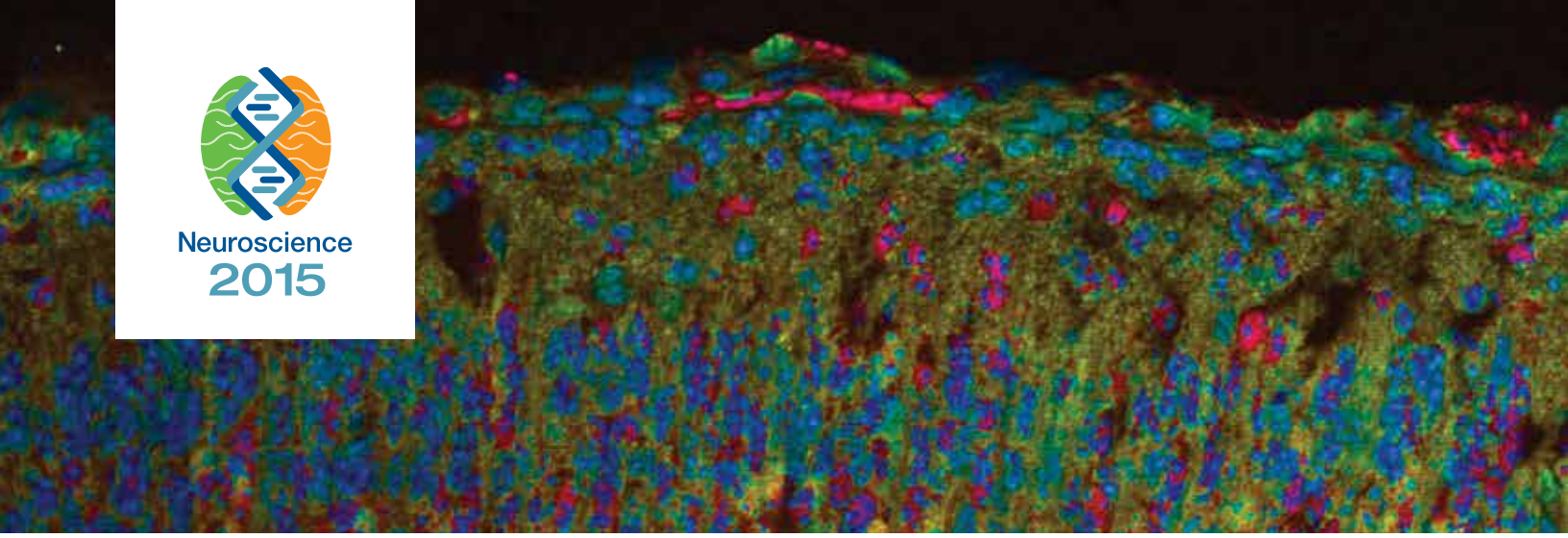
Neuroscience 2015

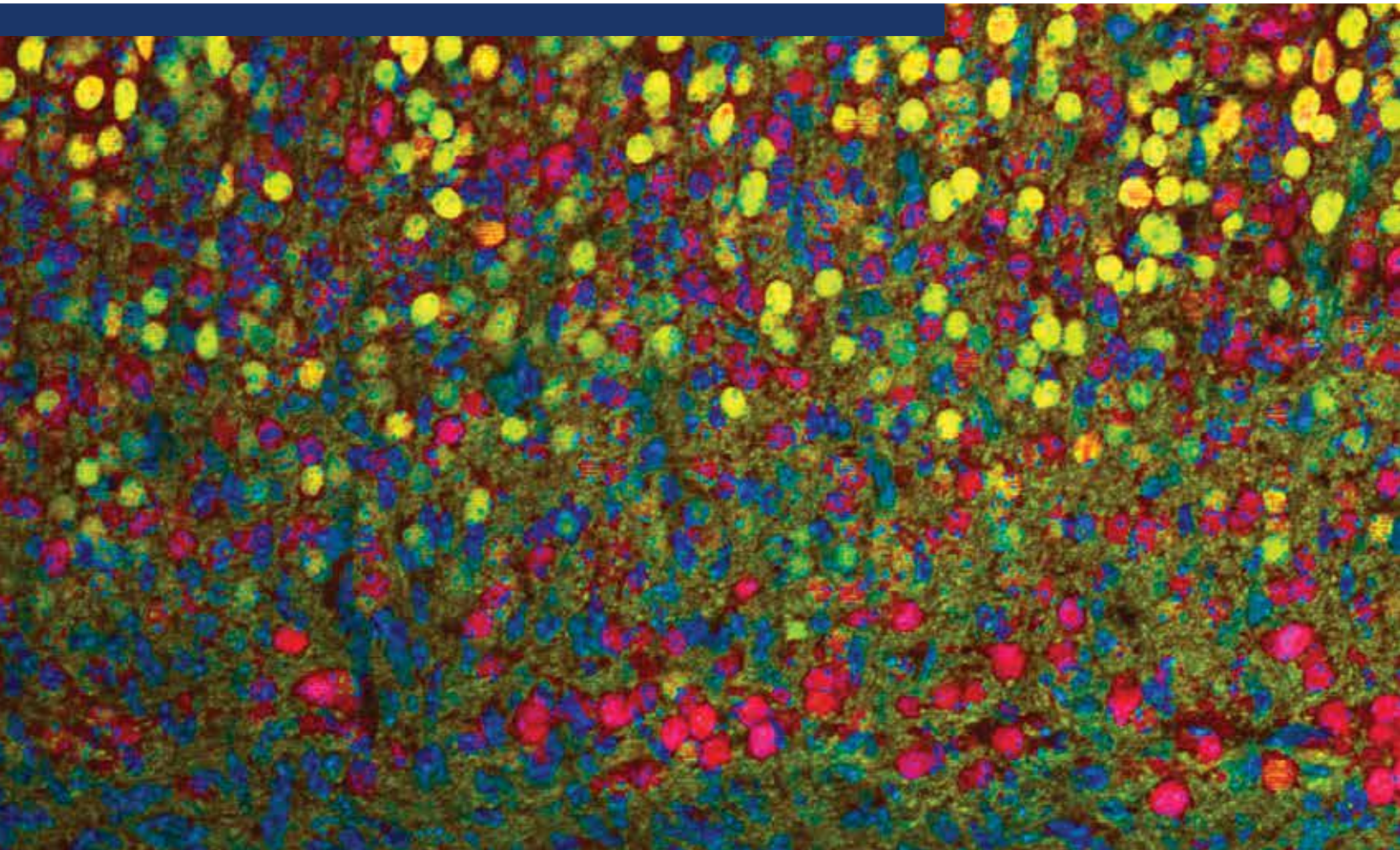


NEUROBIOLOGY OF DISEASE WORKSHOP

Human Brain Malformations: From Genetics to Therapeutics

Organized by Peter Crino, MD, PhD, and Mustafa Sahin, MD, PhD

SfN Society for Neuroscience



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Neuroscience 2015

NEUROBIOLOGY OF DISEASE WORKSHOP

Human Brain Malformations: From Genetics to Therapeutics

Organized by: Peter Crino, MD, PhD, and Mustafa Sahin, MD, PhD
Friday, October 16, 2015 / 8 a.m.–5 p.m.
Location: McCormick Place
Room: S100BC / Chicago, IL

TIME	TALK TITLE	SPEAKER	
7:30–8 a.m.	**Check-In**		
8–8:10 a.m.	**Opening Remarks**	Peter Crino, MD, PhD | Temple University Mustafa Sahin, MD, PhD | Boston Children's Hospital	
8:10–8:40 a.m.	Patient Presentation	Douglas Nordli, MD | Children's Hospital of Chicago	
8:40–9:20 a.m.	Overview and Approaches to Brain Malformations	Joseph Gleeson, MD | Rockefeller University	
9:20–10 a.m.	The mTOR Pathway and Malformations of Cortical Development	Peter Crino, MD, PhD | Temple University	
10–10:40 a.m.	Tuberous Sclerosis	Mustafa Sahin, MD, PhD | Boston Children's Hospital	
10:40–11 a.m.	**Morning Break**		
11–11:40 a.m.	Reelin Pathways in Brain Development	Gabriella D'Arcangelo, PhD | Rutgers University	
11:40–12:20 p.m.	Microtubules, Tubulin Isotypes, and Human Tubulinopathies	Elizabeth Engle, MD | Boston Children's Hospital	
12:20–1 p.m.	**Summary, Discussion, and Q&A**		
1–1:10 p.m.	Breakout Guide		
1:10–2 p.m.	**Lunch**		

AFTERNOON BREAKOUT SESSIONS / PARTICIPANTS SELECT DISCUSSION GROUPS AT 2 P.M. AND 3:30 P.M.			
TIME	**BREAKOUT SESSION**	**SPEAKERS**	**ROOM**
2–3:30 p.m.	Molecular Control of Neurogenesis	Arnold Kriegstein, MD, PhD | University of California, San Francisco Joseph LoTurco, PhD | University of Connecticut	S102A
	Cell Biology of Neuronal Migration	Peter Crino, MD, PhD | Temple University Seon-hee Kim, PhD | Temple University	S102BC
	Cerebellum and Brainstems	Elizabeth Engle, MD | Boston Children's Hospital Mary Hatten, PhD | Rockefeller University	S102D
	Interneurons in Developmental Malformations	Stewart Anderson, MD | University of Pennsylvania School of Medicine Elizabeth Powell, PhD | University of Maryland School of Medicine	S103A
	Somatic Mutations in Developmental Malformations	Joseph Gleeson, MD | Rockefeller University Ann Poduri, MD | Boston Children's Hospital	S103BC
	Animal Models of Brain Malformations	Angelique Bordey, PhD | Yale University Gabriella D'Arcangelo, PhD | Rutgers University	S103D
	Epileptogenesis and Brain Malformations	Eric Marsh, MD, PhD | Children's Hospital of Pennsylvania Jack Parent, MD | University of Michigan	S105A
	Novel Therapies for Brain Malformations	Elizabeth Berry-Kravis, MD, PhD | Rush University Mustafa Sahin, MD, PhD | Boston Children's Hospital	S105BC
3:30–5 p.m.	**Repeat sessions above.** **Select a second discussion group.**		
5–6 p.m.	**Reception**		S106

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Introduction

Malformations of cortical development (MCD) are developmental disorders of the cerebral cortex that are highly associated with intractable epilepsy, and in some cases, with neurobehavioral, cognitive, and sensorimotor disabilities. The broad spectrum of MCD includes small focal cortical dysplasias (FCDs), which affect a few millimeters of the cortex, as well as larger, more pervasive MCDs that affect an entire hemisphere (e.g., hemimegalencephaly [HME]) or the whole brain (e.g., lissencephaly). In some cases, surgical resection of an MCD can be curative for intractable epilepsy, whereas in more extensive cases of MCD, the neurological disabilities are profound and few treatments are available. The aim of this Neurobiology of Disease Workshop is to review the progress in MCD research, including human genomics and animal model systems, and to explore how understanding the molecular pathogenesis of these disorders has provided new insights into normal brain development.

