UNRAVELING THE MOLECULAR AND CELLULAR MECHANISMS OF NEUROLOGICAL DYSFUNCTION IN TUBEROUS SCLEROSIS COMPLEX

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UNRAVELING THE MOLECULAR AND CELLULAR MECHANISMS OF NEUROLOGICAL DYSFUNCTION IN TUBEROUS SCLEROSIS COMPLEX

ONTRAFELEN VAN DE MOLECULAIRE EN CELLULAIRE MECHANISMEN DIE TEN GRONDSLAG LIGGEN AAN DE NEUROLOGISCHE SYMPTOMEN OPTREDEND BIJ TUBEREUZE SCLEROSE COMPLEX

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GENERAL INTRODUCTION

TUBEROUS SCLEROSIS COMPLEX AND ITS CLINICAL PROFILE

Tuberous Sclerosis Complex (TSC) is a multi-organ disorder, which is characterized by the development of benign malformations and specific neurological and psychiatric symptoms[1]. It was first formally described by the neurologist Bourneville in 1880, based on a female patient who presented with epilepsy, skin tags, facial rash, and sclerotic areas in some of the cerebral convolutions on pathological examination of the brain[2].

TSC is a relatively common genetic syndrome with a birth incidence of 1:6000[1]. Although all major organs are susceptible to TSC, the most frequently affected are the brain, skin, kidneys, heart, lung and eye[1]. None of the manifestations associated with TSC is pathognomonic for the disorder and accordingly, diagnosis is made based on the presence of combinations of major and minor disease features, as revealed by clinical, radiological and pathological examination (**Table 1 and Figure 1**)[1].

Though not part of the diagnostic criteria, neurological and neuropsychiatric symptoms, such as epilepsy, intellectual disabilities and autism, are often present and can be a reason to seek medical attention[1]. Other clinically relevant but less frequent complications of TSC include: kidney dysfunction, internal bleedings from large angiomyolipomas, dyspnoe and lung collapse secondary to lymphangiomyomatosis, heart flow and rhythm disturbances caused by rhabdomyomas, and hydrocephalus due to subependymal giant cell astrocytomas[1]. Notably, symptomatology in TSC is profoundly age-dependent with neurological symptoms and heart and skin manifestations generally already present in infancy and young childhood, while kidney and lung manifestations usually develop from late childhood and puberty (Table 1)[1]. This age-specific clinical spectrum and

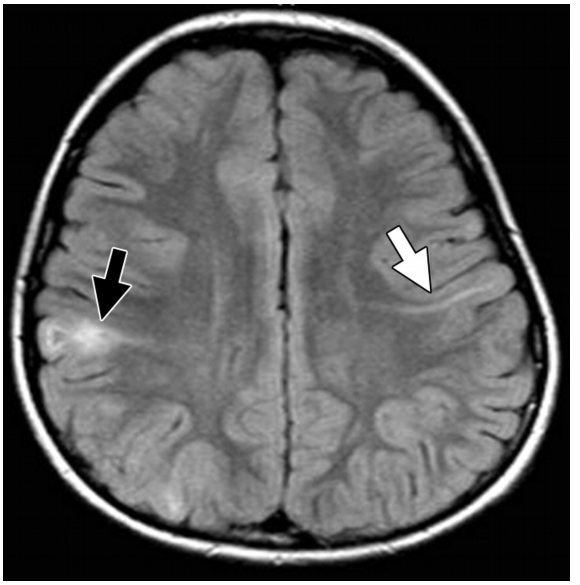

Figure 1. Brain manifestations of TSC. Axial magnetic resonance (MR) image shows cortical and subcortical tuber (*black arrow*) and radial migration line (*white arrow*) in 8-year-old boy with TSC.

Table 1. Clinical features of TSC (Adapted from[1]). Cerebral cortical dysplasia and cerebral white-matter radial migration lines are combined as a single feature of TSC; when both lymphangiomyomatosis and renal angiomyolipomas are present, other features must be present before TSC is diagnosed.

Major Features	Organ involved	Age at onset
Facial angiofibroma	Skin	Infancy to adulthood
Ungual fibroma	Skin	Adolescence to adulthood
Shagreen patch	Skin	Childhood
Hypomelanotic macule	Skin	Infancy to childhood
Cortical tuber	Brain	Fetal life
Subependymal nodule	Brain	Childhood to adolescence
Subependymal giant-cell tumor	Brain	Childhood to adolescence
Retinal hamartoma	Eye	Infancy
Cardiac rhabdomyoma	Heart	Fetal life
Renal angiomyolipoma	Kidney	Childhood to adulthood
Lymphangiomyomatosis	Lung	Adolescence to adulthood
Minor Features*	Organ involved	Age at onset
Multiple pits in dental enamel	Teeth	
Hamartomatous rectal polyps	Digestive tract	
Bone cysts	Skeleton	
Cerebral white-matter radial migration lines	Brain	
Gingival fibromas	Teeth	
Retinal achromic patch	Eye	
"Confetti" skin lesions (groups of small, lightly pigmented spots)	Skin	
Multiple renal cysts	Kidney	

*Data are not available to list the typical age at onset.

Definitive TSC	Probable TSC	Possible TSC
2 major features or 1 major and 2 minor features	1 major and 1 minor feature	Either 1 major feature, or 2 or more minor features

the significant variability in the expression of disease among affected individuals makes TSC challenging to diagnose [1].

TSC GENES AND PROTEINS

TSC is caused by inactivating mutations to the *TSC1* (9q34)[3] or *TSC2* (16p13)[4] genes. In affected families, the disease inherits in an autosomal-dominant fashion, but sporadic cases are most abundant, accounting for 2/3 of the total disease population[1]. Disease-causing mutations can be identified in 75-85% of individuals

meeting the clinical criteria for a definite TSC diagnosis, of which approximately 80% are mutations in the *TSC2* gene[5-9]. Failure of *TSC1* or *TSC2* mutation detection in 15-25% of cases can be attributed to ineffective mutation detection methods, mosaicism and the existence of additional *TSC* genes[10]. A broad spectrum of mutations has been identified in both genes, including frameshifts, large rearrangements, non-sense mutations, missense mutations and small in-frame deletions and insertions[5-9]. In the setting of TSC, in which variable phenotypes and a wide mutational spectrum are often encountered, genetic analysis and functional assays are of significant value to complement the clinical picture and to distinguish between pathogenic and non-pathogenic variants[11-13]. Although there is considerable overlap in disease severity, *TSC1* mutations[5-7, 9] and specific *TSC2* missense mutations[14-18] associate with less severe TSC disease. In addition, large *TSC2* deletions extending into the neighboring *PDK1* gene are correlated with a higher incidence and increased severity of renal cystic disease[19, 20]. In the majority of TSC-associated lesions, *TSC1* or *TSC2* loss of heterozygosity (LOH) has been demonstrated, indicating that somatic inactivation of the wild-type *TSC1* or *TSC2* allele is the most likely trigger for lesion formation[21-25].

The *TSC1*-encoded 130kDa protein hamartin (TSC1) has no significant homology to other known proteins, while the 200kDa protein tuberin (TSC2), encoded by *TSC2,* contains a GTPase activating (GAP) domain at its C-terminus[3, 4]. TSC1 and TSC2 interact to form a functional, predominantly cytosolic heterodimer, the TSC1-TSC2 complex[26, 27]. The TSC2 GAP domain seems to be critical for TSC1-TSC2 complex function as disease-associated missense mutations tend to cluster here[5, 7, 8, 28]. TSC1 is thought to be important for stabilizing TSC2 levels through inhibition of proteasome-mediated TSC2 degradation[29, 30], and for enhancing TSC2-GAP activity[31].

TSC1-TSC2 COMPLEX AND TOR SIGNALING NETWORKS

The TSC1-TSC2 complex acts as a GAP for the small G-protein Ras homolog enriched in brain (Rheb)[31], of which two isoforms exist: Rheb1 and Rheb2. As a GAP, the TSC1-TSC2 complex accelerates the conversion of Rheb-GTP to Rheb-GDP to inactivate Rheb. Together the TSC1-TSC2 complex and Rheb function as an important node in the regulation of target of rapamycin (TOR) signaling[32] (**for a schematic of the TOR pathway see Figure 2**).

TOR is a large protein kinase that in mammalian cells exists in 2 distinct protein complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2)[33]. The two TOR complexes can be functionally differentiated by their sensitivity to the inhibitor rapamycin such that TORC1 is directly and strongly inhibited while TORC2 is only inhibited after prolonged treatments[34, 35].

TORC1 is conserved through evolution and serves to regulate protein translation, cell division and autophagy in a wide range of organisms[36]. Accordingly, ablation of *Tor* or its essential mTORC1 companion *Regulatory-associated protein of TOR (Raptor)* prohibits murine embryonic development beyond 5 days[37-39]. The essential role of TORC1 signaling in regulating cell growth and proliferation is also evident from the many sporadic forms of cancers that show TORC1 gain of function[40].

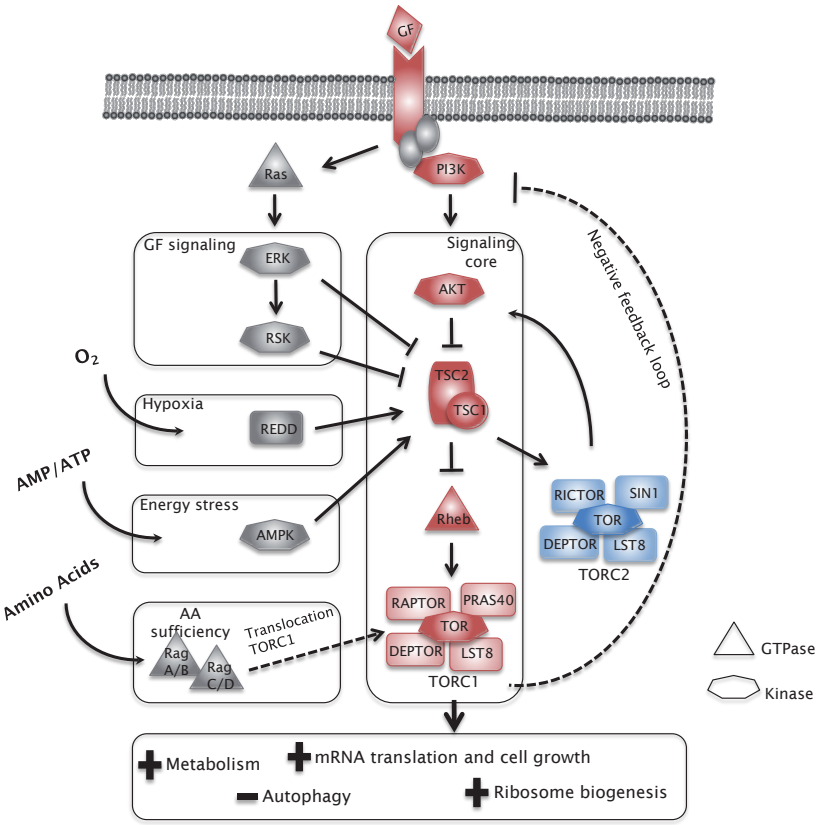

Figure 2. Schematic of TOR signaling pathway (Adapted from[185]). TORC1 is composed of TOR and its associated proteins RAPTOR, proline-rich Akt/PKB substrate 40 kDa (PRAS40), Lethal with SEC13 protein 8 (LST8) and mammalian specific DEP domain containing MTOR-interacting protein (DEPTOR). It integrates various environmental cues relayed via signaling modules to a central 'signaling core'. The TSC1-TSC2 complex acts as an inhibitor of Rheb in the TORC1 signaling core, but also functions as an activator of TORC2. TORC2 is composed of TOR and its associated proteins RICTOR, Mammalian lethal with SEC13 protein 8 (MLST8), mammalian specific DEP domain containing MTOR-interacting protein (DEPTOR) and stress-activated-protein-kinase-interacting protein 1 (SIN1). Up- and downstream effectors of TORC2 are incompletely characterized, but include AKT. TOR signaling is characterized by major negative feedback loops restraining PI3K-AKT signaling in contexts of enhanced TORC1 activity. Dashed lines indicate multistep mechanisms of regulation.

In TSC, insufficient TSC1-TSC2 complex function results in aberrantly activated TORC1 signaling, promoting cell growth[31].

The exact mechanism by which Rheb1-GTP and Rheb2-GTP activate TORC1 has not yet been determined, but they are essential for TORC1 activity in *Drosophila* and *in vitro*[32]. Active TORC1 phosphorylates multiple downstream targets including p70 S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins 1 and 2 (4E-BP1 and 4E-BP2), which are involved in cap-dependent translation[36].

Notably, recent studies indicate that the S6K-S6 signaling axis regulates cell growth, whereas cell proliferation is controlled by 4EBP-eIF4E signaling[41, 42].

Several inputs impinge on the TSC1-TSC2 complex such that it allows Rheb-TORC1 signaling and downstream events in favorable cellular conditions but inhibits Rheb-TORC1 activity in states of depletion and stress[32]. Upstream mediators of the TSC1-TSC2 complex include AMP-activated protein kinase (AMPK), DNA damage response 1 (REDD1), phosphoionositide3-kinase (PI3K)-AKT, mitogen-activated protein kinase (ERK)[32] and p90 ribosomal S6 kinase (RSK), which translate intracellular energy status (AMPK), oxygen levels (REDD1) and growth factor binding (PI3K-AKT, ERK and RSK) into activation (AMPK and REDD1) or inhibition (AKT, ERK and RSK) of the TSC1-TSC2 complex[32]. In addition, the availability of amino acids induces translocation of TORC1 to the lysosomal membrane, resulting in colocalization of Rheb and TORC1[43, 44].

Intriguingly, the TSC1-TSC2 complex also regulates TORC2[36, 45]. This occurs in a Rheb-independent manner, and seems to involve direct binding of the TSC1-TSC2 complex to TORC2 components[33]. Additional crosstalk between TORC1 and TORC2 is mediated by S6K-dependent phosphorylation and inhibition of Rapamycin insensitive companion of TOR (RICTOR)[46]. By incompletely understood mechanisms, TORC2 is activated by growth factors and regulates actin organization, cell motility and cell survival by means of phosphorylation of its downstream effectors, including the prosurvival protein AKT[33]. Remarkably, AKT is regulated by both TOR complexes, though in opposite directions, since extensive feedback loops function to limit AKT activity in contexts of prolonged TORC1 activation[33].

Hence, in *TSC* insufficient cells, TORC1-dependent feedback mechanisms and reduced TORC2 function converge to reduce AKT activity. Although the attenuation of upstream signaling appears to restrict tumor growth and malignant transformation in TSC[36, 47, 48], 'benign metastases' can arise in the form of renal angiomyolipoma cells that gain the ability to spread and cause lymphangiomyomatosis under the influence of estrogen[49].

NEUROLOGICAL AND NEUROPSYCHIATRIC MANIFESTATIONS OF TSC

Brain lesions are found in the vast majority of TSC individuals and are classified as cortical tubers, subependymal nodules and subependymal giant cell astrocytomas[1]. Tubers are primarily associated with the neurological symptoms. A subset of subependymal nodules (~10%) gain the capacity to grow and, when larger than 1cm in size, are classified as subependymal giant cell astrocytomas[1]. When located in narrow regions such as the foramen of Monroe, subependymal giant cell astrocytomas can obstruct cerebrospinal fluid flow eventually leading to hydrocephalus and brain damage[1].

Neurological and neuropsychiatric symptoms are among the earliest signs of TSC and include epilepsy, intellectual impairments, autistic spectrum disorders (ASD), attention-deficit hyperactivity disorder (AHDH), mood and anxiety disorders and specific neuropsychological deficits[50]. Up to 90% of individuals with TSC will develop epilepsy during their lifetime, making it one of the hallmarks of the disease[51]. In most patients the onset of epilepsy occurs in childhood or even infancy

and can involve a wide range of seizure types, including infantile spasms, tonic, clonic, tonic-clonic, atonic, myoclonic, atypical absence, partial, and complex partial seizures[51]. Unfortunately, seizures in TSC are often refractory to standard anti-epileptic drug treatments[51].

Intellectual abilities in TSC are best described by considering a subgroup of patients (~30%) who have a 'profound phenotype', with IQ scores around 20 and often showing arrest in their development, and a 'normal distribution phenotype' group (70%), whose IQ scores follow a normal distribution though with a lower mean than population IQ scores[50, 52, 53] (Fig. 1). Consequently, as a whole, approximately half of the TSC population have IQ scores below 70 and are considered to have global intellectual impairment[50, 52, 53]. Nevertheless, even individuals with normal IQ scores are found to display a range of neuropsychological deficits, mostly in executive functioning and attention[50, 54].

Several psychiatric disorders are associated with TSC and individuals with global intellectual impairment are the most likely to present with them. Developmental disorders such as ASD and ADHD are observed in approximately 40-50% of TSC patients, while in later life, mood and anxiety disorders become apparent with rates of around 30%[50, 55]. In addition, behavorial problems are often reported, including aggressive behavior and impulsivity[50]. Strikingly, it has been estimated that only 10% of TSC patients do not experience significant impairments in daily life because of cognitive and psychiatric symptoms[54].

ANIMAL MODELS FOR TSC

Several mouse models have been generated to gain further insight into the mechanisms governing disease development in TSC. *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ mouse embryos were observed to die at mid-gestation, probably due to a failure in liver development[56-60], while naturally occurring *Tsc2*$^{-/-}$ rat embryos (term Eker rat model is used for heterozygous *Tsc2* knock-out rat line) also show embryonic lethality[61]. Like TSC patients, TSC animal models show an increased propensity to develop lesions, though tumor nature and organ involvement varies between species[62]. For example, *Tsc1*$^{+/-}$ and *Tsc2* $^{+/-}$ mice develop renal cystadenomas instead of renal angiomyolipomas and show hepatic hemangiomas which are rarely seen in TSC patients[56-60]. In contrast, the Eker rat exhibits a distinct lesion profile from both TSC patients and *Tsc1*$^{+/-}$ and *Tsc2* $^{+/-}$ mice, with spleen and uterine hemangiomas, pituitary tumors and a low incidence of brain lesions[62]. In addition to germ-line mutants, a number of mutant mouse lines carrying cell type-specific *Tsc1* or *Tsc2* mutations have been generated. These have proven to be valuable tools for elucidating the etiology of neurological symptoms in TSC. Heterozygous *Tsc2* knock-out mice recapitulate spatial learning deficits[63] and we show in **Chapter 3** that heterozygous *Tsc1* knock-out mice display spatial learning and social behavior deficits. Cell-specific homozygous *Tsc1 and Tsc2* mutants are more runted, have compromised lifespan, extensive neuropathology and exhibit spontaneous seizures[64-69].

ARE TUBERS THE CORRELATE FOR NEUROLOGICAL DYSFUNCTION IN TSC?

Characteristics of tubers

The cortical tubers, after which TSC is named, are abnormalities in cortical architecture formed during early brain development and found in varying numbers in more than 80% of TSC patients[1] (**Figure 1**). They are made up of a heterogeneous mixture of giant cells and dysplastic neurons, and show extensive astrogliosis. Morphological analysis reveals that the tubers reflect defects in neuronal migration, differentiation and proliferation[70]. Giant cells are characterized by dramatic soma size enlargement and the presence of short, thickened processes of unclear identity[70]. Since these cells exhibit a heterogeneous staining pattern, including staining for markers typical of glial cells (GFAP, S100), neurons (NeuN) and immature neuroglial cells (nestin, vimentin, Double-cortin), they are thought to be stalled in differentiation at some ill-defined cell stage[70]. Dysmorphic neurons express mature neuronal markers, but are nonetheless cytomegalic and abnormally shaped with aberrant dendritic arbors[70]. Astrocyte abnormalities consist of gliotic and reactive astrocytes, which are large-sized cells located in the vicinity of giant cells[71]. More research is needed to determine how these focal lesions and their associated abnormal cell types arise.

It remains conducive to debate whether somatic loss of the second *TSC1* or *TSC2* allele is the trigger for tuber formation, as has been shown for other TSC-hamartomas. Classical LOH analyses[22, 23, 72] and sequencing[24, 73] performed on tuber tissue has so far failed to show significant rates of LOH or the presence of somatic mutations in the *TSC* genes. However, a recent study was able to detect small somatic mutations exclusively in microdissected pS6-positive giant cells of tuber specimens[74], raising the possibility that only specific cell types within tubers carry somatic mutations and that they exert non-autonomous effects on neighbouring cells, resulting in a mosaic focal malformation[74]. In agreement with such a two-hit mechanism, Feliciano *et al.* could model tuber-like lesions in the mouse brain by single-cell homozygous *Tsc1* knock-out during corticogenesis[75], and Goto *et al*[68] showed that regulable neural progenitor-specific homozygous *Tsc1* loss yielded giant cells. Furthermore, neuron-specific, astrocyte-specific and neuroprogenitor cell-specific homozygous *Tsc1/2* knock-out mice exhibit global tuber-like pathology throughout the brain[65-67]. Finally, Magri *et al* provided convincing experimental evidence that *Tsc1* null telencephalic neural stem cells show enhanced generative potential, followed by premature differentiation and impaired migration, resulting in pathology reminiscent of tubers[69].

There is also evidence that functional instead of genetic inactivation of the TSC1-TSC2 complex could initiate tuber development. For example, impaired colocalization of TSC1 and TSC2[76], or inappropriate AKT and ERK-dependent inhibitory phosphorylation of TSC2[77-79] may be sufficient to inactivate TSC1-TSC2 complex activity. Interestingly, Eker rats in which irradiation was employed as a second hit strategy, develop neuronal cytopathology reminiscent of that seen in human tubers, but without loss of *Tsc2*, suggesting that mutations in other genes (AKT, ERK) could indeed contribute to pathology by TSC1-TSC2 complex

inactivation[80]. Regardless of the mechanism, abnormal cells within tubers stain intensively for molecular markers of increased TORC1 activity, while staining for these markers is faint in normal-appearing perituberal tissue, indicating that tubers present focal malformations characterized by TORC1 hyperactivity[70].

Tubers and epilepsy

Epilepsy in TSC appears to be related to the presence of tubers. Epileptiform activity often correlates with the location of a tuber[81], and surgical removal of a dominant epileptogenic tuber zone in unifocal and multifocal partial epilepsy frequently has beneficial effects with respect to seizure control[82, 83]. Notably, different subtypes of tubers can be identified, based on their appearance on magnetic resonance imaging (MRI), that show differential propensity to develop epileptogenic activity[84]. In particular, cyst-like tubers are associated with a higher incidence of epileptiform activity[85] and a more aggressive seizure phenotype[86]. However, the exact relationship between tubers and epilepsy in TSC is still not fully understood. For instance, up to 10% of individuals with TSC suffering from intractable epilepsy have normal brain MRIs, with no evidence of cortical tubers, while other patients with a high tuber load do not develop epilepsy [81, 87], indicating that there may be other processes contributing to epilepsy development in TSC. Accordingly, in a few studies morphological abnormalities have been noted in non-tuber cortex, including atypical cells and reduced neuronal counts[88-90]. Furthermore, some of the resected tubers are found to be electrically silent, suggesting that abnormal activity in the surrounding tissue rather than in the tuber itself leads to epileptogenesis [91].

Tubers, epilepsy and neuropsychiatric symptoms

It has been a long-prevailing view that tubers and epilepsy represent the major correlate for the neuropsychiatric symptoms associated with TSC. However, studies assessing the relations between tuber load and intellectual disabilities or autism have yielded contradictory and confusing results (discussed in[92]). The presence of epilepsy is consistently shown to correlate with cognitive deficits. Specifically, early seizure onset, intractable seizures, and infantile spasms significantly enhance the risk for mental retardation[52, 93]. However, tubers and infantile spasms together account for only 47% of the variance in IQ[94], indicating that tubers and epilepsy may have a more limited causal role of in the development of intellectual and behavioral disabilities. These findings emphasize the need to search for alternative mechanisms to explain the cognitive and behavioral deficits in TSC. This topic was further explored in **Chapter 3**.

MOLECULAR AND CELLULAR MECHANISMS OF EPILEPSY AND NEUROPSYCHIATRIC SYMPTOMS IN TSC

A multidisciplinary approach, involving neuroimaging, clinical-pathological, animal and *in vitro* studies have greatly contributed to our understanding of the molecular and cellular mechanisms that underlie the CNS manifestations associated with TSC (**illustrated in Figure 3).**

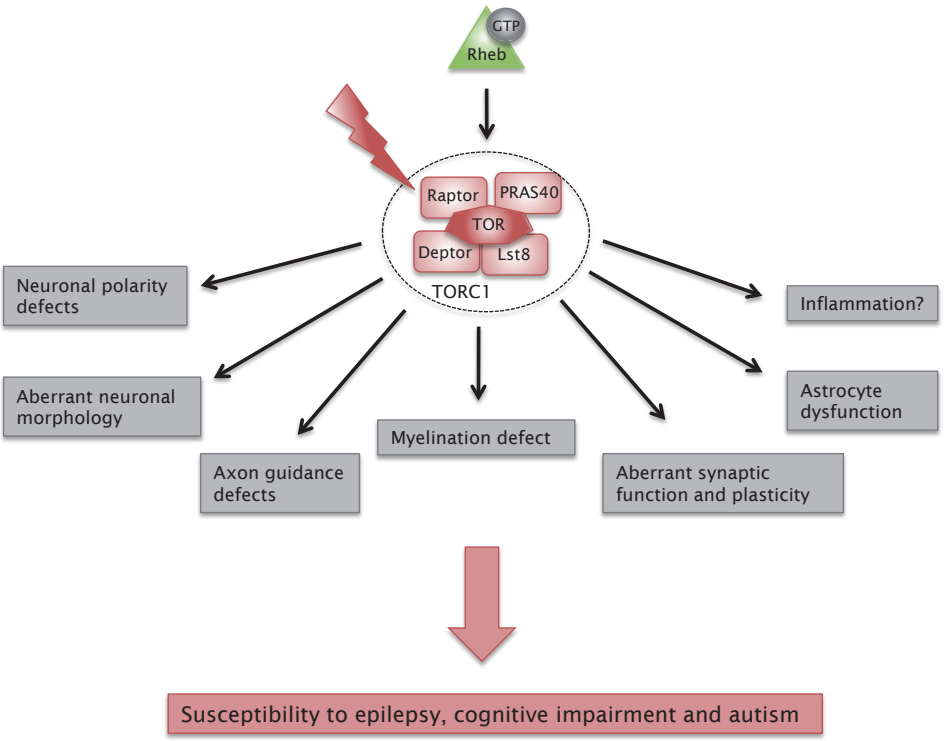

Figure 3. Mechanisms of neurological and cognitive dysfunction in TSC. In TSC, loss of TSC1-TSC2 complex function results in enhanced Rheb-GTP-TORC1 signaling. TORC1 signaling is implicated in many processes in the brain and dysregulation of these processes predisposes TSC individuals to neuropsychiatric phenotypes.

Cell types critically involved in epilepsy and neuropsychiatric dysfunction

Studies performed in different cell-specific *Tsc1* and *Tsc2* mouse mutants have shown that epilepsy can arise from homozygous *Tsc1* or *Tsc2* deletions in progenitor cells[68], including radial glia[67] and telencephalic neural stem cells[69, 95], but also from homozygous deletions in astrocytes[65] or neurons[66] only. Notably, excitatory neuron[63] or inhibitory neuron-specific[96] homozygous *Tsc1* knock-out mice do not develop spontaneous epilepsy, although the latter show a decreased seizure threshold on exposure to the proconvulsant flurothyl[96]. Taken together, these results support a multi-cellular mechanism for epilepsy in TSC, involving glial cells, and both excitatory and inhibitory neurons. Similar studies employing heterozygous *Tsc1* and *Tsc2* mouse mutants could be performed to assess which cell types critically contribute to spatial learning deficits.

Neuronal connectivity

Converging evidence from neuroimaging, clinical-pathological, animal and *in vitro* studies suggest that TSC-TORC1 signaling plays an essential role in establishing proper connectivity in the brain, by regulating neuronal polarity, dendritic and

spine morphology, axon guidance, and myelination. First, dysplastic, cytomegalic neurons in tubers[97], neurons of neuron-specific homozygous *Tsc1* knock-out mice[98] and cultured *Tsc2* null neurons[98] frequently have more than one axon, implicating an important role for the TSC1-TSC2 complex in establishing neuronal polarity. TSC-TORC1 signaling appears to regulate neuronal polarity by controlling the levels of SAD kinase[98]. Notably, elevated SAD levels are also observed in cortical tubers[97].

TORC1 has emerged as a major regulator of dendritic arborization, soma cell size and spine morphogenesis in cultured neurons[99-101]. Accordingly, spine density was found to be decreased and dendrite number increased in neuron-specific homozygous *Tsc1* knock-out mice[102], whereas cell hypertrophy is a uniform characteristic of the neurons and glial cells from all brain-specific homozygous *Tsc1* and *Tsc2* knock-out mice[65-69, 95]. Furthermore, bizarre shaped hypertropic dendritic arbors and cytomegaly are noted for giant cells and dysplastic neurons in tuber tissue[97]. Interestingly, a recent study provides a link between TORC1, cytoplasmic linker protein of 170 kDa (CLIP-170) and the actin-binding protein IQGAP1, suggesting a model whereby TORC1 binds CLIP-170 and regulates its interaction with IQGAP1, to allow the cross talk between dynamic microtubules and the actin cytoskeleton to define dendritic morphology[67]. In addition, TORC1 signaling is also implicated in axon guidance. Repulsive axon guidance in the form of Ephrin-Eph-A signaling was found to inhibit TORC1 signaling, thereby allowing axon retraction and fine tuning of neuronal projections[103]. Accordingly, *Tsc2*$^{+/-}$ mice, exhibiting TORC1 hyperactivity, display aberrant neuronal projections[103].

