Genetic and Environmental Contributions to Autism Spectrum Disorder Through Mechanistic Target of Rapamycin

Atsushi Sato and Kazutaka Ikeda

ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that affects an individual's reciprocal social interaction and communication ability. Numerous genetic and environmental conditions are associated with ASD, including tuberous sclerosis complex, phosphatase and tensin homolog hamartoma tumor syndrome, fragile X syndrome, and neurofibromatosis 1. The pathogenic molecular mechanisms of these diseases are integrated into the hyperactivation of mTORC1 (mechanistic target of rapamycin complex 1). Rodent models of these diseases have shown high mTORC1 activity in the brain and ASD-related behavioral deficits, which were reversed by the mTORC1 inhibitor rapamycin. Environmental stress can also affect this signaling pathway. In utero exposure to valproate caused ASD in offspring and enhanced mTORC1 activity in the brain, which was sensitive to mTORC1 inhibition. mTORC1 is a signaling hub for diverse cellular functions, including protein synthesis, through the phosphorylation of its targets, such as ribosomal protein S6 kinases. Metabotropic glutamate receptor 5–mediated synaptic function is also affected by the dysregulation of mTORC1 activity, such as in fragile X syndrome and tuberous sclerosis complex. Reversing these downstream changes that are associated with mTORC1 activation normalizes behavioral defects in rodents. Despite abundant preclinical evidence, few clinical studies have investigated the treatment of ASD and cognitive deficits. Therapeutics other than mTORC1 inhibitors failed to show efficacy in fragile X syndrome and neurofibromatosis 1. mTORC1 inhibitors have been tested mainly in tuberous sclerosis complex, and their effects on ASD and neuropsychological deficits are promising. mTORC1 is a promising target for the pharmacological treatment of ASD associated with mTORC1 activation.

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AUTISM SPECTRUM DISORDER AND MECHANISTIC TARGET OF RAPAMYCIN

Autism spectrum disorder (ASD) is a major neurodevelopmental disorder that affects around 1% of the general population. ASD symptoms are categorized into 1) social communication deficits and 2) reciprocal and repetitive interests/behaviors (1). Various genetic deficits are identified in individuals with ASD, including single-gene disorders (2), copy number variations in specific loci, and chromosomal alterations (3). Environmental stress, such as premature birth and drug exposure in utero, can also give rise to ASD in offspring (4,5). Despite accumulating knowledge of the etiology of ASD, relevant knowledge that can contribute to the development of effective treatments for ASD is still sparse.

One mechanism of ASD that may reveal new therapeutic strategies is dysregulation of the mTOR (mechanistic target of rapamycin) complex (mTORC). The central component of the complex is mTOR, a protein with serine-threonine kinase activity. mTOR forms complexes with several proteins to form mTORC1 and mTORC2 (6). The most important difference between mTORC1 and mTORC2 is that mTORC1 consists of raptor (regulatory associated protein of mTOR),

whereas mTORC2 consists of rictor (rapamycin-insensitive companion of mTOR), making it insensitive to rapamycin (7,8). mTORC1 activity is under the regulation of environmental signals, such as growth factors, hypoxia, and energy levels (9). Some inputs activate mTORC1, such as the PI3K/Akt (phosphoinositide 3-kinase/protein kinase B) pathway (10) and Ras/MEK/ERK (rat sarcoma/mitogen-activated protein kinase kinase/extracellular signal–regulated kinase) pathway (11), whereas others inhibit mTORC1, such as the hypoxia/AMPK (5′-adenosine monophosphate–activated protein kinase) pathway (12). These signals are transmitted to TSC1/2 (tuberous sclerosis complex 1/2), which is suppressed by inputs from most pathways while activated by the AMPK pathway. The activation of TSC1/2 suppresses downstream Rheb (Ras homolog enriched in brain) by converting the active GTP (guanosine-5′-triphosphate)–bound form to the inactive GDP (guanosine diphosphate)–bound form. Rheb directly stimulates mTORC1, and TSC1/2 activity results in a reduction of mTORC1 activity, and vice versa (13). mTORC1 phosphorylates its targets and modulates their functions, including S6Ks (ribosomal protein S6 kinases, which control global protein synthesis) (14), eIF4E (eukaryotic translation initiation factor 4E)–binding proteins (4E-BPs;

which enhance the initiation of cap-dependent translation) (15), and ULK1 (unc51-like autophagy-activating kinase 1, which suppresses the initiation of macroautophagy) (16).

Several human genetic disorders are known to be associated with mTORC1 activation and ASD (Figure 1). Rapamycin does not directly bind to mTOR but instead binds to FKBP12 (FK506-binding protein 12) to form the FKBP12-rapamycin complex. This complex can then bind to mTOR in mTORC1 and suppress mTORC1 activity, whereas mTORC2 does not interact with the FKBP12 complex because of the presence of rictor (17). mTORC1 inhibitors are currently widely used to treat tumors and drug-resistant epilepsy in TSC (18) and malignancies in different organs (19). Rapamycin and its analogs are expected to reverse mTORC1 hyperactivity and related neuropsychiatric deficits, such as ASD.