Over the past decade, our knowledge of the molecular genetic and cell-signaling abnormalities that lead to MCD has rapidly expanded owing to the identification of single gene defects that cause MCD. This course will focus on the structural and functional consequences of these mutations as well as how investigations into these genes' functional roles have yielded invaluable insights into mechanisms governing normal cortical development. Several MCD subtypes can be functionally linked by association with distinct cell signaling pathways. For example, a subset of focal MCD, including tuberous sclerosis complex, FCD, and HME, has been associated with aberrant activation of the mammalian (or "mechanistic") target of rapamycin (mTOR) cascade. This association with mTOR activation (in these so-called "mTORopathies") reveals logical mechanisms that account for these lesions' histopathological features, which include laminar disorganization, aberrant cell polarity, and enhanced cell size. The evolving association of at least 10 MCD subtypes, including lissencephaly, with mutations in tubulin and tubulin-association genes (so-called "tubulinopathies") and the Reelin pathway has provided insights into more devastating consequences of MCD, such as loss of gyral structure, impaired neural migration, and altered laminar structure. The generation of animal models for these disorders has been an invaluable tool to define how gene mutations affect cortical cytoarchitecture, network integrity, and neuronal excitability.

Course topics, manuscripts, and recommended reviews will provide the foundation for the faculty and attendees to explore new and compelling questions about the pathogenesis of and therapeutic developments for MCD.

mTOR Signaling in Malformations of Cortical Development

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Introduction

Over the past 10 years, enhanced activation of the mammalian target of rapamycin (mTOR) signaling cascade has been identified in focal malformations of cortical development (MCD) subtypes, collectively referred to as "mTORopathies." Mutations in mTOR regulatory genes (e.g., *TSC1, TSC2, AKT3,* and *DEPDC5*) have been associated with several focal types of MCD that are highly associated with epilepsy. mTOR plays important roles in the regulation of cell division, growth, and survival; thus, aberrant activation of the cascade during cortical development can cause dramatic alterations in cell size, cortical lamination, and axon and dendrite outgrowth that are often observed in focal MCD. Although it is widely believed that structural alterations induced by hyperactivated mTOR signaling are critical for epileptogenesis, newer evidence suggests that mTOR activation on its own may enhance neuronal excitability. Clinical trials with mTOR inhibitors have shown efficacy in the treatment of seizures associated with focal MCD.

Malformations of cortical development are highly associated with medically intractable epilepsy as well as intellectual disability and autism spectrum disorders (Sisodiya, 2004; Aronica et al., 2012). There is a broad spectrum of MCD, ranging from small focal cortical dysplasias (FCD), identified on pathological examination of a resected epileptogenic focus, to multifocal or diffuse structural abnormalities, including polymicrogyria and lissencephaly (Guerrini and Dobyns, 2014). During the past 20 years, rapid expansion in our knowledge of molecular genetic abnormalities leading to MCD has identified single gene defects that cause MCD and has clearly demonstrated how understanding MCD can provide invaluable insights into mechanisms governing normal cortical development. While ongoing identification of new genes responsible for MCD is proceeding rapidly, and functional links between these mutations and their structural effects in the cortex are being clarified, the exact mechanisms through which these molecular events lead to epilepsy remain a mystery.

Perhaps the largest conceptual advance during the past 10 years has been the understanding that abnormal activation or inhibition of several distinct cell-signaling cascades is pathogenic and likely responsible for MCD. Furthermore, these same cascades likely contribute to the seizure phenotype that is characteristic of these malformations. Identification of abnormal signaling in a variety of cellular cascades has served as a bellwether for new therapeutic advances in the treatment of epilepsy. For example, many MCD subtypes exhibit abnormal and enhanced activation of the mTOR pathway, a cellular cascade that modulates cell proliferation, growth, motility, migration, and death. The mTOR cascade plays a critical role in normal cortical development and remains functionally active during adulthood, maintaining cell metabolism, synaptic plasticity, and autophagy. A collection of neurodevelopmental disorders characterized by focal MCD has been linked to aberrant mTOR signaling. These disorders include cortical tubers in tuberous sclerosis complex (TSC); focal cortical dysplasia (FCD); hemimegalencephaly (HME); several megalencephaly (ME) subtypes; ganglioglioma (GG); polyhydramnios, megalencephaly, and symptomatic epilepsy syndrome ("Pretzel syndrome"); and familial focal epilepsy with variable foci (Crino, 2011). This group of "mTORopathies" is unified by a clinical phenotype of epilepsy, a spectrum of developmental delay, abnormal cortical cytoarchitecture, and hyperactivated mTOR signaling.

Classification of Focal MCD

Distinct classification schemes have been proposed to define the relevant imaging and histological features of FCD (Mischel et al., 1995; Barkovich et al., 1996). The "Palmini classification system" (Palmini et al., 2004) was restructured and further subdivided FCD into types IA, IB, IIA, and IIB. Recently, a task force extended the classification system for FCD into type IA, IB, IIA, or IIB dysplasia and introduced a new type III dysplasia to account for the detection of FCD in association with other brain pathology, such as vascular malformations or tumors (Blumcke et al., 2011). Individual classification schemes for HME and tubers have not yet been established; HME may in fact represent a "lobar" or "hemispheric" form of FCD. A new and comprehensive classification scheme treats all types of MCD as resulting from distinct developmental and molecular genetic etiologies that have direct effects on cortical development at distinct epochs and within distinct cell types (Barkovich et al., 2012).

Focal MCDs exhibit a range of cytoarchitectural alterations, from subtle cortical dyslamination with malpositioned or heterotopic neurons to grossly disorganized or absent cortical lamination and abnormal cell morphology (Krsek et al., 2008; Blumcke and Muhlebner, 2011; Muhlebner et al., 2012). All focal MCDs exhibit some degree of disorganized cortical lamination. For example, in FCD type IA or IB, the laminar architecture is disrupted in subtle radial (FCD type IA) or tangential (FCD type IB) patterns, whereas in FCD type IIB and tubers, laminar structure is typically lost. Variable

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numbers of reactive astrocytes may be seen in each subtype. The neuropathological findings in HME can be highly variable; some specimens exhibit relatively preserved gyral, lobar, and laminar architecture, while others exhibit dramatic alterations in hemispheric architecture, with no remaining visible normal structure. The mechanisms accounting for these variations are unknown but are likely linked to the effects of specific gene mutations or other molecular events, e.g., modifier genes occurring in select cell populations at defined developmental epochs.

FCD type IIB, tubers, GG, and to a variable extent, HME, exhibit large (cytomegalic) cells in which the cell soma is 1.5 to 2 times larger than normal neurons. For example, dysmorphic neurons (DNs) exhibit a neuronal morphology but feature very large cell soma and aberrant dendrites. DNs may be found in any cortical layer and in the subcortical white matter. Balloon cells (BCs) exhibit a characteristic enlarged ovoid shape, a laterally displaced nucleus, and limited dendritic or axonal projections. BCs are the pathognomonic cell type of FCD type IIB, cortical tubers in TSC (also referred to as "giant cells"), and some HME specimens. In GG, atypical ganglion cells are morphologically similar to BCs and likely form by similar mechanisms.

mTOR Signaling and Focal MCD

There is now solid evidence that hyperactivated mTOR signaling is a feature of several focal MCD subtypes and likely provides a common pathogenic mechanism for the cytoarchitectural abnormalities seen in these MCDs. mTOR is a serione/threonine kinase that is a highly conserved across many organisms. The mTOR signaling cascade is involved in multiple functions, such as maintaining cellular homeostasis and energy metabolism, nutrient cues and oxidative stress, proliferation and survival, response to growth factors, differentiation and migration, cytoskeletal organization, and autophagy (Menon and Manning, 2008; Dobashi et al., 2011; Zoncu et al., 2011). mTOR functions as a kinase in two independent heteromeric complexes: mTOR complex 1 (mTORC1) and mTORC2 (Loewith et al., 2002). mTORC1 is modulated primarily through the growth factor–phosphoinositide-3-kinase (PI3K)–Akt signaling cascade, and rapamycin (a macrolide antibiotic) is a specific mTORC1 inhibitor, functioning through the binding protein FKBP12 (Sabers et al., 1995). mTORC2 plays an indirect regulatory role over mTORC1 by way of Akt signaling and is a relatively rapamycin-insensitive complex. mTORC2 has been linked to actin-mediated cytoskeletal assembly and organization. mTORC1 uniquely contains raptor and PRAS40, while mTORC2 is composed of rictor, protor, and Sin1. Both complexes consist of mTOR and mLST8 proteins. DEPTOR (DEP domain–containing mTOR-interacting protein) directly interacts with and inhibits mTOR in both complexes. mTORC1 is modulated upstream by TSC1 and TSC2 via the Ras homolog enriched in brain (Rheb). The upstream effectors from insulin-dependent and growth factor receptors, cellular energy metabolism, and hypoxia-inducible factors converge on the TSC1/TSC2 complex (Weber and Gutmann, 2012). Insulin-like growth factor receptors signal through PI3K, then PDK1, and then Akt to inhibit the TSC1/TSC2 complex and thereby release inhibition of (i.e., activate) mTORC1. TSC2 acts as a GTPase-activating protein toward Rheb, which results in the inhibition of mTOR signaling. TSC1 protein stabilizes TSC2 by binding to it and preventing its ubiquitination. TBC1 domain family member 7 (TBC1D7) is a third component of the complex that modulates the GAP activity effects exerted on Rheb by TSC2 through binding to TSC1 (Dibble et al., 2012). TBC1D7 knockdown diminishes the association between TSC1 and TSC2, leading to decreased Rheb–GAP activity and increasing mTORC1 signaling.

Several other cellular states affect mTOR signaling. For example, PTEN (phosphatase and tensin homolog deleted on chromosome 10) inhibits the PI3K/PDK1/Akt pathway and releases the inhibition on TSC1/TSC2 while promoting mTOR activity. In the setting of hypoxia–ischemia, REDD1 is expressed and serves to dampen mTOR signaling via interactions with TSC2. Ambient cellular ATP levels signal to mTOR via AMPK (AMP-activated protein kinase), which phosphoactivates TSC2 when energy stores are replete. Levels of amino acids, in particular leucine, modulate mTOR activation through several heteromeric signaling complexes (Ragulator, GATOR1, GATOR2) that include DEPDC5, NPRL3, MIOS, and WDR53 and function at the lysosome (Menon et al., 2014). Thus, numerous regulatory nodes can enhance or inhibit mTOR signaling in response to myriad cellular states.

Focal Cortical Dysplasia and Tuberous Sclerosis: Paradigm mTORopathies

FCD and cortical tubers in TSC are among the most common pathological substrates associated with medically intractable pediatric epilepsy (Tassi et al., 2002; Krsek et al., 2008). TSC is an autosomal

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dominant, multisystem disorder resulting from mutations in either *TSC1* or *TSC2* and characterized by a spectrum of neurological deficits including autism, intellectual disability, and intractable epilepsy (Crino et al., 2006; Chu-Shore et al., 2010). Identification of the *TSC1* and *TSC2* genes and the links to mTOR signaling has provided critical insights into mechanisms of focal MCD and, in fact, has provided the paradigm for studying other focal MCD subtypes.

The molecular mechanisms leading to tuber formation during brain development reflect the effects of loss-of-function mutations in either *TSC1* or *TSC2*, leading to constitutive mTOR activation and altered development of the cerebral cortex (Orlova and Crino, 2010). Numerous studies have demonstrated activated mTORC1 substrates phospho-p70-S6 kinase, phospho-S6, and phospho-4E-BP1 in resected and postmortem TSC tuber samples (Baybis et al., 2004; Miyata et al., 2004; Talos et al., 2008). Indeed, two recent studies have demonstrated mTORC1 activation in fetal tubers (Prabowo et al., 2013; Tsai et al., 2014).