Finally, myelination is observed to be severely impaired in several TSC mouse models, including neuroprogenitor specific[67, 68, 95] and neuronal specific homozygous *Tsc1* and *Tsc2* knock-out mice[66]. Moreover, TSC brain lesions are markedly hypomyelinated[70] and widespread deficits in white matter volume are observed on MRI scans of TSC patients[88]. It remains to be established how these myelination impairments arise, but the presence of myelination deficits in neuron-specific homozygous *Tsc1* knock-out mice[66] strongly suggests that they develop due to a neuronal induction defect.

Recent studies have assessed myelination and white matter integrity in TSC brains using diffusion tensor imaging (DTI). Alterations in diffusion properties within normally-appearing white matter were observed, suggestive of disorganized and structurally compromised axons with deficient myelination[104, 105]. These findings provide strong evidence that the TSC1-TSC2 complex is essential for the proper wiring and myelination of neuronal circuits. Changes in neuronal circuit organization could render the network hyperexcitable leading to seizure development. Wiring defects could also impair information flow, resulting in neuropsychiatric disabilities.

Synaptic function and plasticity

The TORC1 pathway has been shown to be involved in several forms of protein synthesis–dependent synaptic plasticity that are believed to be the cellular correlate for long-term memory. These forms include the late phase (more than one hour after induction) of long-term potentiation (L-LTP)[106] and metabotropic receptor-dependent long-term depression (mGlurLTD), a form of synaptic weakening induced by activation of group I metabotropic glutamate receptors (mGlur1 and mGlur5)[107].

Rapamycin, a selective inhibitor of TORC1, specifically impairs L-LTP and mGlurLTD and causes long-term memory deficits (summarized in ref[108]). Interestingly, all components of the PI3K–TOR pathway and the complete translation machinery are present in dendrites, suggesting that this pathway controls protein translation near the activated synapse (designated as 'local' protein translation)[106]. Indeed, Brain Derived Neurotrophic Factor (BDNF) stimulation is found to initiate TORC1-dependent protein translation in isolated dendrites[109]. Besides BDNF, activation of N-methyl D-aspartate (NMDA)- and beta-adrenergic receptors can also induce TORC1 signaling in neurons[110, 111].

Evidence from studies performed on tuber tissue and in animal models support the notion that synaptic function and plasticity are altered upon *TSC* loss. First, *Tsc2*$^{+/-}$ mice exhibit a decreased threshold for L-LTP[63], while the Eker rat displays impairments in several forms of LTP and LTP[112]. In addition, acute homozygous deletion of *Tsc1* in the hippocampus was observed to result in an inability to express mGlurLTD[113]. Second, tuber tissue has a distinct neurotransmitter receptor expression profile, characterized by a relative excess of excitatory receptor subunits compared to inhibitory receptor subunits[114, 115]. NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression profiles in dysmorphic neurons are suggestive of increased $Ca2^{+}$ permeability (high GluR1/GluR2 and NR2B/NR2A ratios)[115,97], while inappropriately strong immunoreactivity for groupI mGlur receptors, associated with proconvulsant activity, is also observed[116]. Furthermore, dysmorphic as well as normal-appearing neurons show signs of epileptiform discharge patterns in electrophysiological studies performed on resected tuber tissue[97, 117], while enhanced AMPA and NMDA currents were found in *Tsc1* and *Tsc2* null mouse neurons[101, 113, 117]. Since it appears that these neurons have passive properties that make them less likely to reach firing threshold it remains to be determined whether they directly function as seizure instigators[97].

These observed changes in synaptic function and plasticity may impair cognitive and social functioning, but could also disturb the balance between excitatory and inhibitory transmission, predisposing to epilepsy.

Astrocyte dysfunction

Astrocytes control the extracellular levels of ions and neurotransmitters, such as potassium and glutamate, which may directly affect neuronal excitability[118]. Therefore, astrocyte dysfunction could contribute to epileptogenesis in TSC. Indeed, several defects in astrocyte function were observed in astrocyte-specific homozygous *Tsc1* and *Tsc2* knock-out mice. First, *Tsc1* null astrocytes exhibited a decrease in astrocytic GLT-1 and GLAST glutamate transporters, resulting in elevated extracellular glutamate levels and associated neuronal cell death[119, 120]. Notably, stimulating GLT-1 channels by treatment with ceftriaxone suppressed seizure frequency in these mice[121], indicating an important role for these channels in TSC epileptogenesis. Moreover, enhanced expression of GLT-1 channels is also associated with a beneficial effect of rapamycin treatment on seizure incidence[122]. Inwardly rectifying potassium channels and astrocytic connexin protein Cx43 also showed lower expression levels in these astrocytes, leading to impaired potassium buffering and, in addition, contributing to neuronal excitability[123, 124]. It remains to

be determined whether similar channel and connexin abnormalities are present in tuber and non-tuberal tissue from TSC patients and in other TSC mouse models. The contribution of astrocyte dysfunction to learning and social behavior deficits has not yet been explored.

What is the role of inflammation in TSC-epileptogenesis?

An interesting new topic in the etiology of TSC-associated epilepsy is the involvement of inflammatory processes. The presence of microglia/macrophages and T-lymphocytes, and the altered expression of components of the two major pro-inflammatory pathways (IL-β and complement system) in tuber tissue implicate activation of both the innate and the adaptive immune system in these lesions[125]. Notably, gene expression studies show increased expression of genes associated with inflammation in the perituberal tissue[126] which often appears to contain the epileptogenic focus[91]. Enhanced expression of two of these inflammatory genes, *SerpinA3* and *Sparc,* is replicated in neuroprogenitor-specific homozygous *Tsc1* knock-out mice[68]. However, inflammation has not yet been studied in the other available mouse models. Furthermore, inflammation could develop secondary to seizure activity, or could reflect an epiphenomon not related to epileptogenesis. Nevertheless, experimental evidence from other animal epilepsy models suggests that inflammation may relate to seizure initiation or exacerbation (reviewed in Vezzani et al[127]). Furthermore, since rapamycin is an immunosuppressant and effective for treating epilepsy in TSC mouse models, one could speculate that it exerts its anticonvulsant action by reducing inflammation. Obviously, further studies are warranted to investigate the timing of inflammation processes in relation to the start of seizures, and to test the potential of anti-inflammatory therapies in ameliorating epilepsy in these TSC mouse models.

Heterozygosity or homozygosity

A subject for debate in TSC neurobiology is the role of heterozygosity versus homozygosity for *TSC* mutation in development of disease features. TSC patients are heterozygous for a *TSC* mutation, but lesions and pathology are believed to only develop upon loss of the second allele. Nevertheless, studies performed in heterozygous *Tsc2* knock-out mice indicate that some of the disease features are already apparent in heterozygous state, including aberrant neuronal projections[103], synaptic plasticity alterations[63] and spatial learning and social behavior deficits[63]. Collectively, it appears that neurological pathophysiology in TSC results from a combination of brain-wide haploinsufficiency combined with mosaic regions in which homozygosity triggers additional pathology.

TORCOPATHIES

A large body of fundamental and clinical evidence gathered in recent years suggests that TORC1 dysfunction is not only at the root of neurological dysfunction in TSC, but of many more neurological disorders, collectively referred to as TORCopathies[128] (**shown in Figure 4**). Therefore, TSC can be viewed as a model system with relevance to many related disorders.

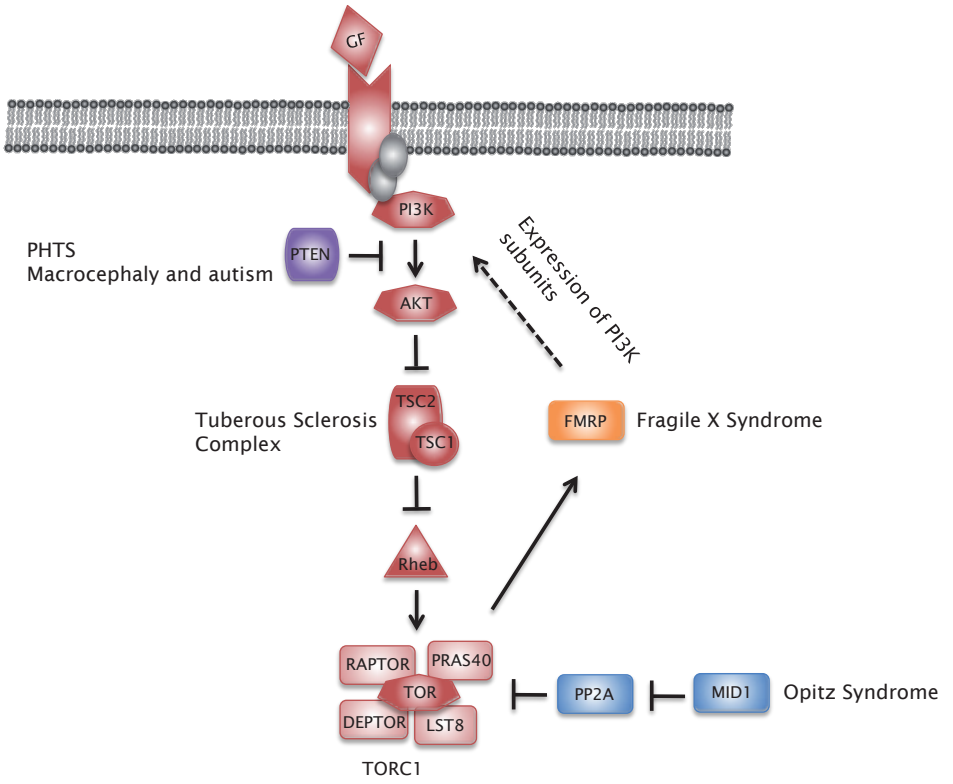

Figure 4. TORCopathies. An increasing number of diseases are associated with deregulated TORC1 signaling, collectively referred to as TORCopathies. The disorders depicted in the figure have putative molecular links to components of the TORC1 signaling cascade. In additional disorders (mentioned in the text) aberrations in TORC1 signaling are found, but the molecular link to the TORC1 pathway is unknown.

Genetic syndromes which link to TORC1

A constellation of genetic syndromes, presenting with clinical spectra that include epilepsy, developmental delay, intellectual disabilities and autism, link to TORC1. Of these, the PTEN-hamartoma tumor syndromes (PHTS) represent a sister group of clinical entities, all resulting from germ-line mutations in the *PTEN* gene, that have been traditionally classified together with TSC as the Hamartoma syndromes[129]. In addition, *PTEN* mutations are a common finding in patients with macrocephaly and autism[129]. Like mutations to *TSC1* and *TSC2*, *PTEN* mutations affect the PI3K-AKT-TORC1 pathway but in a distinct manner, owing to the location of PTEN upstream from the TSC1-TSC2 complex. Consequently, it is less straightforward to determine which signaling events are essential for the neurological dysfunction in PHTS patients. Nevertheless, preclinical animal studies established the reversibility of nearly all neurological features of *PTEN* deficiency with rapamycin[130, 131], suggesting that TORC1 signaling is critical for the pathogenesis of PHTS and is therefore a

promising target for treatment. An open-label trial testing the efficacy of TORC1 inhibitor therapy in treating PHTS-associated hamartomas, with neurocognitive functioning as secondary outcome measure, is currently recruiting[200].

Fragile X Syndrome (FXS) and Down's syndrome (DS) are highly prevalent genetic causes of intellectual impairments and have both been associated with TORC1 hyperactivity[132, 133]. In DS an increase in basal BDNF levels is supposed to trigger TORC1 up-regulation[133]. Recent data suggest that the FXS protein, FMRP functions up- and down-stream of TORC1 to tightly control protein synthesis[132, 134]. Interestingly, FXS and TSC display opposite phenotypes with regard to mGlurLTD, a form of protein-synthesis dependent synaptic weakening induced by activation of group I metabotropic glutamate receptors (mGlur) and considered to be essential to the pathogenesis of FXS[135]. While FXS mice show enhanced mGlurLTD that no longer requires protein synthesis[134], it is impaired in adult mice with an acute homozygous *Tsc1* deletion in the hippocampus[113]. It remains to be tested whether this phenotype is also present in *Tsc1* and *Tsc2* germ-line heterozygous mutants, which carry chronic instead of acute deletions. Nevertheless, this is a remarkable finding that would suggest that FMRP regulates a distinct (sub)pool of proteins.

Two additional genetic syndromes were recently linked to TORC1 signaling: Polyhydramnios, Megalencephaly, and Symptomatic Epilepsy syndrome (PMSE) and Lafora Disease. PMSE is a rare genetic syndrome, characterized by cognitive disabilities and severe epilepsy. It is caused by recessive mutations in the STE20-related kinase adaptor α (*STRADA*) gene. In a mouse model for this disease similar histological and molecular brain abnormalities can be observed as in TSC, including cytomegalic neurons, heterotopia and aberrant activation of TORC1[136]. It is thought that STRADA deficiency leads to the inappropriate nuclear localization of LKB1, impairing its ability to inhibit TORC1 signaling[136]. Lafore Disease is an autosomal recessive progressive late-onset myoclonus epilepsy characterized by the accumulation of polyglucosan inclusion bodies, called Lafora bodies. The onset is usually in puberty and the disease is characterized by developmental delay and regression. In Lafora Disease TORC1 hyperactivity was found in combination with compromised autophagy[137]. This impairment in autophagy is thought to impact on the Lafora body accumulation and cell death seen in the disorder[137].

Symtomatology in the above syndromes is noticeably different and it remains to be determined which mechanisms govern the development of the distinct phenotypes in the context of TORC1 hyperactivity. Nevertheless, TORC1 inhibitor rescue experiments in mouse models should be performed to test causality.

Intruigingly, two genetic syndromes seem to correlate with decreased rather than increased TORC1 activity, namely Opitz and Rett syndrome[138, 139]. Opitz syndrome is a congenital disorder, in which impaired formation of midline ventral structures is seen in combination with mild intellectual disabilities[138]. MID1, the Opitz Syndrome protein, functions as an E3 ubiquitin ligase that targets the catalytic subunit of protein phosphatase 2A (PP2A-C) for ubiquitin-mediated degradation[138]. In Opitz syndrome, increased PP2A levels are associated with disruption of the TOR/Raptor interaction and downregulation of TORC1 signaling[138]. Rett syndrome is a progressive disorder that shows a peculiar disease course. After apparently normal development until 6 months of life, children undergo a rapid regression marked

by a deceleration of head growth rate, the onset of stereotypical hand movements, irregular breathing and seizures[139]. Recently, by an as yet unknown mechanism, loss of the MeCP2 protein in Rett Syndrome was found to result in general dysfunction of AKT-TORC1 signaling and protein synthesis. This was already apparent in the presymptomatic phase of the disease[139]. Obviously, it still has to be established whether the observed reductions in TORC1 signaling constitute the primary defects in these disorders, or whether these are merely epiphenomena.

Focal cerebral cortical malformations

Another group of TORCopathies are the focal cerebral cortical malformations (FCM), the most common cause of medically intractable epilepsy in children[140]. FCM include tubers in TSC, focal cortical dysplasia (FCD), and hemimegalencephaly (HME), which can be seen in conjunction with TSC. All are characterized by a varying degree of cortical dyslamination, loss of neuronal orientation and the presence of dysmorphic, enlarged neurons[140]. In addition, giant cells, also referred to as balloon cells outside TSC, are a typical finding in FCD type IIB, HME and tubers[140]. Interestingly, alongside these morphological similarities, dysmorphic neurons and balloon cells in FCM lesions were recently observed to exhibit a molecular signature that is similar but not identical to TSC. Notably, both cell types show TORC1 hyperactivity[141-143]. Similarly, gangliomas, low-grade brain tumors that share pathologic features with FCM and that are highly epileptogenic, display molecular markers of TORC1 hyperactivity[144], indicating that a common molecular pathogenesis defines this group of lesions. It is thought that FCM may result from somatic mutations that occur in neural progenitor cells during brain development and that affect components of the PI3K-TORC1 pathway[128]. However, mutation studies have thus far failed to identify any pathogenic mutations in FCM specimens[128]. Due to a lack of mouse models for these focal disorders, it is not yet known whether the epilepsy associated with these disorders is sensitive to TORC1 inhibitor therapy. However, their morphological and molecular similarity to TSC would suggest they would do.

Acquired epilepsy

TORC1 dysfunction has recently been implicated in the pathophysiology of acquired epilepsy. Acquired epilepsy is associated with previous brain insults and comprises several stages: an acute phase of brain injury, a latent period and an actual phase of chronic epilepsy[145]. Kainate and pilocarpine injections in rodents model this form of epilepsy, with development of the characteristic stages[145]. Remarkably, TORC1 activation shows a parallel biphasic activation pattern, with high activation states in the acute and chronic phases, indicating that it could mediate the propensity to develop seizures in the chronic period[146]. Indeed, treatment with rapamycin, a specific TORC1 inhibitor, is able to counteract morphological changes associated with epilepsy, including mossy fiber sprouting and cell death in conjunction with a variable but overall positive effect on the development of spontaneous seizures[146-150].

TARGETED TREATMENTS FOR TSC

Until recently, there was not a single therapy that targeted the primary molecular defect in TSC. Rather, individual symptoms were all managed separately and often with unsatisfying results. For instance, whereas vigabatrin is a potent drug for treating infantile spasms, it is associated with a significant risk of visual field restriction[82]. Furthermore, other forms of TSC-associated epilepsy are frequently refractory to pharmacological treatment, and there are few effective interventions available for the cognitive and behavorial disabilities of TSC[82]. Fortunately, advances in our understanding of the molecular biology of TSC led to the realization that TORC1 inhibitors could target the primary molecular defects underlying TSC[32, 151].

The prototype TORC1 inhibitor is the naturally occurring macrolide sirolimus (official generic name for rapamycin), which has been in use for more than a decade as an immunosuppressant for the prevention of renal allograft rejection[152]. In order to improve the pharmokinetic properties of sirolimus, mainly the low bioavailability and poor water solubility, several derivates were developed. These are commonly referred to as rapalogs[152] and include everolimus, temsirolimus and ridaforolimus (formerly deforolimus)[152]. Rapalogs have an allosteric mode of action. In a complex with the intracellular FK506-binding protein (FKBP12) they target TOR's FKBP12–rapamycin binding domain, and interfere with the TOR-Raptor interaction and thereby stop TORC1 signaling to downstream effectors[152].

Sirolimus can limit tumor growth, prevent neuropathology and ameliorate epilepsy and spatial learning deficits in TSC mouse models[63, 102, 122, 153-155]. These studies paved the way for off-label treatment of TSC patients and clinical trials[156]. Sirolimus is the most extensively studied rapalog to date and has shown efficacy in the treatment of several TSC-associated tumors including angiomyolipomas, facial angiofibromas and subependymal giant cell astrocytomas, in addition to its potential to stabilize lung function in patients with lymphangiomyomatosis, and to decrease seizure frequency (reviewed in[156]). Preliminary evidence also suggests that it may improve neurocognitive functioning[157]. Everolimus is an emerging alternative as promising results were obtained in the management of subependymal giant cell astrocytomas, skin lesions and epilepsy and, recently, in cardiac rhabdomyomas[158] (reviewed in[156]). Everolimus has been approved for use with a group of patients with subependymal giant cell astrocytomas[156], who are not eligible for surgical intervention. Common adverse events associated with sirolimus and everolimus treatments include apthous ulcers, diarrea, and upper respiratory infections[156]. Large trials testing the effectiveness of sirolimus and everolimus in ameliorating epilepsy and improving neurocognitive functioning are currently being performed and the results are eagerly awaited[200].

Comparison of sirolimus and everolimus

What would be the drug of choice for TSC patients? Sirolimus and everolimus appear to have similar efficacies for TORC1 inhibition, yet they differ in pharmacokinetic properties. Everolimus shows increased water solubility, slightly improved bioavailability, and a shorter half-life, that could translate to fewer adverse events[152, 156]. Indeed, side-by-side analysis of heart transplantation patients receiving either compound points to a more favorable side-effect profile for everolimus, with

lower incidences of infection, edema and diarrea[159]. For successful treatment of the neurological and neuropsychiatric manifestations of TSC considerable brain penetration is essential and, consistent with its more hydrophobic nature, sirolimus is found to accumulate in higher amounts in the brain after prolonged treatment[102, 160]. Nevertheless, available data is limited to a few rodent studies and no comparative data on the effects of the different analogs in brain cells is available. While the vast majority of future and ongoing trials plan on using everolimus, it remains debatable whether the milder side-effect profile of everolimus outweighs the possibly reduced blood-brain barrier permeability.

Potential drawbacks of rapalog therapy

Rapalog treatment does not induce complete regression of tumors in TSC individuals[157, 161, 162]. Several mechanisms could account for these findings. First, TORC1-independent mechanisms, might significantly contribute to tumor development[163]. Second, rapalogs may act by decreasing cell size rather than inducing cell death, limiting complete regression[62]. In a preclinical model, tumor response was associated with a decrease in cell size and the induction of apoptosis[154, 164]. Nevertheless, mechanisms underlying TSC tumor shrinkage upon prolonged rapalog treatment are poorly understood and deserve further study. As a third possibility, rapalogs may relieve the feedback inhibition on upstream effectors, allowing AKT-dependent pro-survival signaling to counteract treatment. Indeed, paradoxical activation of PI3K-AKT and ERK is observed upon rapalog treatment in different sporadic human cancers as well as in TSC cell lines[165, 166]. Nevertheless, dual PI3K-TORC1 inhibition has equal benefit to sole TORC1 inhibition in tumor reduction in a TSC mouse model[167], questioning the relevance of PI3K-AKT hyperactivity to the lack of therapeutic success. Whether treatment of CNS manifestations will be hampered by similar feedback activation of upstream effectors remains to be determined. Feedback loops are active in brain tissue, and can be modulated by sirolimus[102]. Finally, limited therapeutic efficacy may be associated with specific characteristics of the allosteric mode of action of rapalogs, that may prevent optimal TORC1 inhibition. Indeed, it has been recently shown that rapalogs do not exert complete control over TORC1 signaling, but that one of the signaling branches, to the 4E-BP proteins, gains resistance over time[168].

Second-generation TOR inhibitors

In an effort to completely inhibit TORC1, several labs have developed so-called catalytic TOR inhibitors (reviewed in[40]). These are small-molecule ATP mimetics targeting the catalytic domain of TOR directly, irrespective of whether TOR is complexed with RAPTOR or RICTOR. In preclinical studies these compounds have been shown to antagonize signaling downstream of TORC1, thereby inhibiting cell growth, proliferation and lymphoma growth to a far greater degree than rapalogs[169, 170]. Some catalytic TOR inhibitors also target related kinases such as PI3K, thus offering the additional potential to counteract upstream activation[40]. Several of these 'second-generation TOR inhibitors' are now in clinical development and it will be interesting to determine whether they exhibit greater clinical activity at tolerable doses and schedules than rapalogs.

What is the optimal timing of TORC1 therapy for the treatment of the neurological symptoms of TSC?

There is increasing awareness that a history of infantile spasms predisposes children with TSC to developmental delay and subsequent intractable epilepsy[82]. Since the earliest signs of TSC (cardiac rhabdomyomas and cortical tubers) can be detected by routine fetal echocardiography, diagnosis can be made pre- or perinatally, and children could be followed up prospectively[171]. In TSC, epileptic discharges precede clinical epilepsy in the majority of cases[171]. Therefore, a preventative approach could be employed, according to which treatment is initiated in these children upon the occurrence of EEG abnormalities, but prior to the start of clinical seizures, in order to interfere with this neurological sequelae[171]. Jozwiak *et al* set out to compare such preventative care with standard care and found significantly lower rates of intellectual disabilities and drug-resistant epilepsy in the preventative care group[171], suggesting that this is a valuable therapeutic approach that deserves more detailed follow up.

In the near future we can expect the outcomes of trials assessing the efficacy of sirolimus in treating the neurological symptoms of TSC[200]. Experimental studies have already reported spectacular effects of sirolimus therapy on epilepsy in a number of TSC models[64, 68, 69, 95, 102, 122, 172]. Remarkably, all but one[122] of the studies assessed the preventative rather than the curable potential of the drug, by initiating sirolimus application early in the postnatal period and before the occurrence of the epileptic insults. Only in the study of Zeng *et al*[122], mice were treated when they had already developed epilepsy. Here, treatment resulted in a significant reduction in seizure frequency[122]. Although this is promising preclinical data with regard to treating instead of preventing epilepsy with sirolimus, the mice carried the *Tsc1* deletion in astrocytes only, leaving open the question of whether epilepsy due to *TSC1* or *TSC2* loss in all brain cells can be cured once seizures have started. Clearly, more research into the optimal time window for sirolimus treatment is warranted, particularly in models in which the *Tsc1* or *Tsc2* deletion is targeted to all brain cell types. Based on the available experimental data it cannot be excluded that sirolimus treatment is ideally initiated before the presence of clinical seizures, as pioneered by Jozwiak *et al*[171].

An important question with regard to future TORC1 inhibitor treatment in TSC is whether chronic treatment will be required or whether a critical period exists during which treatment would have long-term beneficial effects. Available evidence from experimental and clinical studies points towards the need for chronic treatment. First, TSC lesions exhibit regrowth upon rapalog withdrawal[161, 162, 173]. Second, rapalog therapy initiated in mouse models before lesion development showed no additional beneficial effects[173]. Furthermore, neurological dysfunction, including epilepsy, reappears in TSC mouse models on cessation of sirolimus treatment despite the fact that therapy was started early in postnatal life[69, 102, 122]. These findings strongly suggest that either chronic rapalog therapy is required to counteract disease features in TSC or, less likely, that there is a critical window for treatment in the prenatal period[172]. Unfortunately, since rapalog treatment is associated with significant side-effects, such long-term therapy is unlikely to always be tolerated well[157]. Perhaps, intermittent dosing schemes, as tested by Lee

et al., may be less toxic[174]. Clearly, there is a clinical need for further investigation into optimal dosing schemes that treat epilepsy but lead to minimal toxicity in TSC mouse models.

Ketogenic diet and low glycemic index diet

Dietary therapies, such as the ketogenic diet (KD), have a longstanding tradition in ameliorating childhood pharmacoresistant epilepsy[175]. The KD, characterized by a low-carbohydrate, high-fat composition, was recently found to decrease insulin levels and hereby inhibit TORC1 signaling in the brain[176, 177]. This finding indicates that the diet may be particularly effective in the setting of TSC-associated epilepsy. Indeed, a few small studies support this notion[178, 179]. However, due to its restrictive composition, many patients find it hard to comply with the KD. As an alternative, the low glycemic index diet (LGID) was developed, which allows a more liberal intake of carbohydrates that only minimally raise insulin levels[180]. The LGID was well tolerated and efficacious in a group of children with intractable epilepsy from different etiologies, but has not yet been tested specifically on TSC patients[180]. The KD and LGID target the primary molecular aberration of TSC and therefore the cognitive and behavioral abnormalities in TSC patients may also improve while on this diet. In summary, the KD or LGID may provide an alternative to drug treatment for the epilepsy and neuropsychiatric symptoms of TSC.

TARGETING TORC1 BEYOND TSC

Inhibition of TORC1 with rapalogs has increasingly been put forward as an attractive treatment for a number of diseases, including TSC and related TORCopathies, in which aberrant TORC1 signaling constitutes the primary molecular defect[128]. Because of its central position in numerous signaling pathways TORC1 inhibition has potential for many applications beyond these TORCopathies. For instance, rapalogs are currently approved for use in sporadic cancers, including renal cell carcinoma and mantle cell lymphoma, in which they exert pro-apoptotic, anti-proliferative and anti-angiogenic effects[181]. In addition, sirolimus has been used for several years as an immunosuppressant to prevent rejection in organ transplantation, while its anti-proliferative action is employed in cardiac sirolimus-eluting stents, to prevent local restenosis[152]. Interestingly, intensive research efforts have recently pointed to rapalogs as promising compounds for the alleviation of symptoms associated with neurodegenerative disorders[182]. In experimental models of disorders, such as Alzheimer's, Parkinson's and Huntington's disease, rapalogs have yielded beneficial effects with respect to survival and behavior, presumably by inducing autophagy with the subsequent removal of disease-associated aggregates[182].

Furthermore, exciting recent studies show that long-term rapalog treatment initiated in adulthood can increase lifespan in mice[183, 184]. For *Drosophila*, but not yet for mammals, it was also found that dietary restriction (DR), the most robust environmental method found to date to slow down aging, mediates life span extension through TORC1 down-regulation[185]. It is hypothesized that modulating TORC1 signaling slows aging through downstream processes including mRNA translation, autophagy, endoplasmic reticulum (ER) stress signaling, stress

responses, and metabolism. However, the relative contribution of these processes remains unclear[185]. The intensive interest in TORC1 inhibition will certainly be beneficial to TSC patients, as drug companies will continue to invest in the search for and development of TOR inhibitors with optimal efficacy and safety profiles.