ASD AND HUMAN DISEASES

Human genetic disorders and environmental factors have provided insights into the critical role of mTORC1 dysregulation in ASD. Although not every disorder is found in all individuals with ASD, these disorders provide insights into the ways in which ASD is caused and how it can be reversed pharmacologically in a molecular manner (Table 1).

Tuberous Sclerosis Complex

Historically, TSC (Mendelian Inheritance in Man [MIM] #191100, #613254) has been defined as a neurocutaneous

syndrome (genetic disorder that affects the skin and brain) and presents with facial angiofibroma, epilepsy, and intellectual disability (20,21). The identification of two genes that cause TSC, *TSC1* (22) and *TSC2* (23), expanded knowledge of phenotypic variations of TSC, such as those that are represented in current diagnostic criteria. Major manifestations of TSC include hamartomatous lesions in different organs and in different age groups (24). TSC is estimated to occur in 1 in 6000 live births, with no regional, ethnic, or sexual differences (25,26). Compared with other neurocutaneous syndromes, TSC is more likely to be associated with neurologic complications, such as characteristic brain tumors (e.g., subependymal giant cell astrocytoma) and drug-resistant epilepsy (27). Neurodevelopmental disorders and psychiatric problems, such as anxiety and depression, are also often found in patients with TSC. These disorders are collectively referred to as TSC-associated neuropsychiatric disorders (28). Up to half of patients with TSC are diagnosed with ASD, equally divided among males and female patients (2,29). This lack of sex differences contrasts with the observation that males are approximately three times more vulnerable to ASD than females (30). This suggests that a diagnosis of TSC may be an independent risk factor for ASD, regardless of sex.

The *TSC1* and *TSC2* genes encode hamartin and tuberin, respectively (22,23). TSC1 and TSC2 form a heterodimer that receives signaling inputs (Figure 1). The activated heterodimer suppresses Rheb, resulting in mTORC1 inhibition. The haploinsufficiency of either *TSC1* or *TSC2* weakens the
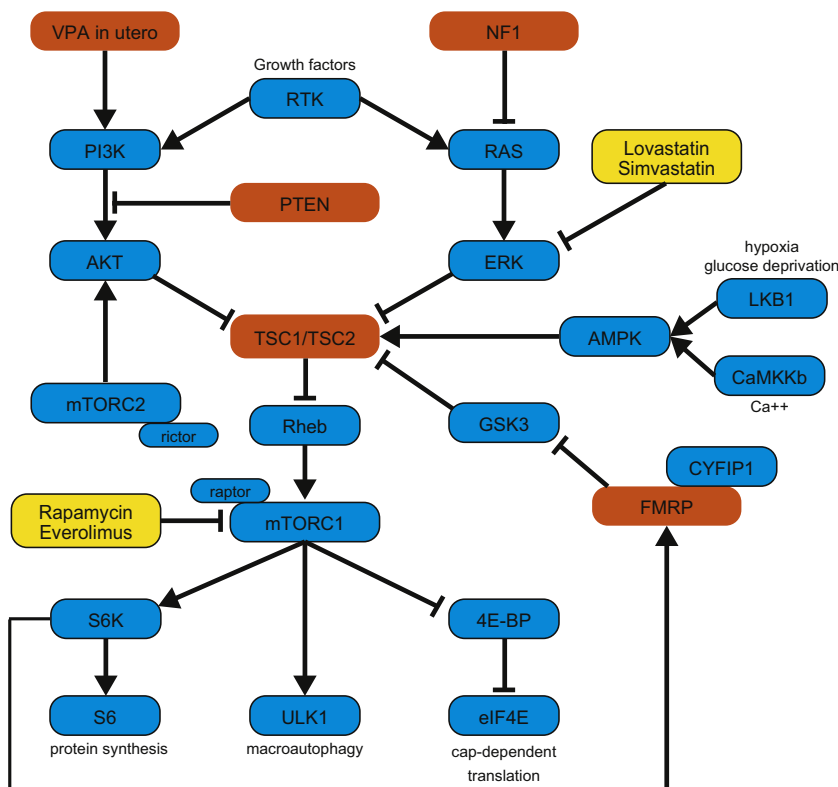
Figure 1. Signaling pathways that involve mTORC1. Physiological inputs to these pathways include growth factors through RTK, the elevation of calcium ion concentrations through CaMKKb, and hypoxia and glucose deprivation through LKB1. These and other signals that stem from FMRP converge in TSC1/TSC2. The activation of TSC1/TSC2 results in mTORC1 inhibition and decreases protein synthesis (ribosomal protein S6), macroautophagy initiation (ULK1), and cap-dependent translation initiation (EIF4E). The net result of the defect that is discussed in the text (highlighted in orange) is mTORC1 activation. A number of drugs that have been evaluated in humans (highlighted in yellow) inhibit each target molecule. Arrows indicate activation. Lack of arrows indicates inhibition. 4E-BP, eukaryotic translation initiation factor 4E-binding protein; AKT, protein kinase B; AMPK, adenosine monophosphate–activated protein kinase; CaMKKb, calcium/calmodulin-dependent protein kinase kinase β; CYFIP1, cytoplasmic fragile X mental retardation protein-interacting protein 1; eIF4E, eukaryotic translation initiation factor 4E; ERK, extracellular signal–regulated kinase; FMRP, fragile X mental retardation protein; GSK-3, glycogen synthase kinase 3; LKB1, liver kinase B1 (serine/threonine protein kinase 11); mTORC, mechanistic target of rapamycin complex; NF1, neurofibromatosis 1; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; raptor, regulatory associated protein of mTOR; RAS, Rat sarcoma; Rheb, Ras homolog enriched in brain; rictor, rapamycin-insensitive companion of mTOR; RTK, receptor tyrosine kinase; S6, ribosomal protein S6; S6K, S6 kinase; TSC, tuberous sclerosis complex; ULK1, unc51-like autophagy-activating kinase 1; VPA, valproic acid.

