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mTOR, a Potential Target to Treat Autism Spectrum Disorder



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Abstract: Mammalian target of rapamycin (mTOR) is a key regulator in various cellular processes, including cell growth, gene expression, and synaptic functions. Autism spectrum disorder (ASD) is frequently accompanied by monogenic disorders, such as tuberous sclerosis complex, phosphatase and tensin homolog tumor hamartoma syndrome, neurofibromatosis 1, and fragile X syndrome, in which mTOR is hyperactive. Mutations in the genes involved in the mTOR-mediated signaling pathway have been identified in some cases of syndromic ASD. Evidences indicate a pathogenic role for hyperactive mTOR-mediated signaling in ASD associated with these monogenic disorders, and mTOR inhibitors are a potential pharmacotherapy for ASD. Abnormal synaptic transmission through metabotropic glutamate receptor 5 may underlie in a part of ASD associated with hyperactive mTOR-mediated signaling. In this review, the relationship between mTOR and ASD is discussed.

Keywords: Autophagy, fragile X syndrome, mammalian target of rapamycin, metabotropic glutamate receptor 5, neurofibromatosis, phosphatase and tensin homolog, tuberous sclerosis complex.

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INTRODUCTION

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that critically regulates important cellular physiology, such as protein synthesis and mRNA translation. This regulation occurs in response to a diverse range of stimuli from outside the cell, such as growth factors, cytokines, energy starvation, and hypoxia, and affects cell growth, cell differentiation, and metabolism. The relevance of mTOR in human diseases is increasingly appreciated in the field of oncology [1], immunology [2], metabolism [3], and neurology [4], including autism spectrum disorder (ASD).

ASD is a neurodevelopmental disorder of which the core symptoms include impairment in reciprocal social interaction and restrictive, repetitive behaviors and interests [5]. Its incidence has been reported to be increasing, and the current prevalence is estimated to be approximately 1% of the general population. Twin studies revealed that the monozygotic concordance reaches as high as 90%, whereas the concordance is substantially lower in dizygotic twins, implying that genetics have significant impact on an individuals' susceptibility to ASD [6]. Thus, individuals with heritable disorders, such as tuberous sclerosis complex (TSC) and fragile X syndrome (FXS), demonstrate ASD symptoms at a particularly high frequency. Recent advances in the genetic research of ASD have revealed that many genetic disruptions are found in individuals with ASD, including single gene mutations and copy number variations, in different chromosomal

regions [7]. Understanding the molecular pathophysiology of ASD-associated disorders, such as TSC and FXS, has also progressed. An accumulating body of evidence highlights the causal role of dysregulated mTOR signaling pathway in a subset of individuals with ASD and provides us with an insight to understand the molecular pathophysiology of ASD and potential pharmacological therapies.

MAMMALIAN TARGET OF RAPAMYCIN

mTOR associates with several protein components to form two distinct complexes. mTOR complex 1 (mTORC1) is characterized by a critical component called raptor [8, 9] and regulates protein synthesis, energy metabolism, cell growth and proliferation, and cap-dependent mRNA translation [3]. mTOR inhibitors, such as rapamycin and everolimus, block mTOR-raptor coupling and inactivate mTORC1 [8, 9]. The other mTOR is mTOR complex 2 (mTORC2), which has a rictor instead of a raptor. The absence of a raptor makes mTORC2 insensitive to mTOR inhibitors [10, 11]. mTORC2 controls cellular functions, such as cytoskeletal rearrangement and cell survival [10, 11].

Upstream of mTORC1, a number of stimuli, such as growth factors, cytokines, energy starvation, and hypoxia, are integrated by TSC 1/2 complex. The *TSC1* product hamartin and the *TSC2* product tuberin form a heterodimer complex [12, 13] (Fig. 1). TSC2 has GTPase-activating protein (GAP) activity that is regarded as the most important of TSC1/2 complex [14, 15]. The central role of TSC1 is to stabilize the complex as TSC1 couples with TSC2 and prevents TSC2 from ubiquitin-mediated degradation [16]. In addition, TSC1 may have mTORC1-independent functions.