SCOPE OF THIS THESIS

In TSC the neurological and psychiatric symptoms are often the most disabling to the patients and their families. However, the etiology of these symptoms is still incompletely understood. In particular, the causal relations between developmental neuropathology, epilepsy, and the cognitive and behavioral symptoms are unclear. Furthermore, clinical-pathological, animal and *in vitro* studies point to additional pathogenic features, that may have causal or additional roles in epileptogenesis and neuropsychiatric symptomatology. Reductionist approaches using mouse models can help to disentangle these disease characteristics and to identify primary and secondary factors in disease progression.

The aim of this thesis is to contribute to the understanding of the CNS manifestations of TSC to, ultimately, come to a tailor-made drug therapy to alleviate the neurological and psychiatric symptoms associated with the disease. In **Chapter 2** the current knowledge about TSC and related neurocutaneous syndromes is reviewed to gain more insight into common pathophysiology. In **Chapter 3**, the relationships between neuropathology, spatial learning and social behavior deficits are investigated using *Tsc1*$^{+/-}$ mice. In **Chapter 4** the connection between neuropathology and epilepsy is studied using a novel *Tsc1*$^{-/-}$ inducible mouse mutant. In **Chapter 5** and **6** the role of Rheb, the direct target of the TSC1-TSC2 complex, in murine development and learning and hippocampal plasticity is investigated.

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ONCOGENES ON MY MIND: ERK AND MTOR SIGNALING IN COGNITIVE DISEASES

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ABSTRACT

Defects in RAS–ERK and PI3K-MTOR signaling pathways have recently been shown to cause several genetic disorders classified in Neuro–cardio-facial-cutaneous (NCFC) and Hamartoma syndromes. Although these pathways are well-known players in cell proliferation and cancer, their role in cognitive function is less appreciated. Here, we focus on the cognitive problems associated with mutations in the RAS–ERK and PI3K-MTOR signaling pathways and on the underlying mechanisms revealed by recent animal studies. Cancer drugs have been shown to reverse the cognitive deficits in mouse models of NCFC and Hamartoma syndromes, raising hopes for clinical trials.

MUTATIONS IN RAS SIGNALING PATHWAYS ARE A LEADING CAUSE FOR COGNITIVE DYSFUNCTION

The RAS (rat sarcoma viral oncogene homolog) signaling pathways are evolutionary conserved pathways, transducing signals from membrane-bound receptors to proteins that regulate fundamental cell processes like cell growth and proliferation. Therefore, it is not surprising that genetic disorders with gain-of-function mutations in the RAS signaling pathways are characterized by benign and malignant overgrowths. This is a common phenotype for two groups of syndromes, classified in Neuro-cardio-facial-cutaneous (NCFC) and Hamartoma syndromes. A high prevalence of mental retardation (see Glossary) and behavioral disturbances is also found among these patients (Table 1). Many of the genes associated with these diseases have been identified in the past few years (e.g. SOS1 (Son of Sevenless, Drosophila, homologue 1) in Noonan Syndrome, RAF-1 (v-raf-1 murine leukemia viral oncogene homolog 1) in Noonan syndrome and LEOPARD, and SPRED-1 (sprouty related EVH1 domain containing protein 1) in Neurofibromatosis type I-like syndrome) [1-11] and all these genes are part of the ERK (extra-cellular signal regulated kinase) and MTOR (mammalian target of Rapamycin) pathways (Figure 1). Thereby, genetic alterations in RAS-ERK and PI3K (phosphoionositide 3-kinase)-MTOR signaling can be considered a leading cause of cognitive and behavioral impairments, collectively affecting ~ 1/1000 people.

Studies on rodents carrying mutations in components of the RAS-ERK signaling pathways indicate that post-mitotic neurons have reprogrammed these signaling pathways to regulate synaptic plasticity (Table 2), believed to be the cellular basis for learning and memory (Figure 2). Combining these neuroscience studies with molecular insights from cancer research has rapidly increased our understanding of the etiology of the cognitive deficits in the affected patients, and offers the opportunity to treat the cognitive deficits in NCFC and Hamartoma syndrome patients.

COGNITIVE DEFICITS ARISING FROM GENETIC IMPAIRMENTS IN RAS-ERK SIGNALING

The NCFC syndromes comprise a constellation of disorders that include Neurofibromatosis Type 1 (NF1), Noonan syndrome, Costello syndrome, Cardio-Facio-Cutaneous (CFC) syndrome, LEOPARD syndrome[1] (an acronym for its cardinal features; lentigines, ECG conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness) and NF1-Like syndrome[2]. All these syndromes are associated with some degree of mental impairment (Table 1). In general, Noonan, LEOPARD, and NF1 are associated with mild cognitive deficits. However, despite low frequencies of mental retardation (IQ < 70) in NF1 and Noonan syndrome [12-15], ~40% of the children require special education [12,13]. In addition, even children with NF1 with a normal IQ can still display specific deficits in multiple cognitive domains, including visuo-spatial skills, attention, executive functioning, and memory which puts them at risk for specific problems at school or work [13,14].

In contrast to these relatively mild phenotypes, patients with Costello or CFC syndrome present with high frequencies of mental retardation (Table 1). It is tempting to speculate that the generally milder cognitive phenotypes in Noonan, LEOPARD, NF1 and NF1-like syndrome are due to the fact that the causative mutations affect regulators of the RAS-ERK pathway. Mutations affecting the RAS, RAF and MEK (mitogen-activated and extracellular-signal regulated kinase kinase) proteins, found in Costello syndrome and CFC, might have a stronger effect on the output of the pathway (Figure 1). However, there are no data directly comparing activity levels of RAS-ERK signaling in brain tissue in the different disorders. Moreover, mutations in the same gene can yield variable phenotypes, such that patients with identical amino acid changes have been diagnosed with different syndromes (see Box 1 for striking examples). Thus, the relationship between genotype and cognitive phenotype is still poorly understood.

RAS-ERK signaling can modulate synaptic plasticity by regulating processes at both sides of the synapse: at the presynaptic side it modulates neurotransmitter release (in the axon terminal of the presynaptic neuron) and at the postsynaptic side it controls protein synthesis (at the dendritic spines of the postsynaptic neuron) (Figure 1, 2).

A PRESYNAPTIC RAS-ERK PATHWAY MODULATES NEUROTRANSMITTER RELEASE

By changing the amount of neurotransmitter released from its axon terminal, the presynaptic neuron can affect the strength of a synaptic connection (Figure 2). Several lines of evidence suggest that the RAS-ERK pathway is involved in this process, which is likely activated by binding of the neurotrophin BDNF (brain-derived neurotrophic factor) to the presynaptic TRKB (tyrosine receptor kinase type B) receptor. Bdnf mutant mice show a decrease in neurotransmitter release [16], whereas stimulation of the RAS-ERK pathway by the application of BDNF, as well as by expression of the active H-Ras(G12V) (Harvey rat sarcoma viral oncogene homolog) gene, results in an ERK-dependent enhancement of neurotransmitter release. This is achieved by ERK phosphorylation of Synapsin-I, a protein that binds to synaptic vesicles containing neurotransmitters [16,17] (Figure 1,2). The presynaptic RAS-ERK signaling pathway is not only controlled by neurotrophins. A recent study showed that also stress can induce presynaptic changes via the RAS-ERK pathway. Activation of this pathway is induced by corticosterone binding to the mineralocorticoid receptor [18], but it is still unclear how activation of this receptor couples to RAS-ERK signaling.

Expression of the active H-Ras(G12V) gene in a subset of neurons that form stimulating synapses on their target neurons (excitatory neurons), resulted in enhanced synaptic plasticity and improved learning in an ERK-Synapsin-I-dependent manner [17]. This observation was surprising, because most of the NCFC disorders are characterized by increased RAS signaling, whereas the patients had learning deficits. The most likely explanation for this apparent paradox is that increased RAS-ERK-Synapsin-I signaling in these diseases is mostly restricted to inhibitory neurons. In contrast to excitatory neurons, inhibitory neurons form repressing

Table 1. Cognitive phenotypes of NCFC and Hamartoma syndromes.

Disease (prevalence)	Prominent phenotypical characteristics	Genes (% of cases associated with gene) [b]	Protein (function)	CNS features [a]		
				Very frequent (75- 100%)	Frequent (25-74%)	Less frequent (up to 25%)
Neuro-cardio-facial-cutaneous syndromes						
Neurofibromatosis type 1 (NF1, 1:3000)	Café-au-lait macules, skin fold freckling, Lisch nodules, cutaneous and plexiform neurofibromas [52]	*NF1* (95%) [49]	Neurofibromin (RAS-GAP) [a]	Low-average IQ [15], specific deficits in attention, executive functioning and visual-spatial skills [13]	Learning disabilities, ADHD [a], social, emotional and behavioral problems, motor problems, speech problems, sleep disturbances, MRI [a] abnormalities, macrocephaly [13,49]	Mild MR, autism, seizures, low grade gliomas [13,47,49]
Neurofibromatosis 1-like syndrome (rare) [a]	Café-au-lait macules, skin-fold freckling, macrocephaly, lipomas; no neurofibromas or Lisch nodules [11]	SPRED1 [11]	SPRED1 (inhibitor of Raf activation by Ras)			Frequency unknown. Some patients with macrocephaly, learning disabilities and/or ADHD [11]
Noonan syndrome (1:2000)	Typcial facial features (hypertelorism, ptosis, low-set posteriorly rotated ears), webbed neck, short stature, cardiac problems [52]	*PTPN11* (50%) [1]	SHP2 (tyrosine phosphatase)	Low-average IQ [12]	Learning disabilities, motor problems, speech problems [69]	Mild MR, social and emotional problems, seizures [12,69,70]
		RAF1 (3-17%) [8,9]	RAF1 (serine/threonine kinase)			
		BRAF (<2%) [9]	BRAF (serine/threonine kinase)			
		KRAS (~2%) [4,10,62]	KRAS (small G-protein)			
		SOS1 (~9-13%) [6,7]	SOS1 (GEF protein) [a]			
		MEK1 (~<2%) [63]	MEK1 (tyrosine/serine/threonine kinase)			
LEOPARD (rare) [c]	Multiple lentigines, cardiac problems, short stature, Noonan-like facies, hearing loss [52]	*PTPN11* (>80%) [71]	SHP2 (tyrosine phosphatase)			Mild MR [52]
		RAF1 (~<7%) [8]	RAF1 (serine/threonine kinase)			

Costello syndrome (rare) [d]	Coarse facial features, deep palmar/plantar creases, papillomata, short stature, cardiac problems [52]	*HRAS* (85-90%) [2,72-74]	HRAS (small G-protein)	Mild to moderate mental MR [a], delay in language and motor development, macrocephaly [75,76]	CNS abnormalities [76]	Irriability in young children, seizures [76]
		BRAF (~4-6%) [63]	BRAF (serine/threonine kinase)			
		KRAS (7%) [62]	KRAS (small G-protein)			
		MEK1 (~2-3%) [63]	MEK1 (tyrosine/serine/threonine kinase)			
Cardio Facio Cutaneous syndrome (CFC, rare)	"Noonan-like" with bitemporal constriction, sparse hair, ulerythaema ophryogenes, cardiac problems [52]	*BRAF* (43-78%) [3,5,77]	BRAF (serine/threonine kinase)	Moderate to severe MR, hypotonia, marked delay in language and motor development [78]	Obsessive behavior, sleep disturbance, failure to thrive, macrocephaly, CNS abnormalities, seizures [78,79]	Aggression [79]
		MEK1 (7-11%) [5,77]	MEK1 (tyrosine/serine/threonine kinase)			
		MEK2 (6-7%) [5,77]	MEK2 (tyrosine/serine/threonine kinase)			
		KRAS (5-8%) [4,77]	KRAS (small G-protein)			
Tuberous Sclerosis Complex (TSC, 1:6000)	Hamartomas, hypomelanotic macules, facial angiofibromas, renal angiomyolipomas	*TSC1* (19%) [80] [f]	Hamartin (binding partner of Tuberin)	CNS abnormalities, seizures [81]	Bimodal IQ distribution: 50% normal IQ, 30% severe MR, specific deficits in attentional-executive skills, memory and language, psychiatric disturbances including autism [32]	Subependymal giant cell astrocytoma [81]
		TSC2 (66%) [80] [f]	Tuberin (RHEB-GAP)			
Bannayan-Riley-Ruvalcaba (BRR, rare)	Macrocephaly, hamartomas (lipomas, hemangiomas), penile macules [82]	*PTEN* (60%) [83]	PTEN (tyrosine phopshatase)	Macrocephaly, developmental delay [84]		Seizures [84]
Cowden Syndrome (rare)	Macrocephaly, Mucocutaneous lesions, high frequency of various types of malignancies [82]	*PTEN* (80-90%) [83]	PTEN (tyrosine phopshatase)		CNS abnormalities [85]	Learning disabilities, autism [82]

[a] Rare: up to a few hundred cases reported in literature; GAP: GTP-ase Activating Protein; GEF: Guanine nucleotide exchange factor; CNS: Central Nervous System; MR: mental retardation (mild: IQ 50-69; moderate: IQ 35-49; severe: IQ ≤ 34); ADHD: Attention Deficit Hyperactivity Disorder; MRI: Magnetic Resonance Imaging

[b] The ~ sign indicates that the mutation is reported in a subgroup of patients that is negative for a combination of other mutations associated with the disease, without specifying the size of the original population. In order to obtain an estimate of the prevalence of this mutation, we have corrected the percentage reported for the percentage in which these other mutations are postulated to occur, as reported in this table

[c] LEOPARD is an acronym for the manifestations of this syndrome: multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness.

[d] In the majority of studies, Costello patients with mutations other than HRAS are re-diagnosed to CFC syndrome.

[e] The Hamartoma syndromes compromise Tuberous Sclerosis Complex, Peutz-Jeghers syndrome, and the subgroup of the PTEN-hamartoma tumor syndromes, consisting of Bannayan-Riley-Ruvalcaba, Cowden syndrome, Proteus syndrome and Lhermitte-Duclos disease. These latter group of diseases are all caused by germ line mutations in the PTEN gene [83]. However, because information on the cognitive phenotypes of Peutz-Jeghers syndrome, Proteus syndrome and Lhermitte-Duclos disease is very limited, these syndromes are not included.

[f] Calculated only for patients with a clinical diagnosis of TSC.

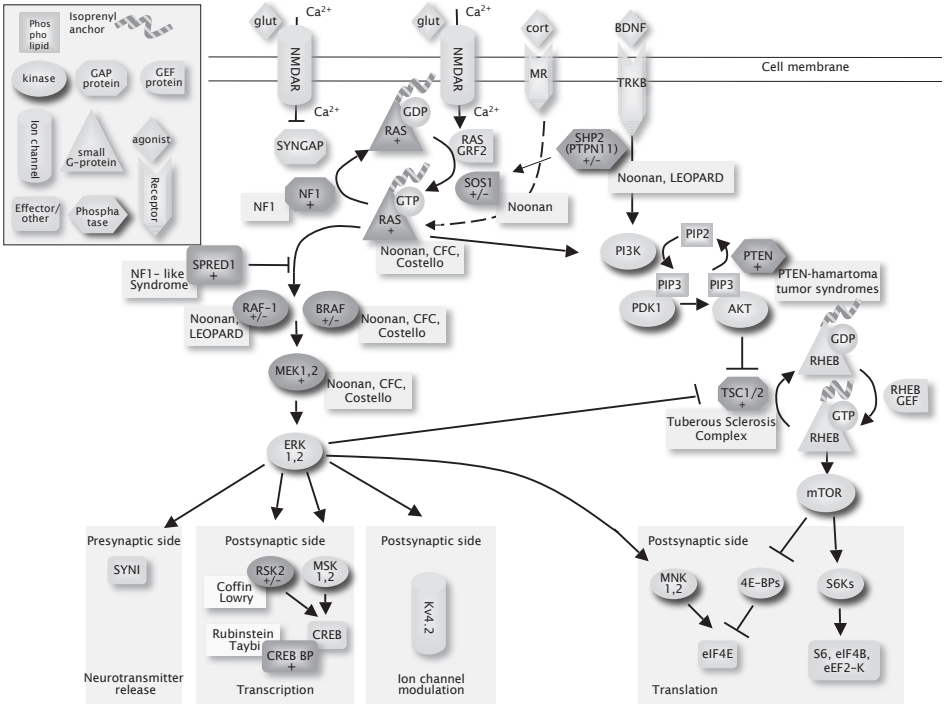

Figure 1: Overview of neuronal RAS-ERK and PI3K-MTOR signaling pathways and the associated NCFC and Hamartoma syndromes. The RAS-ERK and PI3K-MTOR pathways are both regulated by the activity of small GTP-binding proteins (G-proteins) RAS and RHEB, respectively (accentuated in the figure with a thick border). These small G-proteins can reside in two different states: a GTP (guanosine triphosphate)-bound active state and a GDP (guanosine diphosphate)-bound inactive state. Their activity level is determined by interactions with GAP (GTPase activating protein) and GEF (Guanine exchange factor) proteins. The different GEF proteins promote the exchange of GDP for GTP leading to enhanced activity, while GAP proteins catalyze the hydrolysis of GTP to GDP, leading to suppression of activity. Activation of RAS is initiated by calcium influx through N-methyl-D-aspartic acid (NMDA) receptors, which activates RAS-guanine nucleotide-releasing factor (RAS-GRF2) and inactivates synaptic RAS GTPase activating protein (SynGAP) [20-24,26,29]. Alternatively, RAS is activated upon mineralocorticoid receptor (MR) activation by corticosterone (cort), β-adrenergic receptor (β-AR) activation by noradrenaline (NA) or by brain-derived neurotropic factor (BDNF) binding to the tyrosine receptor kinase type B (TRKB) receptor, which initiates RAS signaling by activating the GEF protein: Son of Sevenless, Drosophila, homologue 1 (SOS1) [16-18]. Src homology protein 2 (SHP2) stimulates this activation in as yet undefined ways. Active RAS activates RAF, which induces a phosphorylation cascade ultimately leading to activation of ERK and its downstream targets. At the presynaptic side these include Synapsin-I (SynI), which modulates neurotransmitter release. At the postsynaptic side ERK activates ribosomal S6 kinase 2 (RSK2) and myocardial Snf1 (sucrose nonfermenting 1)-like kinases 1, 2 (MSK 1, 2), which in turn activate the transcription factor cAMP response element-binding (CREB). Also, ERK activates mitogen activated protein-interacting kinases 1, 2 (MNK1, 2), which signal for translation. Finally, ERK directly modulates the dynamics of ion channels including the potassium channel Kv4.2. These processes all influence the strength of the synapse, essential to learning and memory formation. MTOR is a major controller of dendritic translation through its downstream targets S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). MTOR is driven by RAS homolog enriched in brain (RHEB) activity, which is increased by inhibition of Tuberin-GAP (TSC2) activity. This could occur directly by RAS-PI3K signaling ▶

contacts on their target neurons. Indeed, Nf1 mice (Nf1 heterozygous knock-out mice) show increased inhibitory transmission, which is probably mediated by enhanced release of the main inhibitory neurotransmitter in the central nervous system (GABA; Gamma-aminobutyric acid). This increased inhibitory transmission appears to directly cause the impairments in plasticity and learning in these mutants [19] (Box 2). This is an interesting example of how a similar modification of the RAS-ERK pathway, can generate opposite systems-level outcomes by affecting two different types of neurons. However, it is not yet clear how the NF1 mutation affects RAS-ERK signaling preferentially in inhibitory neurons.

Box 1: What's in a name? Diagnosing the NCFC syndromes

A clinical or genetic diagnosis of a syndrome is invaluable to the affected patient and its parents in two aspects. First, they can identify themselves with families with the same disorder, and second, they expect to get a clear prognosis. However, the large overlap in phenotypes of the NCFC syndromes, the desire to diagnose patients at a young age, even though the phenotype might still be obscure then, and the frequent lack of definite diagnostic criteria make it difficult to establish a diagnosis in an affected patient. This is especially true for patients with overlapping characteristics of Noonan, Costello and CFC syndromes [10]. Now that many genes have recently been identified, would a diagnosis based on the identified genetic mutation ensure a more accurate prognosis for the patient? Unfortunately, this is not the case, because even patients with identical mutations often have highly variable phenotypes. For example, identical mutations at D153V in *KRAS* were found in children diagnosed with Noonan syndrome [62], severe Noonan with CFC features [10], and CFC [3]. Likewise, mutations at E501K in *BRAF* are reported in patients with Noonan [9] and CFC [3], and *BRAF* A246P mutations in CFC [3] as well as in Costello (the latter re-diagnosed as CFC) [63]. This indicates that modifier genes, of which none are identified at present, have an important role in shaping phenotypes in these syndromes.

Model organisms like mutant flies and mice are now used to identify these modifier genes. Therefore, future research might lead to a novel classification system based on a 'fingerprint' of a large number of selected genes that segregates patients on the basis of a certain prognosis (*eg,* malignancy risk or cognitive function) rather than on a mutated gene or a syndrome diagnosis.

▶ or indirectly through RAS-ERK signaling. The genes mutated in the different syndromes are depicted in red, with the name of the syndrome(s) in yellow. The genes shown in blue are not (yet) associated with a syndrome, but are plausible candidate genes for cases in which no mutation is identified. The plus and minus signs indicate whether the identified mutations up- or down-regulate ERK or MTOR signaling (based on *in vitro* assays). Note that the vast majority of the mutations encountered in the NCFC and Hamartoma syndromes lead to enhanced ERK or MTOR signaling. Abbreviations: SPRED1: sprouty related EVH1 domain containing protein 1, PIP3: Phosphatidylinositol (3,4,5)-trisphosphate, PIP2: phosphatidylinositol (4,5)-bisphosphate, PDK1: Pyruvate dehydrogenase kinase, isozyme 1, eEF2-K: eukaryotic elongation factor 2 kinase AKT: , S6: , eIF4B: , eIF4E

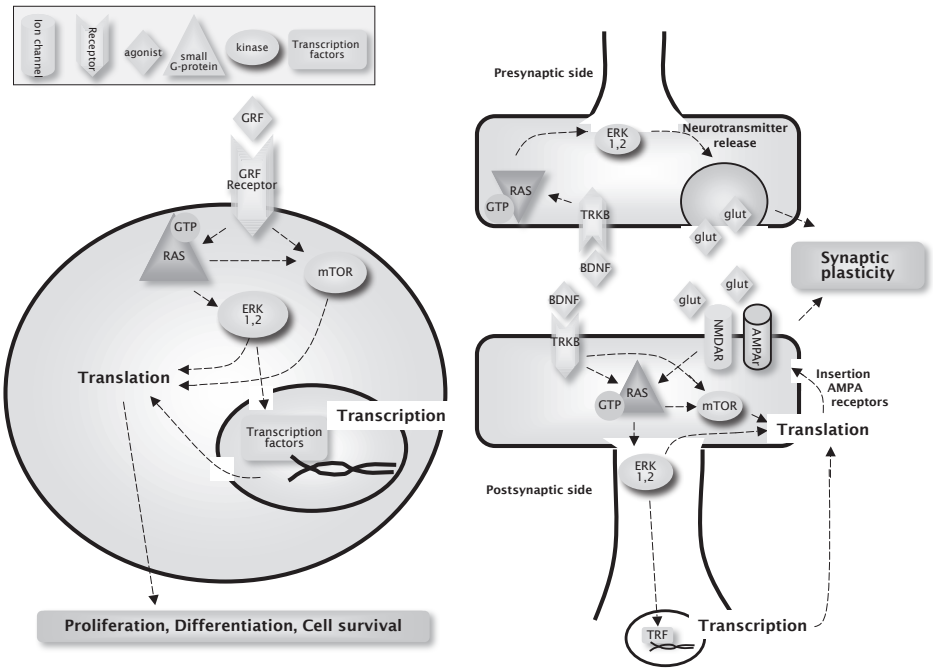

Figure 2: The output of ERK and MTOR signaling in mitotic cells and neurons. (a) In non-neuronal, mitotic cells, extra cellular signals such as growth factors and cytokines induce proliferation, differentiation and cell cycle progression via activation of ERK and MTOR pathways. However, neurons (b) are mostly post-mitotic and the ERK and MTOR pathways are recruited for a process called synaptic plasticity, important in memory formation. Synaptic strength of a synapse is strongly dependent on the amount of neurotransmitter (glutamate) released presynaptically and on the number of glutamate-responsive AMPA receptors present in the postsynaptic cell membrane. Upon different stimuli, including calcium influx via the NMDA receptor and BDNF binding to pre- and postsynaptic TRKB receptors, ERK and MTOR pathways change synaptic strength by both modulating neurotransmitter (glutamate) release and by regulating the insertion of AMPA receptors, which are activated by glutamate. Abbreviations: glut: glutamate, G-protein: guanine nucleotide binding protein, GRF: growth factor, GTP: guanosine triphosphate, TRF: transcription factor.

THE POSTSYNAPTIC RAS-ERK PATHWAY IS AN IMPORTANT SIGNAL INTEGRATOR

Synaptic strength is not only controlled by regulating the amount of neurotransmitter release. In fact, most of the changes taking place during memory formation occur on the postsynaptic side of the synapse. Influx of calcium ions through NMDA (N-methyl-D-aspartic acid) receptors is the pivotal trigger to initiate the process of synaptic strengthening, which can be measured in vitro (then referred to as LTP; long-term potentiation), and which is an absolute requirement for learning and memory. The RAS-GEFs (guanine nucleotide exchange factors) are recognized as major connectors between calcium ions and RAS-ERK activation, as they associate with NMDA receptors, and are activated by the influx of calcium ions through these receptors [20,21]. Genetic studies suggest that RAS-GRF2 (guanine nucleotide-releasing factor) is the

Box 2: Treating cognitive defects in NF1 – Lost in translation?

Similar to NF1 patients, *Nf1* heterozygous knockout mice have problems in learning and attention [19,50]. In addition, they show deficits in synaptic plasticity [19]. Importantly, these deficits can be rescued by genetically reducing the level of N-RAS or K-RAS, suggesting that learning and plasticity deficits in *Nf1* mice are caused by enhanced RAS signaling [19].

RAS activity is critically dependent on its association to membranes, for which it requires the post-translational addition of a farnesyl or geranylgeranyl anchor. Statins decrease the synthesis of cholesterol, farnesyl and geranylgeranyl by inhibiting HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase, the rate-limiting enzyme in the mevalonate synthesis pathway. Interestingly, treatment of *Nf1* mice or flies with statins cures their learning deficits [50,54]. Statins are prescribed widely to treat hypercholesterolaemia, and have an excellent safety profile in adults and children. Therefore, the effect of Simvastatin on cognitive functioning has recently been investigated in a randomized, placebo-controlled trial, involving 62 children with NF1 [64]. Outcome measures included neuropsychological tests, MRI analysis and a neurophysiological test (measuring eye-hand movement control). Unfortunately, a three-month treatment resulted in a significant improvement in only one out of nine neuropsychological outcome measures, when compared to the placebo group. Several factors could have attributed to these disappointing results. First, it is conceivable that reversing deficits in higher cognitive functions in humans is far more difficult than reversing cognitive deficits in mice. This could be due to the greater complexity of the human brain. Second, the statin concentration that was reached in the human brain, could have been significant lower than in mice. This could be due to differences in metabolism, or to differences in blood-brain barrier permeability. Third, there was a large placebo or re-test effect, which brought 3 of the 9 neuropsychological outcome measures back to normal values. Since statins did not improve cognitive function in wild-type mice, it is possible that a ceiling effect was reached for these measures. Finally, it can not be excluded that the tests were not sensitive enough to capture a real improvement (see also Box 3). Because of all these biological and methodological issues, trials involving a longer treatment are now initiated. This would allow the brain more time to undergo changes, and would diminish the placebo and re-test effect by increasing the time in between testing moments. Moreover, a longer treatment would allow inclusion of real-life measures such as school performance.

main GEF that drives RAS-ERK-dependent synaptic strengthening [22,23]. However, an increase in calcium can also activate ERK through CaMKII (calcium/calmodulin-dependent protein kinase 2) mediated inactivation of SynGAP (synaptic RAS GTPase activating protein), a negative regulator of RAS signaling [24] (Figure 1). Besides calcium influx, the postsynaptic RAS-ERK pathway can also be activated by BDNF binding to the TRKB receptor, by the activation of β-adrenergic receptors and by a, more indirect, cAMP (cyclic adenosine monophosphate)-PKA (protein kinase A)-dependent pathway [25-27] (Figure 1). Hence, the postsynaptic RAS-ERK pathway serves as a major signal integrator to control synaptic plasticity.

THE POSTSYNAPTIC RAS-ERK SIGNALING PATHWAY HAS MANY TARGETS

How does the postsynaptic RAS-ERK pathway control postsynaptic plasticity? By changing the number of AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in the cell membrane, the postsynaptic neuron directly controls its sensitivity to glutamate and its probability to fire an action potential (Figure 2). Both over-expression of an active form of RAS in hippocampal neurons and silencing of SynGAP lead to an ERK-dependent increase in the amount of AMPA receptors in the postsynaptic membrane [28,29]. This suggests a direct link between postsynaptic ERK signaling and AMPA receptor dynamics.