Table 1. ASD-Related Behavioral Deficits in Rodent Models of mTORC1-Associated ASD

Mutation/Exposure	Findings	Treatment	Reference
$Tsc1^{+/-}$	(F) ↓ social interaction, ↓ nest building	–	(38)
	(M, F) ↓ social interaction, ↑ rearings	5 mg/kg rapamycin, 2 days	(41)
$Tsc2^{+/-}$	(M, F) ↓ social interaction, ↑ rearings	5 mg/kg rapamycin, 2 days	(41)
	(M) ↓ sociability, ↓ social novelty preference	3 mg/kg rapamycin, 1 week	(117)
	(M) ↓ reversal learning	30 mg/kg MPEP, 2 days	(120)
$Tsc2^{+/-}$, Rats	(M) ↓ social interaction, ↓ rearings	–	(42)
	(M) ↓ social interaction, ↓ social discrimination	1 mg/kg everolimus, 3 times per week	(43)
$L7^{Cre};Tsc1^{flox/flox}$	(M) ↓ sociability, ↓ social novelty preference, ↑ self-grooming, ↑ ultrasonic vocalizations	6 mg/kg rapamycin, daily from P7	(44)
$Pcp2^{Cre};Tsc2^{flox/ko}$	(M, F) ↓ sociability, ↓ social novelty preference, ↑ marble burying	2 mg/kg rapamycin, 3 times per week	(45)
$Pten^{+/-}$	(M, F) ↓ sociability, ↓ social novelty preference, ↑ brain mass	–	(58)
	(F) ↓ sociability, ↓ social novelty preference	0.3 mg/kg SB 242084 (5-HT$_{2C}$ receptor antagonist), 20 min before testing	(59)
	(F) ↓ sociability	75 mg/kg PF-4708671 (S6K1 inhibitor), daily from P4 to P14	(60)
	(F) ↓ sociability, ↓ social novelty preference (M, F) ↑ brain mass	–	(144)
$L7^{Cre};Pten^{flox/flox}$	(M) ↓ social interaction, ↓ sociability, ↑ rearings	–	(145)
$Nse^{Cre};Pten^{+/flox}$	(M) ↓ sociability, ↓ social novelty preference	–	(146)
$Nse^{Cre};Pten^{flox/flox}$	(M) ↓ social interaction, ↓ sociability, ↓ social novelty preference, ↑ brain mass	–	(61)
	(M) ↓ social interaction, ↑ brain mass	10 mg/kg rapamycin, 5 times per week	(62)
$GFAP^{Cre};Pten^{flox/flox}$	(NR) ↓ sociability, ↑ marble burying, ↑ hole-poke, ↑ rearings	–	(147)
$Nestin\text{-}CreRE^{T2};Pten^{flox/flox}$	(M) ↓ social interaction, ↓ sociability, ↑ brain mass	–	(148)
$Oxt^{Cre};Pten^{flox/flox}$	(M) ↓ social recognition (F) ↓ social novelty preference	–	(149)
$Camk2a^{Cre};Pten^{flox/flox}$	(M, F) ↓ reversal learning, ↓ social novelty preference	Genetic removal of rapamycin-insensitive companion of mTOR	(63)
$Nkx2.1^{Cre};Pten^{+/flox}$	(M) ↓ social interaction	–	(150)
Pten-ΔPDZ	(NR) ↓ sociability, ↑ brain mass	–	(151)
$Fmr1$ KO (C57BL/6 Background)	(M) ↓ reversal learning, ↓ social novelty preference, ↑ marble burying	Genetic removal of $S6K1$	(74)
$Fmr1$ KO (C57BL/6J Background)	(M) ↓ social novelty preference	1.75 mg/kg rapamycin, incorporated into mouse chow	(84)
	(M) ↑ perseveration	–	(152)
$Fmr1$ KO (FVB/129P2 Background)	(M) ↑ perseveration	20 mg/kg MPEP, 30 min before testing	(153)
$Nf1^{+/-}$	(M) ↓ long-term social learning	Genetic removal of $Pak1$	(91)
$GFAP^{Cre};Nf1^{flox/flox}$	(M) ↓ ultrasonic vocalizations (M, F) ↓ ultrasonic vocalizations	–	(93)
VPA In Utero Single Dose	(M) ↓ social interaction	10 mg/kg rapamycin, 2 days	(105)
	(M) ↓ sociability, ↑ marble burying	5 mg/kg rapamycin, 5 days	(106)
	(M) ↓ sociability	30 mg/kg BrBzGCp2 (glyoxylate 1 inhibitor), 10 hours before testing	(154)
	(M) ↓ sociability, ↓ social novelty preference, ↑ self-grooming, ↑ marble burying	Clonazepam and baclofen infused into mPFC	(155)
VPA In Utero Single Dose, Rats	(M) ↓ sociability, ↓ social novelty preference	1 mg/kg rapamycin, daily from P23 to P33 5 mg/kg sulindac, daily from P23 to P33	(107)
$S6K1$ KO	(M) ↓ social novelty preference, ↑ marble burying	–	(74)

