mice that had heterozygous loss of *Rptor* from forebrain neurons (*Emx1*-Cre;*Rptor*$^{fl/+}$) [180]. *Pten*$^{+/-}$ mice exhibited neuronal hypertrophy in cortical layer V and a deficit in social approach behavior. Partial downregulation of mTORC1 in *Pten*$^{+/-}$ mice was sufficient to correct both neuronal hypertrophy and social deficits [180]. Chen et al. used *CamK2a*-Cre mice to induce loss of *Pten* in postmitotic forebrain neurons [153]. *Pten*-cKO animals exhibited hyperactivity of both mTORC1 and mTORC2 signaling, macrocephaly, seizures, premature mortality, and behavioral abnormalities. Concomitant deletion of *Rictor* in *Pten*-cKO mice prolonged their lifespan by approximately two-fold (i.e. median survival shifted from ~50 to ~110 days postnatal) but did not completely prevent premature mortality as all mice died between ~P90-P130. In this study, mTORC2 downregulation also prevented seizures and corrected behavioral abnormalities. Postnatal intracerebroventricular injection with an antisense oligonucleotide (ASO) targeting *Rictor* at four weeks of age was sufficient to improve seizures and behavioral deficits in *Pten*-cKO mice. *Rptor* deletion in *Pten*-cKO mice normalized brain size; however, surprisingly, neither seizures nor behavioral impairments were improved, and the median survival was only modestly increased by a few days [153]. These studies show that both mTORC1 and mTORC2 complexes contribute to *Pten*-related pathology in mice. One discrepancy between the ability to prevent behavioral phenotypes with Raptor (Huang et al.) or Rictor (Chen et al.) manipulation might be because Huang et al. used constitutive *Pten*$^{+/-}$ animals while Chen et al. studied homozygous loss of *Pten* from postnatal forebrain neurons. It would be interesting to investigate whether embryonic *Pten* haploinsufficiency can also be rescued via manipulation of mTORC2.

While TSC phenotypes tend to be ascribed to mTORC1 hyperactivity, the studies described above in *Pten* mouse models suggest that there could be a potential contribution of mTORC2. It should be noted, however, that while Akt signaling is elevated in response to *Pten* loss, phosphorylation of Akt at the mTORC2 site (Ser473) is consistently reduced in the context of *Tsc1* or *Tsc2* mutations [164, 181]. The contribution of mTORC1 and mTORC2 signaling to the neuropsychiatric presentations of TSC has not been comprehensively investigated. However, a recent study demonstrated that heterozygous loss of *Rptor* restored several TSC-related phenotypes in mice with loss of *Tsc1* from dopamine neurons (*Slc6a3*-Cre;*Tsc1*-cKO) [158]. These mice exhibited dopamine neuron hypertrophy and impaired striatal dopamine release that led to deficits in cognitive flexibility. Concomitant heterozygous loss of *Rptor*, while not sufficient to prevent somatic hypertrophy, significantly improved striatal dopamine release and prevented cognitive inflexibility. Notably, the authors showed that homozygous deletion of *Rptor* in *Tsc1*-cKO

dopamine neurons caused neuronal hypotrophy and was not able to improve dopamine release deficits [158]. This demonstrates that complete suppression of mTORC1 can be just as detrimental to neuronal function as mTORC1 hyperactivation. These findings underscore the idea that rebalancing rather than completely suppressing mTORC1 signaling may be a preferable therapeutic approach for mTOR-related brain disorders.

The importance of considering tissue- and mTOR complex-selectivity of therapeutic approaches was signified by two studies conducted in a mouse model of fragile X syndrome (*Fmr1*-KO), which exhibits mTORC1 hyperactivity [182]. Yan et al. selectively downregulated mTORC1 by injecting shRNA targeting *Rptor* directly into the hippocampal CA1 region of *Fmr1*-KO mice; rescuing, in part, aberrant spine morphology, synaptic function and memory deficits [183]. In contrast, a different study showed that orally administered rapamycin in *Fmr1*-KO mice did not reverse behavioral deficits and had adverse effects on social behavior and sleep in both control and *Fmr1*-KO mice [184]. These studies suggest that selective manipulation of a specific mTOR complex within a targeted brain region, as opposed to systemic non-specific inhibition, might be a more beneficial strategy for the treatment of disorders with altered mTOR signaling.

Conclusions

While the mTOR pathway has been extensively studied in many systems, it remains enigmatic due to its significant complexity and breadth of actions. Research using *in vitro* and *in vivo* models has revealed a multitude of upstream regulators and downstream targets and shown that these can vary significantly based on cell type and developmental stage. Given this complexity, it is not surprising that results from treatment with rapamycin and its analogues have had mixed success. In both people and in animal models, rapalogs exhibit variability in their efficacy and side effects. While it will be interesting to see how recently developed dual-drug strategies that enable brain-specific targeting of rapalogs affect neuropathophysiology, the development of alternative therapeutic strategies is warranted. One exciting approach currently under development is gene therapy [185]. While this has traditionally meant the delivery of a gene that is lacking via a virus-based carrier, it has expanded to include approaches such as ASOs and CRISPR/Cas9-based systems, including those that modify gene expression without altering the genome sequence [186, 187]. Although there are still several limitations and barriers to the wide-spread implementation of these technologies, gene-based therapies offer potential advantages of selective brain region- and cell type-targeting [188, 189].