Protein synthesis is an absolute requirement to convert transient changes in synaptic strength into stable, long-lasting connections, and hence in stable memories. When measured in vitro, this phase of synaptic strengthening is referred to as late-phase LTP (L-LTP). ERK signaling plays a critical role in controlling protein synthesis by regulating both transcription and translation events. One of the targets of the RAS-ERK pathway is the transcription factor CREB (cAMP response element-binding), which is important for memory formation [26]. The regulation of CREB seems to be sensitive to the application of BDNF [30] and its activation is dependent on several kinases downstream of ERK [26] (Figure 1). One of these, RSK2 (ribosomal S6 kinase 2) [26], is associated with the X-linked Coffin-Lowry syndrome (OMIM 303600) and patients with this disease present with mental retardation. Notably, the CBP (CREB-binding protein) gene, which encodes an essential transcriptional co-activator of CREB, is mutated in Rubinstein-Taybi Syndrome, a disease characterized by severe mental retardation (OMIM 180849), again stressing the importance of proper ERK-dependent signaling in cognitive function.

Besides its roles in transcription, ERK signaling also controls translation in concert with the MTOR signaling pathway and more directly by activating the MNK (mitogen activated protein-interacting kinase) isoforms which in turn activate eIF4E (eukaryotic initiation factor 4E) [31] (Figure 1). Collectively these studies indicate that RAS-ERK signaling plays a critical role in several major aspects of synaptic plasticity.

COGNITIVE DEFICITS ARISING FROM GENETIC IMPAIRMENTS IN PI3K-MTOR SIGNALING

Tuberous Sclerosis Complex (TSC) and the PTEN (phosphatase and tensin homolog)-hamartoma tumor syndromes, a group of clinical entities all resulting from germ-line mutations in PTEN, are classified as the Hamartoma syndromes (Table 1). The PTEN-hamartoma tumor syndromes present with mental impairments, however because of their rare nature detailed descriptions on the cognitive phenotypes are sparse. The cognitive profile of TSC patients is remarkably variable, with half of the patients having a normal IQ and about 30% an IQ below 20 [32]. Similar to NF1, specific deficits in attention, executive functioning, memory and language are also common in TSC patients with a normal IQ [32]. The variation in cognitive abilities can in part be explained by differential effects of TSC1 (Tuberous Sclerosis Complex 1

gene) versus TSC2 (Tuberous Sclerosis Complex 2 gene) mutations (TSC2 mutations tend to aggregate with more severe cases of mental retardation), and the presence of brain hamartomas and epilepsy (Table 1) [32]. The mutational spectrum of the two TSC genes is very broad, complicating research into a possible contribution of modifier genes to the variability in phenotype. Hence, no modifier genes that affect cognitive function have been identified. However, polymorphisms in the Interferon-gamma gene [33] and in the gene encoding the DNA repair agent 8-oxoguanine glycosylase 1 (OGG1) [34] modulate susceptibility to renal angiomyolipomas in TSC patients, pointing to a role for modifier genes in the phenotypical variability of TSC.

PI3K-MTOR SIGNALING CONTROLS PROTEIN TRANSLATION

Rodent models have been developed for both TSC as for the PTEN-hamartoma tumor syndromes, and studies on these mutants reveal an essential role of PI3K-MTOR signaling in learning and memory [35-38] (Table 2). Both Pten homozygous knock-out mice and Tsc1 and Tsc2 heterozygous knock-out mice have impaired learning and show deficits in synaptic plasticity (Table 2) [36-39]. However, unlike the patients with PTEN-hamartoma tumor syndromes, Pten mouse mutants have severe disruptions in brain architecture [37], which is probably related to these mice carrying a homozygous rather than a heterozygous deletion. It is not clear whether the learning deficits are secondary to these developmental brain abnormalities or the direct result of aberrant neuronal plasticity in the absence of PTEN. By contrast, a heterozygous Tsc1 mouse mutant showed learning impairments in the absence of brain pathology or seizures, implying that the TSC proteins have a direct role in synaptic plasticity [38].

Like the RAS-ERK pathway discussed above, the MTOR pathway is involved in the protein synthesis-dependent phase of synaptic strengthening. Rapamycin, a selective inhibitor of MTOR, specifically impairs this phase of synaptic strengthening and causes long-term memory deficits [40,41]. Interestingly, all components of the PI3K-MTOR pathway and the complete translation machinery are present in dendrites [41], suggesting that this pathway controls protein translation near the activated synapse (designated as 'local' protein translation). Indeed, BDNF stimulation is found to initiate MTOR-dependent protein translation in isolated dendrites [42]. Besides BDNF, activation of NMDA- and β-adrenergic receptors can also induce MTOR signaling. [25,43]. MTOR drives local protein translation through phosphorylation of its downstream targets, which include 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein) and S6K (S6 kinase) [44] (Figure 1). In addition to its important role as an initiator of local protein synthesis, MTOR can also suppress local translation of certain proteins, amongst which the Kv1.1 potassium channel [45].

As expected based upon their increased MTOR signaling, Tsc2 mutant mice show increased phosphorylation of S6 ribosomal protein, which is involved in protein translation (Figure 1). Consequently, a relatively weak stimulus is sufficient to recruit the protein synthesis-dependent phase of synaptic strengthening in these mutants. Paradoxically, this causes a learning deficit rather than a learning enhancement probably due to inappropriate storage of unrelated or unprocessed information [39].

It remains to be elucidated which dendritically targeted mRNAs are specifically regulated by MTOR, and how this couples to strengthening of the synapse. However, a direct link has been established between PI3K signaling and AMPA receptor insertion, suggesting that this might be one of the main mechanisms by which MTOR signaling drives long lasting synaptic changes [46]. Taken together, these studies imply that a critical balance of MTOR signaling is required to control neuronal protein translation, which is essential to long-term synaptic changes.

ERK AND MTOR SIGNALING IN AUTISM

So far we have focused on the roles of ERK and MTOR signaling in cognitive function; however, behavioral problems are also commonly associated with both the NCFC and the Hamartoma syndromes. Evidence for a relationship with autism is somewhat limited for both NF1 [47,48] and Noonan syndrome [48], but autism is certainly a prominent characteristic of the Hamartoma syndromes (Table 1). Half of the TSC patients present with autistic features [32]. Conversely, mutations in the TSC genes are found in 1% of autistic individuals and PTEN germ line mutations are found in as many as 17% of patients presenting with both autism and macrocephaly [48]. These are strikingly high percentages in light of the still obscure genetic knowledge on autism.

Mouse models for the PTEN-hamartoma tumor syndromes and TSC are found to recapitulate the social withdrawal phenotype as seen in autistic individuals [36,38]. Taken together, the high incidence of autism in Hamartoma syndromes patients and the autistic phenotypes in mouse models for these syndromes make clear that aberrations in the PI3K-MTOR pathway can cause molecular and cellular changes that lead to autistic behavior. Thus, this pathway might be a major player in causing autism. However hampered by a lack of knowledge of the brain areas involved in autism, insight is limited into the exact mechanisms leading to autism upon enhanced PI3K-MTOR signaling.

COGNITIVE IMPAIRMENTS ARE RELATED TO REVERSIBLE CHANGES IN SIGNALING RATHER THAN GROSS BRAIN ABNORMALITIES

Structural brain abnormalities and seizures are part of the phenotypic spectrum of the NCFC and Hamartoma syndromes (Table 1). It could be argued that the cognitive deficits develop only secondary to these brain abnormalities. However, evidence for this idea is limited and contested. First, although infantile spasms are associated with a poor cognitive outcome in TSC [32], clinical studies fail to show consistent data on a correlation between MRI abnormalities and cognition in TSC and NF1 [49]. Second, cognitive impairments in most of the mouse models for NCFC and Hamartoma syndromes occur in the absence of structural brain abnormalities as seen in patients (Table 2). Third, as outlined in previous sections, the cognitive impairments found in these mouse models seem to arise from disturbances in the balance of neuronal signaling, as treatments with drugs specifically targeting these signaling disturbances can rescue both the cognitive deficits as the impairments in

synaptic plasticity in mouse models for TSC and NF1 (Box 2) [39,50]. Interestingly, recent results show that even though epilepsy correlates with poor cognitive outcome in TSC, this symptom can also be directly attributed to disturbed MTOR signaling, and can be rescued with Rapamycin [51]. These findings suggest that the cognitive impairments are not due to irreversible developmental abnormalities of the brain, but can be attributed to reversible changes in signaling.

TREATING COGNITIVE GENETIC DISEASE – LESSONS FROM CANCER

Cancer research has generated a wealth of knowledge on how to interfere with RAS-ERK and PI3K-MTOR signaling. By exploiting this knowledge we might be able to reverse the cognitive deficits associated with the NCFC and Hamartoma syndromes. However, there are several important aspects that have to be considered when using oncology drugs to treat cognitive deficits. First, animal studies suggest that both increased and decreased ERK or MTOR signaling result in cognitive impairments, indicating that a strict balance is required (Table 2). This is in marked contrast to tumors associated with these diseases, which are always caused by up-regulation of the ERK or MTOR pathway, and often require an additional second hit affecting the other allele (loss of heterozygosity) to become oncogenic [52]. Thus, treating cognitive deficits requires considerably more careful dosing than the treatment of cancer. Importantly, this implies that high doses of these drugs, as used in cancer treatment, might negatively affect cognitive function, which is of considerable concern (Box 3). Second, side-effects are acceptable in treating a life-threatening tumor, especially if the treatment is short. However, the treatment of cognitive deficits would probably be life-long so requires an exceptionally good safety profile. Finally, many of the small molecule inhibitors used in cancer treatments are specifically designed to not be able to cross the blood-brain barrier, which makes them unsuitable to treat cognitive disorders.

Treatment of NCFC syndromes with inhibitors of the RAS-ERK pathway

Inhibiting RAS activity is a potential treatment mechanism for the cognitive impairments in the NCFC disorders (Figure 1). RAS activity can be diminished by attacking its Achilles' heel: its requirement to be post-translationally modified (Box 2). Both Farnesyl Transferase (FTase) inhibitors and statins can reduce RAS signaling in this manner, but although they show anti-proliferation effects in vitro, their success in treating cancer as a monotherapy has been limited (reviewed in [53]). Nevertheless, it is likely that the amount of RAS inhibition required to treat cognitive deficits is significantly lower than for tumor regression. Indeed, both FTase inhibitors and statins were sufficient to rescue cognitive and plasticity deficits of Nf1 mice [19,50], and more recently to rescue learning impairments in Nf1mutant flies [54], suggesting an evolutionary conserved mechanism. Despite these findings, a clinical trial assessing the effects of Simvastatin in NF1 patients showed little effect (Box 2).

It has to be noted that the in vitro anti-proliferative effects of both FTase inhibitors and statins cannot solely be ascribed to their ability to interfere with RAS signaling [55]. Therefore, we cannot rule out that the rescue of the learning deficits of Nf1 mice is the result of other mechanisms. For instance, statins might reduce

Table 2. Mouse and rat mutants of genes associated with the RAS-ERK and PI3K-MTOR signaling pathways and their phenotypes with respect to hippocampal function [a].

		Phenotype			
Gene	**Mutation**	**Hippocampalb-dependent learning**	**Synaptic plasticity[b]**	**Molecular signaling**	**Morphology**
RAS-GRF1	*Ras-Grf1* homozygous knock-out mouse	Impairments in some spatial learning paradigms [86], intact performance in others [22]	Impaired LTD [23], intact LTP in some protocols [22] and slight impairment in others [23]	Intact NMDA-receptor induced ERK phosphorylation [23]	No apparent changes in brain morphology [22]
RAS-GRF2	*Ras-Grf2* inducible homozygous knock-out mouse [23]	Not known	Impaired LTP and decreased presynaptic plasticity	Decreased NMDA-receptor induced ERK phosphorylation	No apparent changes in brain morphology [87]
SynGAP	*Syngap* heterozygous knock-out mouse	Impaired spatial learning [88]	Impaired LTP [29,88]	Increased basal ERK and MEK phosphorylation, increased NMDA-receptor induced ERK phosphorylation [88]	Increased number of AMPA-receptor clusters in neuronal cultures of homozygous knock-out mice [89]
HRAS	*H-Ras* homozygous knock-out mouse	Not known	Enhanced LTP in some protocols [90], intact LTP in others [88]; increased NMDA-receptor mediated responses [90]	Increased phosphorylation of NR2A and NR2B subunits of the NMDA receptor [90]; intact basal ERK and MEK phosphorylation [88]	No apparent changes in brain morphology [88,90]
HRAS	Forebrain and excitatory neuron-specific constitutively active *H-Ras* (*H-RasG12V*) mouse mutant [17]	Enhanced spatial learning	Enhanced LTP and increased presynaptic plasticity	Increased basal ERK and SYNI phosphorylation, intact basal AKT phosphorylation	Increased amount of neurotransmitters ready for release (docked vesicles)
KRAS	*K-Ras* heterozygous knock-out mouse [19]	Impaired spatial learning	Impaired LTP	Not known	Not known
NRAS	*N-Ras* heterozygous knock-out mouse [19]	Intact spatial learning	Not known	Not known	Not known
NF1	*Nf1* heterozygous knock-out mouse (see Box 2)	Impaired spatial learning [19]	Impaired LTP and increased GABA-mediated inhibition [19]	Increased basal ERK and CREB phosphorylation [50,91]; intact basal AKT phosphorylation [91]	Mild astrogliosis [92]
BRAF	Forebrain and excitatory neuron-specific *B-Raf* homozygous knock out mouse [93]	Impaired spatial learning	Impaired LTP	Intact basal ERK phosphorylation; decreased ERK phosphorylation after a learning paradigm	Not known
MEK1	Neuron-specific dominant-negative *Mek1* mutant [94]	Impaired spatial learning	Not known	Not known	No apparent changes in brain morphology

MEK1	Forebrain and excitatory neuron-specific dominant-negative *Mek1* mouse mutant [95]	Impaired long term spatial memory	Impaired late phase LTP	Decreased protein synthesis upon LTP inducing stimuli; decreased ERK, S6 and eIF4E phosphorylation upon LTP inducing stimuli and after a learning paradigm	Not known
ERK1	*Erk1* homozygous knock-out mouse	Enhanced spatial learning in some protocols [96], intact performance in others [97]	Impaired LTP in some protocols [96], intact LTP in others [96,97]	Both increased [96] and intact [97] ERK2 signaling reported.	No apparent changes in brain morphology [96,97]
ERK2	Knock-down mouse mutant with a 20-40% reduction in *Erk2* expression [98]	Impaired spatial learning	Not known	Not known	No apparent changes in brain morphology
PI3K	*p58*α (regulatory subunit of *Pi3k)* knock-out mouse [35]	Impaired spatial learning	Not known	Not known	Decreased synaptic density
PTEN	Mouse mutant with homozygous *Pten* deletion in limited neuronal populations (including hippocampus)	Impaired spatial learning [36]	Impaired basal transmission and LTP [37]	Increased basal AKT, MTOR and S6K phosphorylation [36]	Hypertrophy of cell soma, ectopic dendrites and axonal tracts and increased spine density [36,37]
TSC1	*Tsc1* heterozygous knock-out mouse [38]	Impaired spatial learning	Not known	Not known	No neuronal abnormalities, no lesions by MRI
TSC2	*Tsc2* heterozygous knock-out rat	Intact spatial learning [99]	Impaired LTP and LTD and increased presynaptic plasticity [89]	Intact basal ERK phosphorylation, increased ERK phosphorylation upon LTP inducing stimuli	Adult animals are free of cerebral hamartomas, aged animals develop them at a slow rate [27]
TSC2	*Tsc2* heterozygous knock-out mouse [39]	Impaired spatial learning, was rescued by treatment with Rapamycin	Lower threshold for late phase LTP, was rescued by treatment with Rapamycin	Increased basal S6 phosphorylation, was rescued by treatment with Rapamycin	No apparent changes in brain morphology

[a] Only rodent mutants in the direct RAS-ERK and PI3K-MTOR routes in which hippocampal function is specifically tested are presented.
[b] See glossary.

Box 3: Cognitive function and chemotherapy: the chemobrain

The increasing number of patients surviving cancer has aroused interest in how chemotherapy affects quality of life. Patients receiving conventional chemotherapy that causes DNA damage and cell death (for example platinum compounds), often report transient or even persistent cognitive impairments across various domains including working memory, executive function and processing speed (reviewed in [65]). However, the precise impact of chemotherapy on brain function is a matter of debate for two major reasons. First, some of the studies reported a discrepancy between self-reported problems and objective neuropsychological tests, with no clear correlation between these two measures [66,67]. Second, most studies are cross-sectional studies, hence cognitive performance of the subjects before treatment is not known. Recently, several prospective (longitudinal) studies on this topic have been published, and although most studies suggest that chemotherapy has an impact on cognitive function, it does not seem as dramatic as reported by some earlier cross-sectional studies (for a review, see Ref [66]). Possibly, the effects in these prospective study designs were smaller, because patients are repeatedly assessed with similar tests, which can result in practice effects that might mask a real cognitive decline. But one interesting aspect that was noted in several of these prospective studies, was a greater than expected incidence of cognitive problems in these patients even *before* initiation of chemotherapy (see Ref [65] and references therein). Although several factors including psychological factors (e,g. stress, anxiety, depression after being diagnosed with a life-threatening disease) and biological factors (for example cytokine elevation) could cause this pre-treatment deficit in cognitive functioning, it is tempting to speculate that certain polymorphisms in the genes the function in the RAS-ERK or PI3K-MTOR pathways, result in increased cancer susceptibility as well as decreased cognitive function.

Because of the DNA-damaging nature of conventional chemotherapies, neuronal cell death is more likely to be an important mechanism underlying the induced cognitive problems than direct interference with synaptic plasticity. By contrast, the novel chemotherapies that are based on small molecule inhibitors directed against proteins in the pathways discussed in this review can directly impede synaptic plasticity. Thus, provided that they can cross the blood-brain barrier, they might severely affect cognitive function. For instance, MEK and MTOR inhibitors are found to affect cognitive function in wild type mice [40,68]. Therefore, substantial animal and clinical studies will be required to assess the short-term and long-term effects of these new cancer treatments on cognitive function.

the synthesis of neurosteroids, because the rate-limiting step of steroid synthesis is the conversion of cholesterol to pregnenolone. Because neurosteroids directly activate the inhibitory GABA-A receptor [56], reduction of neurosteroid levels might help to decrease the enhanced inhibition mediated by GABA-A signaling, as observed in Nf1 mice [19].

The RAS-ERK pathway can also be targeted with several newly developed small molecule inhibitors of B-RAF (v-raf-1 murine leukemia viral oncogene homolog

B1) and MEK. Cancer trials with these inhibitors are underway (reviewed in [57]). However, this first generation of small molecule inhibitors is probably not suitable for treating cognitive deficits, because of low blood-brain permeability and significant side-effects.

Treatment of Hamartoma syndromes with inhibitors of the MTOR pathway

The MTOR inhibitor Rapamycin (Sirolimus), applied as immunosuppressant in organ transplant patients, has already been successfully used to treat astrocytomas and angiomyolipomas in TSC patients [33,58,59]. Rapamycin is also shown to have anti-proliferative effects in patients with brain tumors owing to reduced PTEN activity [60]. A recent study revealed that Rapamycin can reverse the cognitive deficits and aberrations in synaptic plasticity in Tsc2 mutant mice [39]. In addition, a clinical trial to measure the effect of Rapamycin treatment on renal hamartomas in TSC patients in conjunction with cognitive function (memory and executive skills) as secondary outcome measure is underway (NCT00490789; http://clinicaltrials.gov). Ideally, all future Rapamycin trials in TSC patients should include some measures to assess cognitive improvements and quality of life. The success of the cognitive improvements (if any) should then be carefully weighed against the drawbacks associated with a long-term treatment with Rapamycin.

Finally, like RAS, the TSC target protein RHEB (RAS homolog enriched in brain) is critically dependent upon farnesylation. This suggests that FTase inhibitors and statins might also help to treat the cognitive deficits in the Hamartoma syndromes [61].

CONCLUDING REMARKS

Here, we have emphasized the importance of the oncogenic RAS-ERK and PI3K-MTOR signaling pathways in cognitive functioning by focusing on the cognitive deficits associated with the NCFC and Hamartoma syndromes. There is a strong connection between genetic alterations in components of these pathways and cognitive dysfunction. Because of pioneering studies in cancer research, these signaling routes are very well characterized and rapid progress has now been made to understand their role in neuronal function. Animal studies revealed that the neuronal RAS-ERK and PI3K-MTOR pathways modulate neurotransmitter release, control synthesis of proteins required for stabilizing synaptic changes, and regulate receptor properties and dynamics. These processes play a pivotal role in synaptic plasticity, required for proper cognitive function. Recent targeted treatments in animal models of NCFC and Hamartoma syndromes, using drugs designed for cancer treatment have been successful, and will undoubtedly stimulate the initiation of many clinical trials.

Box 4: Areas for future research

Compelling proof that the treatments that can rescue the cognitive deficits in mutant mice are also effective and safe in patients.

1. Identification of small-molecule inhibitors of the ERK and MTOR pathways with minimal side-effects and which can cross the blood-brain barrier efficiently so that they can be used to treat cognitive deficits.
2. Do the treatments, which can rescue cognitive functioning in mouse models of the Hamartoma syndromes, also rescue their autistic phenotypes?
3. Investigating how certain mutations only affect a subclass of neurons (eg. NF1 affects predominantly inhibitory neurons).
4. Investigating to what extent the mechanisms underlying the more severe NCFC disorders are different from the mechanisms underlying the NCFC disorders with only mild cognitive deficits.
5. Identification of the mRNAs which translation is controlled by MTOR and defining which are critical for causing the cognitive deficits in the Hamartoma syndromes.

GLOSSARY

AMPA receptor: Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Subtype of the glutamate receptor, mediates fast excitatory synaptic transmission in the central nervous system.

Angiomyolipoma: Benign kidney tumor composed of an abnormal collection of blood vessels *(angio)*, smooth muscle *(myo)*, and fat *(lipoma)*. Found in 70-80% of TSC patients.

Astrocytoma: Benign brain tumor composed of undifferentiated, dysfunctional glial cells. Found in 10-20% of TSC patients.

Autism: A developmental disorder characterized by a triad of symptoms: a qualitative impairment in social interaction, qualitative impairments in communication, and restricted**,** repetitive and stereotyped patterns of behavior.

Dendrite: A neuronal process arising from the cell body that receives synaptic input. From the viewpoint of a specific synapse, this dendrite lies on the postsynaptic side.

Excitatory neuron: A neuron that forms stimulatory contacts on its target neurons, and thereby increases their probability to fire. Glutamate is the most common neurotransmitter released by excitatory neurons.

GABA: Gamma-aminobutyric acid; Most abundant inhibitory neurotransmitter in the central nervous system.

Glutamate: Most abundant excitatory neurotransmitter in the central nervous system.

Hamartoma: A benign tumor-like growth consisting of a disorganized mixture of cells and tissues normally found in the area of the body where the growth occurs.

Hippocampus: Part of the brain essential for memory formation. In rodents its function is typically assessed with maze-tasks.

Inhibitory neurons: A neuron that forms inhibiting contacts on its target neurons, and thereby reduces their probability to fire. In the central nervous system, GABA is the most common neurotransmitter released by inhibitory neurons.

LTD/LTP: Long-term depression/Long-term potentiation; An *in vitro* measure of synaptic weakening and strengthening, respectively. LTP can be subdivided in an early phase, (1-2 hours after LTP induction) requiring posttranslational changes only, and a late phase, which requires the synthesis of new proteins. The protein synthesis-dependent phase of synaptic strengthening is required for long term memory formation.

Mental retardation (MR): The combination of an IQ <70 (normal IQ is 100 ± 15) with significant limitations in at least two areas of adaptive behavior (e.g. communication, daily living skills or social skills), apparent before the age of 18. An IQ of 69–50 is defined as mild; 35–49 as moderate; 20–34 as severe, and <20 as profound MR.

Neurotrophins: Family of proteins, which are important for neuronal survival in the developing brain, and play a role in synaptic plasticity in the mature brain.

NMDA receptor: A subtype of glutamate receptor. Mediates calcium influx during LTP induction.

Plasticity (synaptic/neuronal): The ability of neurons to change the strength of synaptic contacts or their excitability. These processes are required for memory formation.

Postsynaptic: The side of the synapse on the dendrite where the receptors are located which are receptive to the released neurotransmitter molecules.

Presynaptic: The side of synapse on the axon terminal where neurotransmitter molecules are released, which convey signals to the target cell.

Spine: A small protrusion from dendritic branches on which synapses are formed.

Synapse: The site of contact between two neurons, consisting of a presynaptic side and a postsynaptic side.

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3

COGNITIVE DEFICITS IN *TSC1*$^{+/-}$ MICE IN THE ABSENCE OF CEREBRAL LESIONS AND SEIZURES

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ABSTRACT

Objective*:* Tuberous Sclerosis Complex (TSC) is characterized by brain lesions, epilepsy and increased incidence of mental retardation and autism. The causal link between lesion load and epilepsy on cognitive disabilities has been debated and these factors explain only part of the IQ variability. A *Tsc2* rat model of the disease provided evidence that the *TSC* genes are directly involved in neuronal function. However, these lesion and epilepsy free animals did not show learning deficits, leaving open the possibility that the presence of brain lesions or epilepsy is a prerequisite for the cognitive deficits to fully develop. Here, we reinvestigated the relationship between cerebral lesions, epilepsy and cognitive function using *Tsc1*$^{+/-}$ mice.

Methods: We used immunocytochemistry and high-resolution MRI to study the presence of neuronal pathology in *Tsc1*$^{+/-}$ mice. We used the Morris water maze, fear conditioning, social interaction and nest building test to study the presence of cognitive and social deficits.

Results: We observed no spontaneous seizures, nor cerebral lesions in the brains of *Tsc1*$^{+/-}$ mice. In addition, giant dysmorphic cells were absent and spine number and dendritic branching appeared to be normal. Nevertheless, *Tsc1*$^{+/-}$ mice showed impaired learning in the hippocampus sensitive versions of the learning tasks and impaired social behavior.

Interpretation: *Tsc1*$^{+/-}$ mice show social and cognitive deficits in the absence of apparent cerebral pathology and spontaneous seizures. These findings support a model in which haploinsufficiency for the *Tsc* genes leads to aberrations in neuronal functioning resulting in impaired learning and social behavior.

INTRODUCTION

Tuberous Sclerosis Complex (TSC) is caused by a heterozygous mutation in either the *Tsc1* gene or *Tsc2* gene and is characterized by benign tumors arising in different organs and severe neurological and psychiatric symptoms. Up to ninety percent of the patients suffer from epilepsy and almost fifty percent from mental retardation (IQ < 70)[1]. Moreover, in half of the patients pervasive development disorder (PDD) or autism is encountered, which is a fifty fold increased risk as compared to the general population[2]. MRI scans show three kinds of brain lesions: (cortical) tubers, subependymal nodules and subependymal giant cell astrocytoma's. In addition, finegrained abnormalities are observed[3, 4]. Several studies demonstrated a correlation between lesion load and mental retardation or autism (see[5] for an overview), suggesting that these lesions may cause the cognitive deficits. However, some studies failed to replicate this finding[5, 6]. The presence of epilepsy is more consistently shown to correlate with cognitive deficits. Specifically early seizure onset, intractable seizures and infantile spasms significantly enhance the risk for mental retardation[1, 7]. However, tubers and infantile spasms together only account for 47% of the variance in IQ[8], indicating that there may be an association but not a causal role of tubers and epilepsy in the development of mental retardation[9]. These findings emphasize the need to search for alternative mechanisms underlying the cognitive and behavioral deficits.

The TSC proteins function as a GTPase-activating protein complex towards Rheb which controls mTOR signaling[10]. Recent studies have implicated the TSC-mTOR pathway to be important for neuronal functioning[11-13]. This suggests that mental retardation and autism in TSC may result from abnormal neuronal plasticity rather than being caused by the brain lesions and epilepsy. Indeed, the Eker rat, a spontaneous *Tsc2* mutant, shows impaired synaptic plasticity[14]. However, young Eker rats, free from lesions and spontaneous seizures, show no deficits in spatial learning and even an improvement in episodic-like memory[15]. Thus, it seems possible that a reduction in TSC protein levels does not inevitably lead to neurocognitive abnormalities and that the presence of brain lesions or seizures is a prerequisite for the cognitive deficits to fully develop. Here we explore potential cognitive effects of *Tsc1* haploinsufficiency, using *Tsc1*$^{+/-}$ mice.

MATERIALS AND METHODS

Mice

The *Tsc1*$^{+/-}$ mice were generated by replacing exon 6 through to exon 8 of *Tsc1* with a reporter/selection cassette as described previously[16]. Consequently, *Tsc1* null embryos express Tsc1 transcripts in which exon 5 and 9 are fused. This alters the *Tsc1* reading frame, which is predicted to prematurely terminate at a TGA codon in exon 9. Therefore, any protein produced from this allele would lack all of the known functional domains of hamartin including the putative Rho activation domain[13, 17]. The *Tsc1*$^{+/-}$ mutant was crossed 6 times into the C57BL/6J OlaHsD and subsequently crossed at least 3 times into C57BL/6N/HsD. The resulting offspring of this cross, which consists of *Tsc1*$^{+/-}$ mice and their wild-type littermates,

were used for experiments. *Tsc1*$^{+/-}$ mutants in this background were born at the expected Mendelian frequency (62 wild-type mice, 67 mutants), appeared to be healthy and none of the mice died prematurely. Mice were genotyped when they were about 7 days of age, but the experimenter remained blind for the genotype during data collection and the initial analysis. Genotypes were again established after all experiments were done and the code was then broken to perform the final statistical analysis. All behavioral experiments were done with 2.5-6 month old littermates, housed in groups. Single-housed mice were excluded for the behavioral studies. Genotype groups were approximately sex and age matched. The mice were kept on a 12h light/dark cycle, with food and water available ad libitum. The behavioral experiments were performed during the light period of the cycle. All animal experiments were approved by the Dutch Ethical Committee, or in accordance with Institutional Animal Care and Use Committee guidelines.