Table 1. Continued

Mutation/Exposure	Findings	Treatment	Reference
4E-BP2 KO	(M) ↓ sociability, ↓ social interaction, ↑ self-grooming, ↑ marble burying, ↑ ultrasonic vocalizations	Genetic addition of *eIF4E*	(110)
	(M) ↓ sociability, ↑ marble burying	0.3 mg/kg JNJ162596385 (mGlu$_1$ antagonist), 30 min before testing 3 mg/kg fenobam (mGlu$_5$ antagonist), 24 hours before testing	(111)
eIF4E Transgenic	(M) ↓ sociability, ↓ social interaction, ↑ self-grooming, ↑ marble burying	4EGI-1 infused intracerebroventricularly	(112)
CamKII^{Cre};Atg7$^{flox/flox}$	(M) ↓ social interaction, ↓ sociability, ↓ social novelty preference	–	(117)

ASD, autism spectrum disorder; F, female; KO, knockout; M, male; mPFC, medial prefrontal cortex; P, postnatal day; VPA, valproic acid.

suppression of Rheb and renders mTORC1 uninhibited. Activated mTORC1 is considered a central pathomechanism of TSC (Figure 1) (13). Individuals with mutations of *TSC1* or *TSC2* generally present the same phenotype, whereas individuals with *TSC2* mutations are more severely affected in certain aspects, such as more severe skin and kidney involvement (31,32), more severe intellectual disability, heavier seizure burden (32,33), and ASD (33,34). Despite these findings, the mechanisms by which these phenotypes arise are not well understood, especially how *TSC2* mutations cause more severe manifestations.

Soon after the identification of *TSC2*, the Eker rat model of hereditary renal carcinoma was found to have a germline mutation of the *Tsc2* gene (35), followed by the establishment of a mouse model of TSC with the germline haploinsufficiency of *Tsc1* and *Tsc2* (36,37). These rodents, however, lack characteristic brain lesions, such as cortical tubers, that are found in most human patients with TSC (38,39). Similar to patients with TSC, *Tsc1$^{+/-}$* and *Tsc2$^{+/-}$* heterozygous knockout mice exhibited ASD-related deficits in social interaction in both males and females and cognitive deficits (38,40,41). Eker rats also exhibited ASD-related social impairments, regardless of concomitant status epilepticus (42,43). This body of evidence suggests that epilepsy and brain lesions are not essential for developing ASD or cognitive deficits in TSC.

The influence of the complete deletion of *Tsc1* and *Tsc2* on the brain and behavior has been investigated to explain the gap between humans who harbor obvious brain lesions and mice that have no morphological changes in the brain. The loss of *Tsc1* and *Tsc2* in cerebellar Purkinje cells resulted in progressive Purkinje cell loss and ataxia and ASD-related social deficits (44,45). This phenotype was much more severe when *Tsc1* and *Tsc2* were deleted primarily in glial cells. These knockout mice exhibited epileptic seizures, progressive macrocephaly, and early mortality. Histological alterations included diffuse glial cell proliferation and the dispersion of hippocampal pyramidal cells (46,47). Intriguingly, these histological and neurologic changes and the elevation of mTORC1 activation were more severe in *Tsc2* knockout mice (47), consistent with more severe manifestations in human patients with TSC.

These rodent models also provide preclinical data on the potential therapeutic usefulness of mTORC1 inhibitors for neurologic manifestations of TSC. Short-term rapamycin treatment reversed ASD-related behavior (41,43) and cognitive deficits (40) through mTORC1 inhibition. Chronic rapamycin treatment prevented seizures, premature death (47), ASD-related behavioral deficits (44), and related histological abnormalities without serious adverse effects. These findings strongly suggest that hyperactivated mTORC1 is sufficient to cause ASD and other cognitive deficits in TSC.

