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# Rapamycin for treating Tuberous Sclerosis and Autism Spectrum Disorders

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#### Abstract

Tuberous sclerosis (TSC) is a genetic disorder caused by heterozygous mutations in the *TSC1* or *TSC2* genes and is associated with autism spectrum disorders (ASD) in 20–60% of cases. In addition, altered TSC/mTOR signaling is emerging as a feature common to a subset of ASD. Recent findings, in animal models, show that restoration of the underlying molecular defect can improve neurological dysfunction in several of these models, even if treatment is initiated in adult animals, suggesting that pathophysiological processes in the mature brain contribute significantly to the overall neurological phenotype in these models. These findings suggest that windows for therapeutic intervention in ASD may be wider than thought previously.

#### Keywords

Tuberous sclerosis; TSC; mTOR; autism; ASD; rapamycin; pharmacology; treatment

#### Tuberous sclerosis

Tuberous sclerosis (TSC) belongs to the group of phakomatoses (neurocutaneous disorders) and is caused by heterozygous mutations in either the *TSC1* or *TSC2* genes [1,2]. This genetic disorder is inherited in an autosomal-dominant fashion and its birth incidence is approximately 1:6000 [3]. In about 30% of cases, affected individuals inherit their condition from affected parents (familial cases, autosomal-dominant pattern of inheritance). In 70% of the cases, it is caused by *de novo* germ-line mutations in the *TSC* genes. In addition to manifestations in the skin and nervous system (neurocutaneous disorder), TSC is associated with hallmark features in the kidney, lung, heart and liver [4]. In terms of pathology, a common denominator of many disease manifestations in TSC is the involvement of either tissue malformations (so-called hamartomas) or tumor growths. The penetrance of *TSC* gene mutations is thought to be complete [5] and the expressivity of any one TSC phenotype highly variable. Here, we provide an updated and extended version of previous articles on this topic [6–8].

## TSC: Autism spectrum disorders (ASD) and other neuropsychiatric phenotypes

Clinical manifestations associated with brain involvement are common in TSC patient populations and include intellectual disability, specific neuropsychological impairments, ASD, attention-deficit hyperactivity disorder (ADHD), epilepsy and, in adults, additional

psychiatric features, such as anxiety and mood disorders (Box 1). These symptoms indicative of brain involvement occur in various combinations and with variable severity (highly variable expressivity).

The association of TSC with social withdrawal, impaired social contact, stereotypies and abnormal speech was described by Critchley and Earl in the 1932 [9] many years before the description of infantile autism by Leo Kanner [10]. Systematic studies of the association of autism with TSC started several decades later. These studies reported a diagnosis of ASD in approximately 20-60% of individuals affected by TSC [11,12]. On a phenotypic behavioral level, autism associated with TSC is similar to autism of other etiologies, although few studies are available that directly compared these populations. ASD is more common in TSC individuals with cognitive impairments but may also be present in approximately 20% of individuals with IQs in the normal range [11,13,14]. TSC accounts for 1-4% of all cases of autism [15]. Although the majority of individuals with TSC and autism have a history of infantile spasms, there are some who develop autism but have no history of seizures. Additionally, approximately half of all individuals with TSC affected by infantile spasms will not develop autism [16,17]. These clinical observations suggest pathogenetic links between intellectual disability, infantile spasms and ASD in TSC, but also support the notion of distinct cellular or circuit mechanisms in the different phenotypic manifestations of TSC. In contrast to the skewed sex ratio of ASD in general (far more males are affected than females), the sex ratio of individuals with TSC affected by ASD is approximately even [11].

#### Disinhibited mTOR signaling and ASD

The lack of common genetic variants in the etiology of ASD renders identified rare causes of ASD (such as TSC) indispensible models for the study of ASD pathomechanisms. Insights from several different rare ASD-related syndromes suggest that common pathways are involved in the pathogenesis of at least a subset of ASD: dysfunctional mTOR signaling is a theme common to several ASD-related conditions.

First, ASD is commonly associated with TSC [11,12], demonstrating that heterozygous mutations in the *TSC1* or *TSC2* gene substantially elevate an individual's risk to develop ASD; individuals with TSC have an approximately 100-fold increase in the probability of being diagnosed with autism compared to the general population [18]. Alterations in TSC-related cell signaling (Box 2) not only play a role in the pathogenesis of TSC-related ASD but also contribute to autism risk in other conditions.

Mutations in *PTEN* (phosphatase and tensin homolog deleted on chromosome ten), which regulates PI3K (phosphoinositide 3-kinase)/AKT signaling upstream of TSC/mTOR (mammalian target of rapamycin), are also associated with ASD [19], suggesting that disinhibited mTOR signaling may also contribute to autistic phenotypes in these cases. Additionally, mutations in *PTEN* can lead to Lhermitte-Duclos disease (characterized by the presence of intellectual disability, ataxia, cerebellar ganglion cell hypertrophy and seizures) and Cowden syndrome (a multiple hamartoma syndrome) [20].