Morris water maze

Before starting the Morris water maze, mice were handled daily for a week. The pool measures 1.2 m in diameter and contains an 11 cm diameter platform submerged 1 cm below the water surface, of the water (26°C), which was painted milk-white. We used dimmed lighting and SMART version 2.0 (Panlab, Barcelona, Spain) to track the mice. Mice were given 2 trials per day, with a 30s inter-trial interval for 9 consecutive days. At a training session, the mouse was first placed on the platform for 30s, then placed in the water at a pseudo-random start position and given a maximum of 60s to find the platform. If the mouse did not find the platform within 60s, it was placed back on the platform. After 30s on the platform, this procedure was repeated once more. The platform position remained the same during all trials. A probe trial in which no platform was present, was given one hour after the training at day 7 and day 9. In this test, mice were placed at the opposite side of previous platform position and were allowed to search for the platform for 60 seconds. In the *visible water maze test* the platform is flagged to make it visible and mice are tested for 3 days, 2 trials a day as described above. However, in this test the position of the platform changes every day to minimize the spatial learning component.

Fear conditioning

Fear conditioning was performed in a conditioning chamber (Med. Associates) equipped with a grid floor via which the foot shock could be administered. For context conditioning, each mouse was placed inside the conditioning chamber for 180 seconds. A foot shock (2 s, 0.4 mA) was delivered 148 s after placement in the chamber. Twenty-four hours later, context-dependent freezing was measured during 3 minutes. For cued conditioning, each mouse was placed inside the conditioning chamber for 100 seconds before the onset of a conditioned stimulus (CS: 20 seconds, 10 kHz tone), which co-terminated with a 2 seconds 0.3 mA foot shock (unconditioned stimulus; US). This CS-US pairing was repeated after 2 minutes and the mouse was returned to its home cage 30 seconds hereafter. Twenty-four hours later the mice were placed in a distinctly different looking and

smelling chamber. Baseline freezing was measured for 120 seconds, followed by another 100 seconds of measurement during the tone.

Social interaction and nest building

For social interaction we used female mice only, as males often show aggression or sexual behavior when confronted with an intruder. Female test mice were allowed to habituate for 15 minutes in the test cage: a plastic cage of 45x20 cm, filled with normal bedding material placed in dimmed lighting. After habituation, two unfamiliar female stimulus mice (in a hybrid 129P2-C57BL/6 background) were placed in the cage for two minutes. We used the same stimulus mice for all experiments. The time the test mouse spent exploring the stimulus mouse was recorded by an experimenter who was blind for the genotype. Afterwards, two identical trials were performed with an inter-trial interval of 10 minutes.

For measuring nest building, cages with two littermates of the same genotype and sex were arranged and three nestlets (Harlan, iso-Blox, total mass: 7 grams) were provided at day 0. Starting from day 2 nesting material which was not shredded, was weighted daily to calculate the weight of the nest. A nest of 4 grams was considered to be a fully completed nest.

MRI

Mice were deeply anesthetized by an intraperitoneal injection of Nembutal and then perfused transcardially with Phosphate Buffered Saline followed by 4% freshly prepared paraformaldehyde. The head was removed and prepared, keeping the skull intact to prevent brain deformations due to extraction.

The brains were immersed in a perfluorinated polyether (Fomblin, Solvay Solexis) to prevent image distortions due to tissue-air interfaces. The brains were imaged using a vertical 9.4T, 89 mm bore magnet with a shielded 1T/m gradient system and a transmit/receive birdcage radiofrequency coil with an inner diameter of 20 mm (Bruker Biospin, Rheinstetten, Germany). Scan parameters were: T2-weighted 3D SE sequence using a summation of 8 echoes, effective TE = 49.5 ms, TR = 1 s, total scan time per brain = 9 hours. The 3D image resolution was 55 x 55 x 110 µm.

All scans were assessed for lesions by two trained observers, who were blind to genotype.

Haematoxylin/Eosin and Golgi staining

For Haematoxylin/Eosin (HE) staining, perfused brains were embedded in paraffin and standard HE staining was performed on 5 µm thick slices. For Golgi-Cox staining, mice were anesthetized using isofluorane and the brain was quickly removed and placed in distilled water. A rapid Golgi stain was performed as described in the manual (FD Rapid GolgiStainKit, FD NeuroTechnologies Inc). Sections of 100 µm were cut and counterstained with thionin. Spine density and dendrite branching analysis was performed on Golgi stained brain sections.

Cell soma size and cell count

Perfused brains were embedded in gelatin, 40 µm coronal slices were cut and stained using a standard avidin-biotin-immunoperoxidasecomplex method with

NeuN (1:7000, Chemicon) as primary antibody. Images of the stained neocortex at a 20x magnification were used to measure soma size using "Image-Pro Plus 6.2" (Media Cybernetics, Silver Spring, MD, USA) software.

Data analysis and statistics

Statistical tests used are indicated in the results and were calculated using Statview software. Numbers of mice used for statistics are indicated in the figure legends and can be deduced from the F (Fisher) distribution $F_{x,y}$ in which x is number of groups minus 1 and y is number of mice minus the number of experimental groups. The F distribution represents the ratio of the two variances. Thus when F <1 the null hypothesis is true, and it is false when F >1. For one-way ANOVA's F is the same as t^2.

RESULTS

Tsc1$^{+/-}$ mice show no apparent cerebral pathology

To investigate the relationship between cognitive functioning and brain pathology in TSC, we made use of *Tsc1*$^{+/-}$ mice[16] which were back-crossed at least 9 times in C57BL/6, a strain commonly used for behavioral phenotyping. To investigate whether these *Tsc1*$^{+/-}$ mice have noticeable brain pathology, we made high resolution T2-weighted MRI scans of mutant and wild-type brains. Although brain lesions are detected on MRI scans in more than 90% of the TSC patients, no lesions were found in these *Tsc1*$^{+/-}$ mutants as assessed by two independent observers (Figure 1A). In addition, serial sectioning followed by NeuN or Haematoxylin/Eosin staining revealed no astrocytomas or intracranial lesions in the brains of *Tsc1*$^{+/-}$ mice. (Figure 1B, C see Materials and Methods for experimental details).

In vitro studies have shown that acute knock-down of one *Tsc1* allele leads to a 10% decrease in spine density and in a 40% increase of neuronal soma area in cortical slice cultures[11]. Increased soma size was also observed in *Tsc1* mutants carrying a neuron specific homozygous deletion of *Tsc1*and large dysmorphic neurons were observed in aged and irradiated Eker rats[18, 19]. We measured cell soma size of NeuN stained neurons in the neocortex of *Tsc1*$^{+/-}$ and wild-type mice, but failed to detect the presence of a population of abnormally large neurons in the mutants (z=0.49, p=0.97 Kolmogorov-Smirnov, 2 independent samples; Figure 1 C-D). In addition, Golgi/Cox staining revealed no differences in the spine density of hippocampal granule cells ($F_{1,2}$ = 0.06, p= 0.8 ANOVA; Figure 2A), nor did we observe an abnormal branching pattern, as the number of primary ($F_{1,2}$ = 1, p= 0.4 ANOVA) and secondary dendrites ($F_{1,2}$ = 0.3, p= 0.6 ANOVA; Figure 2B) of these neurons was normal. Taken together, these results indicate that *Tsc1*$^{+/-}$ mutants are free of any apparent cerebral pathology.

Tsc1$^{+/-}$ mice show impaired cognitive function

Next, we tested whether heterozygous loss of *Tsc1* in the absence of notable cerebral pathology is sufficient to affect cognitive function. To that end we made use of the Morris water maze task, in which the animal has to learn the location of a hidden escape platform in a pool of water[20]. Mutants and wild-type mice both

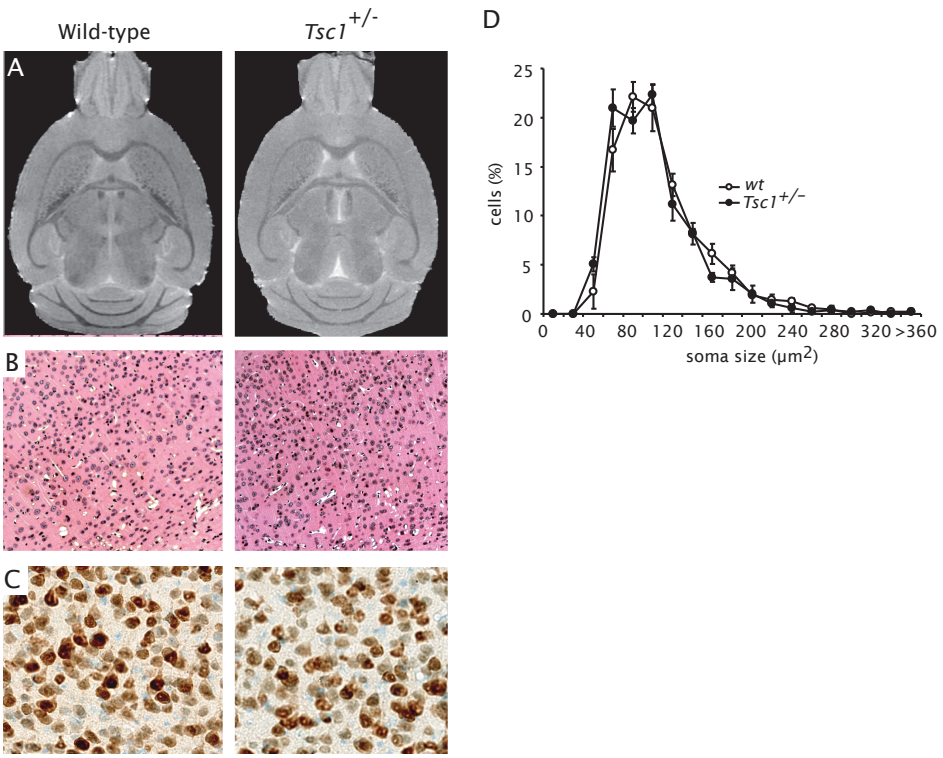

Figure 1. *Tsc1*$^{+/-}$ mice show no discernable brain pathology. (A-C): Absence of brain lesions in *Tsc1*$^{+/-}$ mice using high resolution MRI technology and serial sectioning following HE and NeuN staining. **(A)** Example of a 9.4 Tesla, T2-weighted image of a wild-type brain and a *Tsc1*$^{+/-}$brain. Scans of 5 *Tsc1*$^{+/-}$ and 2 wild-type mice of 6-8 months have been analyzed. **(B)** Example of Haematoxylin/Eosin stained sections of a wild-type brain and a *Tsc1*$^{+/-}$brain. Brains of 6 *Tsc1*$^{+/-}$ and 4 wild-type mice of 6-8 months were used for analysis. (**C**) Example of NeuN stained brain sections of mice of either genotype. **(D)** Soma size quantification of NeuN stained neurons revealed no population of abnormally large neurons in *Tsc1*$^{+/-}$ mice. For the analysis we determined the size of 800 cell soma's of 3 *Tsc1*$^{+/-}$ and 3 wild-type mice, aged 3-8 months. The number of cells belonging to a particular size category was calculated per mouse and these values were averaged to get a value per genotype. Error bars represent SEM.

showed a significant reduction of escape latency upon training with no significant difference between the two genotypes (genotype: $F_{1,16}$=0.9, p=0.4, Repeated Measures ANOVA, Figure 3A). In addition, swim speed between the two groups was not significantly different (WT: 18 cm/s, *Tsc1*$^{+/-}$: 21 cm/s; $F_{1,16}$=1.8, p=0.2 ANOVA). However, to test whether the mice had learned the actual precise location of the platform (hippocampus dependent spatial learning) or just learned to swim in circles at a fixed distance from the rim (hippocampus-independent procedural learning) the platform was removed at a probe trial given at day 7. Wild-type mice searched significantly more in the target quadrant as compared to the other quadrants ($t_{1,6}$=3.7, p<0.01, Paired t-test between target quadrant and average of the other quadrants; Figure 3C,E) and showed significantly more crossings of the platform

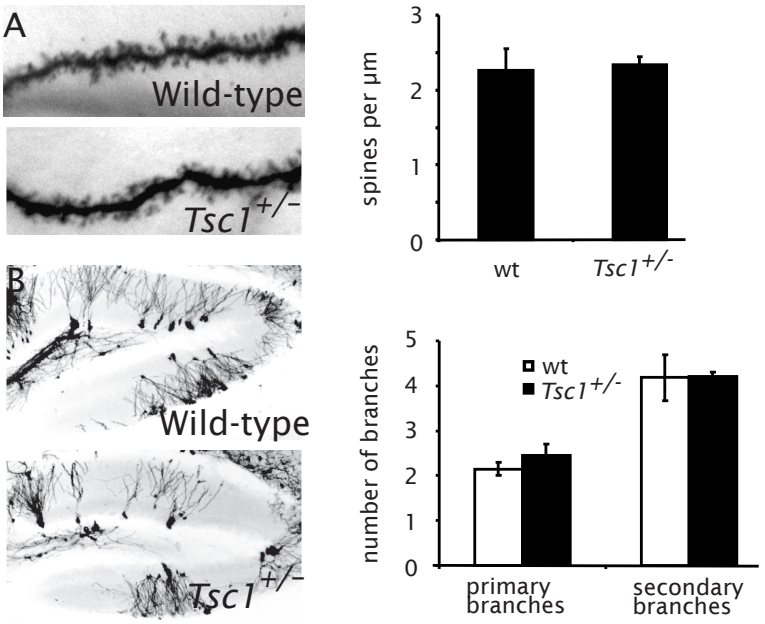

Figure 2. Golgi/Cox staining revealed no differences in spine density and branching of hippocampal granule cells. (A) Golgi/Cox stained neurons of hippocampal granule cells show no differences in the density of spines. **(B)** No changes in the number of primary and secondary dendrites of hippocampal granule cells. Sections from 2 *Tsc1*$^{+/-}$ and 2 wild-type mice age 4 to 7 months were used for quantification. We measured 10 cells of each mouse for dendritic branching, and we counted two stretches of 10µm per cell for the spine density measurement. These values were averaged to obtain a single number per mouse. Error bars represent SEM.

position compared to similar positions in the other quadrants ($t_{1,6}$=3.7, p<0.01, Paired t-test, Figure 3G). In contrast, *Tsc1*$^{+/-}$ mutants showed no preference for the target quadrant ($t_{1,8}$=1.5 p=0.3) nor did they show increased platform position crossings as compared to the other positions ($t_{1,8}$=1.5, p=0.3 Figure 3C,E,G). Two additional training days did not result in any improvement of the mutants, since they still showed no preference for the target quadrant ($t_{1,8}$=0.02, p=1; Figure 3D,F) nor for crossing the target position ($t_{1,8}$=0.2, p=0.8; Figure 3H). This deficit is not due to reduced eyesight, impaired motor performance or motivational problems as escape latency was identical if the mice were trained for three additional days using a visible platform test ($F_{1,16}$=0.3, p=0.6, repeated measures ANOVA; Figure 3B). Taken together these results strongly suggest that *Tsc1*$^{+/-}$ mutants have a spatial learning deficit, which is most likely due to hippocampal dysfunction[20].

Hippocampus-dependent learning can also be tested using contextual fear conditioning, in which the animal has to associate a certain context with an aversive event (foot shock)[21]. Context-dependent memory was tested 24 hours after training. *Tsc1*$^{+/-}$ mice showed significant less freezing than wild-type mice, again indicating a hippocampal learning deficit ($F_{1,18}$=6, p<0.02, ANOVA; Figure

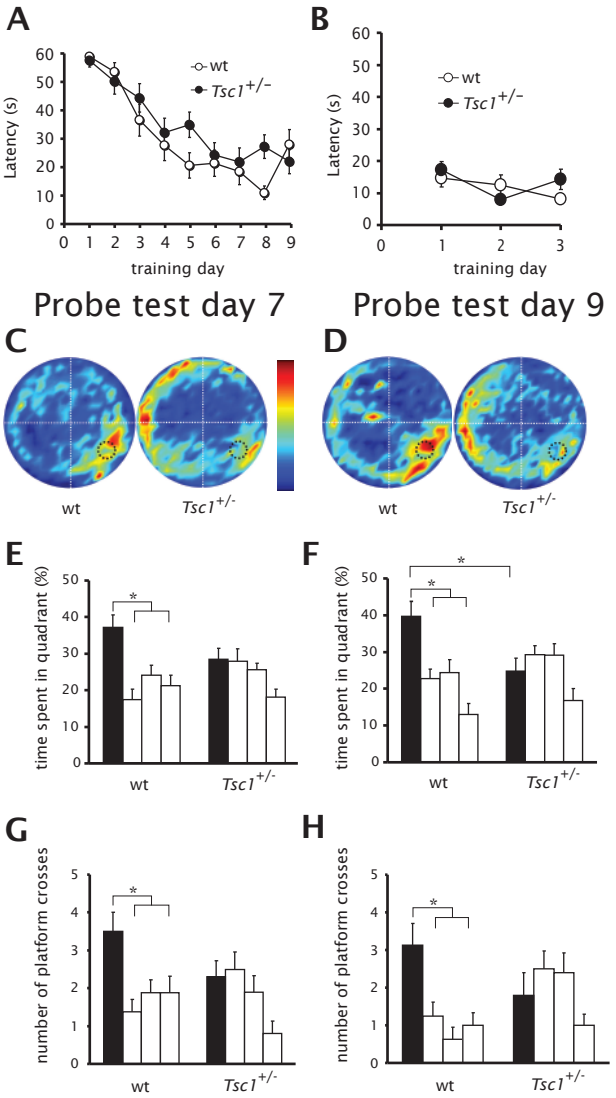

Figure 3. Spatial learning deficits in the Morris water maze. (A, B) Latency to reach the hidden platform **(A)** or visible **(B)** platform during training is not different between the two groups. For both tests, we used 8 wild-type mice and 10 *Tsc1*$^{+/-}$ mice, age 2-4 months. Error bars represent SEM. **(C-H)** Probe trials at day 7 **(C,E,G)** and day 9 **(D,F,H)** in the Morris water maze task. **C,D)** Visual representation of all swimming tracks of either group of mice combined from probe day 7 **(C)** and probe day 9 **(D).** The color indicates the time spent at a certain location (red is high, blue is low; see color bar). This clearly visualizes that wild-type mice search specifically for the platform. In contrast, *Tsc1*$^{+/-}$ mice search randomly, although preferentially at a certain distance from the wall. **(E,F)** Quantification of the time spent in each quadrant at a probe trial given at day 7 **(B)** and day 9 **(F)**. Black bar indicates the target quadrant; white bars indicate adjacent right, opposite and adjacent left quadrants respectively. **(G,H)** Quantification of the number of platform crosses at each of the four (virtual) platform positions at day 7 **(G)** and day 9 **(H)**. Black bar indicates the target position; white bars indicate adjacent right, opposite and adjacent left positions respectively.

4A). This was not due to a decreased anxiety of the mutants, since there were no differences between the two groups in an open field test (time spent in inner zone: $F_{1,15}$=0.15 p=0.7 ANOVA, data not shown). Moreover, the phenotype is not due to decreased sensitivity of the shock, as pre and post-cued fear conditioning, in which the shock is paired with a tone, was normal ($F_{1,22}$=0, p=1.0, repeated measures ANOVA, tested in an independent group; Figure 4B). Taken together these results suggest that these *Tsc1*$^{+/-}$ mutants have a hippocampal learning deficit[21].

Autism or PDD is encountered in half of the TSC patients. Autism is a complex phenotype which is not easily tested for in mice, however profound impairments in social interaction is one of it most striking features and standardized tests have been developed to test social interaction in mice. We tested social interaction using an interaction test in which a female test mouse was introduced to two unfamiliar female stimulus mice. Wild-type and mutant mice showed a normal habituation response to the repeatedly introduced stimulus mice; however *Tsc1*$^{+/-}$ mice interacted significantly less with the stimulus mice during all trials ($F_{1,19}$=5, p<0.05, repeated measures ANOVA; Figure 5A). Moreover, *Tsc1*$^{+/-}$ mutants showed significantly reduced nest building behavior ($F_{1,22}$=6, p<0.05, repeated measures ANOVA, Figure 5 B) which is another commonly used measure of social behavior in mice. After 10 days, only 27% of the *Tsc1*$^{+/-}$ mutants had built a completed nest, whereas nearly 70% of the wild-type mice accomplished this (X^2=4.2, p<0.05Chi Square test, Figure 5 C). Taken together, these results suggest that *Tsc1*$^{+/-}$ mice are also impaired in their social behavior.

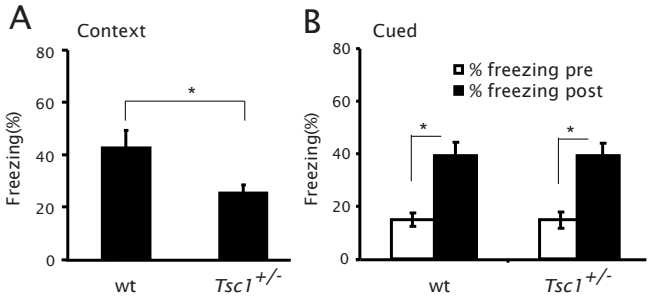

Figure 4. Impaired context conditioning in *Tsc1*$^{+/-}$ mice. Bars represent the amount of freezing behavior, which is used as an index for recollection of the context or tone. Error bars represent SEM. **(A,B)** *Tsc1*$^{+/-}$ mice show impaired contextual conditioning (**A**), but not impaired cued conditioning **(B).** Number of mice tested: WT=8, *Tsc1*$^{+/-}$=12 for contextual conditioning and wt=12 and *Tsc1*$^{+/-}$ =12 for cued conditioning. Age of mice was between 3 and 6 months.

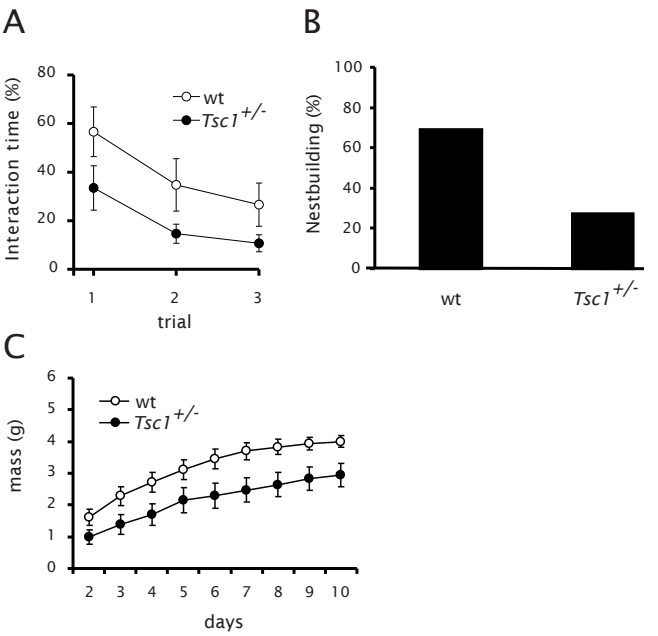

Figure 5. Reduced social functioning in *Tsc1*$^{+/-}$ mice. (A) Social interaction test. Female test mice were introduced to two unfamiliar female mice. The duration of the interaction of the test mouse with the stimulus mice was recorded. *Tsc1*$^{+/-}$ mice interacted significantly less with the stimulus mice during all trials. Number of mice tested: wt= 10, *Tsc1*$^{+/-}$ =11, average age 4.5 months. Error bars represent SEM. **(B)** Nest building behavior is reduced in *Tsc1*$^{+/-}$ mice. Cages with two mice of the same genotype and sex were arranged and nest weight was assessed as a measure of nest building behavior. Number of mice: 13 pairs of wild-type mice and 11 pairs of *Tsc1*$^{+/-}$ mutants, 3 months of age. **(C)** The percentage of animals with a fully build nest (= less than 3 grams of un-shredded nestlets) after 10 days.

DISCUSSION

Pathology

Here we report cognitive and social behavior deficits in *Tsc1*$^{+/-}$ mice in the absence of any apparent brain pathology and spontaneous seizures. The absence of brain pathology and seizures in this TSC mouse model is in line with literature, as virtually no brain lesions have been reported in *Tsc2*$^{+/-}$ mice and in *Tsc2*$^{+/-}$ (Eker) rats nor spontaneous seizures in *Tsc2*$^{+/-}$ (Eker) rats[15, 22]. However (brain) pathology has been demonstrated in *Tsc2*$^{-/-}$ embryos, *Tsc2*$^{-/-}$ neuro-epithelial cells and recently in *Tsc1* neuron specific knock-out mice [18, 23, 24]. Moreover spontaneous seizures are seen in ~10% of the last mentioned mutant, suggesting that both alleles have to be knocked out in the brain for the pathology and seizures to fully develop in TSC rodent models.

Social deficits

TSC patients have a fifty times enhanced risk for autism as compared to the general population[2]. Autism is a developmental disorder characterized by a triad of

symptoms: general inability to form reciprocal social interactions, severe impairment in verbal and non-verbal communication and a markedly restricted repertoire of activities and interests. Studying these features in mice is problematic, but an easy test is available to measure social interaction. This test showed significantly reduced social approach in *Tsc1*$^{+/-}$ mice. Moreover, *Tsc1*$^{+/-}$ mice showed reduced nest building behavior, which is another commonly used paradigm to measure social function in mice. Taken into account the limitations mentioned above we believe this is an important finding as this implicates a direct role for the *Tsc* genes in social behavior. Since only six percent of all autism cases can be attributed to a known genetic alteration[25], the one percent of autism cases that can be attributed to TSC[26] is a remarkable high number. Thus our finding may not only be important in understanding the mechanisms leading to autism in TSC, but may also contribute to the broader knowledge of the cell biological basis of this common developmental disorder.

Cognitive deficits

Our results show that the *Tsc1*$^{+/-}$ mice are impaired in two commonly used hippocampus dependent learning paradigms, the Morris water maze and contextual fear conditioning. Importantly, the non-hippocampus dependent versions of these paradigms (visible platform water maze and cued conditioning) were not affected, suggesting that hippocampal function in *Tsc1*$^{+/-}$ mice, is in particular impaired. Hippocampal function in humans is thought to be essential for declarative memory formation. However, it is likely however that the impairment is not restricted to the hippocampus but also affects (neo) cortical areas, which use similar molecular mechanisms. Thus, these learning deficits observed in *Tsc1*$^{+/-}$ mice may well parallel the decreased overall cognition as seen in many patients with TSC.

Notably, although hippocampal plasticity was impaired in *Tsc2*$^{+/-}$ (Eker) rats[14], these rats showed no deficits in hippocampus-dependent spatial learning tests as the Morris water maze and radial arm maze, and even *enhanced* memory in delayed matching-to-place versions of these two paradigms[15]. There are several possible explanations for this discrepancy: First, as argued by these authors[15], it is possible that the observed enhanced episodic memory in Eker rats is actually the result of reduced recall of previous target positions, thus causing less interference with learning the novel location. However, reduced memory of previously visited locations is not supported by the water-and radial arm maze training data of the Eker rats. Second, the conflicting results between *Tsc1*$^{+/-}$ mice and the *Tsc2*$^{+/-}$ Eker rats could indicate that the *Tsc1* and *Tsc2* genes have a distinct role in neuronal function and that only mutations in *Tsc1* result in impaired cognitive function. However, since the TSC1 and TSC2 proteins operate as a complex in the cell this does not seem to be very plausible. Moreover, cognitive disabilities are present in patients carrying either *TSC1* or *TSC2* mutations. Third, the distinct phenotypes could reflect the different functional consequences of the two mutations. As recently suggested[13], a wide range of mutations is observed in TSC patients and these could yield proteins with different abilities to stimulate or obstruct the various cell signaling pathways in which the TSC proteins are involved, causing high phenotypic variability. This could explain the markedly bi-modal distribution of IQ scores in TSC patients with half of

the patients having an IQ score in the normal range and one third of the patients showing severe mental retardation with an IQ below 20[1, 9]. Although this mechanism could indeed play a role in a subset of patients carrying missense mutations, the great majority of patients carry mutations resulting in premature stopcodons. Because of nonsense mediated mRNA decay mechanisms and increased turnover of truncated proteins this will mostly result in no protein product coming from the mutant allele. In our Tsc1$^{+/-}$ mutant, a transcript containing exon 1-5 is still present, which is predicted to result in a small truncated protein of approximately 200 amino acids[16], lacking all of the known functional domains of hamartin[13]. In contrast, in the Eker rat a 50 kilobase insertion leads to a truncated protein without the GAP domain, but leaving 2/3 of the N-terminus of the protein in tact[27, 28]. However, since the detection of truncated TSC proteins is challenging, it is uncertain to what extend these proteins are expressed and whether they could explain the phenotypic differences in these animal models or in humans. A final explanation for the phenotypic difference between our Tsc1$^{+/-}$ mutant and the Eker rat could be attributed to the effect of modifier genes. Since mice and rats are different species, the genetic background is obviously quite different, and this may have a different effect on the development of cognitive problems[29]. However, even within a single species, phenotypes as embryonic lethality, renal pathology and tumor expression patterns appear to be strongly dependent on genetic background[16, 22, 30]. Thus, it is conceivable that cognitive variability in rodents as well as in humans is at least to some extend caused by modifier genes.