PTEN Hamartoma Tumor Syndrome

The *PTEN* gene was originally discussed with regard to genetic predispositions to various tumors, such as Bannayan-Riley-Ruvalcaba syndrome (MIM #153480; e.g., macrocephaly, intellectual disability, and multiple intestinal hamartomas), Cowden syndrome (MIM #158350; e.g., macrocephaly, mucocutaneous lesions, intestinal polyps, and a higher risk of malignancies) (48,49), and Lhermitte-Duclos syndrome (MIM #158350; e.g., dysplastic gangliocytoma of the cerebellum, ataxia, and increase in intracranial pressure) (50). These disorders share macrocephaly as a common presentation and developmental problems, including ASD, suggesting a relationship between these disorders and ASD. A subset of ASD individuals with extreme macrocephaly were found to have the mutated *PTEN* gene, consequently referred to as "macrocephaly/autism syndrome" (MIM #605309) (51,52). These conditions are now considered to result from *PTEN* gene mutations. They are recognized as a spectrum called PTEN hamartoma tumor syndrome (PHTS) (53). Approximately 7%–17% of individuals with ASD and macrocephaly and 1%–5% of those with ASD carry mutations of the *PTEN* gene (51,54,55). A recent comparative study found that core ASD symptoms were similar between ASD with *PTEN* mutations and ASD with nonsyndromic ASD and macrocephaly. Those with the *PTEN* mutation, however, had less severe symptoms, suggesting distinct pathogenetic mechanisms that underlie PHTS (56).

The *PTEN* gene encodes phosphatase and tensin homolog, a protein that negatively regulates the cancer-related PI3K/AKT/mTOR signaling pathway (57). PI3K is activated by inputs from receptor tyrosine kinase (e.g., insulin and such growth factors as insulin-like growth factor-1) and converts PIP2 (phosphatidylinositol 4,5-bisphosphate) to PIP3 (phosphatidylinositol (3,4,5)-trisphosphate). An increase in PIP3 facilitates

the activation of Akt and inhibition of TSC1/TSC2, leading to the suppression of mTORC1 activity. This explains how deletion of the *PTEN* gene results in mTORC1 activation and ASD. The presence or absence of involvement of the PI3K/Akt pathway may also suggest why TSC and PHTS are distinct with regard to the predisposition to benign and malignant tumors.

Pten deletion in mice revealed the significance of *PTEN* in the pathogenesis of macrocephaly and ASD-related abnormal behavior through constitutive mTORC1 hyperactivation. $Pten^{+/-}$ mice are a model of PHTS in human patients. These mice exhibited ASD-related deficits in social interaction and mild macrocephaly (58–60). Deletion of the *Pten* gene in neurons in the cerebral cortex and hippocampus recapitulated macrocephaly and ASD-related abnormal behavior, such as less interest in other mice, and these effects were associated with an increase in Akt and S6 phosphorylation in the brain (61).

The recovery of these behavioral deficits in these *Pten* mutant mice was associated with mTORC1 inhibition. ASD-related social deficits in $Pten^{+/-}$ mice were reversed by the pharmacological inhibition of S6, similar to mTORC1 inhibition (60). In neuron-specific *Pten* knockout mice, chronic treatment with rapamycin prevented the development of brain hypertrophy and ASD-related abnormal behavior and reduced S6 phosphorylation (62). ASD-like behavior in frontal robe–specific *Pten* knockout mice was improved by the genetic removal of rictor but not raptor (63), suggesting that mTORC1 inhibition by rapamycin may have only a limited effect in correcting the phenotype that is associated with *Pten* mutations. Different brain region involvement and different dosages of the *Pten* mutation may explain these variable effects.

Fragile X Syndrome

Fragile X syndrome (FXS) (MIM #300624) is one of the most common causes of hereditary intellectual disability. Its phenotype includes intellectual disability, ASD, anxiety, and physical features, such as macrocephaly and macroorchidism in males (64). Unlike the diseases that are discussed above, the genetic defect in most FXS cases is the elongation of CGG repeats in the 5′-untranslated region of the *FMR1* gene, located on the X chromosome (65,66). Typically, individuals with FXS have a CGG repeat size greater than 200 in the *FMR1* gene, called a "full mutation" (67), whereas a shorter *FMR1* repeat size (between 55 and 200, premutation) is also relevant to the characteristic movement disorder, called fragile X–associated tremor/ataxia syndrome (68). FXS as diagnosed by detection of the full mutation is found in 1 in 7100 males and 1 in 11,000 females (69). Approximately 30% of male patients with FXS have ASD (2).

In FXS, the elongation of CGG repeats decreases expression of the *FMR1* gene. The protein product of the *FMR1* gene, FMRP (fragile X mental retardation protein), is an RNA binding protein that represses the translation of postsynaptic components of neurons (70,71). One of the binding partners of FMRP is CYFIP1 (cytoplasmic FMRP-interacting protein 1). The FMRP-CYFIP1 complex binds to eIF4E and inhibits the initiation of translation, which is abolished in FXS (72). As noted above, eIF4E-mediated translation initiation is also suppressed

by the sequestration of eIF4E by 4E-BP. mTORC1 hyperactivity reduces 4E-BP binding to eIF4E, resulting in the enhancement of eIF4E-mediated translation initiation. Uninhibited eIF4E-mediated initiation of translation may play a role in the development of ASD in FXS.