The pathological similarities between FCD type IIB and tubers suggest a mechanistic link between these lesions via mTOR signaling. Enhanced mTOR signaling was first identified in FCD type IIB (Baybis et al., 2004; Miyata et al., 2004). This discovery set the stage for subsequent studies showing mTOR activation in HME (Ljungberg et al., 2006; Aronica et al., 2007) and GG (Samadani et al., 2007) and solidifying the concept that FCD and HME may reflect a spectrum of developmental brain malformations linked to aberrant mTOR signaling known as "mTORopathies." Phospho-p70-S6 kinase and phospho-S6 isoforms were detected by immunohistochemistry in resected FCD type IIB specimens. The central hypothesis of these studies was that molecular events leading to abnormal brain development resulted in mTOR activation evidenced by hyperphosphorylation of mTOR, p70-S6K, and S6 proteins. As in tubers, p70-S6K and S6 phosphoisoforms were identified in cells with enlarged somas, i.e., DNs and BCs in FCD type IIB. The discovery of FCD types IIA and IIB, in association with *DEPDC5* (Baulac et al., 2015; D'Gama et al., 2015; Scerri et al., 2015), *PI3K* (Jansen et al., 2015), *NPRL3* (Sim et al., 2015), and *MTOR* (Lim et al., 2015; Nakashima et al., 2015) mutations, suggests that somatic mutations in mTOR regulatory genes are likely causative in FCD. Similarly, a growing body of evidence demonstrates that HME is also linked to mutations in mTOR regulatory genes, including *PI3K* (Lee et al., 2012), *AKT3* (Lee et al., 2012), *PTEN* (Jansen et al., 2015), and *DEPDC5* (D'Gama et al., 2015). Thus, the histological similarities among FCD type IIB, HME, and tubers highlight the common effects of aberrant mTOR signaling during cortical development.

Acknowledgments

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Tuberous Sclerosis Complex

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Introduction

Tuberous sclerosis complex (TSC) is a genetic multisystem disorder that has an impact on many organ systems by causing growth of benign tumors, so-called hamartomas. Because brain, skin, kidneys, heart, liver, lungs, and less frequently retina, gingiva, bones, and gastrointestinal tract can be affected, TSC patients require the coordinated care of many specialists. More than 90% of patients develop neurological symptoms such as epilepsy, autism spectrum disorders (ASDs), intellectual disability, attention deficit hyperactivity disorder (ADHD), anxiety, sleep disorders, and other behavioral problems, referred to as TANDs (TSC-associated neuropsychiatric disorders). TANDs place a significant disease burden on patients and their families. This review focuses on the molecular mechanisms that underlie the neurological symptoms of TSC and discusses current treatment approaches and targeted therapeutic strategies.

TSC is caused by a mutation of either the *TSC1* gene, located on chromosome 9q34, or the *TSC2* gene, located on chromosome 16p13.3. It occurs with a frequency of 1:6000. A heterozygous mutation of either gene leads to loss of its respective gene products, hamartin (*TSC1*) and tuberin (*TSC2*). A mutation can be found in 85% of patients, and to date, more than 1500 mutations have been shown to cause TSC (LOVD Tuberous Sclerosis Database, http://chromium.liacs.nl/LOVD2/TSC/home.php). Thirty percent of these mutations are inherited in an autosomal dominant fashion, and 70% are *de novo* mutations, resulting in a disease with remarkable phenotypic variability among individuals. In general, compared with *TSC1* mutations, *TSC2* mutations occur more frequently *de novo* and result in a more severe phenotype; however, genotype–phenotype analyses have failed to show further correlations, possibly owing to the large number of mutations.

Neurological Manifestations

In the vast majority of TSC patients, the CNS is involved. Magnetic resonance imaging (MRI) of the brain frequently shows structural abnormalities, such as cortical tubers and subependymal nodules (SENs), which can evolve into subependymal giant-cell astrocytomas (SEGAs).

Tubers are focal malformations of embryonic cortical development. They are localized at the subcortical junction zone and are characterized by disorganized lamination and giant cells expressing markers of neuronal and glial differentiation, suggesting a differentiation defect of early progenitor cells (Mizuguchi and Takashima, 2001; Ess et al., 2005). Among TSC patients, 80–90% have tubers; tuber counts range from 5 to 50, with an average count of 18.8 (Kaczorowska et al., 2011).

SENs are small nodules along the lateral ventricle walls. In 5–20% of TSC patients, SENs give rise to SEGAs, which are slow-growing tumors with a mixed glioneuronal phenotype. SEGAs tend to be larger than SENs, occur near the foramen of Monroe, and have the potential to cause obstructive hydrocephalus. It has been suggested that SENs and SEGAs originate from a neural stem/progenitor cell population (Ess et al., 2005; Zhou et al., 2011).

Until recently, these pathological changes were thought to be responsible for the neurological phenotype of TSC patients. However, current studies have suggested that subtler microscopic changes, such as aberrant white matter connectivity, which cannot be visualized with regular MRI techniques, play a role in causing cognitive deficits, behavioral problems, ASD, and epilepsy.

TSC is one of the most frequent genetic causes of epilepsy. Up to 90% of TSC patients develop seizures, most of them during infancy; seizures in TSC pose a considerable challenge to health and quality of life, because they are difficult to treat with conventional antiepileptic drugs. At least one-third of patients develop refractory epilepsy. Early onset of seizures is associated with an increased risk of behavioral problems, intellectual disability, and reduced quality of life. Fifty percent of patients have infantile spasms as the initial seizure manifestation. Complex partial seizures are the most frequent seizure type later in life, although any other form of seizure can be present as well.

The mechanisms by which TSC causes epilepsy are unclear. Tubers and the perituberal cortex have long been associated with epilepsy (Holmes and Stafstrom, 2007; Major et al., 2009). Recent studies have suggested a multifactorial evolution based on the fact that epileptiform discharges can also occur in areas without tubers, and TSC patients without tubers can have epilepsy (Gallagher et al., 2009). Vigabatrin is the mainstay of therapy for early-onset seizures in TSC patients, and is used as a first-line treatment for infantile spasms as well as focal seizures before the age of 1 year. Vigabatrin has been shown to be effective against infantile spasms in up to 95% of children with TSC (Curatolo et al., 2001). Vigabatrin elevates GABA levels by irreversible inhibition of the GABA transaminase. Its exact

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mechanism of action in TSC is unclear, although a recent study suggested that vigabatrin inhibits the mTOR (mammalian target of rapamycin) pathway in mouse models (Zhang et al., 2013).

A wide range of cognitive and behavioral problems are common in TSC (de Vries et al., 2015). ADHD is frequently present in patients with TSC at a rate of 50%, comparable with the prevalence of ASD in TSC. In addition, adult patients with TSC have higher rates of psychiatric comorbidities, such as mood disorders, anxiety, obsessive–compulsive behavior, and alcoholism.

Intellectual disability has a prevalence of 40–50% in TSC. Thirty percent of patients are severely affected, having IQs in the very low range, and 70% have IQs in the normal but slightly left-shifted range. Still, specific impairments of memory, attention, or executive skills are common even in patients with normal intelligence. Early-onset epilepsy, refractory seizures, and autism are associated with poor cognitive outcomes (Chu-Shore et al., 2010).

Sleep disturbances are a common problem in TSC and affect up to 60% of children and 30% of adults (Hunt and Stores, 1994). Polysomnography shows disorganized sleep with reduced REM sleep, frequent awakenings, and sleep instability, even in the absence of nocturnal seizures. In addition, seizures and treatment with antiepileptic drugs may affect sleep.

TSC is one of the major genetically identifiable causes of ASD. Up to 50% of TSC patients have ASD, compared with 1% of the general population. TSC accounts for 1–4% of all autism cases and has a male-to-female ratio of 1:1, compared with 4:1 in the general population. Most TSC patients with autism also have epilepsy. The incidence of epilepsy in autistic patients without TSC is 30%, raising the question of whether epilepsy (especially early-onset seizures and infantile spasms) predispose a person to autism. Growing evidence suggests that ASDs are caused by abnormal neuronal connectivity and that symptoms arise as a result of "local overconnectivity and long-distance underconnectivity" (see "Aberrant Neuronal Connectivity" later on).

The TSC Signaling Pathway

mTOR, a kinase that integrates multiple signals to regulate cellular growth and translation, has been implicated in many functions of the developing as well as the mature CNS. Axonal growth and specification, as well as proper synapse development and synaptic plasticity, require the mTOR pathway. mTOR is activated in response to hormones, mitogens, amino acids, stress, and energy and functions within two distinct multiprotein complexes: mTOR complex 1 and 2 (mTORC1 and mTORC2). The main components of mTORC1 are the proteins mTOR, raptor, and mLST8. mTORC1 mediates control of cell size and protein synthesis through regulation of translation initiation via phosphorylation of its substrates S6K1 and 4E-BP1. In contrast, the main components of mTORC2 are mTOR, rictor, and mLST8. The function of mTORC2 is much less understood, but it appears to be involved in cytoskeletal organization. TSC1 and TSC2 proteins function as a heterodimeric complex to inhibit mTOR signaling by regulating a small GTP-binding protein, Rheb (Ras homolog enriched in brain), which acts as an mTOR activator (Fig. 1). The TSC1/TSC2 complex is activated by low cellular energy levels via AMP kinase. Mutation of either *TSC1* or *TSC2* alone is sufficient to disrupt the complex and disturb its function, leading to constitutive activation of the mTOR pathway (for a review of the mTOR pathway, see Lipton and Sahin, 2014).

Although data on mTOR signaling in the CNS of TSC patients is limited to samples mostly obtained during epilepsy surgery, increased constitutive phosphorylation levels of mTOR pathway substrates have been identified in tubers, confirming *in vitro* models with *in vivo* data.

Aberrant Neuronal Connectivity

Although many studies have tried to correlate tuber number and location with the severity of CNS involvement, the question has been raised as to whether microscopic structural changes, rather than larger brain malformations such as tubers, account for neurological symptoms in TSC patients. Seizures, autism, and intellectual disability can occur in patients irrespective of their tuber burden, while others with a high tuber count may have few or no neurological deficits. The fact that TSC rodent models develop seizures and behavioral abnormalities in the absence of gross structural brain abnormalities supports this hypothesis. Neuronal network development is a complex and highly regulated process that requires multiple steps to acquire proper axon, dendrite, and synapse morphology. Failure of axonal growth, specification and guidance, synapse formation,

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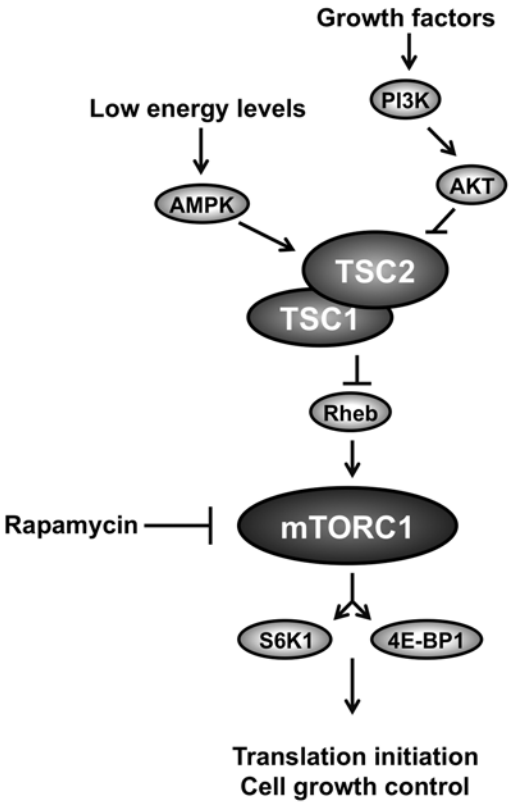

Figure 1. Schematic of the TSC/mTOR pathway. mTORC1 is activated by growth factors, hormones, and nutrients and regulates cell size and translation via its substrates S6K1 and 4E-BP1. The heterodimeric TSC1/TSC2 complex is activated through AMPK by low-energy states and functions via Rheb to inhibit mTORC1 activity. The compound rapamycin inhibits mTORC1 activity downstream of the TSC1/2 complex.

myelination, or circuitry results in impaired neuronal connectivity.