Taken together, this study shows that cognitive dysfunction in a TSC animal model does not depend on overt brain pathology or epilepsy. These findings support a model in which haploinsufficiency for the *Tsc* genes leads to aberrations in neuronal functioning resulting in impaired learning and social behavior. However, further research is needed to study the molecular mechanisms linking *Tsc* haploinsufficiency to the cognitive deficits and to understanding the striking variability in the cognitive phenotypes observed in both TSC patients and in animal models. The *Tsc1*$^{+/-}$ mutant described in this study seems a valuable model to unravel these mechanisms and to test drugs designed to ameliorate the cognitive symptoms of the disease.

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4

DELETION OF THE *TSC1* GENE IN ADULT MICE IS SUFFICIENT TO CAUSE EPILEPSY

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In revision for Annals of Neurology: new experiments planned for 2012

ABSTRACT

Objective: Tuberous Sclerosis Complex (TSC) is characterized by brain lesions, autism spectrum disorders, mental retardation and epilepsy. Seizure development in TSC often appears to be related to specific lesions called cortical tubers. Tubers are complex neuropathological structures, characterized amongst others by *TSC1* null neurons. Recently, *Tsc1* null neurons have been shown to exhibit increased excitatory currents, indicating that these cells could potentially initiate seizure development. Here, we took a reductionist approach and tested whether global and acute deletion of the *Tsc1* gene in adult mice is sufficient to trigger seizure onset in the absence of any developmental neuropathology.

Methods: We studied seizure characteristics and interictal patterns using EEG recordings. Molecular and cellular correlates of epilepsy were investigated using Western blot analysis, immunohistochemistry and hippocampal field recordings.

Results: We observed that within days of the deletion of the *Tsc1* gene, mice developed seizures and abnormal interictal EEG patterns. In addition, an increased number of target of rapamycin complex 1 (TORC1) activated neurons was observed, with occasional giant-cell like properties. TORC1 and Extracellular signal-regulated kinase (ERK) activity, which are both known to be involved in the protein-synthesis-dependent phase of synaptic potentiation, were increased in these mice. In accordance with these biochemical data we observed that deletion of *Tsc1* resulted in increased protein-synthesis-dependent synaptic potentiation.

Interpretation: Taken together, these data indicate that intact levels of TSC1 are required to prevent unrestrained synaptic potentiation and epilepsy. Our data provides novel evidence that loss of TSC can directly contribute to seizure development in TSC patients.

INTRODUCTION

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder characterized by the development of benign lesions in different organs and severe neurological and psychiatric symptoms including epilepsy, mental retardation and autism spectrum disorders [1]. TSC is caused by germ line inactivating mutations in either the *TSC1* or *TSC2* gene [1]. Hamartin (TSC1) and Tuberin (TSC2), the *TSC1* and *TSC2* gene products, form a protein complex (TSC1-TSC2) that functions as a GTPase-activating protein complex (GAP) for the small G-protein Ras homolog enriched in brain (RHEB), which is an essential activator of the target of rapamycin complex 1 (TORC1) [2]. In mitotic cells this multiprotein complex is a major regulator of cell growth and proliferation via phosphorylation of its downstream targets including p70 S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins 1 and 2 (4E-BP1 and 4E-BP2) [2]. As a consequence, TORC1 signaling is aberrantly activated in TSC1 and TSC2 insufficient cells, promoting cell growth [2]. In the majority of TSC-associated lesions, *TSC1* or *TSC2* loss of heterozygosity (LOH) has been demonstrated, indicating that somatic inactivation of the wild-type *TSC1* or *TSC2* allele is the most likely trigger for tumor formation [3-5].

Up to 80 to 90% of individuals with TSC will develop epilepsy during their lifetime[6]. In most patients the onset of epilepsy occurs in childhood or even infancy and can involve a wide range of seizure types, including infantile spasms, tonic, clonic, tonic-clonic, atonic, myoclonic, atypical absence, partial, and complex partial seizures[6]. Epileptogenesis in TSC seems to be related to the presence of cortical tubers and other specific neuropathological features of the disorder [6]. Cortical tubers are abnormalities in cortical architecture formed during early brain development and found in varying numbers in more than 80% of TSC patients [6]. They consist of dysplastic neurons and giant cells, as well as glial components [6]. However, the precise mechanisms underlying epilepsy in TSC are still not fully understood. For instance, up to 10% of individuals with TSC suffering from intractable epilepsy have normal brain MRIs, with no evidence of cortical tubers, while other patients with a high tuber load do not develop epilepsy [6, 7], indicating that there may be other processes contributing to epilepsy development in TSC. In addition, even when a causative tuber can be identified, it is not clear which components of these complex structures trigger the epilepsy. For instance, epilepsy could be caused by aberrations in glutamatergic and GABA-receptor subunit expression, which have been observed in tuber samples, or by astrogliosis, which is invariably found to accompany tubers [1]. However, some of the resected tubers are found to be electrically silent, suggesting that the abnormal activity in the surrounding tissue rather than in the tuber itself leads to epileptogenesis [8].

Heterozygous *Tsc1* and *Tsc2* mouse models do not show any obvious pathology, nor do these mice develop epilepsy [9, 10]. Since recent pathological[11] and experimental studies[12-14] provide evidence that like somatic TSC lesions, brain lesions probably result from biallelic *TSC1/2* inactivation, brain specific *Tsc1/2* homozygous knock-out mice are attractive models to investigate the mechanism underlying the epilepsy. Indeed, such mutants were shown to develop a global tuber-like neuropathology and epilepsy [15-17]. Besides this neuropathology, other pathological

features have also been described which could contribute to the epileptogenesis. For example, *Tsc1* null astrocytes in astrocyte-specific *Tsc1* knock-out mice are impaired in extracellular K^+ and glutamate uptake, while increased excitability has been measured in the neocortex of neuron-specific *Tsc1* homozygous knock-out mice [18-20]. Hence, the precise cause of epileptogenesis in mutants in which the TSC gene is deleted before or during brain development remains unclear. In a recent study this issue was circumvented by deleting the *Tsc1* gene in a subset of adult hippocampal CA1 neurons, *in vivo.* This resulted in increased excitatory currents in the absence of any structural changes, indicating that the TSC1-TSC2 protein complex may be important *in vivo* for maintaining proper synaptic strength and preventing epilepsy [21]. Nevertheless, these mice did not develop spontaneous seizures, which could be due to the targeted region being too limited to see a clear effect of *Tsc1* deletion.

Here, we hypothesized that increased excitatory synaptic strength in *TSC1/2* null cells may be sufficient to trigger epilepsy development without developmental brain pathology being a prerequisite. To test this hypothesis, we took a reductionist approach and globally deleted the *Tsc1* gene in adult mice, thereby preserving normal brain development.

METHODS

Mice

Tsc1$^{+/f}$ mice (*Tsc1*^{tm1Djk}), carrying loxP sequences flanking exons 17 and 18 obtained from the Jackson Laboratory (Bar Harbor, Maine) were crossed with mice expressing the *Cag-cre* transgene [22] to obtain a germ line heterozygous *Tsc1* knock-out line (*Tsc1*$^{+/-}$). Subsequently, *Tsc1*$^{+/-}$ mice were bred with *Cre-ER*^{TM} transgenic mice[23] and the resulting *Tsc1*$^{+/-}$ *Cre-ER*^{TM}*+* mice were used for the F2 cross with *Tsc1*$^{f/f}$ mice to yield the experimental genotypes *Tsc1*$^{f/-}$ *Cre-ER*^{TM}*+* and *Tsc1*$^{f/+}$*Cre-ER*^{TM}*+*, in a mixed-strain background (C57BL/6J, BALB/cJ and 129/SvJae). Throughout the study the two experimental genotypes were treated in parallel with either vehicle (sunflower oil) or tamoxifen dissolved in sunflower oil (10 mg/g; Sigma-Aldrich, St. Louis, MO). For EEG recordings, Western blot analysis, immunohistochemistry and field recordings, independent groups of mice were used (numbers specified in the legends). Mice were treated according to standard procedures [9] and all animal experiments were approved by the Local Animal Experimentation Ethical Committee, in accordance with Institutional Animal Care and Use Committee guidelines.

Western blot analysis

Hippocampi and cortices were isolated by dissection and homogenized in lysis buffer (10mM Tris HCl pH 6.8, 2.5% SDS) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The concentration of the lysates was adjusted to 1 mg/ml and 20 µg was used for Western blot analysis. Bands were visualized using Enhanced Chemo Luminescence (Pierce; Thermo Fisher Scientific, Waltham, MA) and quantification was done using 'ImageJ64' software. For antibodies used see supplementary methods.

Immunohistochemistry

Brains of paraformaldehyde perfused mice were dissected and embedded in paraffin according to standard procedures. Immunostainings were performed on 6 μm slices using an avidin biotin-immunoperoxidase-alkaline-phosphatase complex method (Zymed Laboratories, San Franscisco, CA). For double label immunofluorescent microscopy, a combination of mouse and rabbit antibodies were used, followed by Cy2-labelled goat anti-mouse and Cy3-labelled goat anti-rabbit secondary antibodies (DAKO, Carpinteria, CA, USA). Cells were counterstained with Hoechst's and stained sections were studied using a Zeiss LSM700 confocal microscope. For antibodies used see supplementary methods.

Hippocampal field recordings

Extracellular recordings of fEPSPs in CA1 *striatum radiatum* were performed as described previously[24]. Stimulation during the LTP experiment was performed at 1/3 of the maximum response. During the entire LTP experiment test stimulations were given once a minute; 6 responses were averaged to obtain a single data point for visual representation.

Experimental details on EEG recordings and PCR analysis are included in the Supplementary Methods.

RESULTS

Adult loss of the *Tsc1* gene is sufficient to induce seizures

To investigate whether adult LOH of the *Tsc1* gene is sufficient to induce epilepsy in adult mice, we created an inducible *Tsc1* knock-out line by crossing *Cre-ER*^{TM} transgenic mice[23] with *Tsc1* mutant mice to yield two experimental genotypes: *Tsc1*$^{f/-}$ *Cre-ER*^{TM} *+* and *Tsc1*$^{f/+}$*Cre-ER*^{TM}*+* (hereafter named: *f/-* and *f/+*), Injection of the *f/-* and *f/+* mice with tamoxifen induces translocation of the Cre-ER^{TM} protein to the nuclei to drive recombination of the floxed *Tsc1* allele in all cells, resulting in *Tsc1* null and +/− mice respectively. Prior to tamoxifen injection, *f/−* and *f/+* mice have normal brain morphology and normal levels of TORC1 activation [9, 15] and data not shown).

At adult age (between 2-4 months) two tamoxifen injections at subsequent days were given to induce *Tsc1* deletion. These two tamoxifen injections were found to be sufficient to drive complete genomic recombination in *f/-* mice when measured three days after the second injection (Fig 1A). Similar to germ line homozygous *Tsc1* deletion[25-27], total loss of the *Tsc1* gene in adulthood is also lethal, and *f/-* mice had a median survival of only 7 days after the second tamoxifen injection.

Interestingly, starting the first day after the second tamoxifen injection, *f/-* mice were regularly observed to suffer from spontaneous or handling-induced seizures characterized by repetitive forelimb clonus, sometimes followed by a period of loss of upright posture and generalized repetitive clonus of all limbs. These seizures resembled those found in other TSC mouse models [15, 17, 28]. To further characterize the seizures, EEG measurements were performed in 6 *f/+* and 5 *f/-* mice injected first with vehicle only and then with tamoxifen (see Fig 1B for the procedure). EEG recordings were made during the dark phase of the day/night cycle, using a mobile

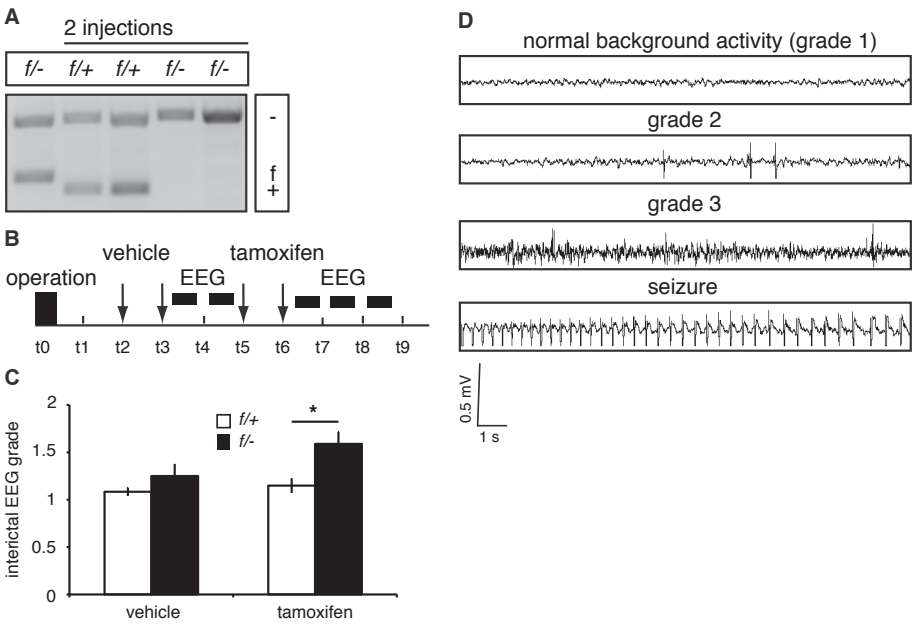

Fig 1. Adult deletion of the *Tsc1* gene induces seizures and interictal abnormalities. (A) Two injections of tamoxifen lead to recombination of the floxed allele in cortical tissue of *Tsc1*$^{f/+}$*Cre-ER*^{TM}*+* (*f/+*) and *Tsc1*$^{f/-}$ *Cre-ER*^{TM}*+* (*f/-*) mice. The wild-type allele is depicted by +, the knock-out allele by – and the floxed allele by f. **(B)** Schematic of the time line of treatments and electroencephalopgraphic (EEG) measurements. (**C)** Average interictal spike grade scored under each treatment (see Supplementary Methods for further details). **(D)** Examples of interictal (grade 1, 2, and 3) and ictal EEG activity observed in *f/+* mice (upper trace) and *f/-* mice (lower traces). EEG patterns corresponding to grade 4 of the grading scale as described in [51, 52] were not identified in our data set. N=6 for *f/+* mice and 5 for *f/-* mice. Error bars represent the standard error of the mean (SEM).

device which allows the animals to move freely. As expected, none of the *f/+* mice showed seizures upon vehicle or tamoxifen treatment. In contrast, all of the *f/-* mice developed seizures upon tamoxifen injection, with an average of 3 seizures per recording period of 16 hours (number of seizures in *f/-* mice: 3 ± 1.5).

In TSC patients with epilepsy and in animal models of TSC, interictal abnormalities including interictal spikes and sharp spike-waveforms are often observed [6]. To quantify possible interictal abnormalities associated with the loss of the *Tsc1* gene we made use of a grading system, as described earlier [29]. After vehicle treatment we observed that the average grade of EEG activity was similar in both genotypes (mostly grade 1 and occasionally grade 2), while upon tamoxifen injections *f/-* EEG patterns showed a significantly higher average grade than *f/+* EEG patterns (effect of genotype; $F_{1,9}$=6.75; p<0.05 two-way repeated-measures ANOVA: Bonferroni post hoc test: tamoxifen treatment *f/+* vs *f/-*: $t_{1,9}$=3.28; p<0.05 see Figure 1C, D). Taken together, these data show that adult deletion of the *Tsc1* gene is sufficient to trigger epileptogenesis and interical abnormalities resembling those seen in TSC patients and other TSC mouse models.

Adult deletion of the *Tsc1* gene increases TORC1 and ERK activation

Next, we investigated the effect of *Tsc1* deletion on TORC1 activity. Genomic recombination of the intact *Tsc1* genomic locus severely reduced TSC1 protein levels in *f/-* tissue (Fig 2A, B). Similar to what is found in TSC tumor tissue [30] and TSC mouse models [15, 28], S6 S235/236 phosphorylation (pS6) was markedly increased in the cortex and hippocampus of *f/-* mice sacrificed 3 days after the last injection, a time point when nearly all *f/-* mice have developed seizures (cortex: $t_{1,12}$=9.66; p<0.01, hippocampus: $t_{1,12}$=8.59; p<0.01 unpaired t-test; see Fig 2A, B). Although 4EBP1 is an established target of TORC1 *in vitro* [31], levels of phosphorylated 4EBP1 have not been regularly explored in the various TSC mouse models described to date. Somewhat surprisingly, the increase in 4EBP1 phosphorylation at residues T37/46 was found to be modest in *f/-* mice and only reached statistical significance in the cortex (cortex: $t_{1,18}$=3.21; p<0.01, hippocampus: $t_{1,19}$=1.11; p=0.28 unpaired t-test; see Fig 2A, B).

It is well established that *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ cells show decreased activity of AKT and other upstream activators of TORC1, due to negative feedback loops in the

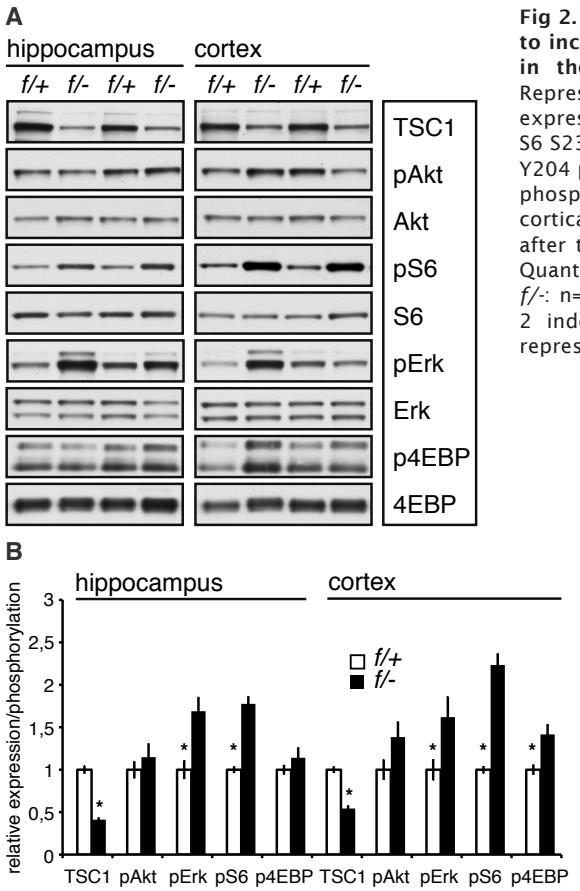

Fig 2. Adult loss of the *Tsc1* gene leads to increased TORC1 and ERK activation in the hippocampus and cortex. (A) Representative immunoblots of TSC1 expression, AKT S473 phosphorylation, S6 S235/236 phosphorylation, ERK T202/Y204 phosphorylation and 4E-BP1 T37/46 phosphorylation in hippocampal and cortical lysates of mice sacrified 3 days after the second tamoxifen injection. **(B)** Quantification of immunoblots. *f/+*: n=4; *f/-*: n=3; all samples were run in at least 2 independent experiments. Error bars represent the SEM.

TORC1 pathway [32, 33]. However, impairments in AKT phosphorylation were not found in tissues of *f/-* mice, indicating that these negative feedback loops are not active in the brain tissue of our model (cortex: $t_{1,11}$=1.80; p=0.10, hippocampus: $t_{1,12}$=0.81; p=0.43 unpaired t-test; see Fig 2A, B). Surprisingly, in addition to increased pS6 levels, we also found increased levels of T202/Y204 phosphorylated ERK in *f/-* mice (cortex: $t_{1,19}$=2.44; p<0.05, hippocampus: $t_{1,14}$=4.21; p<0.01 unpaired t-test; see Fig 2A, B). Although ERK hyperactivation is a feature of some TSC tumor types [34] and crosstalk between ERK and TORC1 signaling is well appreciated [35], aberrations in ERK activation have not described earlier in TSC1/2 deficient murine cells [25, 36, 37]. In conclusion, these data demonstrate that acute deletion of the *Tsc1* gene increases TORC1 and ERK signaling in the hippocampus and cortex.

Adult loss of the *Tsc1* gene increases the number of TORC1 activated neurons and induces the development of enlarged giant cell-like neurons

To estimate TORC1 activation in brain slices from *f/-* and *f/+* mice, we performed immunohistochemistry using an antibody specific for S235/236 phosphorylated S6 (pS6). Three days after the last tamoxifen injection the *f/-* mice had a greater proportion of TORC1 activated cells in both the hippocampus and cortex (Fig 3A). Interestingly, in the cortex of *f/-* mice occasional enlarged cells were seen, reminiscent of the giant cells that have been observed in human tubers and in a global pattern in brain-specific *Tsc1* homozygous knock-out mice [6, 15-17]. We investigated the cell lineage of these TORC1 activated cells by performing double labeling for pS6 in combination with either an antibody for the neuronal marker Neuronal nucleus (NeuN), or for the astrocyte marker S100. Remarkably, we observed that virtually all pS6+ cells, including the enlarged cells, were neurons, indicating that although the *Tsc1* deletion is global, astrocytes do not seem to be affected in terms of TORC1 activation (Fig 3B). To determine whether a subset of the pS6+/NeuN+ neurons were interneurons we performed double labelings for glutamic acid decarboxylase 67 (GAD67) and pS6. As shown in Figure 3B, we observed cells positive for both GAD67 and pS6, indicating that TORC1 is activated in some interneurons upon *Tsc1* deletion.

TSC lesions and brains from neuron-specific *Tsc1* homozygous knock-out mice are characterized by hypomyelination [15]. In our model, in the limited time span of 3 days following *Tsc1* deletion myelination was not affected, indicating that myelination impairments may not be causally related to seizure development (Fig 3C). Subsequently, we tested for the presence of astrogliosis, a hallmark of human TSC tubers and astrocyte-specific *Tsc1/2* homozygous knock-out mice [28]. We did not find evidence for an increase in astriogliosis in *f/-* mice (Fig 3D), most likely due to the limited time span of 3 days following injection. Nevertheless, these data suggest that astrogliosis does not necessarily precede seizure development in our model. Taken together, these data show that shortly after *Tsc1* gene deletion, the number of TORC1 activated neurons increases and some enlarged giant-cell like neurons are encountered, in the absence of myelination deficits and astrogliosis.

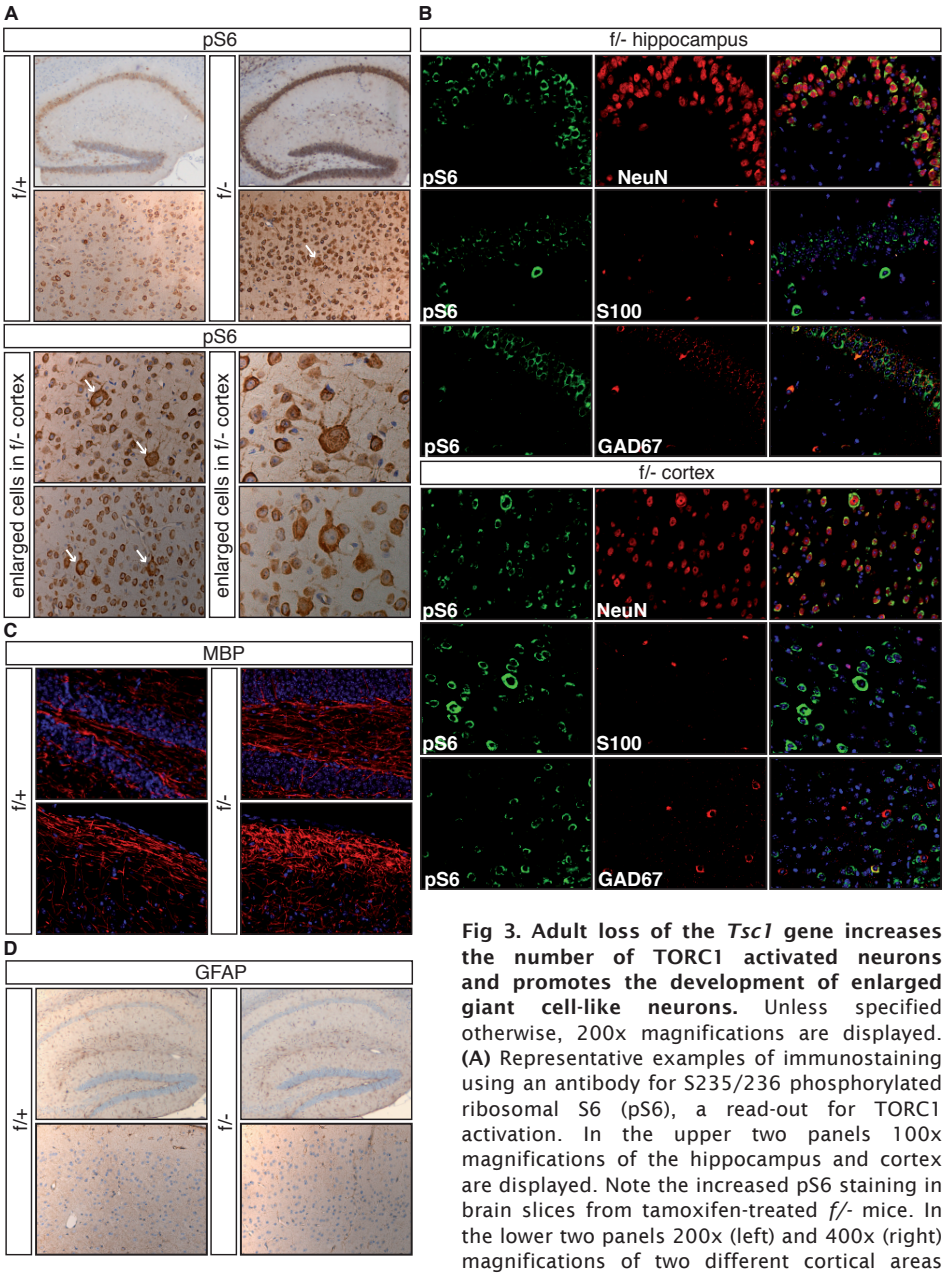

Fig 3. Adult loss of the *Tsc1* gene increases the number of TORC1 activated neurons and promotes the development of enlarged giant cell-like neurons. Unless specified otherwise, 200x magnifications are displayed. **(A)** Representative examples of immunostaining using an antibody for S235/236 phosphorylated ribosomal S6 (pS6), a read-out for TORC1 activation. In the upper two panels 100x magnifications of the hippocampus and cortex are displayed. Note the increased pS6 staining in brain slices from tamoxifen-treated *f/-* mice. In the lower two panels 200x (left) and 400x (right) magnifications of two different cortical areas containing enlarged cells are shown. Enlarged cells are indicated with the arrows. **(B)** Representative examples of double label stainings using an antibody for pS6, to indicate TORC1 activated cells, in combination with either an antibody for NeuN, a marker for neurons, for S100, a marker for glial cells, or for glutamic acid decarboxylase 67 (GAD67), a marker for interneurons. Note that virtually all pS6 positive cells are neurons, of which some are interneurons. **(C)** Representative examples of immunostaining using an antibody for myelin basic protein (MBP), the major component of the myelin sheath. Note that myelination is not affected in either the hippocampus (upper panel) or the cortex (lower panel) in *f/-* mice after tamoxifen treatment. **(D)** Representative examples of immunostaining using an antibody for glial fibrillary protein (GFAP), a marker for reactive astrogliosis (100x magnification). Note that this staining fails to show enhanced astrogliosis in *f/-* mice. Slices of 3 mice of each genotype, sacrificed 3 days after the second tamoxifen injection, were stained.