Associations between common variants of the receptor tyrosine kinase (RTK) MET and ASD [21] have been reported. Like other RTKs, MET signals through the PI3K/AKT and Ras/MAPK (mitogen-activated protein kinase) pathways [22], and thereby involves some of the same signaling pathways implicated in TSC and the other conditions discussed here.

Neurofibromatosis type I is a single-gene disorder caused by heterozygous mutations in the *NF1* gene and has also been linked to autism [23,24]. The *NF1* gene encodes neurofibromin, a GTPase activating protein that dampens Ras/MAPK signaling and is also involved in the regulation of mTOR signaling [25].

In addition to these upstream regulators, a downstream effector of TSC/mTOR signaling has also been implicated in ASD. Single nucleotide insertions in the eIF4E (eukaryotic translation initiation factor 4E) promoter that enhance promoter activity have been observed in individuals with autism from two different families [26]. When bound to 4E-BPs (eIF4E binding proteins), eIF4E, which controls a rate-limiting step for translational initiation, is not available. Upon phosphorylation of 4E-BP by mTORC1, eIF4E is released from 4E-BP-mediated repression.

Mutations in the FMR1 (Fragile X mental retardation 1) gene cause Fragile X syndrome (FXS) and associate with mTOR-dependent protein synthesis abnormalities [27] and ASD. The phenotype of t h i s X-linked disorder also includes intellectual disability, specific neuropsychological impairments, ADHD and epilepsy [28]. CGG triplet repeat-expansions in the 5' untranslated region (UTR) of the FMR1 gene are thought to lead to hypermethylation, which blocks transcription of the FMR1 gene and reduces expression of the FMR protein (FMRP). FMRP is an RNA-binding protein involved in the local dendritic regulation of protein synthesis downstream of metabotropic glutamate receptor (mGluR) signaling, thereby contributing to the control of spine morphology and synaptic function [29]. Specifically, increased group I mGluR signaling appears to play a central role in the pathophysiology of FXS, including associated cognitive dysfunction [30,31], because an mGluR5 germ-line heterozygous mutation rescued a wide range of behavioral and physiological phenotypes in the *Fmr1* knockout (KO) mouse model of FXS. The *Fmr1* KO mouse model showed disinhibited mTOR signaling in the hippocampus, which appeared to be related, at least in part, to increased translation of the mRNAs encoding P110beta (a subunit of PI3K) and PIKE-S (PI3K enhancer-S), two regulators of the PI3K/AKT/mTOR pathway [32].

Collectively, these convergent findings strongly implicate disinhibited mTOR signaling in the pathogenesis of ASD. There is additional indirect evidence that also points towards dysregulation of growth regulatory pathways in ASD (Box 3).

### From genes to behavior: neurobiological consequences of disinhibited mTOR signaling and relevance for ASD

A fundamental question is how disinhibited mTOR signaling generates ASD-related phenotypes. Components of the mTOR signaling pathway are widely expressed in the nervous system and this signaling pathway plays a role in regulating numerous cellular processes in the developing and mature brain. An important task therefore is the dissection of temporal (when during development and/or adulthood?) and spatial (which cell types?) requirements for TSC/mTOR signaling dysfunction in the emergence of certain behavioral, cognitive and neurological pathologies. Obviously, this knowledge has important implications for developing treatments (Box 4).

TSC/mTOR signaling is involved in regulating cell proliferation, synaptogenesis and growth of dendrites and axons [33–38] and, hence, is important during brain development. Alterations in the mTOR signaling pathway during critical developmental windows could lead to improper connectivity in the brain, which may play a role in the pathogenesis of autism-related behavioral symptomatology [39]. Infantile spasms, mentioned above, could also contribute to perturbing the establishment of proper circuitry in TSC [12], perhaps even by further exacerbating disinhibited mTOR signaling in the TSC brain [40]. These considerations suggest that any treatment aimed at correcting the behavioral pathology would have to be initiated during these important developmental time windows (i.e. early in the ontology of an individual).

Importantly, however, TSC proteins are not only expressed during development but are also highly abundant in the adult central nervous system (CNS) [41]. This is in contrast to findings in other tissues, where expression levels decrease after cessation of development, and suggests that TSC-mediated inhibition of mTOR signaling is required not only for development but also for proper function of the adult brain. In the mature brain, one of the key known roles of TSC/mTOR signaling is the regulation of synaptic function, including plasticity [42–45]. Additionally, the TSC/mTOR pathway also regulates glial functions in the mature CNS [46–48].