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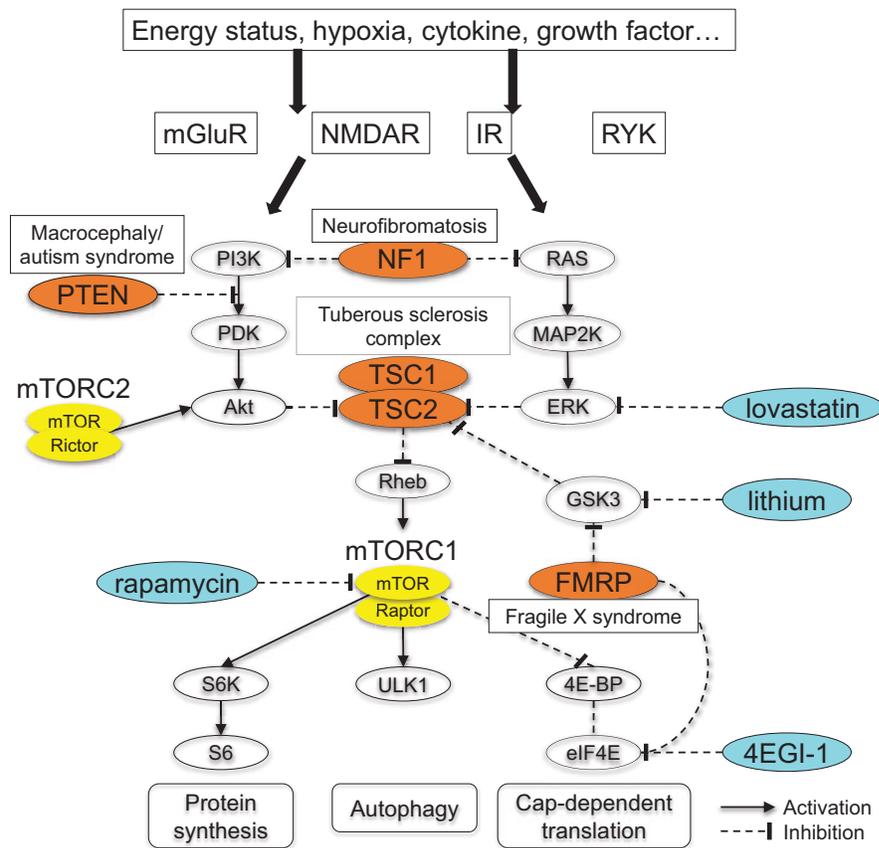


Fig. (1). Signaling pathway involving mTORC1. Various stimuli converge on mTORC1 pathway via a number of routes. mTORC1 activation impacts on different cellular physiology, such as protein synthesis, cap-dependent mRNA translation, and autophagy. Here mutations in *TSC1/2*, *PTEN*, *FMR1*, and *NF1* cause constitutive mTORC1 activation and syndromic ASD. Akt, protein kinase B; eIF4E, eukaryotic initiation factor 4E; ERK, extracellular signal-regulated kinase; 4E-BP, 4E-binding protein; FMRP, fragile X mental retardation protein; GSK3, glycogen synthase kinase 3; IR, insulin receptor; MAP2K, mitogen-activated protein kinase kinase; mGluR, metabotropic glutamate receptor; mTOR, mammalian target of rapamycin; NF1, neurofibromin; NMDAR, NMDA receptor; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; RAS, rat sarcoma; Rheb, Ras homolog enriched in brain; RYK, receptor-like tyrosine kinase; S6K, p70 ribosomal S6 kinase; TSC, tuberous sclerosis complex; ULK1, unc51-like kinase 1.

Tsc1-deficient cells showed deficient rearrangement of the actin cytoskeleton that did not respond to rapamycin, and this phenotype was rescued by inhibiting the Rho-associated protein kinase (ROCK), a downstream effector of the small GTPase RhoA [17]. Its significance is, however, less clear particularly with respect to human disorders. The TSC1/2 complex decreases the GTP-bound active form of the small G-protein Ras homolog enriched in brain (Rheb) and results in constitutive activation of mTORC1 [18].

mTORC1 exerts its kinase activity on target proteins ribosomal protein S6 kinases (S6Ks) and eukaryotic translation initiation factor-4E (eIF4E)-binding proteins (4E-BPs). Two S6K proteins S6K1 and S6K2 exist in mammalian cells. mTORC1 phosphorylates both S6K1 and S6K2, whereas other regulators phosphorylate either S6K1 or S6K2. For example, Neurabin acts on S6K1 and ERK on S6K2. Most of the known regulators activate S6Ks while S6K1 dephosphorylation by PP2A inhibits S6K1. The active form of S6Ks then phosphorylates ribosomal protein S6 and increases general protein synthesis. S6K1 has several isoforms, and p70-S6K1 is most extensively studied [19]. There are

three paralogues of 4E-BPs (4E-BP1, 4E-BP2, and 4E-BP3) [20], and 4E-BP2 is the major form in the mammalian brains. 4E-BP1 and its phosphorylated form are used as markers of mTORC1 activity. 4E-BPs bind to the cap-dependent transcription factor eIF4E to repress cap-dependent mRNA translation. mTORC1 phosphorylates 4E-BPs and make them lose their binding activity to eIF4E. This leads to the deregulation of eIF4E and enhances the subsequent initiation of cap-dependent mRNA translation [21-23]. In this manner, the two genes critically control mTORC1-mediated protein synthesis. The third major substrate of mTORC1 is Unk-51-like kinase 1 (ULK1) that acts as a repressor of autophagy [24, 25], a process that removes damaged organelles and generates energy. mTORC1 activation suppresses ULK1 and enhances autophagy.

