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Cerebellar Development and Autism Spectrum Disorder in Tuberous Sclerosis Complex

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Abstract

Approximately 50% of patients with the genetic disease tuberous sclerosis complex (TSC) present with autism spectrum disorder. Although a number of studies have investigated the link between autism and TSC, the etiology of autism spectrum disorder in these patients remains unclear. Abnormal cerebellar function during critical phases of development could disrupt functional processes in the brain, leading to development of autistic features. Accordingly, we review the potential role of cerebellar dysfunction in the pathogenesis of autism spectrum disorder in TSC. We also introduce conditional knockout mouse models of *Tsc1* and *Tsc2* that link cerebellar circuitry to the development of autistic-like features. Taken together, these preclinical and clinical investigations indicate the cerebellum has a profound regulatory role during development of social communication and repetitive behaviors.

Keywords

magnetic resonance imaging; mouse models; mTOR; Purkinje cell; cerebellum

TSC and Autism Spectrum Disorder

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by significant neurologic disability.¹ TSC results from a mutation of either the *TSC1* or *TSC2* gene, whose protein products, hamartin and tuberin, form a heterodimeric complex that negatively regulates mechanistic target of rapamycin (mTOR) signaling. The mTOR pathway is a critical signaling network that regulates protein synthesis, cell proliferation, cell metabolism, and cell growth.^{2,3} More than 80% of TSC patients have cortical tubers (hamartomas), which contain large glial cells and dysplastic neurons and have loss of normal architecture in cerebral cortex.⁴

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Author Contributions

Drs. Sundberg and Sahin wrote the article together and are sole authors.

Some studies have shown that tuber formation affects the severity of neurological symptoms in TSC, although patients without tubers may also have significant developmental deficits, including intellectual disability and autism.^{5–10} In addition, seizures that emerge during critical developmental periods profoundly impact the severity of the neurological, cognitive, and psychological symptoms of TSC patients.^{10–12} Several studies have demonstrated that *TSC2* mutations are associated with a more severe TSC phenotype compared with mutations in *TSC1*.^{13,14} In addition, several studies indicate that disruption of the TSC pathway contributes to autism spectrum disorder, and approximately 50% of TSC patients fulfill criteria for autism spectrum disorder.^{15,16} This makes TSC one of the most common monogenetic causes of autism spectrum disorder.

Autism spectrum disorder is highly heterogeneous and can usually be diagnosed as early as 2 years of age. The condition presents with a wide range of deficits, including difficulty with social communication, repetitive behaviors, and restricted interests. In addition, patients with autism spectrum disorder often have intellectual disability and epilepsy. Neuropathological studies have consistently demonstrated a loss of cerebellar Purkinje cells in individuals diagnosed with autism spectrum disorder versus typically developing controls.^{17–20} Imaging studies have shown that patients diagnosed with autism spectrum disorder have gray and white matter abnormalities in the cerebellum, dating to early childhood.^{21–25} Significantly, premature infants with isolated cerebellar hemorrhage have a higher incidence of autism spectrum disorder, suggesting that cerebellar dysfunction early in development contributes to the pathogenesis of autism.^{26,27} These findings have raised the hypothesis that abnormal cerebellar function could disrupt higher-order social cognitive processes and lead to autism spectrum disorder.

In the case of individuals with TSC, several lines of evidence suggest a correlation with cerebellar involvement and presence of autism spectrum disorder symptoms. A relationship between cerebellar tubers and autism was first reported in 2000.²⁸ Imaging studies indicate the presence of abnormal cerebellar findings in TSC patients, with 4 to 13% of these patients displaying cerebellar atrophy.^{29–31} Interestingly, cerebellar tubers have unique a morphology and specialized structure compared with supratentorial tubers. In one study, 33% of TSC patients had cerebellar lesions, which were described as having a pyramidal/wedge shaped appearance.³⁰ All the cerebellar tubers studied had increased apparent diffusion coefficient values, and most of them had “zebra-like” contrast enhancement. Despite this presentation, these patients did not have any ‘typical’ cerebellar symptoms on neurological examination.³⁰ There also appears to be an association between the presence of cerebellar tubers and occurrence of cerebellar atrophy.^{29,32} Taken together, these studies indicate that 24% to 33% of TSC patients have cerebellar lesions.^{30,31,33}