Information transmission and processing in the CNS require the formation of polarized neurons with axons transmitting, and dendrites receiving, signals. This process is called "axon specification." Interestingly, components of the Tsc/mTOR pathway appear to be preferentially expressed in nascent axons during early neuronal development (Haddad et al., 2002; Choi et al., 2008). Neuronal loss of *Tsc1* or *Tsc2* in a rat hippocampal tissue culture model resulted in multiple ectopic axons and failure to develop into polarized cells *in vitro*. On the other hand, overexpression of Tsc1 and Tsc2 in rat hippocampal neurons led to axonal loss. Interestingly, the formation of ectopic axons in *Tsc1*-deficient neurons was rescued by treatment with the mTOR inhibitor rapamycin. The TSC/mTOR pathway is also important for proper guidance of axons. Local protein synthesis has been shown to be involved in growth cone dynamics, and components of the mTOR pathway are active in growth cones (Martin, 2004). *Tsc2* haploinsufficiency in mice caused aberrant retinogeniculate projections *in vivo*, and in *Drosophila*, loss of *Tsc1* in the developing retina disrupted axon guidance in a similar fashion (Knox et al., 2007; Nie et al., 2010).

With conventional MRI techniques, white matter appears mostly normal in TSC patients. However, diffusion tensor imaging (DTI), which is designed to detect white matter tract abnormalities, has contributed to the findings of aberrant connectivity in TSC. Initial studies found abnormal ADC (apparent diffusion coefficient, a measure of total diffusion), FA (fractional anisotropy, a measure of diffusion directionality), and RD (radial diffusivity, measuring diffusion perpendicular to axon tracts) values adjacent to cortical tubers and within epileptic zones. Later reports also showed DTI changes in other areas, such as the corpus callosum, in TSC patients compared with controls. Further support for white matter irregularities derives from rodent models, where immunostaining of mouse brains with conditional neuronal and glial *Tsc1* or glial *Tsc2* knockout showed myelination defects that resulted in severe seizures and early death of the animals.

Activity-dependent changes in synapse function, such as long-term depression (LTD) and long-term potentiation (LTP), are presumed to be major mechanisms of learning and memory. The TSC/mTOR pathway plays a role in synapse plasticity through the regulation of protein translation of protein involved in late-phase LTP (Tang et al., 2002). Hippocampal neurons of $Tsc2^{+/-}$ rats showed alterations in synapse plasticity in response to high-frequency tetanization, as LTP and LTD were significantly impaired compared with wild-type controls (von der Brelie et al., 2006), although these rats did not display obvious deficits in learning and memory (Waltereit et al., 2011). Likewise, postnatal hippocampal deletion of *Tsc1* in mice abolished protein synthesis–dependent metabotropic glutamate receptor (mGluR) LTD, whereas a protein synthesis–independent form of NMDA receptor–mediated LTD remained unchanged (Bateup et al., 2011). Interestingly, $Tsc2^{+/-}$ mice do have disrupted hippocampal-dependent functions, such as impaired

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spatial learning and contextual discrimination, in the absence of seizures (Ehninger et al., 2008).

In summary, multiple studies have shown involvement of the mTOR pathway in many steps of neuronal development and maturation, and a failure of proper network formation with mTOR dysregulation. Increasing evidence suggests that TSC is a disorder of impaired neuronal connectivity in the brain caused by aberrant mTOR pathway signaling (Fig. 2).

TSC as a Model for Autism

ASDs are characterized by impaired social interaction, communication deficits, and behavioral problems. TSC is one of the most frequently identified monogenic causes of autism and a promising model to study its pathogenetic mechanisms. Autism is a complex disorder, and animal models are only recently emerging. Although several TSC animal models display behavioral abnormalities, few researchers so far have reported autism-like features in their animals. A likely explanation is the fact that many of these knockout mice have frequent and severe seizures and die early on, obscuring behavioral studies. However, mice either overexpressing a dominant-negative form of Tsc2, or those bearing heterozygous deletion of *Tsc1*, appear to have impaired social interactions (Goorden et al., 2007; Chevere-Torres et al., 2012).

Recent studies have suggested both syndromic and nonsyndromic ASD to be a "developmental disconnection syndrome" caused by aberrant neuronal network formation. A consistent finding in human postmortem ASD studies is a loss of cerebellar Purkinje cells (PCs), suggesting the cerebellum to be involved in ASD pathogenesis. Recently, cerebellum-specific TSC mouse models have been created to model ASD. Homozygous deletion of *Tsc1* or *Tsc2* in PCs caused cell death in the PC layer and reduced excitability of the *Tsc1*-deficient PC. These mice displayed an autism-like phenotype characterized by social impairment, restrictive behavior, and abnormal vocalizations in the absence of seizures and brain malformations such as tubers (Tsai et al., 2012; Reith et al., 2013).

Further evidence for autism as a connectivity disorder comes from imaging studies designed to evaluate white matter tracts. TSC patients with ASD had abnormalities of corpus callosum projections on DTI imaging, whereas TSC patients without ASD had imaging values comparable with those of controls (Peters et al., 2012). Graph theory–based analysis of functional networks via EEG found a globally decreased functional connectivity in individuals with TSC irrespective of the presence of ASD, whereas individuals with nonsyndromic ASD had a more complex pattern characterized by decreased long-range over short-range connectivity and increased network resilience (Peters et al., 2013).

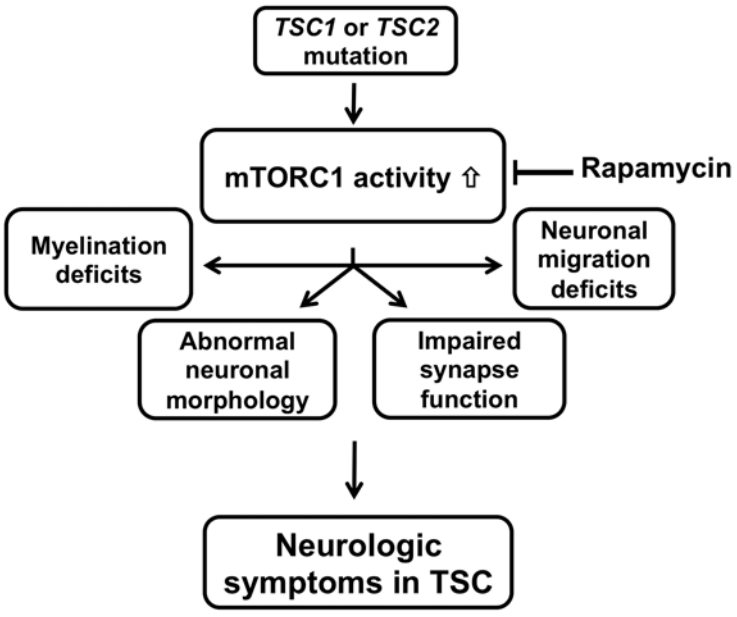

Figure 2. Proposed model of TSC pathomechanism in the brain. Mutation of either TSC1 or TSC2 disrupts the inhibitory function of the TSC1/2 complex. This disruption leads to constitutively increased mTORC1 activity, which in turn results in abnormal neuronal connectivity, characterized by abnormal neuronal morphology and migration, abnormal white matter, and impaired synapse function.

Not only dysregulated mTOR signaling is involved in TSC. Other monogenic causes of ASD, including mutations of NF1 (neurofibromin 1), PTEN (phosphatase and tensin homolog deleted on chromosome 10), the tyrosine kinase MET, and eIF4e are all involved in the mTOR signaling pathway (Lipton and Sahin, 2014), raising the question of whether dysregulation of the TSC/mTOR pathway predisposes

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to autism. This theory opens up exciting potential therapeutic options for the future, as mTOR inhibitors are already available and approved for other manifestations of TSC.

Therapeutic Approaches

Neurological symptoms such as epilepsy, ASD, and cognitive and behavioral problems remain an area of great worry for parents of children with TSC. Despite the presence of many antiepileptic drugs on the market, seizures in TSC can be devastating and difficult to control, and there is an association between early seizure onset and worse developmental outcomes in TSC. Translational research has laid the foundation for promising therapeutic approaches. Rapamycin (sirolimus), a compound originally identified from *Streptomyces hygroscopicus* on Easter Island (Rapa Nui gave the compound its name) and used as an immunosuppressant after kidney transplant for many years, inhibits mTORC1 but not mTORC2 activity. mTOR inhibitors have been used in murine TSC models, resulting in improved myelination and cytopathological architecture as well as restoration of synaptic function (Zeng et al., 2008; Goto et al., 2011; Carson et al., 2012; Tsai et al., 2012), suggesting their potential to prevent the development or progression of neurological symptoms.

When initiated early in life, rapamycin treatment prolonged survival and prevented epilepsy in mice with a glial or a neural progenitor *Tsc1* deletion; when initiated after seizure onset, rapamycin caused cessation of seizures (Meikle et al., 2008; Zeng et al., 2008; Goto et al., 2011). Rapamycin also reverted the autism-like phenotype in cerebellar PC *Tsc1*-knockout mice (Tsai et al., 2012) and reversed learning deficits in $Tsc2^{+/-}$ mice (Ehninger et al., 2008).

Sirolimus (rapamycin) and everolimus (a rapamycin analogue) have successfully been used to treat renal angiomyolipomas and SEGAs in studies in TSC patients. Everolimus recently received FDA approval for the treatment of SEGAs at any age, and for renal angiomyolipomas in patients older than 18 years. Interestingly, patients treated with everolimus for SEGAs had a clinically relevant reduction in their seizure frequency and an improved quality of life, but no changes in cognition, which were assessed as a secondary study outcome (Krueger et al., 2010). Subgroup analysis of patients from this study revealed improved FA measures as a marker for white matter integrity on DTI imaging in everolimus-treated patients (Tillema et al., 2012). Recently, the first prospective phase I/II trial was published that assessed the effect of everolimus on seizures as a primary outcome in 20 TSC patients with refractory epilepsy. In this trial, 12 weeks of treatment (4 weeks' titration, 8 weeks' maintenance) resulted in a statistically significant reduction in seizure frequency and duration, with 20% of enrolled patients achieving seizure freedom, as well as an increased quality of life and improved behaviors, as reported by parents using the Nisonger Child Behavior Rating Form and the Quality of Life for Children with Epilepsy survey (Krueger et al., 2013). A randomized, placebo-controlled phase II study assessing the effect of everolimus on neurocognition as a primary outcome, with effects on seizures, sleep, ASD, behavior, and academic skills as secondary outcomes, recently completed recruiting patients, and the analysis is pending (Trial of RAD001 and Neurocognition in Tuberous Sclerosis Complex, clinicaltrials.gov/show/NCT01289912).