SYNDROMIC ASD ASSOCIATED WITH mTORC1 HYPERACTIVATION

As noted above, the mTORC1 signaling pathway has a central role in cell growth and proliferation. A number of human disorders that are caused by mutations of the genes

involved in this pathway, such as TSC and neurofibromatosis type 1 (NF1), are characterized by the high susceptibility to tumor development (Fig. 1). The associated tumors are basically benign in TSC [26] and NF1 [27], whereas those seen in Cowden syndrome caused by mutations in the phosphatase and tensin homolog (*PTEN*) gene are at a high risk of malignancies [28]. Tumor susceptibility in these disorders can be explained by the germline loss of heterozygosity and additional mutations in the other allele. In contrast, FXS individuals are not at an increased risk of tumor development [29], and enlarged testes are a common physiological feature in the male patients.

ASD and intellectual disability are common neurological features in TSC, FXS, NF1, and a set of disorders caused by *PTEN* mutations. Considering that ASD manifests from infancy when other neurological and neoplastic symptoms do not yet appear, it is speculated that germline haploinsufficiency and consequent mTORC1 activation are sufficient for the development ASD in these disorders. In the following context, the above ASD-related monogenic disorders are discussed with respect to dysregulated mTORC1-mediated signaling.

There are several of genes in this signaling pathway that are associated with ASD [7]. Of note, four human monogenic disorders, TSC, FXS, macrocephaly/autism syndrome caused by a mutation in *PTEN*, and NF1, are caused by mutations in the genes upstream of mTORC1. These findings imply that mTORC1 overactivation is one of the common pathological events underlying ASD.

Tuberous Sclerosis Complex

TSC (MIM#191100, #613254) is an autosomal dominant disorder presenting with disease manifestations in different organs, such as skin, brain, and kidney. Long before the discovery of the causative genes, the disorder was recognized in individuals presenting with the classical “triad” of a facial skin lesion (angiofibroma), epilepsy, and intellectual disability [30]. Later *TSC1* [31] and *TSC2* [32] were identified as the causative genes. TSC is estimated to occur in 1 in 6,000 live births [33], according to the former diagnostic criteria [34]. The recently revised criteria include the results of genetic testing in addition to the original clinical hallmarks including skin features (facial angiofibroma, hypopigmented macules, shagreen patch), neurological features (cortical tuber, subependymal nodule), and hamartomatous lesions in different organs (renal and retinal angiomyolipoma, cardiac rhabdomyoma, lung lymphangiomyomatosis) [26]. *TSC1* and *TSC2* mutations basically cause similar features although individuals with *TSC2* mutations are more severely affected in certain aspects: more severe skin and kidney involvement [35, 36], more severe intellectual disability and heavier seizure burden [36, 37], and ASD [37, 38].

TSC is characterized by various CNS pathological complications, many of which are also specific including cortical tubers and subependymal giant cell astrocytoma [26]. Neuropsychiatric manifestations of TSC are also diverse: epilepsy that is often intractable [39], intellectual disability, ASD, attention-deficit hyperactivity disorder, and anxiety [40]. These manifestations are not specific, but they are highly prevalent and make daily life difficult for individuals

with TSC. The concept of TSC-associated neuropsychiatric disorders is thus introduced in the latest guideline [41, 42]. As for ASD, approximately half of the individuals with TSC have ASD [40, 43]. TSC accounts for 1%–4% of all ASD cases and is a typical monogenic ASD [44].

As described above, *TSC1* and *TSC2* coordinate to inhibit downstream Rheb and mTORC1 (Fig. 1). Somatic mutations of *TSC1* and *TSC2*, mainly the loss of heterozygosity, abolish the inhibition of mTORC1 [35, 36]. The consequent constitutive overactivation of mTORC1, particularly the enhanced phosphorylation of S6Ks and 4E-BPs leading to increased global protein synthesis and cap-dependent mRNA translation, is considered to constitute the central molecular pathology of TSC.

Two groups of rodent models of TSC are currently available, each resulting from different genetic manipulations (Table 1). The first group includes germ-line haploinsufficient models, such as *Tsc1*^{+/-} [45], *Tsc2*^{+/-} mice [46], and Eker rats [47]. Conditional knockout (KO) mice that lack *Tsc1* or *Tsc2* in glial fibrillary acidic protein (GFAP)-positive cells [48, 49] or in Purkinje cells [50, 51] have also been analyzed in detail. Haploinsufficient models present with impaired social interaction, the core behavioral feature of ASD [52–54], and learning deficits in the domains of spatial and working memory [55]. Of note, these animals basically have no epileptic seizures and less severe morphological changes in the brain compared with human TSC individuals [45, 46, 52]. Mice lacking *Tsc1* or *Tsc2* in Purkinje cells also show no evidence of epileptic seizures, but more autistic-like behavioral alterations, such as social impairment, restrictive behavior, and abnormal ultrasound vocalizations, are observed [50, 51]. The neurological phenotype is much more severe in mice with astrocyte-specific deletion of *Tsc* genes. The affected animals frequently suffer from premature death. The animals that survive develop spontaneous seizures and progressive macrocephaly and die before 5 months of age. Intriguingly and in accordance to clinical observation, *Tsc2* KO mice experience more frequent seizures and more premature death with higher levels of mTORC1 activity compared to *Tsc1* KO mice [56]. However, the precise mechanism relating the higher mTORC1 activity with the *Tsc2* deletion is yet to be determined.