Many TSC-related symptoms are expressed in an age-dependent fashion, such that a range of symptoms (e.g., several hamartomas) tends to emerge after 3 years of age [49]. CNS manifestations (seizures and neuropathological findings), however, may be present earlier [49]. To our knowledge, no study has been published that systematically describes the age-dependent expression of neurocognitive and neurobehavioral phenotypes in TSC patient populations.

Although heterozygous mutations of *Tsc* genes in mice did not cause obvious abnormalities in brain structure indicative of developmental pathology (e.g. no tuber-like pathology), these mutations cause learning and memory impairments in  $Tsc1^{+/-}$  and  $Tsc2^{+/-}$  mice [45,50]. Remarkably, a relatively brief treatment of adult  $Tsc2^{+/-}$  mice with the mTOR inhibitor rapamycin reversed the learning and memory impairments [45]. These findings suggest that at least some of the TSC-related cognitive impairments are caused by disinhibited mTOR signaling in adults and are the consequence of functional changes, rather than irreversible structural defects caused during development.

In addition to learning and memory impairments,  $Tsc1^{+/-}$  mice also exhibited reduced levels of social exploration [50], while social behavior appeared to be normal in  $Tsc2^{+/-}$  mice [45]. A recent study reported alterations in an ultrasonic vocalization paradigm of pup/dam interaction in the  $Tsc2^{+/-}$  mutant line [52]. Notably, as discussed above, deficits in these behavioral models of ASD were present in mouse lines without gross structural brain abnormalities, again suggesting that the underlying neurobiological alterations may be functional in nature.

ASD is also associated with stereotypic/restricted behaviors. Interestingly, neuron-specific deletion of *Fkbp12* (*Fk506-binding protein 12*) in mice led to perserverative behaviors on several tasks in addition to memory and plasticity phenotypes [53]. Of note, neuronal *Fkbp12* deletion was also associated with disinhibited mTOR signaling [53]. Although FK506-binding proteins regulate a range of cellular processes [54], disinhibited mTOR signaling in the postnatal brain may have contributed to abnormal perseverative behaviors observed in conditional *Fkbp12* knock-out mice.

Of potential relevance for ASD pathogenesis and treatment are also observations regarding other neurological phenotypes and macroencephaly in *Tsc* and in *Pten* mutant mice. *Pten* mutant mice show a cluster of neurological abnormalities, behavioral changes and seizures, which are of interest because of their similarity to phenotypes associated with ASD in humans. Studies with *Pten* mutant mice have shown a surprising recovery of behavioral and neurological dysfunction in adult animals after disease onset [38,55]. Treatment with rapamycin, starting at 10–12 weeks of age and lasting for 4–6 weeks, reversed macroencephaly and neuronal hypertrophy and significantly improved structural neuroanatomical findings in neuronal-specific *Pten* mutant mice [38]. Moreover, pharmacological inhibition of mTOR also reduced anxiety, improved social behavior, controlled seizures and ameliorated macroencephaly in these mutant mice [38,55]. Importantly, most of these phenotypes were at least partially restored with the mTOR

inhibitor rapamycin even when treatment was initiated in adult mice [38,55]. However, it is important to point out that recovery was not complete; some abnormalities remained, including compression of the CA1 region of the hippocampus and abnormalities in neuronal polarity [38]. It is possible that the efficacy of these treatments can be further improved by targeting other pathways potentially involved in the disorder. For example, PTEN also regulates GSK-3 $\beta$  signaling and it is possible that therapies that target both GSK-3 $\beta$  and mTOR signaling would have higher efficacy. Also, it is important to consider the possibility that prolonged rapamycin treatment could have effects independent of mTOR inhibition. For instance, through an interaction with FK506 binding proteins, rapamycin could alter ryanodine receptor-mediated release from intracellular calcium stores [56].

Macroencephaly was also observed in mice with a homozygous neuron-specific *Tsc1* deletion [45,57]. In addition, these mice showed poor postnatal weight gain, severely compromised survival and neurological abnormalities, including severe hypoactivity, the presence of a pathological hindlimb clasping reflex upon tail suspension, tremor, kyphosis and aberrant tail position [45,58]. Early postnatal inhibition of mTOR with rapamycin substantially improved survival in neuronal *Tsc1* mutant mice [45,57]. Moreover, rapamycin-treated neuronal Tsc1 mutant mice showed a substantially improved neurological phenotype in adulthood [45,57], indicating that neurological findings in these mice are largely attributable to disinhibited mTOR signaling. Rapamycin improved neurological abnormalities despite the persistence of some structural neuronal abnormalities (abnormal neuronal polarity in the cerebral cortex) [57]. Improvement in myelination in the neuronal *Tsc1* mutants upon rapamycin treatment showed the best temporal correlation with the restoration of neurological impairments [57], indicating that myelination deficits may significantly contribute to neurological demise in this model. Conceivably, myelination deficits could contribute to altered network function and cognitive deficits [59,60] in TSC and may also represent a neurobiological mechanism underlying perturbed long-range connectivity potentially relevant to ASD pathogenesis [39].