Research conducted by Drs. Diane and Harry Chugani and their colleagues demonstrate that cerebellar lesions detected on MRI scans also have functional correlates on PET scans.³³ They characterized features of cerebellar lesions in a functional imaging study of 78 TSC patients as part of an epilepsy surgery evaluation. Twenty-seven percent of these patients had cerebellar lesions with increased alpha-[(11)C]methyl-L-tryptophan (AMT) uptake and decreased glucose metabolism. In addition, this study showed that those with right-sided lesions were more likely to have communication problems and autism spectrum disorders.³³

Furthermore, it has been previously demonstrated that cerebellar cortical lesions may play a role in the dentatothalamofrontal circuits. Positron emission tomography (PET) scans showed that children with TSC and autism spectrum disorders had increased glucose metabolism in the deep cerebellar nuclei bilaterally. Moreover, glucose hypermetabolism in the deep cerebellar nuclei and occurrence of infantile spasms were both associated with disturbances in behavior and communication skills, and difficulties in social interaction.³⁴ As the cerebellar cortex inhibits the deep cerebellar nuclei, these findings indicate hypofunction of the cerebellar cortex. These findings may not be restricted to autism spectrum disorders associated with TSC. In fact, in a study of a series of individuals with 'idiopathic' autism spectrum disorder, PET scans also displayed increased AMT uptake in the right dentate nucleus and decreased AMT uptake in the left cortical and thalamic regions,³⁵ supporting the notion that the role of the cerebellum in autism spectrum disorders may be more generalizable.

In summary, in several cohorts of TSC patients the presence of cerebellar lesions correlates with a diagnosis of autism spectrum disorder.^{28,33} Although a number of clinical investigations have been performed for TSC patients, the mechanistic link between cerebellar dysfunction and autism spectrum disorder remains to be determined.

Cerebellar Development

The cerebellum is a highly specialized brain region with carefully detailed architecture and cell layer formations, first described by Cajal. Although the cerebellum has been studied for several decades, our understanding of its functional roles and connectivity to other brain regions remains incomplete. Anatomically the cerebellum can be divided into separate lobes, white matter, and cortical layers. The cerebellum consists of special classes of cerebellar neurons: granule cells, Purkinje cells, and interneurons. We will focus on describing development and differentiation of Purkinje cells, whose dysfunction and reduced number have been previously linked to autism occurrence.^{17–20}

There is a close resemblance in cerebellar development among vertebrates. Mice cerebellar primordium consists of 2 distinct germinal matrices, including the ventral ventricular zone and the dorsal rhombic lip, which are formed between embryonic days 8.5–9.5. This is a birthplace for cerebellar GABAergic neuronal precursors, including the Purkinje cells and glutamatergic neurons.³⁶ The concentration of transcriptional factors, cell position, and migration affect the final fate of the cells in the developing cerebellum.^{37–39}

Purkinje cell precursors in the murine cerebellum develop between embryonic days 11–13. During the differentiation process, Purkinje cells migrate along the radial glial fibers out beyond the mantle of postmitotic precursors. After birth, Purkinje cells continue to migrate along the radial glial system to form a zone (molecular layer) overlying the germinative zone. In addition, the granule neurons migrate via the radial glial pathway through the zone of immature Purkinje cells. This migration process facilitates the formation of another neuronal layer of the cerebellar cortex, the internal granule cell layer.³⁹