In summary, building off the initial success of rapalogs as therapeutic treatments for mTORopathies, new insights into the basic biology of the mTOR complexes and their functions in different neural cell types will facilitate the generation of improved treatments for mTOR-related disorders. Based on our current information, therapeutic strategies that 1) target the most relevant mTOR complex for disease phenotypes, 2) are given at the optimal age and stage of disease progression, 3) act on disease-relevant cellular targets with minimal off-tissue activity, and 4) rebalance signaling to physiologic levels, are likely to be most successful.

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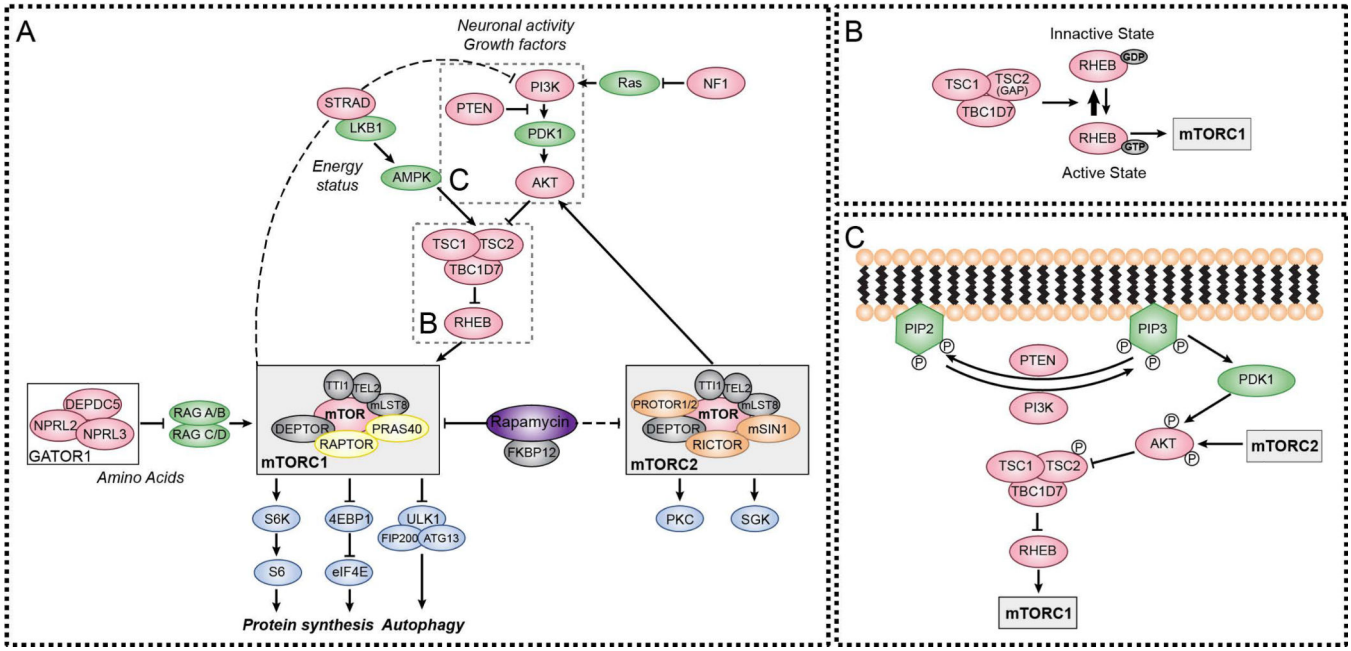

Figure 1. Mutations in regulators of mTOR signaling cause mTORopathies.

(A) mTOR is a protein kinase found in two distinct multiprotein complexes, mTORC1 and mTORC2, which are composed of shared and unique protein components. Several upstream regulators collectively work to control the activity of the two complexes in response to various stimuli including growth factors and nutrients. Mutations in genes that encode for mTOR regulators (denoted in pink) result in neurodevelopmental disorders, collectively termed mTORopathies (see Table 1). Current treatments for mTORopathies include rapalogs, which are derivatives of rapamycin that suppress mTORC1 activity and indirectly inhibit mTORC2 signaling when administered chronically.

(B) The TSC complex functions as a GTPase activating protein (GAP) for the small GTPase Rheb, which is a direct activator of mTORC1.