Fmr1 knockout animals have been extensively investigated to clarify their behavioral similarity to human patients with FXS (73). ASD-related impairments in social interaction and repetitive behavior were observed in different studies of *Fmr1* knockout mice and rats, along with audiogenic seizures and other cognitive deficits (74,75). The molecular pathophysiology of FXS was first revealed with regard to $mGlu_5$, one of two group I metabotropic glutamate receptors (mGluRs), along with $mGlu_1$. Phenotypic alterations in *Fmr1* knockout mice, such as impairments in memory and excessive protein synthesis in the hippocampus, were prevented by reducing $mGlu_5$ expression (76). The pharmacological inhibition of $mGlu_1$ and $mGlu_5$ also effectively reduced repetitive behavior in *Fmr1* knockout mice. $mGlu_5$ inhibition was superior to $mGlu_1$ inhibition in improving motor learning in the rotarod test and abolishing audiogenic seizures (77).

The dysregulation of eIF4E-mediated translation initiation is involved in FXS and TSC (78), suggesting that FXS and TSC may share a molecular mechanism that leads to ASD. Analyses of the brain in *Fmr1* knockout mice revealed higher messenger RNA levels of a set of FMRP target genes, including *Mtor* and *Nf1* (79). The brains of *Fmr1* knockout mice showed increases in PI3K (80), ERK (81), and mTORC1 activity (79,82). These alterations were mediated by an increase in $mGlu_5$ activity in *Fmr1* knockout mice (83). The associated increase in basal protein synthesis was normalized by ERK inhibition but not by PI3K or mTORC1 inhibition (81,83). The long-term administration of rapamycin also failed to correct behavioral deficits in *Fmr1* knockout mice (84). Although there is a molecular pathophysiology interaction between FXS and TSC, these findings suggest that the pharmacological treatment of neuropsychiatric manifestations of FXS should be directed toward inhibiting $mGlu_5$ rather than mTORC1.

Neurofibromatosis 1

Neurofibromatosis 1 (NF1) (MIM #162200) is the most prevalent neurocutaneous syndrome, which is diagnosed in 1 in 3000–4000 individuals. NF1 and numerous other genetic disorders, such as Noonan syndrome, share activation of the RAS/MEK/ERK pathway; thus, they are collectively called RASopathies (85). Its phenotype includes characteristic skin lesions and tumors that occur mainly in peripheral nerves (86). A higher risk of ASD has long been suspected in individuals with NF1. Recent observational studies reported that 15%–30% of patients with NF1 have ASD (87,88).

The *NF1* gene product neurofibromin functions as a GTPase-activating protein for RAS. Neurofibromin negatively regulates the signaling pathway from RAS by converting the active GTP-bound form to the inactive GDP-bound form (89). Mutations of the *NF1* gene thus result in RAS activation and downstream ERK activation. The activation of ERK, in turn, represses the TSC1/2 complex, finally leading to mTORC1 hyperactivity (86). $Nf1^{+/-}$ mice were found to have cognitive

deficits (90), social impairments (91,92), and an increase in ultrasonic vocalizations (93). To date, the efficacy of reversing the NF1 phenotype has been investigated by inhibiting ERK (discussed below), whereas mTORC1 inhibition has not been tested.

In Utero Exposure to Valproic Acid

Exposure to antiepileptic drugs and psychotropic drugs during pregnancy is also associated with ASD (see the Supplement). Valproic acid (VPA) has long been used as an antiepileptic drug, particularly for generalized epilepsies (94), and several medical conditions, such as migraine (95) and bipolar disorder (96). The use of VPA during pregnancy can be considered an environmental stressor that increases the risk of ASD. Compared with use of other antiepileptic drugs, in utero exposure to VPA was significantly associated with ASD (4), a lower IQ at age 6 years (97), and a higher risk of congenital malformations in offspring (98). Females who are able to be pregnant are now recommended to avoid using VPA as much as possible (99).

The condition in which in utero exposure to VPA results in ASD has been replicated in rodents by giving a single high dose of VPA during pregnancy (100). One of the major mechanisms that causes these ASD-related deficits is through the inhibitory actions of VPA on histone deacetylase (HDAC) (101). The critical role of HDAC inhibition in development of the ASD-related phenotype was demonstrated by comparing mice that were exposed to VPA and valpromide, a VPA analog that is devoid of HDAC activity (102). VPA also activates the PI3K/Akt/mTORC1 pathway (103). Mice and rats that were exposed to VPA in utero exhibited an increase in mTORC1 activity in the brain and ASD-related social deficits. These deficits were reversed by postnatal treatment with rapamycin (104–106), indicating that constitutively active mTORC1 disrupts social behavior in these rodents.