Epilepsy is common in ASD populations in general; seizures and ASD also co-occur in TSC. Seizures are a prominent phenotype of mice with a conditional homozygous deletion of *Tsc1* in astrocytes. These mice showed behavioral and electroencephalographic seizures starting at 1–2 months of age [46,61] and died between three and six months of age [46]. Astroglial homozygous deletion of *Tsc1* perturbed astrocytic functions: mutant mice have reduced expression of the astrocytic glutamate transporter Glt-1 (glutamate transporter-1) [47]. As a consequence, these mice have elevated levels of extracellular glutamate [48], which contribute to seizure development in this model [62]. Notably, rapamycin treatment increased Glt-1 transporter levels and also rescued seizure phenotypes in astroglial homozygous *Tsc1* mutant mice [63], suggesting that disinhibited mTOR signaling underlies reduced Glt-1 expression and seizures. Rapamycin was effective in seizure prevention (treatment started before seizure onset) but also showed beneficial effects when treatment was initiated after seizure onset [63].

Altered Glt-1 function could also be of relevance for synaptic function and cognition in TSC models. Glt-1 KO mice showed impairments in long-term potentiation (LTP) [64]. LTP deficits were rescued by the administration of low doses of an N-methyl-D-aspartic acid (NMDA) receptor antagonist, suggesting that excessive NMDA receptor stimulation accounted for LTP impairments [64]. Interestingly, LTP deficits in astrocytic homozygous *Tsc1* mutant mice were rescued by applying an NMDA receptor antagonist [48]. Conceivably, decreased Glt-1 expression could contribute to plasticity defects and memory impairments [48] in this model of TSC.

Synaptic pathology is an important common denominator of ASD across different etiologies [65,66]. For instance, several single-gene mutations implicated in ASD affect genes encoding factors important for the establishment and/or maintenance of synapses, including NLGN3, NLGN4X, NRXN1 and SHANK3. Similarly, synaptic impairments also associate with disorders affecting the PTEN/TSC/mTOR pathway, such as TSC. Neuronal deletion of Tsc1 in mice not only led to neuronal hypertrophy and reduced spine density but also caused enlarged dendritic spines with increased AMPA (a-amino-3-hydroxyl-5-methyl-4isoxapropionic acid)/NMDA currents [44]. AMPA receptor surface expression decreased in response to the mTOR inhibitor rapamycin [67]. Heterozygous deletion of *Tsc2* resulted in abnormally low thresholds for the stabilization of synaptic plasticity in the mouse hippocampus [45], consistent with the role of mTOR signaling in the protein synthesisdependent late phase of LTP [42]. Such inappropriate synaptic consolidation may increase the signal-to-noise ratio and degrade the specificity of synaptic modifications that occur during normal learning and thereby contribute to cognitive impairments. Rapamycin treatment restored late-phase LTP thresholds in  $Tsc2^{+/-}$  hippocampus to levels that roughly corresponded to those of controls [45]. Synaptic abnormalities were also observed in the astrocyte-specific Tsc1 model and in the Eker rat model of TSC [68]. Despite synaptic abnormalities, the Eker rat showed enhancements (rather than impairments) on some memory-associated measures [68]. An interesting observation in human ASD individuals is the finding of cognitive impairments coexisting with relatively spared or even enhanced cognitive function in other domains [69]. It remains to be studied systematically whether this is also the case in ASD associated with TSC.

Disinhibited mTOR signaling could lead to altered synaptic function in several other ways. Importantly, although mTOR signaling is thought to upregulate the synthesis of many proteins, it downregulates the biosynthesis of others, including Kv1.1, a potassium channel mediating important computational functions of dendrites [70]. As observed with the Kv1.1 study [70], upregulating the translation of some mRNAs may compete with the translation of others, thereby resulting in their translational repression [71]. In addition to translationally mediated effects, mTOR can also modulate protein abundance through the regulation of protein degradation (via autophagy) [72] and by modulating gene expression on a transcriptional level [73]. The mTOR-dependent downregulation of Kv1.1 is predicted to increase neuronal excitability [74,75] and impair learning and memory [76,77]. Consequently, downregulation of Kv1.1 could potentially contribute to both seizures and cognitive impairments associated with TSC. Increased TSC-mTOR signaling may also lead to synaptic plasticity defects and cognitive impairments [78] by causing the unfolded protein response [79], thereby leading to increased phosphorylation of eIF2a (eukaryotic translation initiation factor-2 alpha) with consecutive suppression of the translation of plasticity-related proteins.