(C) PI3K converts PIP2 into PIP3 via phosphorylation at the cell membrane. PIP3 recruits the kinase PDK1 that, along with mTORC2, phosphorylates and activates AKT. In turn, AKT phosphorylates TSC2, inhibiting the TSC1/TSC2/TBC1D7 complex, and promoting mTORC1 activity. PTEN is a phosphatase that negatively regulates mTOR signaling by dephosphorylating and converting PIP3 to PIP2.

Table 1.

mTORopathy genes, diseases and clinical manifestations

Gene mutations	Associated Diseases and Syndromes		Clinical Manifestations*		Refs
		Abbreviation	Neurological	Psychiatric	
TSC1 *TSC2*	Tuberous Sclerosis Complex	TSC	Tubers, SENs, SEGAs, Epilepsy, Infantile spasms, Altered white matter	ID, ASD, ADHD, other behavioral conditions	[36, 38, 42, 51–58]
PTEN	PTEN hamartoma tumor syndrome (incl. Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome and Lhermitte-Duclos disease)	PHTS	Macrocephaly	ID, ASD	[61, 63–67]
MTOR *PIK3CA* *RHEB* *AKT3* *TSC1* *TSC2* *DEPDC5* *NPRL2* *NPRL3*	Malformations of cortical development	MCD	FCD, HME, ME, Epilepsy, Infantile spasms	ID	[68–74, 85–87]
NF1	Neurofibromatosis type 1	NF1	Macrocephaly, Epilepsy, Ataxia, Altered white matter	ID, ASD, ADHD, Learning disabilities	[91]
STRADA	Polyhydramnios, megalencephaly, and symptomatic epilepsy syndrome	PMSE	ME, Epilepsy	ID, Psychomotor retardation	[90]
TBC1D7	-		Macrocephaly/ME	ID	[59–60]

(ADHD) Attention deficit hyperactivity disorder, (ASD) Autism spectrum disorder, (FCD) Focal cortical dysplasia, (HME) Hemimegalencephaly, (ID) Intellectual disability, (ME) megalencephaly, (SENs) subependymal nodules, (SEGAs) subependymal giant cell astrocytomas

*Listed are the primary neuropsychiatric presentations of these diseases as listed in the references noted, the NIH Genetic and Rare Diseases Information Center, and OMIM database. Other manifestations may be present in these disorders and not all patients may present with all manifestations listed here.

Table 2.

Summary of mouse models discussed in this review

Disorder	Animal model	Refs
TSC	*Tsc1*$^{-/-}$ (germline KO)	[136]
	Tsc2$^{-/-}$ (germline KO)	[137,138]
	Tsc1$^{+/-}$ (germline Het)	[136,140–141],
	Tsc2$^{+/-}$ (germline Het)	[137–138, 140,145,147]
	Tsc1$^{fl/fl}$;*Emx1*^{Cre}	[150]
	Tsc1$^{fl/fl}$;*Syn1*^{Cre}	[149]
	Tsc1$^{fl/fl}$;*Camk2a*^{Cre}	[151–152]
	Tsc1$^{fl/fl}$;*GFAP*^{CreER}	[154]
	Tsc1$^{fl/fl}$;*L7*^{Cre}	[155, 172]
	Tsc2$^{-/fl}$;*L7*^{Cre}	[156]
	Tsc1$^{fl/fl}$;*Slc6a3*^{Cre}	[158]
	Tsc1$^{fl/fl}$;*Slc6a4*^{Cre}	[159]
	Tsc1 $^{fl/fl}$;*Drd1a-Cre*	[160[
	Tsc1 $^{fl/fl}$;*Adora2a-Cre*	[160]
	Tsc1$^{fl/fl}$;*Gbx2*^{CreER}	[161]
	Tsc1$^{fl/fl}$ + Cre *in utero* electroporation	[173]
	Tsc1$^{flox/-}$ + Cre *in utero* electroporation	[162]
PHTS	*PTEN*$^{-/-}$ (germline KO)	[139]
	PTEN$^{+/-}$ (germline Het)	[139,143–144, 180]
	PTEN $^{fl/fl}$;*CamK2a*^{Cre}	[152–153]
	PTEN $^{fl/fl}$;*L7*^{Cre}	[157]
	PTEN$^{fl/fl}$;*Slc6a3*^{Cre}	[143]
	PTEN$^{fl/fl}$ + AAV-Cre	[171]
PMSE	*Strada*$^{-/-}$ (germline KO)	[133]
	Strada shRNA *in utero* electroporation	[132]
MCD	*Depdc5*$^{fl/fl}$;*Syn1*^{Cre}	[135]
	Rheb^{CA} *in utero* electroporation	[163, 170]
	Tsc1 CRISPR/Cas9 editing *in utero* electroporation	[82]
	Tsc2 CRISPR/Cas9 editing *in utero* electroporation	[82]

Listed are the primary mouse models discussed in this review. Comprehensive reviews of animal models of mTORopathies can be found in references [12, 134, 142, 148]