Adult deletion of the *Tsc1* gene reduces the threshold for late-phase long-term potentiation (L-LTP)

The effect of adult *Tsc1* deletion on hippocampal physiology was investigated by using field potential recordings. Although sparse deletion of the *Tsc1* gene in the adult hippocampus caused an increase in excitatory AMPA and NMDA currents [21], similar increases in basal transmission were not apparent in our tamoxifen injected *f/-* mice, as assessed by measuring the presynaptic fiber volley and field excitatory post synaptic potential (fEPSP) (fiber volley: effect of genotype: $F_{1,32}$=1.95; p=0.17. fEPSP: effect of genotype: $F_{1,32}$=0.03; p=0.86 repeated measures ANOVA; see Fig 4A, B). However, like *Tsc2*$^{+/-}$ mice [10], *f/-* neurons appeared to have a lower threshold for inducing the late-phase of long term potentiation (L-LTP), a long lasting form of LTP that is protein-synthesis dependent. A sub-threshold protocol for inducing L-LTP was already effective in slices of *f/-* mice, while it only resulted in early-phase LTP

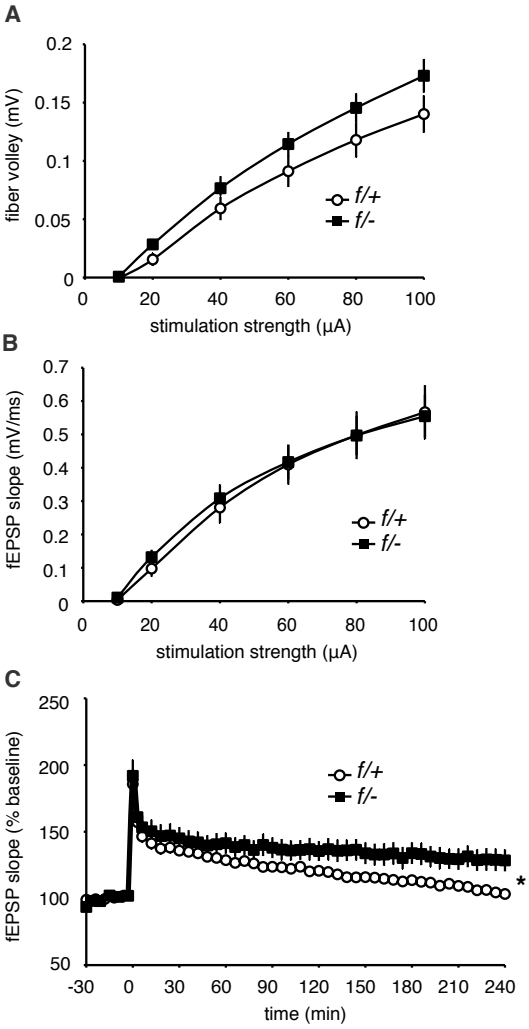

Fig 4. Adult deletion of the *Tsc1* gene decreases the threshold for late phase long-term potentiation (L-LTP) at hippocampal CA3-CA1 synapses. (A-B) Basal synaptic transmission at hippocampal CA3-CA1 synapses is not affected in tamoxifen-treated *f/-* mice. Plots show the presynaptic fiber volley as a function of stimulation strength **(A)** and the field excitatory post synaptic potential (fEPSP) as a function of the stimulation strength **(B)**. **(C)** A single train 100 Hz protocol induces E-LTP at hippocampal CA3-CA1 synapses in tamoxifen-treated *f/+* mice, but induces L-LTP in *f/-* mice. Slices from 5 *f/+* mice (synaptic transmission: 14 slices; LTP: 9 slices) and 6 *f/-* mice (synaptic transmission: 20 slices; LTP: 12 slices) sacrificed 3 days after the second tamoxifen injection are included for analysis. Error bars represent the SEM.

in *f/+* mice (average fEPSP slope in the last 10 minutes: $t_{1,19}$=2.34; p<0.05 unpaired t-test; see Fig 4C). Thus, while basal transmission remains intact upon adult loss of the *Tsc1* gene, *f/-* neurons show a reduced threshold for inducing L-LTP.

DISCUSSION

In this study we show that adult, global induction of *Tsc1* LOH is sufficient to induce seizures and interictal abnormalities. Seizures developed in the context of increased TORC1 and ERK activation in both hippocampus and cortex. In addition, a greater proportion of TORC1 activated neurons, with occasional enlarged neurons were seen in mutant mice, while we could not detect any apparent myelination- or astrocyte pathology. Mutant synapses showed a decreased threshold for the induction of protein synthesis-dependent L-LTP. These data suggest that TORC1- and ERK-dependent increases in synaptic potentiation in *TSC1/2* null cells may contribute to seizure development associated with TSC.

There are some differences in the nature of the seizures and EEG abnormalities observed in our mice as compared to other mouse models. In neuron-specific *Tsc1* mutant mice the seizures were severe, invariably leading to death, while our mice could develop several seizures before experiencing a lethal one [15]. In addition, burst suppression interictal patterns, as seen in astrocyte-specific *Tsc1* mutant mice, were absent in the EEG recordings from our mice [28].

In accordance with findings regarding human TSC tumor tissue [34] and several TSC mouse models [15, 28], we found enhanced TORC1 activation in tissues from our mutant mice. Interestingly, TORC1 signaling to S6K and S6 was the most pronounced, with relatively limited activation of TORC1 signaling to 4EBP1. Because these two targets of TORC1 were not routinely assessed in the other mouse models of TSC described to date, it is hard to say whether this is a general feature of TSC brain tissue. Surprisingly, in addition to TORC1, ERK activation was also enhanced in our model. Although *Tsc1/2* null murine cells were previously reported to have normal phosphorylated ERK levels [25, 36, 37], ERK activation upon forskolin treatment was shown to be higher in the Eker rat model of TSC [38] and some TSC tumor types exhibit ERK hyperactivation [34]. Paradoxically, early studies identified RHEB as an inhibitor of B-RAF [39], so lower ERK activation is to be expected in TSC1/2 deficient cells. More research is needed to find out whether hyperactive ERK is a common finding in TSC1/2 deficient brain tissue. Whether the observed abnormalities in ERK signaling are related to seizure development remains to be tested. Although individuals suffering from syndromes characterized by increased ERK signaling like Neurofibromatosis type 1, Costello Syndrome and Noonan Syndrome, may encounter epilepsy, the incidence is clearly lower in these syndromes than in TSC [40].

We observed the development of occasional enlarged neurons in the cortex upon *Tsc1* gene deletion. Similar enlarged cells are features of human tubers and are also seen in several TSC mouse models. [13, 15, 17, 21]. Apparently, soma size increase is an early and acute event. However, we did not observe apparent astrocyte pathology: TORC1 activated astrocytes were rarely encountered and astrogliosis was not noted in either hippocampal or cortical sections. Our data does not exclude that astrocyte pathology develops upon adult *Tsc1/2* deletion, since we only considered

a three-day period following gene deletion. However, it indicates that astrocyte pathology may not be a prerequisite for epileptogenesis in TSC. In line with our observations, astrogliosis did not develop in epileptic neuronal-specific mutant mice [15], nor in a recent tuber model [13], which exhibited a decreased threshold for seizure induction, raising the possibility that astriogliosis may develop locally, secondary to an extended period of seizure activity.

Bateup *et al.* have observed increases in excitatory AMPA and NMDA currents upon acute sparse deletion of the *Tsc1* gene in the adult hippocampus [21]. These differences could arise from the fact that we performed field recordings instead of measuring isolated currents, and therefore any effects on excitatory currents may have been masked by concomitant increases in inhibitory currents. Alternatively, and more likely, the limited time span following gene deletion in our study (3 days vs 10-14 days in Bateup *et al.*) may have prevented the observation of changes in transmission. Indeed, we could replicate the decreased threshold for L-LTP that was previously reported for *Tsc2*$^{+/-}$ mice [10], and it is conceivable that this would eventually result in increased synaptic transmission. Interestingly, increased synaptic plasticity following brain trauma is linked to epileptogenesis in adult-onset epilepsy [41].

Significant variability exists in epilepsy characteristics such as type, onset, frequency and recurrence rate among TSC patients [6, 7]. Genotype-phenotype studies indicate that *TSC1* mutations and some *TSC2* missense mutations are associated with less severe TSC disease, showing that the nature of the mutation can explain part of the phenotypic variability [42-48]. However, within families, TSC individuals carrying identical mutations can differ tremendously in seizure phenotype [6, 7]. Probably, modifier genes influence disease severity, as has been shown for kidney angiomyolipomas [49]. In addition, some of the variability could be due to the stochastic nature of second-hit events (LOH) occurring during brain development and maturation. These events are associated with the different lesion types found in the brains of individuals with TSC, like tubers and subependymal giant cell astrocytomas [4, 5, 11, 49, 50]. We hypothesize that LOH events during late brain development could also result in sub-radiological aberrations in brain morphology. We speculate that increased synaptic potentiation in these *TSC1* or *TSC2* LOH cells, either belonging to a tuber or having a more diffuse localization, may contribute to epilepsy in TSC, especially in those individuals for whom no causative epileptogenic tuber can be identified.

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SUPPLEMENTARY METHODS

PCR analysis

Mice were genotyped when they were around 7 days of age, but the experimenter remained blind to the genotype during data collection and the initial analysis. For genotyping the following primers were used: Forward (F): 5'-GTCACGACCGTAGGAGAAGC-3', F: 5'-AGGAGGCCTCTTCTGCTACC-3' and Reverse: 5'-GAATCAACCCCACAGAGCAT-3', yielding a 190 bp product from the wild-type allele, a 230 bp product from the floxed allele and a 400 bp product from the knock-out allele.

EEG recordings

Mice were anesthetized (O_2, Isoflurane (~1.5%) and Buprophin (0.01 mg/kg, subcutaneously) and an incision was made in the skin and fascia overlying the skull. After exposing the skull, six holes were drilled (0.5 mm diameter). The first two holes were placed respectively +1 mm anterior and posterior to Bregma, and 1 mm lateral from the midline, above the motor cortex. The second pair of holes were drilled respectively -1 mm anterior and posterior to Bregma and 4 mm lateral from the midline, above the sensory cortex. The final two holes were drilled in the interparietal bone above the cerebellum for the reference and ground screws. In the holes, electrodes in the form of RVS-screws, soldered to pins, were lowered 1-1.5 mm below the skull to touch the dura mater. The pins attached to the screws were inserted in a connector and the assembly was anchored to the skull using dental cement (Kemdent, Swindon, UK). Recordings with a duration of 16 hours started the fourth night after the surgery. At the start of the dark phase of every subsequent day mice were gently restrained and connected with a wireless EEG recorder data logger (NewBehavior AG, Zurich, Switzerland) and were synchronically videotaped by the PhenoTyper® (EthoVision 3.0, Noldus Information Technology, Wageningen, The Netherlands).

Seizures were scored during the entire 16 hour interval by an observer blinded to genotype and were easily identified by repetitive spike discharge followed by a progressive evolution in spike amplitude that usually culminated in a bursting pattern and postictal suppression, very similar to those described in [22]. Seizure numbers observed under the same treatment (2 or 3 nights) were averaged to obtain an average number per mouse.

Interictal spike analysis was performed as described earlier[22]. In short, for each 16 hour interval 6 time samples were taken, separated by 3 hour intervals. Every time sample was scored for its interictal grade. Subsequently, the 6 grades scored per 16 hour interval were averaged to obtain a mean score per mouse, and a

mean per treatment condition was calculated for each mouse and, finally, for each genotype.

Originally, the scoring of the interictal background activity was based on a four-grade scale: 1—normal background activity (6–8 Hz sinusoidal theta rhythm), no epileptiform spikes; 2—mostly normal background activity, few epileptiform spikes; 3—mostly abnormal background activity, many spikes; 4—burst-suppression pattern [22]. However, since we did not observe grade 4 activity in our sample we only made use of the first 3 grades.

Initially, 7 *f/+* and 6 *f/-* mice were operated. Although all these mice were seizure-free before surgery, one mouse of each genotype developed epilepsy shortly after surgery. The development of epilepsy in these mice could be caused by the brain surgery or, in the case of the *f/-* mutant, by leakiness of the *Cag-Esr-cre* system. Since we aimed to investigate the effect of acute, total loss of *Tsc1* on seizure development we excluded both mice and continued with 6 *f/+* and 5 *f/-* mice.

Antibodies

For Western blot analysis the following primary antibodies were used: TSC1 (#4906), pAKT^{S473} (#4060), AKT (#2920), pS6$^{S235/236}$ (#2211), S6 (#2217), p4EBP1$^{T37/46}$ (#2855), 4EBP1 (#9644), pERK$^{T202/Y204}$ (#9101) and ERK (#9102) from Cell Signaling Technology (CST, Danver, MA). Primary antibodies used for immunohistochemistry: anti-pS6$^{S235/236}$ (#2211; CST), anti-NeuN (MAB377; Millipore, Billerica, MA), anti-S100 (S2532; Sigma-Aldrich, St. Louis, MO), anti-MBP (MAB386; Millipore) and anti-GFAP (G6171, Sigma-Aldrich).

5

RHEB IS ESSENTIAL FOR MURINE DEVELOPMENT

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ABSTRACT

Ras homolog enriched in brain (Rheb) couples growth factor signaling to activation of the target of rapamycin complex 1 (TORC1). To study its role in mammals, we generated a *Rheb* knock-out mouse. In contrast to *mTOR* or *regulatory-associated protein of mTOR* (*Raptor)* mutants, inner cell mass of *Rheb*$^{-/-}$ embryos differentiated normally. Nevertheless, *Rheb*$^{-/-}$ embryos died around mid-gestation most likely due to impaired development of the cardiovascular system. *Rheb*$^{-/-}$ embryonic fibroblasts showed decreased TORC1 activity, were smaller and showed impaired proliferation. *Rheb* heterozygosity extended the lifespan of *tuberous sclerosis complex 1 (Tsc1)*$^{-/-}$ embryos, indicating that there is a genetic interaction between the *Tsc1* and *Rheb* genes in mouse.

INTRODUCTION

The *Ras homolog enriched in brain* (*Rheb*) gene is ubiquitously expressed in mammalian cells, with the highest expression levels found in brain and muscle (2). *Rheb* was initially identified as a gene whose expression is rapidly induced upon synaptic activity in the rat hippocampus (26). The *Rheb* gene encodes a small GTPase, closely related to Ras, that exists either in an active GTP-bound state or an inactive GDP-bound state (26). Although the molecular mechanism has not yet been clearly defined, Rheb-GTP activates the target of rapamycin complex 1 (TORC1). TORC1 consists of multiple protein components, including the mammalian target of rapamycin (mTOR) itself and regulatory-associated protein of mTOR (Raptor), and is a major regulator of cell growth that phosphorylates multiple downstream targets including p70 S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins 1 and 2 (4E-BP1 and 4E-BP2) (10). Rheb is inactivated by the Tuberous Sclerosis Complex 1-2 (TSC1-TSC2) GTPase activating protein (GAP) complex that catalyses the conversion of Rheb-GTP to Rheb-GDP and thereby downregulates TORC1.

Although the function of Rheb has been extensively characterized *in vitro* (10), less is known about its function *in vivo*. Disruption of *Drosophila* Rheb (*dRheb*) arrested growth at the first larval stage, indicating an essential role for Rheb in development (16, 19, 20). However, *Drosophila* have only one *Rheb* gene, while mammalian cells also express the closely related Rheb-like1 (*RhebL1*) (16), which is also able to enhance TORC1 signaling *in vitro*, though in a less potent way (21).

In humans, inactivation of the TSC1-TSC2 complex leads to inappropriate activation of Rheb, and results in the disease Tuberous Sclerosis (Complex) (TSC) (1, 22, 23). Studies into the biology of the TSC1-TSC2 complex have significantly advanced our understanding of the mechanisms governing cell growth control, cognitive development and TSC pathogenesis (10).

To gain further insights into the TSC1-TSC2-Rheb-TORC1 signaling axis we inactivated the *Rheb* gene in mouse. We show that deletion of the *Rheb* gene in mouse leads to embryonic lethality during mid-gestation, most likely due to circulatory failure. Furthermore, *Rheb*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were much smaller than control cells and severely impaired in their ability to proliferate. Finally, we show that there is a genetic interaction between the *Rheb* and *Tsc1* genes, resulting in a delay in the lethality of *Tsc1*$^{-/-}$ embryos.

METHODS

Generation of *Rheb* mutant mice

The *Rheb* targeting construct was generated as follows. The mouse *Rheb* genomic sequence (ENSMUSG00000028945) was used to design primers for the targeting constructs. A PCR fragment (680 bp) containing exon 3 and flanking intronic sequence was amplified using the following primers:
Forward (F): 5'-ATGCATGTGAATTATGGCCTGACTGCAG-3'
Reverse (R): 5'-GTCGACCATCACAGAATCTAACCAATCTG-3'

The 5' flanking intronic arm (4.4 kb) (F: 5'-GGTACCTTGAGGAGCACCCTGCTC-3'; R: 5'-CTCGAGACTGCTCTGTGGAGAACACTTCC-3') and 3' flanking intronic arm (3.3 kb) (F: 5'-GCGGCCGCCCTTTAACAGCTTAGCTGCCTTG-3'; R: 5'-CCGCGGC-CTCACTCAAACATGCTATG-3') were amplified using High Fidelity Taq Polymerase (Roche, Basel, Switzerland) on ES cell genomic DNA and cloned in a vector containing a PGK-Neomycin selection cassette and loxP sites, so that exon 3 was flanked by loxP sites and the selection cassette was placed in the 5' intronic sequence. Exon 3 was sequenced to verify that no mutations were introduced during cloning. For counter selection, a gene encoding Diphtheria toxin chain A (DTA) was inserted at the 5' end of the targeting construct. Recombination of the loxP sites will result in deletion of exon 3 and introduce a premature stopcodon at codon 43. The targeting construct was linearized and electroporated into E14 ES cells (derived from 129P2 mice). Cells were cultured in BRL cell conditioned medium in the presence of Leukemia inhibitory factor (LIF). After selection with G418 (200 μg/ml), targeted clones were identified by long-range PCR from the *NEO* gene to the region flanking the targeted sequence and transfected with a plasmid encoding Cre recombinase. A transfection was done with a plasmide containing cre. Clones with a normal karyotype were injected into blastocysts of C57Bl/6 mice to obtain chimeric mice. A male chimera was crossed with female C57BL/6N/HsD mice (Harlan Laboratories, Indianapolis, IN) and the resulting offspring were back-crossed with C57Bl/6 mice (at least nine crosses). *Tsc1*$^{-/-}$ mice (25) were maintained for at least 9 crosses in C57BL/6N/HsD. All animal procedures were approved by the local Ethics Committee.

Immunohistochemistry and HE staining

E11.5 embryos were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Standard HE and immunostainings were performed on 6 μm slices. For the immunostainings a standard avidin biotin-immunoperoxidase-alkaline-phosphatase complex method (Zymed Laboratories, San Franscisco, CA) was used in combination with primary antibodies against pS6$^{S235/236}$ (#2211) and cleaved caspase-3 (#9661) from CST. The TUNEL assay was performed on paraffin sections using the Apoptag peroxidase in situ apoptosis detection kit from Millipore (Billerica, MA; product nr: S7100). For the isolectin staining, 20 μm cryoslices were prepared from E11.5 embryos. These sections were incubated overnight with FITC-labeled isolectin from Invitrogen (San Diego, CA catalogue nr: I21411)

Western blot

Whole embryos were isolated by dissection and homogenized in lysis buffer (10mM Tris HCl pH 6.8, 2.5% SDS) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). The concentration of the lysates was adjusted to 0.5 mg/ml and 15 μg was used for Western blot analysis. The following antibodies were used: Rheb (#4935), pAkt^{S473} (#4060), Akt (#2920), pS6$^{S235/236}$(#2211), S6 (#2217), p4EBP1$^{T37/46}$ (#2855), 4EBP1 (#9644) from Cell Signaling Technology (CST, Danver, MA) and Actin from Chemicon/Millipore (Billerica, MA; catalogue nr: MAB1501R). Bands were visualized using Enhanced Chemo Luminescence (Pierce; Thermo Fisher Scientific, Waltham, MA) and quantification was done using 'ImageJ64' software.

Cell culture and cell surface measurement

E11.5 embryos were minced and incubated in Trypsin/EDTA solution at 37°C for 5-15 minutes. Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), was added to the cells and the suspension was filtered. Several rounds of trypsinisation were performed to obtain an optimal yield. The final cell suspension was then centrifuged (930 x g for 5 minutes) and the cell pellet resuspended in DMEM containing 10% FCS and antibiotics prior to plating out.

To estimate the surface area of individual cells, phase-contrast images of cultured MEFs at 5x magnification were analysed using 'ImageJ64' software.

FACS analysis

Cells were prepared for FACS analysis according to standard procedures. Briefly, ~1 x 10^6 washed cells were resuspended in PBS containing 1% w/v BSA, 0.01% w/v NaN_3, 40 µg/ml RNAse A and 50 µg/ml propidium iodide, and ~2 x 10^5 cells were run through a FACS Aria I flow cytometer (BD Biosciences, San Jose, California). Cell cycle analysis was performed using a Dean-Jett-Fox model on FlowJo software (version 9.0.2).

Quantitative PCR (QPCR) analysis

Total RNA was extracted from E11.5 embryos using Trizol according to a standard protocol (Invitrogen, San Diego, CA catalogue nr: 15596026), and reversed transcribed into DNA (RevertAid Premium First strand cDNA synthesis kit from Fermentas Ammersbek, Germany; catalogue nr: K1652). QPCR analysis was performed using real-time fluorescence determination in the iCycler iQ Detection System (Biorad, The Netherlands). Primers were designed using primer3 on-line software. Target gene expression levels were expressed relative to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT).

Primer sequences: HPRT: TCAGGAGAGAAAGATGTGATTG (F), CAGCCAACACT-GCTGAAACA (R); VEGFA: AATGCTTTCTCCGCTCTGAA (F), CAGGCTGCTGTAACGAT-GAA (R).

RESULTS

Rheb is essential for murine development beyond E12

To investigate the role of Rheb *in vivo* we generated a *Rheb* knock-out mouse by inserting loxP sites in the intronic sequences flanking exon 3 of the *Rheb* gene through homologous recombination (Fig 1a). Subsequent expression of Cre recombinase in the targeted embryonic stem (ES) cells resulted in deletion of exon 3 and germ-line inactivation of the *Rheb* gene. $Rheb^{+/-}$ mice were born in Mendelian ratio from wild type - heterozygous crosses ($Rheb^{+/+}$: 56; $Rheb^{+/-}$: 44; χ^2= 1.44; p= 0.23; χ^2 test) and adult $Rheb^{+/-}$ mice showed no differences in survival (measured from weaning till 8 months of age) or in general health, compared to wild type mice.

Genotypes obtained from 7 day old pups from heterozygous crosses revealed that none of the homozygous pups survived the neonatal period ($Rheb^{+/+}$: 9; $Rheb^{+/-}$: 17; χ^2= 8.69; $p < 0.05$; χ^2 test for Mendelian distribution). To determine the timing of lethality of $Rheb^{-/-}$ embryos, E10.5-E14.5 embryos were analyzed. Homozygous

mutants were observed in Mendelian ratio at E10.5 (*Rheb*$^{+/+}$: 12; *Rheb*$^{+/-}$: 21; *Rheb*$^{-/-}$: 10; χ^2= 0.21; p= 0.90; χ^2 test) and appeared relatively normal. At E11.5, 9 empty decidua (13% of the total), 5 resorbed embryos (7% of total) and one *Rheb*$^{-/-}$ embryo with a clear apoptotic appearance (1.5% of total) were identified. However, *Rheb*$^{-/-}$ embryos were still found in Mendelian ratio (*Rheb*$^{+/+}$: 12; *Rheb*$^{+/-}$: 35; *Rheb*$^{-/-}$: 9; χ^2= 3.82; p= 0.15; χ^2 test). As shown in Figure 1b, the viability of the homozygous mutants declined sharply around E12.5. All homozygous E12.5 embryos were clearly apoptotic and from E13.5 on only resorbed homozygous embryos were observed.

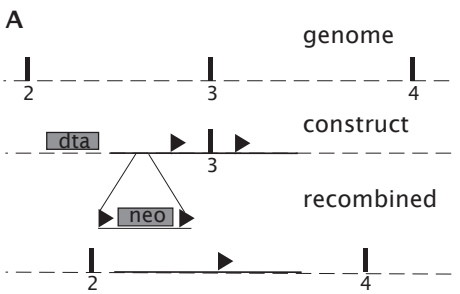
Rheb is required for proper development of the cardiovascular system

Although *Rheb*$^{-/-}$ E10.5 embryos tended to be smaller than controls, their gross morphology appeared normal (Fig 1c). In contrast, *Rheb*$^{-/-}$ E12.5 embryos were clearly smaller than controls and invariably had an apoptotic appearance (size: *Rheb*$^{+/+}$: 0.88 cm; *Rheb*$^{+/-}$: 0.89 cm; *Rheb*$^{-/-}$: 0.68 cm; K=13.94 $p < 0.05$ Kruskal Wallis test; post hoc test: *Rheb*$^{+/+}$ versus *Rheb*$^{+/-}$: $p > 0.05$; *Rheb*$^{+/+}$ versus *Rheb*$^{-/-}$: $p < 0.05$; *Rheb*$^{+/-}$ versus *Rheb*$^{-/-}$: $p < 0.05$; Dunn's multiple comparison test, see Fig 1c). To investigate the cause of death of *Rheb*$^{-/-}$ embryos we performed Haematoxylin Eosin (HE) stainings on 3 E11.5 embryos (Fig 1d). Although there was some variation between the E11.5 *Rheb*$^{-/-}$ embryos examined, they were all less well developed than control littermates. In particular, heart development was impaired. In 2/3 *Rheb*$^{-/-}$ embryos, pericardial hemorrhaging was observed (Figure 1d) in combination with thinning of the ventricular walls, pointing towards cardiorrhexis. In the remaining embryo, pericardial hemorrhaging was not observed, but this embryo also showed notable thinning of the ventricular walls. No obvious defects were observed in the vasculature of *Rheb*$^{-/-}$ embryos, as assessed by high magnification HE images and isolectin staining (Figure 1d, e).

A failure in heart development may lead to circulatory failure and hypoxia in different organs. To determine the causes of cell death in the *Rheb*$^{-/-}$ embryos in more detail we examined caspase and DNA fragmentation as markers of apoptosis (Fig 1e). Cleaved caspase was detected in groups of cells throughout the *Rheb*$^{-/-}$ embryos, particularly in the liver (Fig 1e), and patches of apoptic cells containing fragmented DNA were detected in different organs of the *Rheb*$^{-/-}$ embryos using the TUNEL assay (Fig 1e). Control *Rheb*$^{+/+}$ and *Rheb*$^{+/-}$ embryos did not show such patches of apoptotic cells using either the TUNEL assay or cleaved caspase as a marker.In line with this, cells with fragmented nuclei could be observed in different organ systems.

In addition to areas of apoptotic cells, swollen cells containing vacuoles could be identified in the *Rheb*$^{-/-}$ embryos, indicating necrosis (data not shown).

Taken together, these results suggest that *Rheb*$^{-/-}$ embryos probably die due to circulatory failure, secondary to a poorly developed heart. Notably, *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ embryos die around the same embryonic day with similar pathological features, indicating that both activation and inhibition of growth factor signaling to TORC1 result in comparable phenotypes (11, 15, 25).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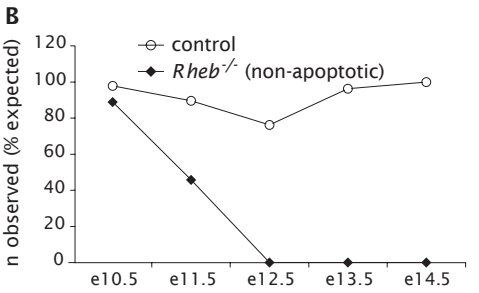
B
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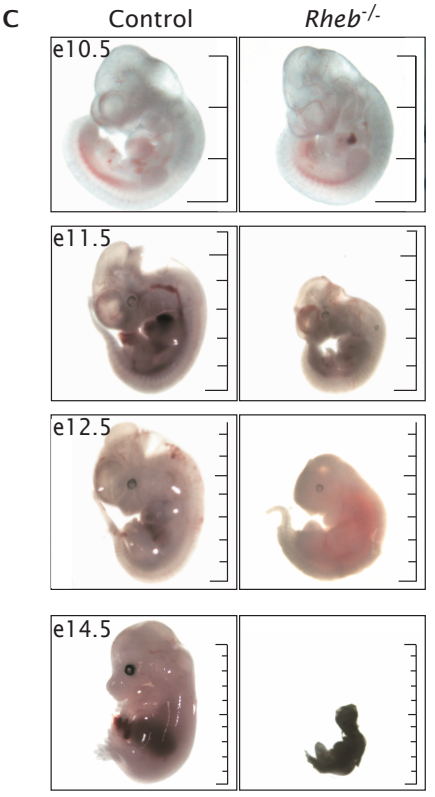
C
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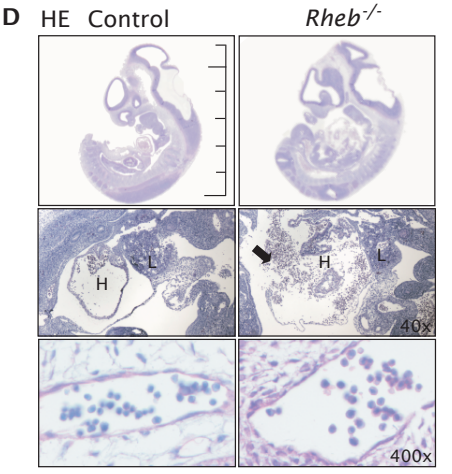
D
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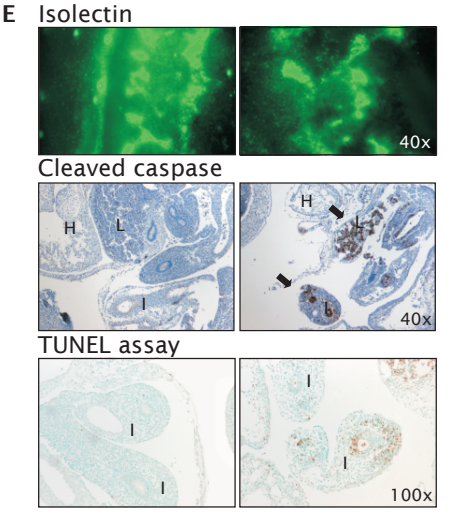
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Rheb deletion impairs TORC1 activity

Rheb is a robust activator of the TORC1 complex *in vitro* and in *Drosophila*. To test whether this was also the case in mice, we stained E11.5 embryos for S235/236-phosphorylated ribosomal protein S6 (S6), a read-out for TORC1 activation (17). As shown in Fig 2a, S6-S235/236 phosphorylation was virtually absent in *Rheb*$^{-/-}$ embryos. To quantify the effect of Rheb deletion on TORC1 activity we measured S6-S235/236 and 4E-BP1 T37/46 phosphorylation in E11.5 embryonal lysates. Phosphorylation of S6 at positions S235/236 was significantly reduced in *Rheb*$^{-/-}$ E11.5 embryos compared to wild type littermates, comparable to the decrease found in hepatocytes of S6K1$^{-/-}$S6K2$^{-/-}$ mice (17) (pS6: 31% of control; $F_{2,32}$= 22.0 $p < 0.0001$; Tukey's multiple comparison test: *Rheb*$^{+/+}$ versus *Rheb*$^{-/-}$: $q = 8.45$, $p < 0.05$; see Figure 2a and b). In addition, phosphorylation of 4EBP1 at positions T37/46 was decreased (p4EBP1: 64% of control; $F_{2,32}$= 6.27 $p < 0.01$; *Rheb*$^{+/+}$ versus *Rheb*$^{-/-}$: $q = 4.64$, $p < 0.05$). Taken together, these results show that the TORC1 pathway is less active in *Rheb*$^{-/-}$ embryos. In contrast, no differences could be detected between heterozygous and wild type tissues, indicating that Rheb levels have to be decreased by more than 50% to significantly affect TORC1 activity (pS6: *Rheb*$^{+/+}$ vs *Rheb*$^{+/-}$: $q = 1.66$, $p > 0.05$ and p4EBP1: *Rheb*$^{+/+}$ vs *Rheb*$^{+/-}$: $q = 0.28$, $p > 0.05$).