The directed migration of interneurons and postmitotic precursors of Purkinje cells results in the formation of the cerebellar cortex. The expansion and development of the cerebellum

depends on the proliferation rate of granule cell progenitors related to the ratio of developing Purkinje cells, which in the human cerebellum is around 400 granule cells per one Purkinje cell.^{37,38} Purkinje cells form connections with basket and satellite cells that provide inhibitory input to other Purkinje cells. Of note, Purkinje cells are the only output neuron of the cerebellar cortex. Purkinje cells' inhibitory neurotransmission establishes the patterning of the cerebellum during development and has a crucial role in the neuronal activity in the cerebellum.⁴⁰

Several transcription factors and growth factors affect the development of Purkinje cells and granule cells with canonical manner. For example, isthmus organizer at the midbrain-hindbrain boundary secretes fibroblast growth factor 8 (FGF8), which plays an important role in the earliest stage of cerebellar development.^{41–44} FGF8 regulates expression of several transcription factors, including region-specific OTX2, EN1/2, PAX2/5/8, and GBX2. Importantly, FGF8 secretion regulates Wnt1 expression at the midbrain-hindbrain boundary, forming a positive feedback loop, which also activates EN2 and PAX2 expression.^{45–49}

The *En1* and *En2* expression in developing precursor cells affects the formation of the cerebellar region, and together with the bone morphogenic proteins, dorsalizes cells within this region.^{50,51} In the granule cell layer, the dorsal genes *Zic1/2* and *Math1*, play important roles in the regulation of the granular neurons' specification and migration.⁵² In the ventricular zone, *Ptf1a*- (pancreas transcription factor 1a), which encodes a bHLH transcription factor, and *Neph3* (nephrin-like protein 3), also known as *KIRREL2*, a membrane molecule and a product of a *Ptf1a* target gene, expression gives rise to all Purkinje cells.^{53–55} The neuroepithelium in the developing mice cerebellar plate expresses *E-cadherin*, *Neph3*, *Ptf1a*, and *Corl2* with a spatiotemporal pattern that overlaps with Purkinje cell generation (embryonic days 12–14).

Purkinje cell precursors in the subventricular zone and postmitotic stage express lineage-specific marker *Corl2* (SKOR2).^{53,55–57} Mature Purkinje cells can be characterized by their expression of Calbindin, L7, and PCP2.^{55,58,59} Granule cells migrate on glial scaffolds, and this process is mediated by a family of neuron-glial adhesion molecules, known as astrotactins. ACTN2 (astrotactin 2) has been implicated in ASD in at least 2 genetic cohorts,^{60,61} supporting the role for abnormal cerebellar development contributing to ASD.

Current Knowledge of Cerebellar Dysfunction and Autism

A number of studies have described that the cerebellum regulates cognitive function, and a loss of cerebellar cells has been linked to autism spectrum disorder.^{19,21,62,63} To establish solid proof that cerebellar deficits link to development of autism spectrum disorder, several research groups have investigated patients with syndromic forms of autism who have cerebellar vermis hypoplasia.^{64–67} According to these studies, the decreased number of cells in the cerebellum appeared to result from developmental hypoplasia rather than neuronal degeneration during later stages of development.^{64–67}

In addition, postmortem analyses have revealed that patients with autism spectrum disorder had reduced Purkinje cell numbers, reduced number of cells in the cerebellar nuclei, excessive number of Bergmann glia, and activated microglia and cytokine production in

cerebellar white matter.^{17,68,69} Related to this, several diffusion tensor imaging studies to analyze cerebral white matter tracts have been performed in children with autism spectrum disorder, who were then compared with typically developing children.^{70–72} These studies indicate that children with autism spectrum disorder who have impairment in white matter microstructure, increase in the diffusivity of bilateral superior cerebellar peduncles and reduced connectivity in the corpus callosum and internal capsule.^{70–72}

In addition, behavioral assessment has shown that cerebellum and brainstem dysfunction can lead to saccadic eye movement abnormalities in patients with autism spectrum disorder.⁷³ Growing evidence suggests that the cerebellum has an important regulatory role in language, cognition, and emotions.^{74–77} The cerebellum also contributes to visual perceptual learning.⁷⁸ According to this study, patients who had focal damage to the posterior cerebellum displayed strongly diminished learning capacities compared with age- and gender-matched controls, in terms of both rate and amount of improvement over time.⁷⁸