At the neuronal level, the *TSC1/2* complex regulates neuronal morphology and synaptic function in the brain. Complete deletion of *Tsc1* and *Tsc2* in the Purkinje cells leads to progressive Purkinje cell loss and abnormal dendritic morphology including increased spine density, and heterozygous deletion is sufficient to reduce excitability of Purkinje cells [50, 51]. GFAP-positive cell-specific loss of the genes causes unregulated astrocyte proliferation, neuronal death independent of seizures, and neuronal disorganization in the brain [48, 56]. Altered structure, an increased number of dendritic spines, and impaired axon guidance are also associated with TSC [57, 58]. An interesting key to link synaptic deficits and ASD is deficient mTORC1-mediated autophagy. Distorted synaptic morphology was observed in the brains of *Tsc2*^{+/-} mice and human sporadic ASD, and impaired autophagy was detected in both conditions [59]. The mechanism of autophagy controlled by the mTORC1/ULK1 pathway may be complicated as autophagy was unexpectedly enhanced in *Tsc1/2* deleted dividing cells [60].

Table 1. mTOR-related disorders and their animal models discussed in this review.

Genes	Human Disorders	Animal Models
<i>TSC1</i> <i>TSC2</i>	Tuberous sclerosis complex <i>TSC1</i> or <i>TSC2</i> haploinsufficiency Skin (e.g. facial angiofibroma) Brain (e.g. cortical tuber) Hamartomas (e.g. renal angiomyolipoma) Often presents with epilepsy, intellectual disability. ASD in approximately half of the patients.	Haploinsufficient mice No brain lesions, no epilepsy ASD-like social behavior Learning deficits Eker rats (<i>Tsc2</i> haploinsufficiency) Brain lesions in rare cases, no epilepsy Reduced social interaction Purkinje cell-specific KO mice Progressive loss of PCs ASD-like social behavior GFAP-positive cell-specific KO mice Seizures, progressive brain enlargement Early mortality
<i>PTEN</i>	Macrocephaly/autism syndrome PTEN haploinsufficiency Marked macrocephaly Found in 7%-17% of ASD with macrocephaly. Note: also the cause of Bannayan-Riley-Ruvalcaba syndrome Lhermitte-Duclos syndrome Cowden syndrome	Haploinsufficient mice ASD-like social behavior Increased brain weight NSE-positive cell specific KO mice Reduced social interaction Seizures, increased anxiety, increased locomotor activity Early mortality
<i>FMR1</i>	Fragile X syndrome > 200 CGG repeats in <i>FMR1</i> X-linked inheritance Intellectual disability, macrocephaly, macroorchidism in males ASD more often in males (30%)	<i>Fmr1</i> KO mice (only males studied) ASD-like social behavior Increased anxiety, increased locomotor activity, cognitive deficits
<i>NF1</i>	Neurofibromatosis type 1 <i>NF1</i> haploinsufficiency Skin (e.g. café-au-lait spots) Neurofibromas ASD in 15%-30% of the patients	Haploinsufficient mice Learning deficits, attention deficits Impaired social discrimination

NSE, Neuron Specific Enolase.

The TSC/mTORC1 pathway also controls synaptic plasticity and long-term memory via metabotropic glutamate receptor (mGluR)-mediated long-term potentiation (LTP) [55, 61] and long-term depression (LTD) [62, 63]. Impaired LTP in the hippocampus of *Tsc1* KO mice is due to elevated levels of extracellular glutamate [61]. Deregulation of mTORC1 perturbs neural excitatory/inhibitory balance at the

network level by exaggerating excitability and reducing inhibitory synaptic transmission [64]. The above synaptic and cellular alterations may ultimately converge on the development of neurocognitive impairments in TSC including ASD.

The therapeutic implication of TSC-associated neurological problems is raised by the observation that the mTOR inhibitor rapamycin has effects for reversing behavioral and

molecular abnormalities. In haploinsufficient models, transient treatment with rapamycin is sufficient to effectively suppress mTORC1 [53, 55], rescue normal LTP [55] and LTD [62], and correct learning deficits [55] and autistic-like behavioral deficits [53]. Rapamycin shows similar benefits in conditional KO model; prevention of brain enlargement, epileptic seizure, and early mortality [56]; autistic-like deficient social approach [50]; and Purkinje cell loss and abnormal morphology [50, 51]. Moreover, the clinical use of mTOR inhibitor improves cognitive function and relieves behavioral problems, as will be discussed later in more detail.