TRAJECTORIES FROM mTORC1 DYSREGULATION TO ASD

The abovementioned causes of ASD, both genetic and environmental, share the dysregulation of mTORC1-mediated signaling. However, other mechanisms may also be involved in the development of ASD, such as elevations of Akt activation in PHTS. Several proteins that are targeted by mTORC1 may give rise to ASD-related phenotypes.

S6Ks—Protein Synthesis

One of the central functions of mTORC1 is to control, likely by upregulating, protein synthesis through the phosphorylation of S6Ks (14). The hippocampus in $Fmr1$ knockout mice showed higher rates of protein synthesis (107). Crossing $Fmr1$ knockout mice with $S6K1$ knockout mice eliminated the dysregulation of protein synthesis and reversed ASD-related behavioral deficits (74). These findings suggest that the elevation of S6K activity elicits excessive protein synthesis and ASD-related deficits in FXS. In contrast, hippocampal slices from $Tsc2^{+/-}$ mice had lower rates of global protein synthesis (108). A reduction of the protein synthesis rate was observed in different cerebral regions in freely moving $Tsc2^{+/-}$ mice (109),

despite the fact that S6K was activated in these mice (40,41). These inconsistent findings raise the possibility of complex pathways that regulate protein synthesis that is associated with mTORC1 and S6K1 activation.

4E-BPs—Initiation of Translation

The translation of a set of synaptic proteins is repressed by 4E-BPs that bind to eIF4E. The mTORC1 phosphorylation of 4E-BPs releases its inhibition of eIF4E and stimulates translation initiation (15). The germline knockout of $4E-BP2$, the major form of 4E-BP in the mammalian brain, engendered ASD-related behavioral deficits that were associated with the exacerbation of mGlu$_5$ long-term depression (LTD) (110,111). ASD-related social impairment was also observed in $eIF4E$ transgenic mice, along with an increase in cap-dependent translation and the enhancement of LTD (112). These behavioral, synaptic, and translational defects were normalized by administration of the eIF4E inhibitor 4EGI-1 (110,112) and an mGlu$_5$ inhibitor (111). Interestingly, cognitive impairment in $Fmr1$ knockout mice was also rescued by 4EGI-1 (113), possibly through suppression of the eIF4E-mediated increase in CYFIP1-induced translation. The above evidence suggests a causal role for eIF4E hyperactivity in cognitive deficits and ASD.

ULK1—Autophagy

Autophagy is a cellular pathway that removes unnecessary proteins and damaged organelles. One of the most notable regulatory pathways is the PI3K/Akt/mTORC1 pathway. Inputs from this pathway suppress autophagy initiation by phosphorylating ULK1 and autophagy factor Atg13, which is necessary for autophagosome formation (19,114,115). Once initiated, autophagy progresses through the involvement of several other ATG proteins (114,116).

The comparison of postmortem brain samples from individuals with ASD and those without ASD revealed higher mTORC1 activity and impairments in autophagy in ASD (117). A similar suppression of autophagy was replicated in the brain in $Tsc2^{+/-}$ mice (117) and neurons that lacked $Tsc1$ and $Tsc2$ (117,118). VPA exposure in utero also caused mTORC1 activation and ULK1 phosphorylation, resulting in a decrease in autophagy in the brain. Rapamycin rescued social behaviors and restored ULK1 activity and autophagy (106). These findings suggest that mTORC1 hyperactivation through different mechanisms result in deficits in autophagy and social behaviors.

Interestingly, $Tsc2$-null neurons exhibited an increase in AMP (adenosine monophosphate) levels AMPK activation, implying energetic stress in the absence of $Tsc1/2$. This then led to direct ULK1 stimulation and mTORC1-independent autolysosome accumulation. Similar changes were observed in mice that lacked $Tsc1$ in Purkinje cells and human cortical tuber samples (118). Autophagy regulation in response to energetic stress may be an mTORC1-independent backup mechanism, suggesting the potential of therapeutically targeting this mechanism using adenosine triphosphate–competitive mTOR inhibitors and the biguanide metformin (119).

mGlu$_5$-Mediated Synaptic Function

As discussed above, the abnormal enhancement of mGlu$_5$ activity was first revealed in FXS (76) and then studied in TSC because these two diseases share ASD and mTORC1 hyperactivity. To date, the findings with regard to mGlu$_5$ activity in TSC is inconsistent. First, the $Tsc2^{+/-}$ hippocampus was found to have a decrease in mGlu$_5$ LTD and reduced basal protein synthesis as well as cognitive deficits, in clear contrast to FXS. Deficient mGluR LTD and associated abnormalities were restored by rapamycin and a positive allosteric modulator of mGlu$_5$ (108). Later, however, an conflicting finding was reported. $Tsc2^{+/-}$ hippocampus showed an increase in mGlu$_5$ LTD and impairments in reverse learning. These deficits were reversed by an mGlu$_5$ inhibitor, similar to $Fmr1$ knockout mice (120). Another strain of $Tsc2$ mutant mice was investigated, in which the level of TSC2 protein was reduced to approximately 7% relative to wild-type mice. These mutant mice exhibited hyperactivity and epileptic seizures, which were improved by mGlu$_5$ inhibition with an mGlu$_5$ negative allosteric modulator and aggravated by mGlu$_5$ potentiation with another mGlu$_5$ positive allosteric modulator (121). mGluR LTD was maintained in $S6K1$ knockout mice but enhanced in $S6K2$ knockout mice with or without $S6K1$ deletion (122). mGlu$_5$ dysfunction likely underlies cognitive deficits, including in ASD. Further clarification is required to ascertain whether mGlu$_5$ LTD is enhanced or deficient in response to mTORC1 hyperactivity.