Additionally, synaptic pathology may result from negative feedback mechanisms that functionally uncouple upstream components of the TSC-mTOR signaling pathway from downstream effectors. In *Tsc1*- and *Tsc2*-null cells, Akt activation was abolished following receptor tyrosine kinase stimulation. Reduced phosphorylation of Akt (S473) has also been found in the brains of homozygous with neuronal-specific deletions of *Tsc1* in mice, suggesting that similar mechanisms are at work in neurons [57]. Functional uncoupling of RTKs (e.g. TrkB receptor) and downstream effectors (i.e. mTOR signaling) could render neurons unresponsive to learning- and plasticity-related signals, although downstream effectors (i.e. phospho-Akt) of receptor tyrosine kinases in *Tsc*-null cells and homozygous SynI-Cre neuronal *Tsc1* mutant mice [57], indicating that negative feedback mechanisms are mTORC1-dependent.

Clues about the role of altered mTOR-dependent translation in ASD also come from animal model studies related to FXS. Perturbing Fmrp function specifically in adult flies impaired a protein synthesis-dependent but not a protein synthesis-independent version of an olfactory learning and memory paradigm [80]. This is consistent with a role of altered protein synthesis in FXS-related memory impairments. Treatment of adult Fmr1 mutant flies with mild doses of the protein synthesis inhibitors puromycin or cycloheximide restored these memory impairments [80]. Notably, the same doses that rescued the mutants did not affect memory formation in wild-type flies, even though higher doses blocked memory formation. Protein synthesis inhibitors were also effective against audiogenic seizures in a Fmr1 KO mice [81]. These findings suggest excessive protein synthesis in FXS-related contributes to neurological impairments and highlight the potential therapeutic value of drugs targeting these mechanisms. Although the therapeutic potential of inhibitors of eukaryotic protein synthesis is limited by their toxicity profiles, other pharmacological modulators of protein synthesis may prove useful for FXS-related neurological impairments. The mTOR inhibitor rapamycin, for instance, blocks protein synthesis and is suitable for use in humans. In fact, dysregulated mTOR signaling has been implicated in FXS [27,32]. mTOR signaling was disinhibited in the hippocampus of Fmr1 KO mice [32] and is involved in mGluR-dependent synaptic plasticity [82]. Although acute bath application of rapamycin did not block exacerbated long-term depression (LTD) in the *Fmr1* KO hippocampus [32], further research should evaluate whether more long-term applications of rapamycin ameliorate synaptic and/or behavioral phenotypes in animal models of FXS.

Excessive protein synthesis in FXS is thought to occur downstream of mGluR receptors. One study, using a fly model of FXS, specifically addressed the developmental versus adult contributions of altered mGluR signaling in the pathogenesis of FXS-related behavioral impairments [83]. Treatment with several mGluR antagonists, including MPEP [6-methyl-2-(pheylethynyl)pyridine], and lithium during both development and adulthood restored impaired courtship behavior, deficient memory in a conditioned courtship task and CNS structural abnormalities in a Drosophila model of FXS [83]. Treatment given specifically during development rescued abnormal courtship behavior, memory impairments and neuroanatomical defects in mushroom bodies in adult FXS flies [83]. By contrast, treatment only during adulthood did not reverse structural brain abnormalities in mushroom bodies but rescued partially abnormal courtship behavior and memory impairments [83]. Notably, the treatment with mGluR antagonists that improved FXS flies impaired wild-type flies [83], confirming the specificity of the mechanisms targeted. In summary, these findings show that rescue of the FXS-related signaling deficits in either development or adulthood resulted in significant reversal of the behavioral and memory impairments in adult FXS flies, suggesting that these impairments are caused by abnormal mGluR signaling during both development and adulthood. Studies in mice have also provided evidence that acute pharmacological treatment with mGluR5 antagonists in adults improved some of the FXSrelated phenotypes, including audiogenic seizures and open field behavioral abnormalities [84,85]. Importantly, however, mouse studies have also shown that some FXS-related phenotypes do not respond to mGluR5 inhibitor-based interventions; whole cell recordings from lateral amygdala neurons showed that mGluR-dependent LTP was impaired at thalamic inputs in *Fmr1* mutant slices, a finding that was paralleled by a decreased postsynaptic surface GluR1 expression [86]. Additionally, a decrease in mEPSCs (miniature excitatory postsynaptic currents) suggested an impairment of presynaptic function at the thalamic-lateral amygdala synapse in Fmr1 mutant mice [86]. Acute MPEP application modulated the presynaptic alterations but had no effect on postsynaptic amygdala abnormalities in *Fmr1* mutant mice [86]. This is in contrast to findings in the hippocampus and illustrates that treatment response may vary in a circuit-dependent fashion.