Macrocephaly/Autism Syndrome Caused by *PTEN* Mutation

Increased head size is an occasional physical feature of individuals with ASD, particularly in early life [65]. Mutations in the *PTEN* gene were identified in those with ASD accompanied by extreme macrocephaly, and this condition was named thereafter “macrocephaly/autism syndrome” (MIM#605309) [66–68]. With respect to human diseases, the *PTEN* gene was originally found as the cause of Bannayan–Riley–Ruvalcaba syndrome (MIM#153480) (manifestation: macrocephaly, intellectual disability, multiple intestinal hamartoma, etc.), Lhermitte–Duclos syndrome (manifestation: cerebellar ataxia, seizure, etc.), and Cowden syndrome (MIM#158350) (manifestation: macrocephaly, mucocutaneous lesions, intestinal polyps, increased risk of malignancies) [69, 70]. As these disorders and macrocephaly/autism syndrome share the *PTEN* gene mutations, they are now recognized as the spectrum called PTEN hamartoma tumor syndrome [28]. From a neurological aspect, macrocephaly and developmental problems, including ASD, are common features along the spectrum, whereas hamartomatous and other features are characteristic in each disorder [28]. *PTEN* mutations can be detected in 7%–17% of individuals with ASD with extreme macrocephaly [62, 66, 67], and they are estimated to be found in 1% or more of all individuals with ASD.

The *PTEN* gene product negatively regulates the phosphoinositol 3-kinase (PI3K), which decreases the levels of phosphatidylinositol (3,4,5)-triphosphate (PIP₃; see figure). This then suppresses PDK and Akt, activates the TSC1/2 complex, and keeps mTORC1 repressed, constituting the PI3K/Akt/TSC/mTORC1 signaling pathway [71]. Mutations in the *PTEN* gene abolish inhibition of PI3K that results in Akt activation, TSC1/2 complex inhibition, and constitutive mTORC1 activation (Fig. 1).

The neurological issues accompanied by *PTEN* mutations have been extensively studied using haploinsufficient mice [72, 73] and conditional KO mice [74, 75] (Table 1). Similar to the mouse models of TSC, *Pten*^{+/-} mice show a mild neurological phenotype: increased brain weight, impaired prepulse inhibition, and altered social behavior reminiscent of ASD [76–78]. Most of neuron-specific enolase-positive cell-specific *Pten* conditional KO mice die in early postnatal weeks. The mice who survive exhibit macrocephaly that is progressive and more severe than that observed in *Pten*^{+/-} mice impaired prepulse inhibition and prominent behavioral abnormalities, such as diminished reciprocal social interac-

tion, increased locomotor activity, more anxiety, memory deficit, and spontaneous seizures [74, 75].

Neurons from *Pten* KO mice display increased phosphorylation of Akt and S6, indicating the overactivation of the PI3K/Akt/TSC/mTORC1 signaling pathway [74, 75]. Neuronal alteration includes increased soma size and axonal growth, hypertrophic and ectopic axonal projections, and abnormal synapses with increased presynaptic varicosities. Dendrites are also hypertrophic and accompanied by increased spine density [74]. According to Zhou, *et al.* [75], chronic administration of rapamycin from an early postnatal period onward prevented brain enlargement and neuronal soma hypertrophy and alleviated axonal and dendritic hypertrophy. This treatment kept the mutant mice in a generally healthy condition as long as the treatment continued, and it prevented phenotypic abnormalities observed in the nontreated mutants, such as impaired social interaction, elevated anxiety, and the development and worsening seizures [75]. Older mice also displayed improvements in neuronal abnormalities and seizures although the behavioral effect was difficult to assess because of handling-related death. It is therefore suggested that the abnormal features of *Pten* conditional KO mice are due to elevated mTORC1 activity and preventable by keeping mTORC1 inhibited.

Fragile X Syndrome

FXS (MIM#300624) is the most common cause of inherited intellectual disability [79]. It is an X-linked disorder with intellectual disability, ASD, increased anxiety, and physical features including macrocephaly and macroorchidism in males [80]. Unlike other disorders mentioned in this review, the genetic defect in most FXS cases is abnormal elongation of CGG repeats in the 5'-untranslated region of fragile X mental retardation 1 (*FMR1*) gene [81]. Typical FXS individuals have a CGG repeat size more than 200, called as “full mutation” [82]. An *FMR1* repeat size between 55 and 200 (premutation) is shorter than that in typical FXS individuals but longer than that in the normal population that has a repeat size <55. Those with such a repeat size do not show typical FXS manifestation, whereas some are affected with the characteristic movement disorder named fragile X-associated tremor/ataxia syndrome (FXTAS) in later life [83]. An individual with a premutation with or without FXTAS is at risk of neuropsychiatric disorders, such as a mood disorder or a panic disorder [84]. FXS as diagnosed by detection of the full mutation is found in 1 in 7,100 males and 1 in 11,000 females [85]. Twenty-two percent of individuals have ASD, and the prevalence rises to 30% in males [43]. Approximately 1%–5% of cases of ASD are accompanied by FXS, and it is yet one of the most common syndromic ASD [44].

FMR1 encodes the protein fragile X mental retardation protein (FMRP), an RNA-binding protein involved in gene translation and enhancing global protein synthesis. Its binding targets are very diverse and include postsynaptic density-95, the GluR1 and GluR2 subunits of the AMPA glutamate receptor, and several other synaptic proteins important for neurotransmission and structure [86, 87]. Although the precise mechanism is less clear, deficiencies in FMRP are associated with increased mTORC1 signaling [88]. CGG repeat

elongation silences the *FMR1* gene via hypermethylation of CpG island in its promoter region. Point mutations in the *FMR1* gene (for example, nonsense mutation, missense mutation, and frameshift mutation) are rarely found in FXS. Both FXS-causing mutations, CGG repeat elongation and point mutation, commonly lead to the development of FXS by abolishing the production of FMRP.