Taken together, inhibition of the mTOR pathway using rapamycin or rapamycin analogues has been shown to be effective in murine models of TSC and has shown promising first results for seizure control in patients with TSC. As mentioned earlier, a phase II study to assess the effects on other neurological complications is under way, but there is a long and winding road ahead, along which many questions still need to be answered. We still do not know why a TSC mutation causes severe neurological impairment in one patient but not in others, and what factors might be contributing. It has been shown that early-onset and refractory seizures are associated with worse developmental outcomes, indicating that infancy is a critical period for the course of the disease. This association opens up the question of whether the timing of treatment initiation is crucial, and whether preventive treatment might be a possibility. If so, how would we decide whom to treat preventively, given the wide spectrum of disease manifestations? Would a short-term treatment be sufficient, or would patients require long-term courses and potentially develop more side effects? Would patients with "mild" disease benefit the same way as those who are severely affected, and vice versa? Having surrogate markers that could predict response to treatment would be extremely helpful, and investigations are in progress to develop such biomarkers.

In TSC, seizures and cognitive and behavioral problems greatly increase disease burden. Conventional therapies are often insufficient to control them, because they do not address the underlying mechanism. The prospect of being able to pursue a mechanism-based treatment approach for neurological manifestations of TSC is both promising

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and exciting, one that we hope will contribute to helping patients and their families in the future to improve their quality of life.

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Reelin Pathways in Brain Development and Function

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Introduction

The extracellular protein Reelin critically controls radial neuronal migration and layer formation in the developing mammalian brain. The molecular mechanisms that mediate this essential function have been partially elucidated by a series of studies that, over the course of two decades, exploited a combination of genetic, biochemical, and cell biological approaches. Together, this body of work revealed that the positioning of neurons within the radial dimension of cortical structures is achieved by the activity of a canonical signaling pathway involving two lipoprotein receptors. These molecular events modulate distinct steps in cellular layer formation, such as neuronal motility, orientation, branching or collapse of cellular processes, and consolidation of cell–cell adhesion. Furthermore, work in recent years revealed that Reelin controls not only neuronal migration, but also neuronal maturation, synaptic activity, and plasticity in the postnatal and adult brain. An additional noncanonical signaling pathway may contribute to the postnatal activities of Reelin on synaptic activity. In parallel with advances in basic science, human genetic studies implicated Reelin and its signaling molecules in a variety of neurological disorders. Knowledge of the molecular mechanisms that underlie the different biological functions is essential to understand the contribution that mutations in Reelin signaling make in human neurological and cognitive disorders.

The *Reelin* Gene and Protein

Reelin was identified as the gene disrupted in the classic neurological mutant mouse *reeler* (D'Arcangelo et al., 1995). Homozygous *reeler* mice exhibit tremors and ataxia, and their neuroanatomical phenotype is characterized by cerebellar hypoplasia and by the disruption of cellular layers in cortical structures, such as the cerebral cortex, the hippocampus, and the cerebellum. Heterozygous *reeler* mice, on the other hand, appear normal but exhibit subtle defects in neuronal maturation and behavioral deficits. In addition to the mouse gene, *Reelin* orthologs have been identified in several vertebrate species, and mutations have been identified in the rat and in humans. Based on a multitude of anatomical studies, mainly performed in the homozygous *reeler* mouse, it is clear that, during brain development, the *Reelin* gene product critically regulates neuronal migration and the inside-out formation of cellular layers in cortical structures during brain development. Additionally, the Reelin protein is required for the assembly of neuronal cell bodies in some brain stem nuclei and autonomic columns in the spinal cord and the formation of synaptic layers in the retina.

Reelin is a large glycoprotein that, during early brain development, is secreted mainly in superficial layers of developing cortical structures, where it functions as a positional cue to control radial neuronal migration. In the postnatal brain, Reelin is secreted throughout cellular layers, where it functions to modulate synaptic function and plasticity. The full-length Reelin protein has a modular structure consisting of a series of eight repeats containing a cysteine pattern commonly found in extracellular proteins: the epidermal growth factor–like repeat. The repeats are unique to Reelin and are flanked by distinct N-terminal and C-terminal regions. The N-terminal region, which is required for homodimer formation, contains a signal peptide required for secretion and a small region of similarity to the extracellular protein F-spondin. The C-terminal region of Reelin contains a positively charged stretch of amino acids that is required for optimal signaling. After secretion in the extracellular environment, full-length Reelin is rapidly cleaved by proteases at two sites, generating three major proteolytic fragments. Among these, the central fragment composed of repeats 3 to 6 is the only one that appears to be necessary and sufficient to induce cellular layer formation in a cortical slice culture assay. However, the signaling activity of the central fragment is reduced compared with that of the full-length Reelin moiety, which can form homodimers via the N-terminal domain, suggesting that proteolytic processing attenuates the signal.

Reelin Function in Brain Development and Function

Reelin is expressed by multiple organs in the developing and adult organism, including the brain, spinal cord, and liver. Because it is a secreted protein, Reelin can be detected in the CSF and in the blood of rodents and humans. Within the developing forebrain, high levels of Reelin protein expression were detected by immunostaining with monoclonal antibodies in and around Cajal–Retzius cells, which populate the embryonic marginal zone of the cerebral cortex, the stratum lacunosum moleculare (SLM) hippocampus, and the outer molecular layer (OML) of the dentate gyrus (Alcantara et al., 1998). Lower levels of Reelin expression have also been detected within the developing cortical plate. It is currently thought that Reelin expression in superficial as well as deeper locations promotes the radial migration of principal (excitatory) cortical neurons. Specifically, Reelin

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promotes their movement by somal translocation toward superficial layers, enabling newborn neurons to bypass older ones and thus establishing an inside-out pattern of corticogenesis. This theory explains why excitatory neurons in the *reeler* cerebral cortex and hippocampus fail to completely form orderly cellular layers. Inhibitory forebrain neurons are not directly affected by Reelin since they use tangential rather than radial migration to enter the pallium from the basal forebrain. In the dentate gyrus, where neurogenesis and neuronal migration extend beyond the prenatal period of development, Reelin is expressed mostly by Cajal–Retzius cells in the OML and promotes the radial migration of dentate granule cells throughout life. In the embryonic cerebellum, Reelin is expressed mainly by granule cell precursors in the external granule layer, where it promotes the radial migration of Purkinje cells and the formation of the Purkinje cell layer. After birth, granule cells migrate inwardly past the Purkinje cell layer to form the internal granule cell layer as the cerebellum continues to grow in size and becomes foliated. In *reeler* mice, the absence of the Purkinje cell layer in the cerebellar cortex leads to secondary defects in granule-cell proliferation and causes the hypoplasia of the entire structure and the motor deficits that are typically seen in mutant mice.

In addition to cellular layer formation, Reelin promotes the branching of some axonal terminals, as well as dendrites and apical processes in the developing hippocampus and cerebral cortex. In the early postnatal hippocampus, Reelin is still abundant in the SLM and the OML of the dentate gyrus, where the axons of the entorhinohippocampal pathway terminate. Reelin is not essential for targeting these axons to the SLM and OML, but it is required to promote their branching in these layers, which ultimately stimulates the formation of synapses by entorhinal afferents onto distal dendrites of hippocampal and dentate neurons. The effect of Reelin on axonal branching appears to be limited to the entorhinohippocampal pathway at early postnatal ages, as no defects have been reported so far in other axonal projections or in the adult *reeler* brain. The effect of Reelin on dendrite processes, however, seems to be more widespread as hippocampal, dentate, and cortical dendrites are severely defective in *reeler*. Culture studies demonstrated that Reelin promotes the growth of apical dendrites of hippocampal neurons, which *in vivo* grow toward Cajal–Retzius cell–containing marginal layers at early postnatal ages. Similarly, in the developing cerebral cortex, Reelin promotes the outgrowth of apical dendrites originating from cortical neurons and contacting Cajal–Retzius cells in the marginal zone. Other studies have further demonstrated that Reelin induces the extension of the Golgi apparatus in apical dendrites, thereby stimulating dendrite growth and cell polarization. However, as for axonal processes, the role of Reelin appears to be limited to developmental stages, since this activity is not required for the maturation of hippocampal neurons in long-term *in vitro* cultures.

In the developing postnatal brain (2–4 weeks after birth in rodents), Reelin also affects the formation of excitatory synapses. However, at these later times, the expression of Reelin in the forebrain is no longer localized to superficial layers, but is widespread throughout all cellular layers owing to the gradual appearance of a subset of Reelin-expressing interneurons in addition to residual Cajal–Retzius cells. In postnatal hippocampal structures, Reelin promotes the development of dendritic spines and thus affects their density. As for the branching of axons and dendrites, spine density is only transiently impaired in young adult Reelin-deficient mice (less than 1 month old), but is not significantly affected in the adult. However, other aspects of synapse structure and function are severely affected by Reelin in the adult brain. For example, Reelin overexpression increases the size of hippocampal spines, and its supplementation in slice cultures strongly enhances hippocampal long-term potentiation. Electrophysiological studies further showed that Reelin enhances neurotransmission, affects the maturation and trafficking of glutamate receptors of the NMDA receptor (NMDAR) and AMPA receptor families, and increases calcium influx (Herz and Chen, 2006).

Taken together, the evidence so far indicates that Reelin plays multiple roles in brain development: it plays a unique and major role in controlling radial neuronal migration in cortical structures during embryonic ages, and contributes to promoting the growth of some axonal projections in the hippocampus, apical dendrite development, and synapse formation in forebrain cortical structures at postnatal ages. Finally, in the adult brain, Reelin modulates synaptic function and plasticity, affecting cognition, learning, and memory.

Reelin Signaling Pathways

Because Reelin is an extracellular protein, it functions as a ligand for cell-surface receptors to initiate a signaling cascade in target cells. During brain development, well-established Reelin target cells are radially migrating neurons, such as excitatory

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cortical neurons, hippocampal pyramidal neurons, dentate granule cells, and cerebellar Purkinje cells. These cells likely receive the signal mostly through their apical dendrite/leading edge contacting the Reelin-rich marginal layers. Other cell populations may respond to Reelin exposure at different stages of development or in the adult brain, to carry out other aforementioned biological activities.

The molecular mechanisms of Reelin activity in the control of radial migration, axon and dendrite branching, and dendritic spine formation have been at least in part elucidated using a combination of genetic and biochemical approaches. These studies provide strong evidence for the existence of a canonical signaling pathway initiated by two high-affinity cell-surface Reelin receptors—the apolipoprotein E receptor 2 (ApoER2) and the very-low-density lipoprotein receptor (VLDLR)—and the intracellular adaptor protein Dab1. The components of the canonical pathways are absolutely essential for Reelin signaling during brain development. ApoER2 and VLDLR are members of the lipoprotein receptor superfamily, and their involvement in Reelin signaling was first suggested by the observation that ApoER2/Vldlr double-knockout mice exhibit a *reeler*-like neuroanatomical phenotype. Single-knockout mice displayed a milder layer phenotype, suggesting that they have partially overlapping functions in mediating the activity of Reelin on neuronal migration. ApoER2 and VLDLR are each capable of binding Reelin with similar affinity. Recent expression studies conclusively demonstrated that both these receptors are expressed in the leading processes of migrating cortical neurons contacting the marginal zone. This observation is consistent with these receptors' function in mediating the somal or terminal translocation of these neurons toward the top of the developing cortex. In addition, ApoER2 was detected near the intermediate zone, where it likely mediates the ability of Reelin to promote the transition from multipolar to bipolar morphology, facilitating radial migration.