Neurofibromatosis type I is another ASD-related condition with links to mTOR signaling [25]. Individuals affected by this disorder often exhibit learning disabilities, which may include difficulties with visuospatial skills, memory and attentional-executive function. Mice with a heterozygous deletion of the *Nf1* gene (*Nf1<sup>+/-</sup>* mice) displayed spatial learning deficits [87] and impairments in attentional-executive function [88]. Neurofibromin accelerates the inactivation of Ras and suppresses Ras/MAPK signaling. Reduced Ras/MAPK signaling associated with impaired synaptic plasticity owing to increased inhibition in the hippocampus of the *Nf1<sup>+/-</sup>* mice [89]. Notably, two different strategies that pharmacologically reduce Ras/MAPK signaling restored deficient synaptic plasticity and learning and memory impairments in this developmental disorder could be corrected with targeted pharmacological interventions in adulthood. It remains to be determined what the contribution of altered mTOR signaling is to neurological phenotypes associated with this disorder.

#### **Concluding remarks**

All together the studies reviewed here demonstrate that disruptions of mTOR signaling could be responsible for a significant number of cases in autism and ASD. Nevertheless, it remains an important goal to assess whether mTOR signaling is also altered in ASD outside the cluster of genetic disorders discussed above (TSC, FXS, NF1, ASD associated with *PTEN* mutations). Determining how mTOR signaling affects phenotypes associated with autism, such as disruptions in social-emotional function, has emerged as a compelling and tangible goal for efforts to better understand this complex condition. Animal models are valuable tools to determine when and where perturbations of mTOR signaling result in relevant behavioral and cognitive phenotypes associated with TSC and other ASD-related disorders. Results in animal models also indicate that interventions in adults may be effective in ameliorating or even reversing key phenotypes associated with neurodevelopmental disorders such as autism. Translational research efforts have begun to explore whether interventions that target mTOR signaling are also effective in the context of clinical populations affected by neurodevelopmental disorders.

#### Box 1. TSC: neuropsychiatric phenotypes

Approximately 50% of individuals with TSC are affected by intellectual disability (IQ<70) [90]. IQs are distributed bimodally in TSC populations, such that 30% of individuals have very low IQs (too low to be accurately measureable with standardized IQ testing; profound phenotype), whereas IQs of the remaining 70% are normally distributed about a mean that is slightly shifted to the left as compared to the general population (normal distribution phenotype) [90,91]. The bimodal distribution of cognitive abilities in populations of TSC patients suggests the existence of distinct subgroups, which may differ with respect to underlying pathogenetic mechanisms. It is important to note that even TSC individuals with an IQ in the normal range often show specific cognitive impairments, which include difficulties with memory and attentional-executive dysfunction [14,92], suggestive of dysfunction of hippocampal and corticostriatal circuits.

Epilepsy is also an important and common clinical feature of TSC and affects approximately 70–80% of subjects over their lifetime [90,93]. Infantile spasms, a form of early childhood epilepsy, are diagnosed in approximately 50% of individuals with TSC [90,93] and constitute a risk factor for ASD. Psychiatric disorders, including depression and anxiety, are also frequently encountered in TSC populations [13,94].

Few studies provide cues regarding the circuit pathophysiology involved in TSC-related neuropsychiatric phenotypes, including autism. With respect to autism, one study found a correlation between tuber localization in the temporal lobe and ASD symptomatology [95]. Cortical tubers are the type of hamartoma found in cortical and subcortical regions of the brain in individuals with TSC and are characterized by developmental tissue malformations. Others found an association of tubers in the cerebellum and ASD phenotypes in TSC [96,97]. Consistent with the involvement of temporal lobe and cerebellar pathophysiology in TSC-related ASD, a positron emission tomography (PET) study showed glucose metabolism in the temporal lobe and cerebellar nuclei of TSC individuals affected by ASD relative to IQ-matched TSC individuals unaffected by ASD [98]. Additionally, increased uptake of the PET tracer alpha-[<sup>11</sup>C]methyl-L-tryptophan (AMT) was reported in the caudate nuclei of TSC individuals affected by autism [98]. Glucose hypermetabolism in the deep cerebellar nuclei and increased AMT uptake in the caudate nuclei were related to stereotypical behaviors and impaired social interaction [98], suggesting that functional alterations in subcortical circuits may be linked to these ASD-associated phenotypes in TSC.

#### Box 2. Molecular cell biology of TSC

The Tsc1 (hamartin) and Tsc2 (tuberin) proteins form a heterodimer and play important roles in cell signaling. Tsc1 stabilizes Tsc2, preventing its degradation [99], and has additional functions, such as interacting with actin-binding proteins [100]. Tsc2 is a GTPase activating protein (GAP) and accelerates the inactivation of Rheb and other small G proteins [101]. Rheb activates mTOR, a kinase with important regulatory functions with respect to translation, transcription, autophagy and other cellular processes [101].