Regarding animal models of FXS, *Fmr1* KO mice have been extensively analyzed [89] (Table 1). Several kinds of independently generated *Fmr1* KO mice exhibit prominent behavioral abnormalities (see reviews [90, 91]). With regard to autistic-like behavioral deficits, a number of studies using the three-chambered social approach test report normal behavior, whereas impaired sociability, the absence of a preference to explore the novel mouse over the novel inanimate object, has been observed in other research [92, 93]. Detailed analysis reveals that *Fmr1* KO mice display fewer affiliative behaviors during social interactions with female mice [94]. Repetitive behaviors, the other major behavioral alteration in ASD, are also observed *Fmr1* KO mice. For example, the mutant animals exhibit higher levels of self-grooming behavior [94]. *Fmr1* KO mice show diverse abnormal features, such as increased anxiety, increased locomotor activity, cognitive deficits in different behavioral tasks, and deficient prepulse inhibition (see reviews [90, 91]).

At the neuronal level, the brain of young *Fmr1* KO mice shows increased spine density and length, and increased immature, thin spines are observed in the brain of adult mice [95]. This parallels the findings from postmortem analyses of the brain from individuals with FXS [96]. FMRP depletion also affects synaptic function, such as LTP and LTD, involving mGluR. An analysis of synaptic plasticity in *Fmr1* KO mice revealed that LTD triggered by mGluR was enhanced, whereas NMDA-dependent LTD was normal [97], leading to the proposal of “mGluR theory of FXS” [98]. In line with this theory, *Fmr1* KO mice exhibited persistent of mGluR-mediated LTD [99] and deficient LTP [100]. The causal role of mGluR5 in the FXS phenotype was suggested by the observation that the mGluR5 negative allosteric modulator MPEP inhibited audiogenic seizures in the mutant [101]. An analysis of *Fmr1* KO mice crossed with *Gmr5*^{+/-} mice, mutants that express half the normal level of mGluR5, demonstrated the critical role of enhanced mGluR5 activity in a wide range of neurological symptoms of FXS [102]. Increased dendritic spine density and elevated levels of protein synthesis in the hippocampus of *Fmr1* KO mice was rescued by reducing mGluR5 expression. These findings were accompanied by the reversal of exaggerated memory extinction and susceptibility to audiogenic seizures [102]. Thus “mGluR theory of FXS” is now widely accepted to explain the diverse neurological phenotype in FXS [103]. Moreover, inhibitors of mGluR5 are being investigated as candidate agents to improve cognitive deficits in human FXS [104].

Recently, attention is being paid to the link between the FMRP and mTORC1 pathway as another pathomechanism in neurocognitive deficits in FXS. Increased levels of phosphorylated S6K1, indicating hyperactivation of mTORC1, were found in the hippocampus of *Fmr1* KO mice [88]. In line with this, evidence of mTORC1 activation was also identified in fibroblasts, lymphocytes, and postmortem brain

tissue of human individuals with FXS [105, 106]. Accumulating evidence suggests a connection between the deletion of FMRP and deregulation of mTORC1 in FXS. Upon FMRP depletion, exaggerated activity of PI3K and Akt was found in *Fmr1* KO mice, suggesting a pathological role of excess signaling through the PI3K/Akt pathway to enhance mTORC1 activity [107]. Increased expression of the FMRP binding protein CYFIP1 was another finding in *Fmr1* KO mice to link FXS to excess protein synthesis via eIF4E [108]. To test whether mTORC1 activation contributes to the development of ASD-like phenotype in FXS, S6K1 KO mice were crossed to *Fmr1* KO mice to genetically suppress mTORC1-mediated protein synthesis. The *Fmr1*-S6K1 double mutants displayed a reversal in excessive protein synthesis and autistic-like behavioral deficits in *Fmr1* KO mice [109]. *Fmr1* KO mice exhibited increased glycogen synthase kinase 3 (GSK3) activity that lowers TSC1/2 complex activity, which likely results in mTORC1 activation [110, 111]. The pathogenic role of GSK3 activation in autistic-like deficient social recognition of *Fmr1* KO mice was demonstrated by pharmacological and genetic intervention. The abnormal behavior was improved by lithium, which acts as a GSK3 inhibitor, and also corrected by knocking-in the *Gsk3b* gene with an FMRP-resistant inactive mutation [110, 111]. Another example for discussing the relationship between mGluR5 and mTORC1 is the BTBR T + tf/J (BTBR) inbred strain of mice. This strain of mouse has autistic-like behavioral abnormalities that were rescued by an mGluR5 antagonist, as seen in *Fmr1* KO mice [112]. Strikingly, rapamycin treatment also alleviated impaired social behaviors of BTBR mice [113]. The preclinical evidence suggests the therapeutic possibility of mTOR inhibitors to correct autistic-like behavioral features in FXS.