Both ApoER2 and VLDLR bind Reelin through a similar extracellular domain that contains a conserved lysine residue (Yasui et al., 2007). It is thought that the strongest signal activation is achieved when the receptors bind multimeric aggregates of full-length Reelin, presumably leading to massive receptor clustering and Src-family kinase (SFK) activation. Upon Reelin binding, ApoER2 and VLDLR cluster and internalize the ligand, causing the activation of the SFKs Fyn and Src, which then phosphorylate the adaptor protein Dab1 and initiate downstream signaling events crucial for migration. The essential function of Dab1 in mediating Reelin signaling is demonstrated by the fact that spontaneous mutations in the *Dab1* gene in *scrambler* and *yotari* mutant mice, or knockout deletion of the gene, all result in a *reeler*-like phenotype. Reelin rapidly induces the phosphorylation of Dab1 on multiple tyrosine residues in cultured cortical neurons, an event that is mediated mainly by Fyn and Src. Dab1 phosphorylation on tyrosine residues is essential for signal transduction, because point mutations at these sites result in the appearance of a *reeler*-like phenotype *in vivo*. Dab1 tyrosine phosphorylation is also coupled to its ubiquitination by the Cbl ligase, and to its degradation by the proteasome system. This degradation represents a mechanism for signal termination and explains why Dab1 protein levels are elevated in the brains of *reeler* as well as Fyn/Src double-knockout mice. Taken together, these findings indicate that Reelin promotes the activation of Dab1 in target cells, but protein degradation mechanisms ensure that the signal is transient. This sequence of activation and shutoff may be crucial for the execution of multiple steps in neuronal migration. Indeed, different models have been proposed to explain how the Reelin function may change dynamically from a permissive, attractive clue during the initial phases of migration to a detachment from radial glia and thus a stop signal during later phases.

In the canonical signal transduction pathway, other kinases mediate Reelin activity downstream of Dab1. Phosphoinositide-3-kinase (PI3K) activation results in the phosphorylation and activation of Akt and mTORC1 (the mammalian target of rapamycin complex C1). Pharmacological studies using cultures of cortical slices suggested that the activation of PI3K and Akt may be important for the control of neural migration during early brain development, whereas the activation of mTORC1 may promote dendrite elongation. Other studies provided evidence that mTORC1 is involved in the NMDA-dependent rescue of synaptic plasticity and behavioral defects in juvenile *reeler* heterozygous mice. Furthermore, basal levels of Akt phosphorylation were reduced in adult forebrain-specific conditional Dab1 knockout mice, which also exhibit plasticity and behavioral defects. Together, these findings suggest that activation of the PI3K/Akt/mTORC1 pathway by Reelin may be involved in the postnatal control of neuronal maturation and synaptic plasticity.

Early efforts to identify potential downstream effectors of Dab1 in the control of migration pointed to components of the platelet-activating factor (PAF)–

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acetyl hydrolase 1b (Pafah1b) complex. This complex functions as a phospholipase and catalyzes the PAF lipid through the activity of two catalytic α-subunits. The complex also contains a regulatory subunit, Lis1, which is involved in neuronal migration and human lissencephaly. Genetic interactions and biochemical experiments indicated that Reelin promotes the interaction between Dab1 and Lis1, and that the absence of these two proteins cooperated to increase the frequency of hydrocephalus (Assadi et al., 2003). Further studies also demonstrated that the catalytic subunit of Pafah1b α2 binds to Dab1 and suppresses the hydrocephalus phenotype of Reelin/Lis double-mutant mice. However, Lis1 controls many aspects of neurogenesis and neuronal migration through a mechanism that involves the coupling of the centrosome to the nucleus. This mechanism appears to be independent from both the catalytic activity of the Pafah1b complex and Reelin. Furthermore, genetic ablation of the catalytic subunits of Pafah1b did not result in neuronal migration defects. Thus, this complex does not appear to be involved in the control of migration or corticogenesis. However, it may play a role in as yet unidentified mechanisms that contribute to normal brain development by preventing the onset of hydrocephalus.

Other biochemical studies identified several molecules that bind Dab1 and thus may potentially function in Reelin signaling in cortical development. These include the Crk family of proteins, which play a significant role in Reelin-dependent migration, likely by binding the GTP exchange factor C3G, which then activates Rap1. Three members of this family (CrkI, Crk II, and CrkL) were shown to bind phospho-Dab1, and Reelin was shown to induce the phosphorylation of C3G and the activation of Rap1. This study suggested that the CrkL/C3G/Rap1 pathway operates downstream of Dab1 in Reelin-stimulated neurons. The biological relevance of Crk family proteins in neuronal migration was demonstrated *in vivo* by the conditional double knockout of Crk and CrkL in neural progenitor cells, which results in a *reeler*-like cortical phenotype. The role of Rap1 in Reelin signaling and radial migration was further explored in multiple *in vivo* studies. In one study, Rap1 was shown to regulate the expression of cell adhesion molecules (CAMs) of the cadherin family in migrating neurons, an event required for glia-independent migration. Specifically, in this study it was proposed that Rap1 promotes cadherin expression, which in turn favors the extension and attachment of leading processes of migrating neurons and enables terminal translocation near the upper cortical plate. In a second study, Rap1 was shown to regulate the membrane localization of N-cadherin. This event was linked specifically to the acquisition of proper cellular orientation, which is also required for glia-independent neuronal migration, but occurs in the deeper regions of the developing cortical plate.

Building on these observations, recent studies further investigated the role of the Crk/C3G/Rap1 pathway in Reelin-dependent migration and provided a comprehensive view of its molecular mechanisms (Sekine et al., 2012; Gil-Sanz et al., 2013). This work suggested that Rap1 has a dual function in migration: in deep regions of the cortical plate, Rap1 functions through N-cadherins to orient cell bodies and enable entry into the cortical plate, but this activity may not be stimulated by C3G. Near the upper cortical plate, Reelin activates C3G and Rap1, and this in turn leads to the activation of α5β1 integrins, which mediate the attachment of apical processes to fibronectin in the marginal zone and the recruitment of CAMs, such as nectins and cadherin 2, which mediate cell–cell interactions in the marginal zone. Together, these secreted and contact-dependent mechanisms enable terminal translocation and the completion of radial migration near the Reelin-rich marginal zone.

In the postnatal and adult forebrain, Reelin is expressed and secreted by multiple cell types, including many GABAergic interneurons, throughout all cortical and hippocampal layers. The ability of Reelin to modulate synaptic activity and plasticity is mediated at least in part by ApoER2 (apolipoprotein E receptor 2), also known as LRP8 (low-density lipoprotein receptor–related protein 8), which interacts with the NMDAR at the synapse in a complex that includes PSD95 (Beffert et al., 2005). NR2 subunits of the NMDAR are phosphorylated upon Reelin exposure, resulting in increased calcium influx and increased glutamatergic activity (Chen et al., 2005). In addition to this acute regulation of NMDAR function, Reelin affects the subunit composition of the NMDAR, favoring its maturation during postnatal brain development. Consistent with these findings, abnormal synaptic and membrane expression of NR2 subunits have been reported in heterozygous *reeler* mice.

In the past few years, new potential Reelin signaling systems have surfaced implicating ephrin ligands and their EphB receptor family. One study found that ephrin-B1 interacting with EphB2 controls the migration of dentate progenitor cells into the dorsal half of the developing dentate gyrus, perhaps in part by affecting Reelin expression. Another study

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showed that the N-terminal region of Reelin binds to the extracellular domains of EphB transmembrane proteins, inducing receptor clustering and activation of EphB forward-signaling in neurons, independently of ApoER2 and VLDLR (Bouche et al., 2013). However, mice lacking EphB receptors displayed a very mild migration phenotype that was limited to a modest positioning defect of CA3 hippocampal pyramidal neurons. Thus, even though there appears to be some signal integration between Reelin and ephrin/EphB receptors, the physiological significance of this interaction for neuronal migration is rather limited. Based on a recent study, it appears that ephrin/Eph signaling may be more important for mediating contact repulsion among Cajal–Retzius cells, to ensure the even distribution of these neurons in the brain.

A recent study utilizing purified recombinant Reelin demonstrated that the full-length protein moiety induces, in addition to canonical signaling, a novel noncanonical signaling pathway that involves MAPKs (mitogen-activated protein kinases) Erk1 and Erk2 and leads to the activation of several activity-dependent immediate-early genes (IEGs) via p90RSK activation (Lee et al., 2014). Treatment of cortical neurons with purified Reelin resulted in the rapid induction of Erk1/2 phosphorylation and IEG induction, which were blocked by the SFK and MEK inhibitors, but not by the lipoprotein receptor inhibitor RAP, suggesting that this noncanonical signaling is mediated by an unknown receptor that is not a member of the lipoprotein receptor family. Another recent study, also utilizing purified Reelin, confirmed the activation of Erk1/2 signaling and IEG expression and further demonstrated that Reelin affects the activity of neuronal enhancer elements that are important for neuronal maturation and synaptic plasticity (Telese et al., 2015). However, this study suggested that Reelin affects the activity of enhancer elements via ApoER2 binding and NMDAR activation. Further investigation is required to clarify the molecular mechanisms controlling synaptic function in the postnatal and adult brain.

Reelin Signaling and Neurological Disorders

The complete loss of Reelin signaling during human development causes a severe neuronal migration disorder known as lissencephaly with cerebellar hypoplasia (LCH), a disease condition that causes severe ataxia, epilepsy, and cognitive delay, and at the neuroanatomical level, closely mimics the mouse reeler phenotype. So far, only subjects carrying homozygous *REELIN* (Hong et al., 2000) or *VLDLR* have been reported in connection with the appearance of LCH. It is presently unclear why *APOER2* or *DAB1* mutations have not been detected in this patient population, despite the critical role of their encoded proteins in neuronal migration. LCH patients represent only a small subset of subjects with lissencephaly, and the majority of cases result from heterozygous mutations in the *LIS1* gene (*PAFAH1b1*). Genetic interaction between the Reelin effector Dab1 and Lis1 was demonstrated by the appearance of a cortical-layer phenotype in *Lis1* and *Dab1* double-heterozygous mice that is not apparent in single mutants (Assadi et al., 2003). In addition to a dramatic worsening of the layer-formation phenotype, double-heterozygous mutations in the *Dab1* and *Lis1* genes resulted in the appearance of progressive hydrocephalus at a high frequency. Interestingly, a high frequency of hydrocephalus was also observed when *Lis1* mutations were combined with mutations in other genes in the Reelin pathway, such as *Reelin* itself, *VLDLR*, and *ApoER2* (Assadi et al., 2003). These results suggested that Reelin and Lis1 signaling cooperate not only in the control of neuronal migration but also possibly in the control of ependymal layer formation.