#### mTORC1

mTOR occurs in two distinct protein complexes. The mTORC1-signaling complex includes mTOR, raptor (regulatory-associated protein of mTOR) and mLST8. mTORC1 regulates a rate-limiting step of translation, the initiation of protein synthesis [102]. eIF4E is required for the translational initiation of mRNAs with a highly structured 5' UTR and can be repressed by 4E-BPs. By phosphorylating 4E-BPs, mTORC1 derepresses eIF4E and, hence, stimulates the translation of mRNAs with a highly structured 5' UTR. Multiple mRNAs that are translated in an mTORC1-dependent fashion encode proteins with important synaptic functions, such as glutamate receptor subunits,  $\alpha$ CaMKII (Ca2+/calmodulin-dependent protein kinase II), PSD-95 (postsynaptic density protein-95) [67,103].

A second pathway through which mTORC1 regulates protein synthesis involves the downstream effectors p70S6 kinase and ribosomal protein S6 (so-called S6-directed translation) [102,104]. S6 directs the translation of mRNAs that contain a 5' terminal oligopyrimidine tract (5'TOP mRNAs), many of which encode components of the translational machinery (such as ribosomal proteins). Thus, by increasing the number of ribosomal proteins and translation factors, S6-directed translation can increase translational capacity.

The TSC proteins are downstream effectors of the PI3K/AKT [105,106] and RAS/MAPK [25,107] pathways that can activate mTORC1 downstream of various neuronal growth factor receptors and neurotransmitter receptors [103,108]. Additionally, the TSC proteins are downstream of AMPK and relay information regarding cellular energy status and nutrient availability to the mTORC1 pathway [109].

#### mTORC2

The other protein complex that contains mTOR is mTORC2 [110]. In this complex, mTOR is associated with rictor (rapamycin-insensitive companion of mTOR), GβL and mSIN1 (mammalian stress-activated protein kinase interacting protein). Unlike mTORC1, mTORC2 is upstream of AKT: phosphorylation of AKT at serine 473 by mTORC2 is required for the complete activation of AKT [111]. It remains to be determined if AKT-mediated phosphorylation of Tsc2 inhibits the ability of the TSC proteins to promote mTORC2 activation [112]. In this case, the TSC proteins, mTORC2 and AKT would form a negative feedback loop [113]. In contrast to mTORC1, which is inhibited by rapamycin, mTORC2 is rapamycin-insensitive. Prolonged rapamycin treatment, however, inhibits mTORC2 indirectly [114].

There are also some feedback mechanisms for TSC/mTOR signaling (distinct from the potential feedback loop between TSC proteins, mTORC2 and AKT). Cells lacking TSC proteins do not activate AKT downstream of receptor tyrosine kinases [57]. Reduced phosphorylation of Akt (S473) has also been found in the brains of homozygous SynI-Cre neuronal *Tsc1* mutant mice, indicating that similar mechanisms are at work in the CNS [57]. Inhibition of mTORC1 with rapamycin reversed this block of Akt activation [57], indicating that negative feedback mechanisms were mTORC1-dependent.

The functions of the TSC proteins (inhibiting mTORC1, alleviating mTORC1-dependent negative feedback mechanisms that limit AKT activation and promoting mTORC2-dependent AKT activation) cooperate to divert AKT signaling away from mTORC1 to other downstream targets. The loss-of-function of TSC proteins may negatively affect AKT signaling in at least two ways: First, increased mTORC1 activity can abolish AKT activation via negative feedback regulation; in addition, loss-of-function of TSC proteins can negatively affect mTORC2-mediated AKT activation. The former mechanism is sensitive to correction by rapamycin or other mTORC1 inhibitors [57], but attenuation of mTORC2-mediated AKT activation should not be responsive to rapamycin treatment. Accordingly, rapamycin treatment would not be expected to restore TSC-related phenotypes caused by mTORC2 hypoactivation. As a matter of fact, prolonged rapamycin treatment could even further exacerbate TSC-related phenotypes caused by mTORC2 hypoactivation of mTORC2.

#### Box 3. ASD and macrocephaly

Approximately 10–30% of ASD cases are associated with macrocephaly [115]. Head circumference might be normal at birth but macrocephaly often manifests within the first four years of life [116,117]. In the subsequent course of development, macrocephaly may disappear or persist into adulthood. This early overgrowth phenotype is a well-replicated finding, although study populations are typically small in size and presumably heterogeneous with respect to etiology, suggesting that a large subset of ASD risk factors converge on this shared phenotype. Brain overgrowth occurs during a period of synapse formation, pruning and myelination, which take place during these early postnatal periods. Additionally, changes in neuron numbers or soma volume, dendritic and axonal arborization as well as glial changes could contribute to the observed increased brain volume. The cellular and molecular mechanisms of ASD-related macrocephaly remain to be elucidated; it is possible that alterations in TSC-related cell signaling pathways contribute to this apparently common phenomenon in ASD populations.