Animal Models for Describing Cerebellar Dysfunction and Autism Symptoms

Despite pathological, imaging, and genetic studies in patients, all indirectly point to an “upstream” role for the cerebellum in autism; direct mechanistic proof of the involvement of cerebellar neurons in autism spectrum disorder had been lacking. Animal models have started to fill this gap. Several genetic models have been established for studying autism spectrum disorder in mouse models. The genes commonly linked to the development of autism spectrum disorder are: TSC genes *TSC1* and *TSC2*, Fragile X mental retardation gene (*Fmr1*), neuroligin 3 and 4 (*NLGN*), *SCA1*, and methyl-CpG-binding protein type 2 gene (*MECP2*).^{79–86} In addition, mutations in *Engrailed*, *PTEN*, *Reelin*, and *DLX* are linked to development of autism spectrum disorder.^{87–91} These mouse models have clear behavioral and neurophysiological features linked to autism spectrum disorder and cerebellar development, including aggressive behavior, anxiety, seizures, gastrointestinal problems, motor deficits, abnormal sleep, and sensory problems. Because *TSC1/2* mutations cause wide penetration of autism for these patients, we will focus here on *Tsc1/2* mice models with cerebellar deficits.

Despite the complex origin of autism spectrum disorders and their heterogeneous cerebellar deficits, recent studies of *Tsc1* and *Tsc2* mutant mice show clear evidence of these deficits contributing to development of behavioral abnormalities similar to the core features of these disorders.^{83–85} Our lab has generated a mutant mouse model in which the *Tsc1* deletion was restricted to the cerebellum, specifically Purkinje cells. Most notably, the *Tsc1*-deficient mouse model showed core characteristics of autistic-like behaviors: lack of interest in socializing, repetitive behaviors, restricted interests, and abnormal communication.⁸³ This provides clear evidence that neurodevelopmental deficits restricted to the cerebellum can result in a wide range of typical autistic-like behaviors.

At the cellular level, deletion of *Tsc1* only from Purkinje cells during postnatal development resulted in the decreased number of Purkinje cells in mice cerebellum (in the null mutants but not in heterozygous), and the cells demonstrated hypo-excitability (in both null and

heterozygous mutants). Together, these results indicate that Purkinje cell hypofunction (not necessarily number) is associated with the behavioral deficits in this model. Interestingly, increased spine density in Purkinje cells was linked to altered behavioral phenotype associated with autism spectrum disorder, and treatment of these mice with rapamycin prevented development of these autistic-like behaviors, providing strong evidence that the TSC/mTOR pathway underlies these defects.

Independent studies by Gambello and colleagues demonstrated that the loss of *Tsc2* in mice Purkinje cells caused a progressive increase in cell size and subsequent apoptotic cell death. In *Tsc2*-deficient mice, Purkinje cell death was associated with motor deficits. Remarkably, oxidative stress and endoplasmic reticulum stress were both increased in *Tsc2* null Purkinje cells. In line with the studies of *Tsc1* knockout mice models, cell death and endoplasmic reticulum stress phenotypes were rescued by treatment with the mTORC1 inhibitor rapamycin in the *Tsc2* knockout model.^{84,85} Together, these results indicate that Purkinje cells are a crucial part of the neural circuitry underlying autistic-like behavior in mice and alteration of the cerebellar function can result in profound defects in behavior that are typically thought to be related to cerebral cortical dysfunction in autism spectrum disorder.