A reduction in Reelin activity has been associated with multiple cognitive and psychiatric disorders, including schizophrenia and autism. Following the initial observation that *REELIN* mRNA levels are reduced in patients with schizophrenia (Impagnatiello et al., 1998), several investigators reported a deficiency in Reelin expression in different groups of psychiatric subjects, including those with bipolar disorder. The reduction in Reelin expression occurs most likely through epigenetic mechanisms that affect promoter methylation (Veldic et al., 2004; Grayson et al., 2006), although evidence for genetic association between schizophrenia and *REELIN* polymorphisms also exists in patient subpopulations. In addition to schizophrenia, reduced expression and *REELIN* polymorphisms have been reported in some groups of autistic patients. Recent genetic studies identified *REELIN* as one of 33 genes that represent major risk factors for autism, incurring *de novo* loss-of-function mutations in more than 5% of autistic subjects (De Rubeis et al., 2014). Animal studies support the association between Reelin and complex brain disorders, particularly schizophrenia. Heterozygous *reeler* mutant mice that express reduced levels of Reelin exhibit behavioral, synaptic plasticity, and dendrite spine abnormalities that are reminiscent of those found in schizophrenia patients. These mice do not exhibit apparent seizures.

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Surprisingly, however, heterozygous mutations in *REELIN* have been recently identified in patients with autosomal-dominant lateral temporal epilepsy, a genetic epilepsy syndrome clinically characterized by focal seizures with prominent auditory symptoms (Dazzo et al., 2015). This finding provides genetic evidence for the role of Reelin signaling in the control of neuronal excitability.

In addition to developmental brain disorders, defective Reelin signaling has been implicated in neurodegenerative disorders such as Alzheimer's disease (Krstic et al., 2013). In particular, the data available thus far suggest that reduction in Reelin signaling, owing to selective loss of Reelin-expressing cells in the hippocampus and entorhinal and frontal cortex, leads to synaptic loss in these brain regions and accelerates cognitive decline.

Together, these findings emphasize the role of Reelin signaling in supporting brain development and function throughout life, and provide insights into the neuropathology of multiple human neurological disorders. A detailed knowledge of the molecular mechanisms that underlie the biological functions of Reelin in the prenatal and postnatal brain may thus lead to the development of novel therapies for these disorders.

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Microtubules, Tubulin Isotypes, and Human Neurodevelopmental Tubulinopathies

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Background

Microtubules (MTs) are dynamic polymers that comprise tandem repeats of α- and β-tubulin heterodimers, with each heterodimer containing an α- and β-tubulin isotype encoded by distinct genes (Lopata and Cleveland, 1987). Heterodimers assemble in a head-to-tail fashion at the growing ends of MTs to form a sheet of longitudinal protofilaments. Lateral interactions between neighboring protofilaments cause the sheet to close, thereby forming the hollow, cylindrical MT body (Lowe et al., 2001; Li et al., 2002). The structural conformation of longitudinal protofilaments is tightly regulated, and α- and β-tubulin are highly conserved throughout eukaryotes.

The human genome encodes eight α- and nine β-tubulin genes. The α- and β-tubulin isoforms have high degrees of sequence homology to one another and diverge primarily at their very carboxy termini. They also have different, but often overlapping, spatial and temporal expression patterns. Each α- or β-tubulin isotype comprises N-terminal, intermediate, and C-terminal domains that are formed by beta sheets that alternate with alpha helices; the three domains correspond to residues 1–205, 206–381, and 382–451 in α-tubulin and to 1-229, 230-371, and 372-450 in β-tubulin (Nogales et al., 1998; Lowe et al., 2001). Residues in the N-terminal domain form the GTP binding pocket; GTP binding is important for protein folding, structure, and stability of tubulin heterodimers, and conformation of longitudinal protofilaments. Hydrolyzation of GTP bound by β-tubulin results in MT depolymerization, while GTP bound by α-tubulin is nonexchangeable and is thought to act as a structural cofactor (Nogales and Wang, 2006). The intermediate domain interacts with the N-terminal domain and mediates longitudinal and lateral interactions necessary for heterodimer and MT stability. The C-terminal domain contains H11 and H12 alpha helices on the external surface that interact with and mediate interactions with kinesin and dynein motors (H12), as well as other microtubule-associated proteins (MAPs) (H11) that extrinsically regulate MT dynamic properties. Other residues found in this domain are also important for the stability of longitudinal protofilaments. Tubulin isotypes undergo posttranslational modifications that affect MT dynamics, stability, and interactions with motors and maps.

Tight regulation of the dynamic behavior and function of the MT cytoskeleton is essential for the development and survival of neurons. MTs are polarized and, in neurons, their "minus ends" are usually oriented toward the centrosome in the cell body, while their "plus ends" project toward the edge of the cell body or the tip of the axons (Gordon-Weeks, 2004). MT polarity serves important functions in both differentiating and adult neurons. Frequent transitions between periods of growth and shortening at the dynamic "plus ends" permit differentiating neurons to extend or retract neurites and growing axons. Dynein and kinesin motors transport protein vesicles and organelles toward the plus and minus ends of MTs, respectively, and are necessary for the regulation of MT dynamics. Their activities are essential for cell migration, axon growth and guidance, and function and viability of adult neurons (Hirokawa and Takemura, 2004; Chevalier-Larsen and Holzbaur, 2006; Ayala et al., 2007; Nadar et al., 2008).

During the past eight years, multiple human neurodevelopmental syndromes have been discovered to result from mutations in genes encoding α- and β-tubulin isotypes. Cortical malformation syndromes have been reported to result from often *de novo* heterozygous missense mutations in *TUBA1A, TUBB2A, TUBB2B, TUBB3, TUBB5,* and *TUBG1* (Keays et al., 2007; Jaglin et al., 2009; Breuss et al., 2012; Poirier et al., 2013; Cushion et al., 2014), and possibly from a homozygous deletion in *TUBA8* (Abdollahi et al., 2009). Mutations result in a spectrum of cortical malformations ranging from lissencephaly, pachygyria, and polymicrogyria to "simplified gyral patterns" (SGPs). Although clinicians cannot predict which isotype is mutant and/or what residue is altered in a patient with a cortical malformation, predominant patterns of cortical dysgenesis allow some phenotype–genotype correlations. In addition to the cortical malformations, different heterozygous familial and *de novo* mutations in *TUBB3* and *TUBB2B* cause disordered cranial and central axon guidance in the absence of detectable cortical malformations, and these do lend themselves to phenotype–genotype correlations (Tischfield et al., 2010; Cederquist et al., 2012). Both the cortical malformation and the axonal tubulinopathies have high prevalence of dysmorphic basal ganglia (DBG), corpus callosum agenesis (ACC), and mild-to-severe cerebellar (CH) and brainstem (BSH) hypoplasia or dysplasia. Finally, hypomyelination and dystonia can arise from heterozygous missense mutations in *TUBB4A* (Hersheson et al., 2013; Lohmann et al., 2013; Simons et al., 2013), while susceptibility to amyotrophic lateral sclerosis (ALS) has been reported in association with variants in *TUBA4A* (Smith et al., 2014).

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The functional consequences of tubulin isotype mutations have typically been examined by determining how well mutant heterodimers fold and how well they incorporate in polymerizing MTs *in vitro*. Such studies demonstrate that tubulin mutations alter both the overall abundance and the function of mutant heterodimers. Approximately half of the amino acid substitutions in TUBA1A, TUBB2B, and TUBB3 that have been tested result in a significant decrease in the production of functional tubulin heterodimers owing to the failure to properly interact with one or more chaperone proteins that capture and fold nascent tubulin polypeptides (Tian et al., 2008; Jaglin et al., 2009; Poirier et al., 2010; Tian et al., 2010; Tischfield et al., 2010). Although it is concluded, in some cases, that these mutations act through haploinsufficiency, with overexpression in mammalian cells, many of the poorly folded mutant heterodimers incorporate into interphase MTs at levels equivalent to wild type. Moreover, recurrent *de novo* heterozygous missense mutations in the absence of nonsense, frameshift, or genomic deletions support altered protein function as a primary genetic etiology, and this theory is reinforced by phenotype–genotype correlations associated with a subset of recurrent mutations (Tischfield et al., 2010; Chew et al., 2013). Thus, it seems most likely that while some mutations do alter the overall abundance of heterodimers, all result in mutant heterodimers that can incorporate into MTs and alter function.

Once incorporated, different substitutions have been shown to have varying effects on the stability and dynamic properties of MTs, and on their association with MAPs and motor proteins. The substitutions that cause cortical malformations are widely distributed among the three domains of tubulin and are predicted to perturb different MT functions according to their structural locations. Subsets of mutations alter residues that interact directly with the GTP nucleotide, while others are located at contact surfaces between the intraheterodimer and interheterodimer, and thus are predicted to alter longitudinal protofilament interactions. Both these regions contain stretches of absolutely conserved residues; thus, a single amino acid substitution could significantly impede the formation of heterodimers, the overall ability of MTs to polymerize, or the conformational changes essential to the dynamic properties of MTs (Lowe et al., 2001). Another subset of mutations affects residues that lie in proximity to the interface between the N-terminal and intermediate domains. These substitutions may also affect heterodimer stability and/or the dynamic properties of MTs by altering the structure of tubulin during nucleotide exchange and hydrolysis. Overall, most of these types of mutations cause a range of gyral malformations, suggesting that MT stability may be diminished during key processes of cell migration, such as the coupling of the nucleus with the centrosome (Ayala et al., 2007; Higginbotham and Gleeson, 2007).

Other mutated residues fall within or immediately adjacent to the regions that participate in lateral interactions. Lateral interactions occur primarily between N-terminal loops of one heterodimer with the large loops in the intermediate domains of an adjacent heterodimer in the flanking longitudinal protofilament. These interactions permit the assembly of MTs, regulate their growth and shortening properties, and provide the stabilizing force that allows them to curve and bend without breaking. This is particularly important in neurons because MTs are arranged in dense networks and, in order to maintain their structural integrity, they must be resistant to forces that cause bending or buckling as is commonly seen *in vivo* (Tischfield and Engle, 2010; Tischfield et al., 2011).

Finally, some amino acid substitutions mutate residues on the surface of MTs that mediate protein interactions with kinesin, dynein, and other MAPs. Intriguingly, multiple TUBB3 substitutions and one TUBB2B substitution directly or indirectly alter β-tubulin residues on H12 that are implicated in motor protein binding, reduce kinesin interactions in yeast and in mouse, and result in a paralytic strabismus syndrome, referred to as congenital fibrosis of the extraocular muscles (CFEOM). CFEOM results from aberrant oculomotor axon growth or guidance, and is often accompanied by additional peripheral and central axon guidance defects (Tischfield et al., 2010; Cederquist et al., 2012).

What follows is a brief summary of the human tubulinopathies reported to date. Particularly relevant reviews for the reader include those reported by Tischfield and Engle, 2010; Tischfield et al., 2011; Bahi-Buisson et al., 2014; Breuss and Keays, 2014; Oegema et al., 2015; and Romaniello et al., 2015.

A Brief Catalog of Tubulinopathies

TUBA1A: Cortical dysgenesis

Reports: Many independent cases reported (Keays et al., 2007; Fallet-Bianco et al., 2008; Lecourtois et al., 2010; Cushion et al., 2013; Bahi-Buisson et al., 2014; Fallet-Bianco et al., 2014; Oegema et al., 2015).

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Phenotype: Typically microcephaly, motor and intellectual disabilities, and seizures. Broad range of delayed psychomotor development.