Macrocephaly is also present in the ASD-associated single-gene disorders, including TSC, Neurofibromatosis type I and Lhermitte-Duclos syndrome. Accelerated early head growth has also been observed in individuals affected by FXS [118]. Patients with FXS

and ASD have a further increase in head circumference relative to those with FXS but no ASD. Disinhibition of mTOR signaling is common to all of these disorders, and it plays an important role in regulating cell size and organ size, including brain size. Disinhibited mTOR signaling contributes to brain enlargement via cellular hypertrophy and, to some extent, hyperplasia [37,38,45,46,55,58,119]. Neuron-specific homozygous deletion of the *Tsc1* or *Pten* genes in mice led to substantial brain enlargement, at least in part because of massive neuronal hypertrophy and enlargement of the dendritic tree [37,38,45,55,58], which was also accompanied by astrogliosis [45]. Homozygous deletion of *Tsc1* in astrocytes caused astrocytic hypertrophy and hyperplasia [46,119,120]. It remains to be determined if altered TSC/mTOR signaling plays a role in other types of ASD that have associated macrocephaly.

#### Box 4. mTOR inhibitor-based treatments for ASD in humans?

The data from animal models of TSC and other ASD-related disorders show that a sizeable portion of the neurological phenotypes associated with pathogenic mutations is caused by altered mTOR signaling and can be corrected using mTOR inhibitor-based treatment approaches, even if treatment is initiated in adult animals. These findings may have important therapeutic implications for TSC-related ASD and other autism-related conditions associated with altered mTOR signaling.

Rapamycin is an FDA-approved drug that has been used in transplantation medicine for many years. Discovered in soil samples from the Easter Islands, rapamycin occurs naturally as antibiotic synthesized by *Streptomyces hygroscopius*. The mechanisms by which rapamycin inhibits mTOR is not fully understood but it is known that rapamycin associates with FKBP12 to bind to mTOR's FRB (FKBP12-rapamycin-binding) domain. Binding of the rapamycin-FKBP12 complex to mTOR may destabilize the mTORC1 complex [121] and interfere with the activation of mTOR by phosphatidic acid [122]. A number of new compounds are available to inhibit mTOR, either by interfering with complex formation (FKBP12-dependent or FKBP12-independent) or by directly inhibiting mTOR's catalytic domain [123].

To date, mTOR inhibitors were tested against brain and kidney tumors associated with TSC, as well as a TSC-related lung condition (lymphangioleiomyomatosis) and had beneficial effects [125–127]. There are planned and ongoing clinical trials to evaluate rapamycin's effects on cognition, behavior and neurological symptoms in TSC [128]. Although it is unclear how rapamycin would modulate cognitive disability associated with cortical tubers or infantile spasms, findings in animal models suggest that rapamycin could ameliorate at least some aspects of cognitive dysfunction in individuals with TSC, even if treatment is started after cessation of development [8].

Preliminary clinical studies have already been performed in other ASD-related disorders to evaluate whether correcting the underlying molecular pathology results in functional improvements in human populations. The effects of mGluR5 inhibitors and lithium on FXS-related neurological phenotypes have been tested in initial studies. A pilot, open-label trial suggested that lithium improves several different outcome measures in adult FXS individuals [129]. In addition, a pilot, open-label, single-dose study using fenobam, an mGluR5 antagonist, suggested improvements in FXS-related pre-pulse inhibition (PPI) deficits [130]. Limitations of these initial studies are small sample sizes and the lack of placebo control groups. Nevertheless, these pilot studies justify further clinical trials to determine whether mGluR5 inhibitors have beneficial effects on neurological impairments in human FXS populations. The potential value of mTOR inhibitors for treating FXS-related neurological phenotypes should be assessed in preclinical models.

With respect to NF1, a placebo controlled clinical study using a brief 12-week simvastatin treatment did not find significant overall effect on the cognition of individuals with Neurofibromatosis type I [131]. Nevertheless, treatment resulted in dramatic improvement in an object assembly task [131]. Notably, the treatment had the biggest impact on individuals with the poorest performances, whereas it did not affect the performance of subjects with scores within the normal range. Although promising, longer treatments with larger sample sizes are needed to confirm that statins have a beneficial impact on the overall cognition of patients with Neurofibromatosis type I.

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#### Figure 1.

Schematic illustration of TSC-mTOR signaling. For detailed information, please refer to the main text and Boxes 2 and 4.