Because Purkinje cells are the only output of the cerebellar cortex, Purkinje cell abnormalities could influence the rest of the brain via the projections of the deep nuclei to and from specific regions of the thalamus, basal ganglia, and (via polysynaptic connections) the neocortex. In fact, anatomical tracing in non-human primates demonstrates long-range connections between prefrontal cortex and cerebellar hemispheres, consistent with a nonmotor role for the cerebellum.⁹² Future studies are needed to understand how Purkinje cell dysfunction and/or hypo-excitability lead to autistic-like symptoms in these animal models.

mTOR Pathway and Rapamycin Treatment

mTOR is a conserved serine/threonine kinase and it affects downstream of the PI3K/AKT pathway, where it activates S6K1 and 4EBP1, which are involved in mRNA translation (see review³). mTOR consists of 2 multiprotein complexes: mTORC1 and mTORC2.^{93–95} The mTORC1 pathway is sensitive to acute rapamycin treatment. Regulation of the mTORC1 pathway occurs via neurotransmitters, nutrients, growth factors, and axon guidance cues. Compared with mTORC1, mTORC2 is generally resistant to rapamycin treatment and not as well-studied in the brain. Several extracellular growth factors affect mTOR pathway activation (e.g., brain-derived neurotrophic factor [BDNF], ciliary neurotrophic factor [CNTF], glutamate, insulin, insulin-like growth factor 1 [IGF1], and vascular endothelial growth factor [VEGF]).^{96–98} mTOR regulates cell survival, cell growth, proliferation, autophagy, and synaptic function. It is also linked to cellular stress pathways in TSC models.^{99–104}

TSC1 and TSC2 proteins form a heterodimeric complex that negatively regulates mTORC1. The *TSC1* gene, located on chromosome 9q34, encodes a transcript that contains 23 exons.¹⁰⁵ The *TSC2* gene is located on chromosome 16p13, where it encodes a large transcript that contains 41 exons.¹⁰⁶ TSC1 and TSC2 function together with TBC1D7 to

activate GTPase-activating protein (GAP). This complex inhibits mTOR pathway by converting active Rheb-GTP to inactive Rheb-GDP form.^{107,108} In the case of tuberous sclerosis, *TSC1* or *TSC2* are mutated and their protein products are not functional, leading to unregulated activation of the mTOR pathway, and subsequently causing abnormal protein synthesis, cellular growth and proliferation.^{2,99–104} Thus, inhibition of the mTOR pathway is a potential target for pharmacotherapy treatment of TSC. In addition to rapamycin, there are other drugs that are molecular analogues of rapamycin, called ‘rapalogues’ (e.g., temsirolimus, deforolimus, and everolimus).¹⁰⁹ Also, dual PI3K-mTOR inhibitors are being tested for mTOR regulation¹¹⁰ and for TSC treatment.¹⁰⁹

Clinical Trials for TSC with Rapalogues

Rapamycin (also known as sirolimus), temsirolimus, deforolimus (also known as ridaforolimus), and everolimus (also known as RAD001) are currently being studied in clinical trials for a variety of indications, including neurocognition, autism, angiofibromas, angiomyolipomas, and multiple cancers occurring with TSC (see study details in www.clinicaltrials.gov). Here we briefly introduce 3 clinical trials with rapamycin and rapalogues that are ongoing for treatment of neurocognitive symptoms and autism in patients with TSC.

We conducted a clinical study for evaluation of the safety of everolimus (RAD001) on TSC patients (www.clinicaltrials.gov; NCT01289912). In 2011, we initiated a Phase II placebo-controlled clinical trial at Boston Children’s Hospital and Cincinnati Children’s Hospital Medical Center for patients with TSC ages 6–21 years old with an IQ of 60 or greater. Primary outcomes of the study were: 1) safety of everolimus compared with placebo in patients with TSC and 2) assessment of the efficacy of everolimus on neurocognition in patients with TSC in comparison with a placebo group of patients, as measured by well-validated, standardized, direct, and indirect neurocognitive tools. This study also included secondary outcomes, which were frequency of seizures, sleep disturbances, autism spectrum disorder features, academic skills, and behavioral problems. This study was completed at the end of 2014, and data analysis is currently in process.