DEVELOPMENT OF PHARMACOTHERAPY FOR mTOR-RELATED ASD IN HUMANS

Challenging Experiences in FXS and NF1

The development of pharmacological therapeutics for neuropsychiatric problems that are associated with the aforementioned diseases were first attempted in FXS. The first candidate was an mGluR inhibitor, such as MPEP, and another was a GABA$_B$ (gamma-aminobutyric acid B) receptor agonist. Audiogenic seizures in $Fmr1$ knockout mice were inhibited by the GABA$_B$ receptor agonist baclofen and worsened by a GABA$_B$ receptor antagonist (123). This line of preclinical evidence prompted researchers to conduct clinical trials with mGlu$_5$ inhibitors and a number of compounds, including GABA$_B$ receptor agonists, for neuropsychiatric symptoms in FXS (124). Despite the clinical expectations based on preclinical results, no therapeutic efficacy was found in randomized clinical trials with FXS participants who were treated with the selective mGlu$_5$ inhibitor mavoglurant (125) and GABA$_B$ receptor agonist arbaclofen (126). There was one promising report of another clinical trial of mavoglurant. In this trial, visual attention and pupil reactivity while viewing photographs of faces were used as primary outcomes, and mavoglurant treatment resulted in significant improvement (127).

As noted above, the central pathophysiology of NF1 is disinhibition of the RAS/MEK/ERK pathway. The HMG-CoA (β-hydroxy-β-methylglutaryl–coenzyme A) inhibitors lovastatin and simvastatin inactivated ERK (128) and ameliorated cognitive dysfunction in $Nf1^{+/-}$ mice (129). Based on these findings, independent randomized clinical trials were conducted with lovastatin and simvastatin. Some of these trials reported improvements in cognitive function (130,131), but others failed to find therapeutic efficacy in ASD and neuropsychiatric symptoms (132–135).

mTORC1 Inhibitors in Clinical Studies of ASD

Despite the abundance of evidence in rodent models, clinical studies are limited with regard to the effects of mTORC1 inhibitors on neuropsychiatric phenotypes. Anecdotal case reports have described that ASD and behavioral problems were improved by everolimus that was used for subependymal giant cell astrocytoma (136) and drug-resistant epilepsy (137). Changes in ASD symptoms and cognitive function were evaluated as secondary outcomes in clinical studies of mTORC1 inhibitors for TSC-associated lesions. Some studies observed improvements in cognitive function and ASD-related behavior. Patients with TSC-associated renal and pulmonary lesions exhibited improvements in neurocognitive performance after sirolimus therapy (138). Studies of everolimus for drug-resistant epilepsy in individuals with TSC revealed improvements in ASD-related behavior in an open-label study (139) and a randomized study (140). Another open-label study of subependymal giant cell astrocytoma reported a negative effect of everolimus on neuropsychological function (141). Later, a randomized controlled trial was conducted, in which children with TSC who had ASD or other neurodevelopmental disorders but no drug-resistant epilepsy were enrolled to investigate the effect of everolimus for these conditions. Everolimus did not improve ASD or neuropsychological deficits (142).

At the time of writing this article, we found no reports of changes in ASD-related behaviors by mTORC1 inhibitors in patients with PTEN-related disorders. One randomized controlled trial will be conducted for PHTS and comorbid ASD using everolimus (143).

Concerns about using mTORC1 inhibitors for ASD include their adverse effects. Some are mild (e.g., stomatitis and hyperlipidemia), but others can be serious (e.g., immunosuppression and interstitial lung disease) (137–143). The benefits of treating ASD and risk of adverse effects should be cautiously weighed.

CONCLUSIONS

In this review, the central role of constitutively active mTORC1-mediated signal transduction was discussed in the context of ASD and its molecular pathophysiology. We presented rodent models of human disorders and the current state of developing pharmacological therapeutics. Currently, mTORC1 inhibitors are the most promising drugs that can improve ASD, which have been widely used in patients with TSC and other diseases. However, it remains unclear whether their beneficial effects on ASD and related neurocognitive conditions surpass their adverse effects, which can be severe. Preclinical evidence is abundant, but further clinical experience is needed with regard to the efficacy of mTORC1 inhibitors and other promising agents, such as mGlu$_5$ inhibitors, in ameliorating ASD.

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