Neurofibromatosis Type 1

NF1 (MIN#162200) is an autosomal-dominant and common neurocutaneous disorder (a group of diseases whose features primarily include the brain and skin) that occurs in 1 in 2,500–3,000 live births [114]. A clinical diagnosis of NF1 is made depending on the criteria that mainly require various skin manifestations [27]. Detailed information about neurocognitive features is not required for the diagnosis although the entire phenotype is much more diverse than described in the criteria [114]. With respect to neurological symptoms, NF1 individuals frequently show signs of visuospatial and visuomotor deficits, attention deficit/hyperactivity disorder, and ASD. According to recent epidemiological studies, the frequency of ASD in NF1 is estimated to be as high as 15%–30% [43] [115–117]. Based on these observations, NF1 is now one of the most prevalent causes of monogenic ASD.

With respect to signaling cascades, NF1 is a RASopathy, a set of disorders associated with deregulation of the rat sarcoma (RAS)/MAPK pathway. Other RASopathies include Noonan syndrome and Costello syndrome [118]. This pathway begins with the activation of receptor tyrosine kinase and downstream RAS proteins (H-RAS, K-RAS, N-RAS). This then stimulates mitogen-activated protein kinase kinase and extracellular signal-regulated kinase (ERK). The TSC1/2 complex, which is located downstream of ERK, receives inhibitory inputs from ERK. In this pathway, NF1 works as a negative regulator of RAS proteins. The heterozygous loss of

the *NF1* gene abolishes the inhibition of the RAS signaling pathway and results in constitutive activation of ERK1/2 and mTORC1 [120, 121], which are considered as the central mechanism underlying NF1 (Fig. 1). With respect to mTORC1, NF1 has another major function as a repressor of PI3K/Akt pathway. Analysis of *NF1*-deleted primary cells revealed that the NF1 loss was associated with enhanced activity of Akt and S6K1. The PI3K inhibitor wortmannin inhibited both Akt and S6K1, whereas rapamycin suppressed S6K1 but not Akt [119], suggesting that NF1 deletion affects different signaling pathways that converge on the common effector, mTORC1 (Fig. 1).

Mice with a heterozygous loss in the *Nf1* gene show neurocognitive deficits compatible with those of human NF1 individuals (Table 1): spatial learning deficits [121], attention deficits [120], and impaired discrimination of social stimuli relevant to ASD [122]. These behavioral changes in mice are associated with the activation of ERK and Akt, both of which enhance signal transmission mediated by mTORC1 [119, 120]. With this regard, *Nf1*^{+/-} mice have decreased hippocampal LTP, both in the early and late phases [121, 123]. Studies using several lines of *Nf1* conditional KO mice suggest that neurocognitive features of NF1 are centrally attributable to neuronal loss of the gene and consequent increases in GABA release [124]. Lovastatin, a farnesylation inhibitor that negatively acts on RAS and ERK, reverses the behavioral deficits seen in *Nf1*^{+/-} mice [120, 121], and GABA receptor antagonists also restored hippocampal LTP in the mutants [121]. This body of evidence led to clinical trials evaluating the neurocognitive effects of the drugs. In spite of clear preclinical findings, individuals with NF1 showed minimal beneficial changes after treatment with simvastatin, whereas the participants with intellectual disabilities responded substantially [125]. To effectively prevent abnormal signaling in NF1, a molecular target that is even more downstream in the signaling pathway may need to be identified and studied. Considering that both the RAS/MAPK and PI3K/Akt pathways finally converge on mTORC1, the evaluation of mTOR inhibitors for neurocognitive deficits will be anticipated.

mTORC1 TARGET PROTEINS THAT INCREASE AUTISTIC-LIKE PHENOTYPES

There is a large body of evidence showing that the heterozygous loss of different genes, *TSC 1/2*, *PTEN*, *FMRI*, and *NF1*, is linked to ASD through unregulated mTORC1-mediated signal transduction. The next question is what actually causes ASD when mTORC1 is unregulated. One possible explanation is that mTORC1 activation suppresses 4E-BPs and subsequent cap-dependent mRNA translation is elevated (Fig. 1). 4E-BPs normally inhibit translation initiation by repressing eIF4F complex formation [126]. The complex includes eIF4E (the cap-binding protein), eIF4A (the RNA helicase), and eIF4G (the scaffolding protein bridging RNA to ribosome) [127]. eIF4E preferentially promotes a set of mRNAs with extensive secondary structure [124, 128]. The causal role of eIF4E in ASD was first pointed out by the discovery of individuals with ASD whose *eIF4E* gene had an activating mutation in its promoter region [129]. Further studies revealed that the 4E-BP2 KO and

eIF4E overexpression generated an autistic-like behavioral manifestation, impaired social approach, and repetitive behaviors in mice [130, 131]. The ASD-like behavioral alteration was mediated by enhanced cap-dependent translation and protein synthesis, resulting in an imbalance in excitatory/inhibitory transmission and mGluR-LTD [130, 131]. Furthermore, pharmacological inhibition of this cap-dependent translation using eIF4E/eIF4G interaction inhibitor 4EGI-1 rescued the behavioral, translational, and synaptic deficits [119, 120], emphasizing the pathogenic role of exaggerated cap-dependent mRNA translation in ASD.