A similar clinical trial has recently been initiated at the Erasmus Medical Center in Rotterdam, Netherlands, to study everolimus efficacy for treatment of autism and neuropsychological deficits in children with TSC (NCT01730209), with expected completion by 2016. The study focuses on TSC patients between 4–15 years of age with an IQ estimated <80 and autism spectrum disorder and/or learning disabilities that require assistance and remedial teaching. The primary outcome of this 12-month study is cognitive ability measured by IQ levels. The secondary outcomes include evaluation of autistic features, social and communicational skills, working memory and attention, visual-motor integration, executive functioning, sleep problems, sensory-related difficulties, overall health, school success, and seizure frequency.

Lastly, a feasibility study of “Rapalogues for Autism Phenotype in TSC” began in 2014 at the Kennedy Krieger Institute, Baltimore (NCT01929642). The aim of this study is to assess the safety and feasibility of administering rapalogues sirolimus or everolimus in 2- to 30-

year-old patients with TSC and self-injury and to measure cognitive and behavioral changes, including reduction in autistic symptoms, aggressive behaviors, and self-injury as well as improvements in cognition across multiple domains of cognitive function. Primary outcome measures are assessments of parental stress, caregiver burden, and family compliance to study protocol. Secondary outcomes include evaluation of whether treatment of TSC patients with sirolimus reduces the frequency, severity, or duration of repetitive, self-injurious, and aggressive behavior.

In summary, these clinical trials will provide valuable information on the safety and potential effects of rapalogues on neurocognitive symptoms, learning difficulties, and autism in patients with TSC. Clinical trials such as these will reveal which treatments may be most beneficial for ameliorating symptoms of TSC, at what age treatments should begin, and how long treatment must continue for long-lasting neurocognitive benefit. Moreover, identifying treatments effective for autism and neurocognitive deficits in TSC may be effective more broadly for other individuals with autism or conditions caused by genes in the same or similar gene pathways.

Future Prospects

Based on preclinical studies, rapalogues hold great promise for future treatment of TSC-related behavioral and physiological symptoms. However, effective therapy directed at the cognitive impairments and autism associated with TSC is still in the early stages of clinical research. Although the underlying etiology for development of autism spectrum disorder remains to be defined, continued research towards this goal is ongoing. In the future, the use of new imaging technologies can be utilized for finding novel correlations of network activity with individual symptoms in individual TSC patients and will enable development of targeted therapies for these patients.

Recent progress in studies with *Tsc1* and *Tsc2* knockout animal models have shown that genetic dysfunctions in physiological contents can provide insights on the developmental regulators, synaptic interactions, neurotransmitters, and novel transcriptional pathways that contribute to autism spectrum disorder susceptibility in the presence of *Tsc1/2* dysfunction.^{83,84} Although the mTORC1 inhibitor rapamycin has been shown to alleviate some of the behavioral deficits in Purkinje cells-specific *Tsc1/2* knockout mice, it may not be an ideal therapeutic for the chronic treatment of TSC patients because of its side effects.

We acknowledge that the human patient brain is different structurally and functionally from mice models. Thus, ideally, we need to have access to patient cerebellar tissue to understand the cellular and circuitry abnormalities leading to autistic behaviors as well as for the development of novel therapies for TSC patients. However, nervous system tissues from patients are rarely accessible for these *in vitro* types of experiments. To overcome this problem, Yamanaka and colleagues have developed cell reprogramming technologies that allow production of human induced pluripotent stem cells (iPSCs) from any patient somatic tissues.^{111,112} Neural differentiation of human iPSC allows characterization of neurodevelopmental disease phenotypes with *in vitro*-platform in laboratories.^{113–115} Thus, in the future researchers can use TSC patient-derived iPSCs for identification of novel

molecular targets to rescue TSC-deficient neural cells. By targeting cerebellar cells and especially Purkinje cells, novel therapeutic strategies for TSC can be established. In the future, development of the iPSC-derived Purkinje cells in combination with studies of *Tsc1/2* knockout mouse models with clear autistic-like phenotypes will provide a powerful system to develop new treatments for TSC and autism.

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