Fetopsy: Most often microlissencephaly or classical lissencephaly, but polymicrogyria may occur. Accompanied by ACC, DBG, and variable CH and BSH.

Neuroimaging: Primarily lissencephaly and microlissencephaly. Spectrum includes SGP and polymicrogyria. At the extreme, CH and BSH are seen with only minimal SGP, as well as ACC and DBG.

Expression: Postmitotic differentiating neurons but not glia (Braun et al., 2010; Breuss et al., 2012).

Mutation type: Heterozygous *de novo* missense variants.

Locations: More than 40 unique substitutions reported, approximately five of which are recurrent. They are spread across the isotype, but none directly alters residues implicated in motor protein–MT interaction.

Functional studies: Variable reduction in heterodimer formation and MT incorporation are seen.

TUBA8: Cortical dysgenesis (low confidence of pathogenicity)

Reports: One report of two consanguineous families (Abdollahi et al., 2009).

Phenotype: Developmental delay, seizures, hypotonia, and optic nerve hypoplasia.

Neuroimaging: Bilateral polymicrogyria, dysplastic or ACC, brainstem dysmorphisms, and normal basal ganglia.

Expression: Extremely low levels in the developing brain (Braun et al., 2010).

Mutation type: Homozygous 14 bp intronic deletion upstream of exon 2.

Functional studies: Altered splicing and reduced TUBA8 levels in patient-derived lymphoblastoid cells.

TUBB2A: Cortical dysgenesis

Reports: One report of two patients (Cushion et al., 2014).

Phenotype: Infantile-onset epilepsy.

Neuroimaging: One patient had dysmorphic corpus callosum, normal cortex. One patient had dysmorphic corpus callosum, SGP, DBG, and mild CH/BSH.

Expression: Developmental expression in human and mouse is much lower than for TUBB2B, TUBB3, and TUBB5 (Braun et al., 2010; Leandro-Garcia et al., 2010; Breuss et al., 2012).

Mutation type: Heterozygous *de novo* missense variants.

Locations: Adjacent amino acids located in a highly conserved loop, proposed to directly associate with the alpha (GTP) molecule, impairing intradimer interface and heterodimer formation.

Functional studies: Variable MT incorporation seen.

TUBB2B: Cortical dysgenesis and rarely primary axon guidance disorder (CFEOM)

A. Cortical dysgenesis

Reports: Many independent cases reported (Jaglin et al., 2009; Cederquist et al., 2012; Guerrini et al., 2012; Cushion et al., 2013; Bahi-Buisson et al., 2014; Fallet-Bianco et al., 2014).

Phenotypes: Typically microcephaly, severe motor and intellectual disabilities, and seizures. Broad range of delayed psychomotor development.

Fetopsy: Typically polymicrogyria or microlissencephaly.

Neuroimaging: Primarily centrally predominant polymicrogyria-like cortical dysplasia. Spectrum includes lissencephaly, pachygyria, and schizencephaly typically accompanied by DBG, ACC, CH, and BSH.

Expression: Highly expressed in postmitotic neurons at key developmental time points, but unlike TABA1A, also expressed in progenitor cells and in glia (Braun et al., 2010; Leandro-Garcia et al., 2010; Breuss et al., 2012).

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Mutation type: Heterozygous *de novo* missense variants and somatic mutations (Jamuar et al., 2014).

Location: At least 27 unique substitutions reported, of which at least three are repetitive. R380 has been altered to three different residues. None is predicted to alter motor protein binding.

***In vitro* studies:** Variable reduction in heterodimer formation and MT incorporation. In vivo knockdown in rat impairs radial migration (Jaglin et al., 2009).

B. Disordered axon guidance and CFEOM
Reports: One family (Cederquist et al., 2012).

Phenotype: Microcephaly, moderate intellectual disabilities, and CFEOM (paralytic strabismus).

Neuroimaging: Perisylvian to diffuse PMG, HCC, DBG, CH, and hypoplastic extraocular muscles. Heterotopic CC connectivity by DTI.

Mutation type: Heterozygous *de novo* missense variant.

Location: E421K on H12 alters residue with which kinesin motor proteins directly interact.

Functional studies: Folds and incorporates into MTs, alters yeast MT dynamic instability and disrupts kinesin–MT interactions. *In vivo* electroporation studies revealed perturbed layer-specificity of ipsilateral cortical microcircuitry and perturbed homotopic connectivity across the midline.

TUBB3: Cortical dysgenesis or primary axon guidance disorder (CFEOM)

A. Cortical dysgenesis
Reports: Multiple cases reported (Poirier et al., 2010; Bahi-Buisson et al., 2014; Oegema et al., 2015).

Phenotypes: Referral at school age for developmental delay and intellectual disabilities, hypotonia, spasticity, and nonparalytic strabismus; no cranial or peripheral nerve findings.

Fetopsy: Microlissencephaly, ACC, DBG, CH, and BSH (Poirier et al., 2010; Fallet-Bianco et al., 2014).

Neuroimaging: Mild focal or multifocal polymicrogyria-like cortical dysplasia or SGP. ACC, DBG, CH, BSH, and corticospinal tract hypoplasia.

Expression: Encodes the neuronal-specific β-tubulin isotype (Braun et al., 2010; Leandro-Garcia et al., 2010; Breuss et al., 2012).

Mutation type: Heterozygous *de novo* missense variants.

Locations: At least seven unique substitutions, of which one is repetitive. None is predicted to alter motor protein binding.

Functional studies: Variable reduction seen in heterodimer formation/MT incorporation. Destabilizes MTs.

B. Disordered axon guidance and CFEOM
Reports: Many familial and *de novo* cases reported (Tischfield et al., 2010; Chew et al., 2013; Balasubramanian et al., 2015).

Phenotypes: Referral at various ages for isolated CFEOM (R262C, R62Q, A302T), CFEOM and progressive axonal sensorimotor polyneuropathy (PN) (R417N), or CFEOM with peripheral neuropathy and various combinations of Kallmann syndrome, lower cranial nerve dysfunction, contractures, and intellectual and social disabilities (R262H, R380C, E410K, D417H).

Neuroimaging: Corresponds to specific mutation. Isolated CFEOM with or without PN has oculomotor nerve hypoplasia, agenesis of the anterior commissure, and mild CC posterior thinning. Among more severe mutations, subsets have ACC, DGB, and corticospinal tract hypoplasia. CH and BSH are rare. There is an absence of detected cortical malformations.

Mutation type: Heterozygous familial, *de novo*, and germline mosaic missense variants. Milder phenotypes are often inherited, whereas the more severe cases are typically *de novo*.

Locations: Eight unique substitutions are reported, of which five are repetitive. Primarily but not exclusively located on helix H12 and H11 and in residues that interact with these helices, thereby predominately altering residues important for kinesin–MT and potentially MAP–MT interactions.

Functional studies: Variable reduction in heterodimer formation and MT incorporation; better folding and higher incorporation correlate with more severe phenotypes. Mutations stabilize MTs, and a subset disrupts kinesin–MT interactions in yeast.

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Tubb3$^{R262C/R262C}$ mouse model: Defects in cranial nerve and commissural axons, increased MT stability, and decreased kinesin–MT interactions.

TUBB5: Microcephaly

Reports: Three cases (Breuss et al., 2012).

Phenotypes: Microcephaly; cognitive impairment with motor and language delay.

Neuroimaging: Dysmorphic basal ganglia and corpus callosum and brainstem hypoplasia. Two patients had no cortical malformations, while one had focal polymicrogyria and localized band heterotopia.

Expression: Expressed in radial glial progenitors, intermediate progenitors, and postmitotic neurons.

Mutation type: Heterozygous *de novo* missense variants.

Locations: M299V, V353I, and E401K.

Functional studies: Variable effect on heterodimer folding and incorporation. Increased mitotic index. Depletion of mouse *Tubb5 in utero* by shRNA knockdown perturbed the cell cycle of progenitors and altered the position of migrating neurons. *In utero* electroporation of mutations altered neuronal differentiation and dendritic spine formation *in vivo* (Breuss et al., 2012; Ngo et al., 2014).

TUBG1: Cortical dysgenesis

Reports: Three patients (Poirier et al., 2013).

Phenotypes: Severe microcephaly, moderate-to-severe developmental disabilities, spasticity, and early-onset epilepsy.

Neuroimaging: Posterior predominant pachygyria to laminar heterotopia; dysmorphic thick CC; normal basal ganglia, cerebellum, and brainstem.

Expression: Constitutively expressed throughout the body and highly expressed in the fetal brain.

Mutation type: Heterozygous *de novo* missense variants.

Locations: L387P, T331P located in buried sites in two distinct α-helices; likely to destabilize helices. Y92C is located in the vicinity of the GTP binding site.

Functional studies: The γ-tubulins form a structural component of the centrosome known as the γ-tubulin ring complex, which plays a role in MT nucleation and regulation of the spindle during mitosis. L387P impairs chaperone-mediated folding of TUBG1, whereas W92C results in decreased frequency of MT nucleation from the spindle body. TUBG1 knockdown by *in utero* electroporation drastically impairs neuronal migration.

TUBB4A: H-ABC and DYT4

Hypomyelinating leukoencephalopathy in isolation or with atrophy of BG and cerebellum (H-ABC)

Reports: Multiple individuals (Simons et al., 2013; Hamilton et al., 2014; Miyatake et al., 2014; Carvalho et al., 2015; Erro et al., 2015).

Phenotypes: Degenerative symptoms start in infancy or childhood and include cognitive deficits, spasticity, ataxia, extrapyramidal movement disorders (dystonia, choreoathetosis, rigidity), and seizures.

Neuroimaging: Hypomyelination with atrophy of the basal ganglia and cerebellum, absent or disappearing putamen, and highly variable cerebral atrophy.

Expression: Low levels in the developing CNS, but highly transcribed in adult cerebellum, brainstem, and striatum (Leandro-Garcia et al., 2010; Breuss et al., 2012).

Mutation type: Heterozygous missense variants, *de novo* in most cases.

Locations: Distributed across isotype. Common amino acid substitution D249N results in less rapidly progressive disease course; growing evidence of phenotype–genotype correlations.

DYT4 or whispering dystonia

Reports: Several reports (Hersheson et al., 2013; Lohmann et al., 2013).

Phenotypes: Whispering dysphonia, generalized dystonia, and a characteristic ataxic gait.

Mutation type: Inherited heterozygous missense variants.

Locations: Recurrent R2G in the highly conserved autoregulatory domain; A271T in one family.

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Functional studies: R2G has reduced mutant mRNA in different cell types compared with controls. R2G disrupts the autoregulatory capability of the wild-type peptide.

Conclusions

While phenotype–genotype correlations among tubulinopathies are becoming less distinct, and the range of brain developmental defects is widening as more cases are reported, some key features of these disorders can help with diagnosis. Although not universal, these features include dysmorphic basal ganglia, hypoplasia or agenesis of the corpus callosum, and brainstem and cerebellar hypoplasia. In the coming years, additional studies will determine whether any of the tubulin phenotypes arise from haploinsufficiency, or whether they all result from altered function. They will also determine whether differences in these disorders reflect simply a combination of each isotype's temporal and spatial expression pattern and the specifically altered residue. Finally, such studies will help us to understand whether different isotypes are necessary for specialized MT functions and/or make unique contributions to neuronal development.

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