The development of ASD via 4EBP/eIF4E-mediated gene translation may be attributable to the expression of specific genes. The deletion of the *4E-BP2* gene preferentially enhanced mRNA translation of synaptic proteins neuroligins (NLgns), and its significance was demonstrated by the observation that *Nlgn1* knockdown alleviated autistic-like behaviors in *4E-BP2* KO mice [130]. The causal role of altered 4E-BP/eIF4E-mediated translational control in ASD is further supported by the loss of FMRP/CYFIP1-mediated translational repression that results in eIF4E-dependent translation in FXS [93]. Another example is the matrix metalloproteinase 9 (*MMP9*) gene. *Fmr1* KO mice exhibit overexpression of the *Mmp9* gene dependent on FMRP and eIF4E and pharmacological suppression of *Mmp9* translation rescued FXS-like phenotypes [121].

Altered mGluR5-LTD is another clue to connect mTORC1 activation to ASD. The idea derives from the observation that the mouse FXS phenotype was rescued by two ways: genetic suppression of mGluR5 expression [102] and genetic deletion of S6K1 mimicking mTORC1 inhibition [109]. The evidence in *Fmr1* KO mice led to the speculation that mTORC1 activation causes ASD via excess mGluR5-LTD. However, it is controversial whether mTORC1 activation exaggerates or attenuates mGluR5-LTD. In one study, *Tsc2*^{+/-} mice exhibited reduced mGluR5-LTD and impaired memory. This deficit was rescued pharmacologically by mTORC1 inhibition and mGluR5 activation and furthermore genetically by crossing with an *Fmr1* KO mice [62]. In contrast, in another report in which *Tsc2*^{+/-} mice were examined, the deficient mGluR5-LTD was observed in the juvenile period, but it was restored in adulthood. Moreover, the learning deficit and perseverative behavior of the mice improved in response to mGluR5 inhibition [132, 133]. Further research is mandatory to overcome these conflicting findings and to better understand the pathophysiology of TSC with respect to mGluR5-LTD.

PHARMACOLOGICAL INTERVENTIONS FOR mTORC1-RELATED ASD

Based on the above discussion emphasizing the pathogenic role of mTORC1 dysregulation, mTOR inhibitors are expected to ameliorate ASD or cognitive impairment in selected sets of individuals. This issue is addressed most intensively in TSC. In the clinical trial of sirolimus for renal angiomyolipomas accompanied by TSC and lymphangio-myomatosis with or without TSC, immediate recall memory and executive function of the participants showed a substantial improvement after sirolimus treatment [134]. Everolimus reduced seizure frequency in individuals with TSC with or

without subependymal giant cell astrocytoma. Moreover, quality-of-life scores, particularly on items related to ASD symptoms, significantly increased after the treatment [135-137]. Based on these promising results, several clinical trials are under way testing mTOR inhibitors for neurocognitive deficits in TSC [104]. Considering the large body of pre-clinical evidence reviewed above, other disorders that are associated with ASD and increased mTORC1 activity may benefit from mTOR inhibitors, such as FXS, NF1, and macrocephaly/autism syndrome with the *PTEN* mutation. Clinical investigation is anticipated to examine the therapeutic efficiency of mTOR inhibitors for mTORC1-related ASD in these disorders.

CONCLUSION

Constitutive mTORC1 activation observed in single-gene, ASD-associated disorders TSC, PHTS, FXS, and NF1 offers insight in understanding the as of yet unclear molecular pathophysiology of ASD. Rodent models of these disorders display autism-related behavioral phenotype and morphological and electrophysiological abnormalities. Evidence is accumulating that mTOR inhibitors mostly rescue these neuronal and behavioral alterations, suggesting the possibility of pharmacological intervention of mTORC1-associated ASD using mTOR inhibitors. On the basis of this preclinical evidence, new translational research will be expected to elucidate the therapeutic efficacy of mTOR inhibitors for ASD in the near future.

LIST OF ABBREVIATIONS

ASD	=	Autism Spectrum Disorder
eIF4E	=	Eukaryotic Initiation Factor 4E
4E-BP	=	Eukaryotic Initiation Factor 4E-Binding Protein
FMRP	=	Fragile X Mental Retardation Protein
FXS	=	Fragile X Syndrome
KO	=	Knockout
LTD	=	Long Term Depression
LTP	=	Long Term Potentiation
mGluR	=	Metabotropic Glutamate Receptor
mTOR	=	Mammalian Target of Rapamycin
mTORC1	=	Mammalian Target of Rapamycin Complex 1
NF1	=	Neurofibromatosis Type 1
NSE	=	Neuron Specific Enolase
PTEN	=	Phosphatase and Tensin Homolog
Rheb	=	Ras Homolog Enriched in Brain
S6K	=	Ribosomal Protein S6 Kinase
TSC	=	Tuberous Sclerosis Complex

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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