In vitro, the TORC1 pathway is subject to negative feedback. For example, *Tsc2*$^{-/-}$ cells are less responsive to insulin, while cells pretreated with rapamycin show increased Akt activation upon insulin stimulation (9). The negative feedback loops in the pathway are directed towards different upstream components, including the insulin receptor substrate (IRS) proteins, and converge on Akt (9). To test whether this feedback loop operates *in vivo*, we estimated IRS-1 and Akt S473 phosphorylation levels in *Rheb*$^{-/-}$ E11.5 embryos. Consistent with the existence

◀ **Fig. 1: *Rheb*$^{-/-}$ embryos die in mid-gestation and show impaired development of the circulatory system. (A)** Strategy to generate a *Rheb* knock-out mouse. (Top) Wild type *Rheb* locus with exons 2-4 depicted as black boxes. (Middle) Targeting construct used for the generation of *Rheb*$^{-/-}$ mice with the gene encoding the Diphteria Toxin A Chain (DTA) outside the homologous recombination sites, the Neomycin resistance gene (*NEO*) inserted in intron 2-3 and the loxP sites in the intronic regions flanking exon 3. (Bottom) Mutant *Rheb* locus after homologous recombination and the expression of Cre recombinase. **(B)** Embryonic survival curve of control and *Rheb*$^{-/-}$ embryos. Control embryos are either wild type or *Rheb*$^{+/-}$ embryos as no differences could be detected between them. From E12.5 on only apoptotic *Rheb*$^{-/-}$ embryos were observed. **(C)** Gross appearance of *Rheb*$^{-/-}$ embryos compared to controls. Embryos were sacrified at the indicated time points. Control embryos are either wild type or *Rheb*$^{+/-}$ as no differences could be detected between them. The total scale bar corresponds to 1 cm. **(D)** Representative examples of Haematoxylin Eosin (HE) stainings performed on E11.5 embryos. The scale bar corresponds to 1 cm. In the middle panel a 40x magnification of the abdominal region is displayed; in the lower a 400x magnification of a brain vessel. Note the absence of a myocardial lining of the heart. Erythrocytes in the abdominal cavity of the *Rheb*$^{-/-}$ embryo are indicated (arrow). Stainings were performed on 3 *Rheb*$^{-/-}$ and 5 control embryos. **(E)** Representative examples of immunostainings performed on E11.5 embryos using antibodies for isolectin, an endothelial marker and for the cleaved form of caspase-3, an apoptosis marker. In the lower panel representative images of a TUNEL assay, a detection method for DNA fragmentation are displayed. The arrows indicate patches of apoptotic cells in the liver and intestinal lumen in the *Rheb*$^{-/-}$ embryo. Immunostainings were performed on 3 *Rheb*$^{-/-}$ and 5 control embryos. H=heart, L=liver, I=intestine.

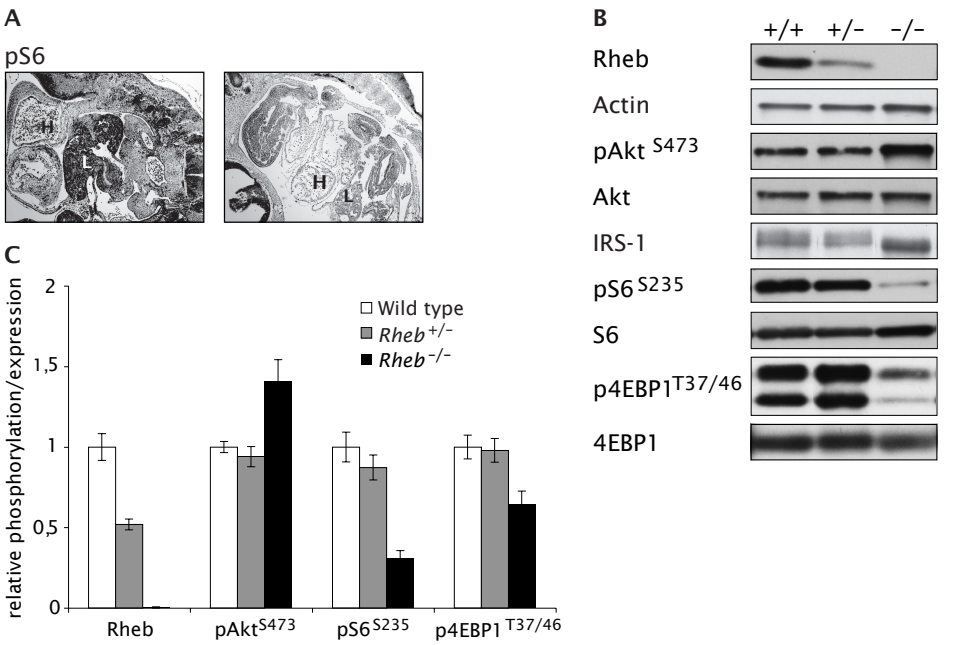

Fig. 2: Akt-TORC1 signaling is affected in *Rheb*$^{-/-}$ embryos (A) Representative examples of an immunostaining performed on E11.5 embryos using an antibody for S235/236 phosphorylated ribosomal S6 (pS6 at Ser235/236), a read-out for TORC1 activation. Note the decreased staining for S235/236 phosphorylated S6 in the *Rheb*$^{-/-}$ embryo. Immunostaining was performed on 3 *Rheb*$^{-/-}$ and 5 control embryos. **(B)** Representative immunoblots of Rheb expression, Akt S473 phosphorylation, IRS-1 mobility, S6 S235/236 phosphorylation and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 T37/40 phosphorylation in lysates prepared from whole E11.5 *Rheb*$^{-/-}$ and control embryos. Note that the increased mobility of IRS-1 corresponds to decreased phosphorylation of this protein. **(C)** Quantification of immunoblots. Wild type: n = 4; *Rheb*$^{+/-}$: n = 6; *Rheb*$^{-/-}$: n = 4; all these samples were run in at least 2 independent experiments. Error bars represent the SEM.

of a negative feedback loop, IRS-1 mobility and Akt S473 phosphorylation were increased in *Rheb*$^{-/-}$ embryos compared to wild type and *Rheb*$^{+/-}$ littermates ($F_{2,39}$= 8.01 $p < 0.01$; *Rheb*$^{+/+}$ versus *Rheb*$^{+/-}$: $q = 0.62$, $p > 0.05$; *Rheb*$^{+/+}$ versus *Rheb*$^{-/-}$: $q = 4.35$, $p < 0.05$; *Rheb*$^{+/-}$ versus *Rheb*$^{-/-}$: $q = 5.36$, $p < 0.05$; Figure 2b) (8).

Rheb is required for cell growth and proliferation

In *Drosophila* deletion of *dRheb* affects both cell size and the cell cycle (16, 19, 20). To investigate whether deletion of *Rheb* has similar effects in murine cells we cultured mouse embryonic fibroblasts (MEFs) from E11.5 *Rheb*$^{-/-}$ and control embryos. MEFs isolated from *Rheb*$^{+/+}$ and *Rheb*$^{+/-}$ embryos grew with the same characteristics (data not shown) but, although it was possible to obtain *Rheb*$^{-/-}$ cells, they hardly proliferated (Fig 3a). Notably, the surface area of *Rheb*$^{-/-}$ cells was significantly reduced compared to control cells (*Rheb*$^{+/+}$: 240 μm^2; *Rheb*$^{-/-}$: 61, 4 μm^2; $F_{1,108}$= 28 $p < 0.01$ ANOVA see Figure 3b). Fluorescence-activated cell sorting

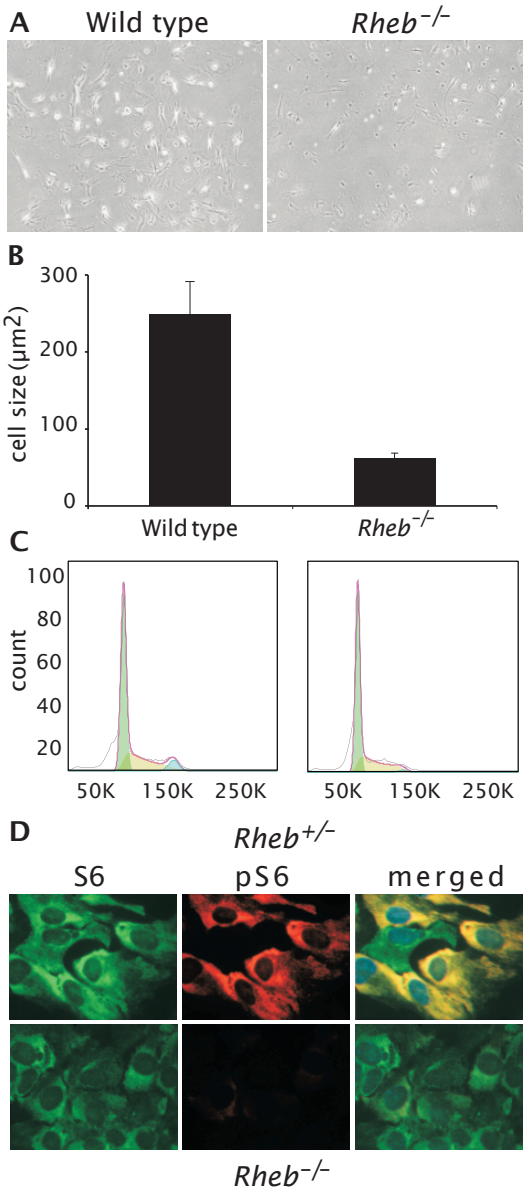

Fig. 3: *Rheb*$^{-/-}$ mouse embryonic fibroblasts (MEFs) are small and do not proliferate. (A) Representative phase contrast pictures of wild type and *Rheb*$^{-/-}$ MEFs. **(B)** *Rheb*$^{-/-}$ MEFs are reduced in size ($p < 0.01$). Cell areas of 45 wild type and 65 *Rheb*$^{-/-}$ MEFs were measured. Error bars represent the SEM. **(C)** Fluorescence-activated cell sorting (FACS) analysis of control and *Rheb*$^{-/-}$ MEFs. Populations of cells in different phases were estimated using the Dean-Jett-Fox model (FlowJo software version 9.0.2). **(D)** Immunostaining of *Rheb*$^{+/-}$ and *Rheb*$^{-/-}$ MEFs with antibodies for S6 and S235/236 phosphorylated S6 (pS6). Although most of the *Rheb*$^{+/-}$ cells stain positive for phosphorylated S6, virtually none of the *Rheb*$^{-/-}$ MEFs show S6 phosphorylation.

(FACS) analysis indicated that fewer *Rheb*$^{-/-}$ MEFs than control MEFs entered G2, and a higher percentage of *Rheb*$^{-/-}$ cells were found in G1 (G2: *Rheb*$^{+/+}$: 5.79%; *Rheb*$^{-/-}$: 1.29%; G1: *Rheb*$^{+/+}$: 50.0%; *Rheb*$^{-/-}$: 54.5% see Fig 3c). In addition, TORC1 signaling was severely decreased, as assessed by the S235/236 phosphorylation status of S6 (Fig 3d).

Rheb heterozygosity extends *Tsc1*$^{-/-}$ embryo lifespan

In *Drosophila*, heterozygosity for *dRheb* partially rescued the lethality of *dTsc1*$^{-/-}$ flies (20, 27). To investigate whether a similar genetic interaction could be observed in mice, we set up *Tsc1*$^{+/-}$ *Rheb*$^{+/-}$ intercrosses (*Tsc1*$^{+/-}$ *Rheb*$^{+/-}$ x *Tsc1*$^{+/-}$ *Rheb*$^{+/-}$). *Tsc1*$^{-/-}$ embryos were reported to die between E10.5 and E13.5 (25). Indeed, at E11.5 we only found severely apoptotic *Tsc1*$^{-/-}$ *Rheb*$^{+/+}$ embryos (data not shown). In case there is a full rescue of the *Tsc1*$^{-/-}$ embryonic lethality, *Tsc1*$^{-/-}$ *Rheb*$^{+/-}$ pups would be expected to be born with a frequency of 1/8. However of the 43 pups analyzed, none had this genotype (significantly different from the expected ratio: $p < 0.01$).

Therefore, embryos from the same intercrosses were analyzed at E15.5, to detect a possible partial rescue of the lethality. Although these intercrosses should result in 9 different genotypes, *Rheb*$^{-/-}$ embryos were not observed with any combination of *Tsc1* alleles, indicating that Rheb forms an essential link to TORC1 signaling. In addition, *Tsc1*$^{-/-}$ embryos with *Rheb*$^{+/+}$ or *Rheb*$^{-/-}$ alleles were not found (Fig 4a). However, *Tsc1*$^{-/-}$ *Rheb*$^{+/-}$ embryos were identified at Mendelian ratios (37 decidu: 5 *Tsc1*$^{-/-}$ *Rheb*$^{+/-}$ embryos observed; 4.63 expected) see Fig 4a), although they were smaller, developmentally retarded and apoptotic compared to the control embryos (Fig 4b). Collectively, these results indicate that there is a genetic interaction between the *Tsc1* and *Rheb* genes in mice, and that *Rheb* heterozygosity extends the lifespan of *Tsc1*$^{-/-}$ embryos by approximately 4 days.

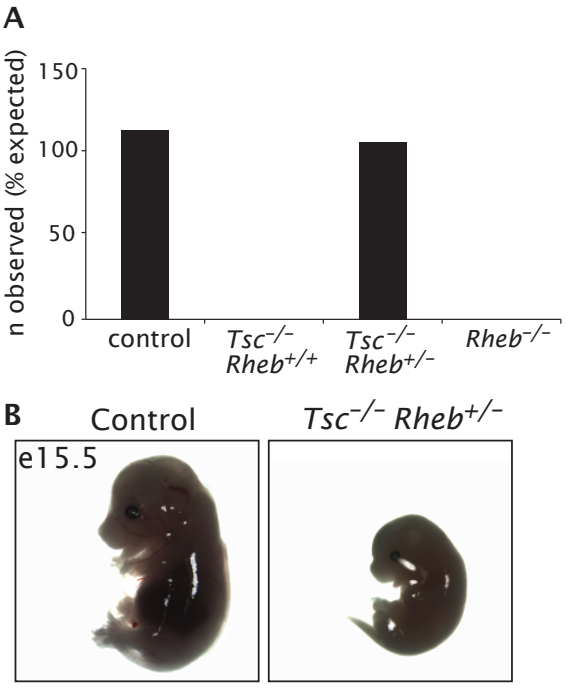

Fig. 4: *Rheb* heterozygosity partially rescues the lethality of *Tsc1*$^{-/-}$ embryos (A) Survival beyond E15.5 of different genotypes resulting from *Tsc1*$^{+/-}$ *Rheb*$^{+/-}$ intercrosses. Control embryos are embryos with all possible combinations of wild type and heterozygous alleles. *Rheb*$^{-/-}$ embryos may have a wild type, heterozygous, or homozygous genotype for *Tsc1;* however none of these combinations were observed. Note that *Tsc1*$^{-/-}$ *Rheb*$^{+/-}$ embryos were observed in Mendelian ratio. **(B)** Example of a control (*Tsc*$^{+/+}$ *Rheb*$^{+/-}$) and a *Tsc1*$^{-/-}$ *Rheb*$^{+/-}$ embryo at E15.5. The *Tsc1*$^{-/-}$ *Rheb*$^{+/-}$ embryo is clearly smaller and developmently retarded compared to the control embryo. In addition, it is already apoptotic, suggestive of resorption within one day.

DISCUSSION

Here we show that Rheb, a vital component of TORC1 signaling, is essential for murine development beyond E12. Interestingly, *mTor*$^{-/-}$ and *raptor*$^{-/-}$ embryos, which lack core TORC1 components die before E8.5, significantly earlier than *Rheb*$^{-/-}$ embryos (6, 7, 14). Therefore, our results indicate that Rheb is not necessary for full TORC1 activation during early embryonic development. Possibly, RhebL1 partially compensates for the loss of Rheb. Alternatively, TORC1 exhibits Rheb-independent activity *in vivo*.

Rheb has been shown to activate growth in adult cardiomyocytes (24) and increased TORC1 activity in the adult heart is involved in cardiac hypertrophy (18). In line with these observations, the midgestational lethality of *Rheb*$^{-/-}$ embryos is most likely due to impaired heart development, implying that TORC1 signaling is critical for heart physiology during embryonal development. The expression of vascular endothelial growth factor A (VEGFA), an essential factor for embryonic cardiogenesis and angiogenesis, is under the control of TORC1 via the hypoxia inducible transcription factor (HIF-1) (3, 4, 28). Although it is possible that the defects in cardiovascular development in the *Rheb*$^{-/-}$ embryos are due to decreased VEGFA expression, equal expression levels of VEGFA were found in *Rheb*$^{-/-}$, *Rheb*$^{+/-}$ *and Rheb*$^{+/+}$ embryos (qPCR analysis; data not shown), making it unlikely that decreased VEGFA levels are the key to the cardiovascular impairments in the *Rheb*$^{-/-}$ embryos. Next to VEGFA, other factors like angiopoietin and ephrins are critical for cardiovascular development and dysregulation of these might possibly underly the lethality of the *Rheb*$^{-/-}$ embryos (5).

Our studies in *Rheb*$^{-/-}$ MEFs indicate that Rheb is essential for cell growth and cell cycle progression. Consistent with these data, increased Rheb levels induce cell cycle progression in Drosophila (19). In contrast to *Rheb*$^{-/-}$ MEFs, cell proliferation in *S6k1/2*$^{-/-}$ MEFs is not impaired, probably because TORC1 signaling towards other targets is still intact (17).

Finally, we show that *Rheb* heterozygosity extends the life span of *Tsc1*$^{-/-}$ embryos. However, like *Drosophila*, decreasing Rheb levels does not lead to a total rescue from *Tsc1*$^{-/-}$ lethality (20, 27). This implies that Rheb may not be the only target of the TSC1-TSC2 complex during murine embryonic development. Possibly, the interaction of Hamartin with ERM proteins, regulating cell adhesion, is important for proper embryonic development (12). Alternatively, aberrant β-catenin signaling, as implicated in TSC, may play a role in the embryonic lethality of *Tsc1*$^{-/-}$ embryos (13).

ACKNOWLEDGEMENTS

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6

INTACT PLASTICITY AND LEARNING IN *RHEB1* MUTANT MICE: IMPLICATIONS FOR RAPAMYCIN TREATMENTS

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ABSTRACT

Rapamycin and other TORC1 inhibitors hold great promise for treating a number of diseases, including genetic syndromes, aging-related disorders and epilepsy. However, such treatments are likely to be long lasting and could involve young children. Therefore it is vital that the effects of sustained TORC1 inhibition on cognitive function are determined. Here we addressed this by using heterozygous *Rheb1* knock-out mice which show a significant reduction of hippocampal TORC1 activity, but develop normally and show intact synaptic plasticity and learning. Reducing TORC1 activity further also had no effect on these measures. Our findings indicate that sustained TORC1 hypo-activity does not necessarily lead to impaired cognitive function.

INTRODUCTION

Inhibition of target of rapamycin complex 1 (TORC1) with rapamycin or its analogs, has increasingly been put forward as an attractive treatment for a number of diseases, including genetic syndromes[1], sporadic cancers[2], epilepsy[3], and aging-related disorders[4]. Long-term treatment is currently being considered for individuals with Tuberous Sclerosis Complex (TSC), for whom it might also be advantageous to start treatment early in life[5]. However, it is not yet clear how sustained reduction of TORC1 activity (i.e. beyond normal levels) could affect brain development and/or cognitive function.

In the brain, TORC1 is important for long-term memory formation and its cellular correlate, the late protein synthesis dependent phase of long-term potentiation (L-LTP)[6]. This is accomplished by phosphorylation of its downstream targets, including p70 S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins 1 and 2 (4E-BP1 and 4E-BP2)[6]. Hence, long-term treatment with TORC1 inhibitors could potentially lead to serious cognitive side effects if TORC1 activity was to be reduced too much. This would severely limit the potential of TORC1 inhibitors in clinical applications. Unfortunately, no good clinical data addressing this concern is available. Moreover, even in animal studies the consequences of TORC1 hypo-activity on synaptic plasticity and learning are not fully understood. Acute treatment with rapamycin was shown to block L-LTP *in vitro*[7, 8], whereas L-LTP measured *in vivo* was resistant to acute rapamycin treatment[9]. In addition, systemic administration of a high dose of rapamycin impaired learning and memory formation in some studies[10, 11], but not in others[12]. To study the effects of TORC1 suppression, while avoiding using high concentrations of rapamycin, a recent study has employed an elegant pharmacogenetic approach using $Tor^{+/-}$ mutants[13]. Under normal conditions $Tor^{+/-}$ mice showed no decrease in phosphorylated p70 S6 kinase (pS6K) levels (a readout for TORC1 activity). However, administration of a sub-threshold dose of rapamycin reduced pS6K activation by 50% compared to wild-type, and resulted in impaired L-LTP[13]. In addition, a single sub-threshold dose of rapamycin impaired memory in $Tor^{+/-}$ mutants but not in wild-type mice[13].

Apart from the scarce and conflicting data on acute rapamycin treatment, even less is known about the effects of long-term suppression of TORC1 signaling, which is most relevant for TORC1 inhibitor treatments for chronic conditions. Genetic approaches to decrease TORC1 activity have been limited owing to the embryonic lethality of many model phenotypes. Although *S6k1/2* knockout mice, which lack one of the main outputs of TORC1, show normal L-LTP and only subtle alterations in learning[14], it cannot be ruled out that other downstream targets of TORC1 are more critical for these processes. To investigate the consequences of long-term TORC1 hypo-activity, during and after brain development, on plasticity and learning we made use of *Rheb1* heterozygous mutant mice. Rheb1 is an essential activator of TORC1 and $Rheb1^{+/-}$ mice show significantly decreased TORC1 activity.

METHODS

Mice

Mice carrying a floxed *Rheb1* allele and the corresponding *Rheb1* knock-out line were generated as described[15]. To create an inducible *Rheb1* knock-out line, *Rheb1*$^{+/-}$ mice were bred with *Cre-ER*^{TM} transgenic mice[16] and the resulting *Rheb1*$^{+/-}$ *Cre-ER*^{TM}*+* mice were crossed with *Rheb1*$^{f/+}$ mice to yield the experimental genotypes *Rheb1*$^{f/-}$ *Cre-ER*^{TM}*+* and *Rheb1*$^{+/-}$ *Cre-ER*^{TM}*+*. Mice were genotyped when they were around 7 days of age. The experimenter remained blind to the genotype during data collection and the initial analysis.

Throughout the study both experimental genotypes were treated in parallel with tamoxifen dissolved in sunflower oil (10 mg/g; Sigma-Aldrich, St. Louis, MO). Mice tested for fear conditioning were later sacrificed for field recordings. PCR and Western blot analysis were performed on independent groups of mice. Mice between 2-5 months of age were used for all experiments and were treated according to standard procedures[15]. All animal experiments were approved by the Local Animal Experimentation Ethical Committee, in accordance with Institutional Animal Care and Use Committee guidelines.

Western blot analysis

Western blot analysis was performed as described previously[15], except that 20 μg total protein was loaded. Antibodies against Rheb1 (#4935), pS6$^{S240/244}$ (#5364), S6 (#2217) (all from Cell Signaling Technology, Danver, MA) and Actin (MAB1501R; Chemicon/Millipore, Billerica, MA) were used.

Hippocampal field recordings and behavioral analysis

Extracellular recordings of fEPSPs in CA1 *striatum radiatum* were performed as described[17]. For 100 Hz stimulation protocols, stimulation was performed at 1/3 of the maximum response during the LTP experiment and at 2/3 of the maximum response for the theta-burst experiment. Test stimulations were given once a minute during early LTP (E-LTP) experiments, and once every five minutes during L-LTP experiments. Watermaze and contextual fear conditioning tests were carried out as described[18], but with a foot shock of 0.6 mA.

PCR analysis

For genotyping the following primers were used: Forward (F): 5'-AGTGTTCTCCACAGAGC-3', Reverse (R): 5'-ACAGGGACAGATTCAGTC-3' and R: 5'-CCGCTGTGTCTACAAGC-3', yielding a 360 bp product from the wild-type allele, a 500 bp product from the floxed allele and a 209 bp product from the knock-out allele.

RESULTS

Plasticity and learning is intact in *Rheb1*$^{+/-}$ mice despite a significant reduction in TORC1 activity

Whereas homozygous *Rheb1* deletion results in mid-embryonic lethality, *Rheb1*$^{+/-}$ mice develop and breed normally, look healthy, have a normal life span and are phenotypically indistinguishable from their wild-type littermates[15, 19]. Importantly, TORC1 activity, as estimated by assessing S6 phosphorylation (pS6), a widely-used read-out of this pathway, is significantly decreased in the hippocampus of these mice ($t_{1,18} = 6.31$; $p < 0.0001$, unpaired t-test; Fig 1A). Thus, *Rheb1*$^{+/-}$ mice serve as a good model to test the effects of long-term suppression of TORC1 activity (from germ-line on) on learning and plasticity. First, we found that *Rheb1* heterozygosity did not affect basal transmission in the CA3-CA1 subfield of the hippocampus, as assessed by measuring the presynaptic fiber volley and field excitatory post synaptic potential (fEPSP) (fiber volley: effect of genotype: $F_{1,45} = 1.03$; $p = 0.31$; fEPSP: effect of genotype: $F_{1,45} = 0.63$; $p = 0.43$, two-way repeated-measures ANOVA; Fig 1B-C), or several forms of E-LTP induced either by theta-burst or single train 100 Hz stimulation (average fEPSP slope in the last 10 minutes: theta-burst: $t_{1,17} = 0.03$; $p = 0.97$; 100 Hz: $t_{1,17} = 0.95$; $p = 0.36$, unpaired t-test; Fig 1D-E). While E-LTP is thought to be the cellular correlate for short-term memory and seems to be exclusively mediated by post-translational mechanisms, L-LTP is considered to underlie long-term memory formation and in some studies is reported to be sensitive to protein synthesis inhibitors (including rapamycin). We did not observe a reduction in L-LTP in *Rheb1*$^{+/-}$ mice compared to control littermates (average fEPSP slope in the last 30 minutes: $t_{1,15} = 0.79$; $p = 0.44$, unpaired t-test; Fig 1F). Water maze learning and contextual fear conditioning have also been shown to be sensitive to TORC1 activity in some studies [1]. We tested *Rheb1*$^{+/-}$ mice using both paradigms. In the Morris water maze, in which the animal has to learn the location of a hidden escape platform in a pool of water, both genotypes showed a similar decrease in the time required to find the submerged platform upon training ($F_{1,20} = 0.71$; $p = 0.41$, two way repeated-measures ANOVA; Fig 2A). They also showed a similar preference for the target quadrant in a probe trial (in which the platform is removed) given after 7 days of training ($t_{1,20} = 0.71$; $p = 0.48$, unpaired t-test; Fig 2B), and were both able to retain a memory for the platform location over a long period, as shown by the probe trial given 14 days after the last training ($t_{1,20} = 0.70$; $p = 0.49$, unpaired t-test; Fig 2C). In addition, both mice had a similar freezing response in the contextual fear conditioning paradigm, indicative of equal recall of the conditioned context ($t_{1,22} = 1.11$; $p = 0.28$ unpaired t-test; Fig 2D). Taken together, these data show that learning can take place regardless of a significant reduction in hippocampal TORC1 activity.

Rheb1 inducible knockout mice show intact L-LTP and learning, despite a large reduction in TORC1 activity

To investigate the effect of a sudden, dramatic reduction in TORC1 activity during adult life, we created an inducible *Rheb1* knock-out line, by crossing *Rheb1*$^{+/-}$*Cre-ER*^{TM} transgenic mice[16] with *Rheb1*$^{f/+}$ mice to yield two experimental genotypes: *Rheb1*$^{f/-}$ *Cre-ER*^{TM}*+* and *Rheb1*$^{+/-}$*Cre-ER*^{TM}*+* (hereafter named: *f/-* and *+/-*). Injection of

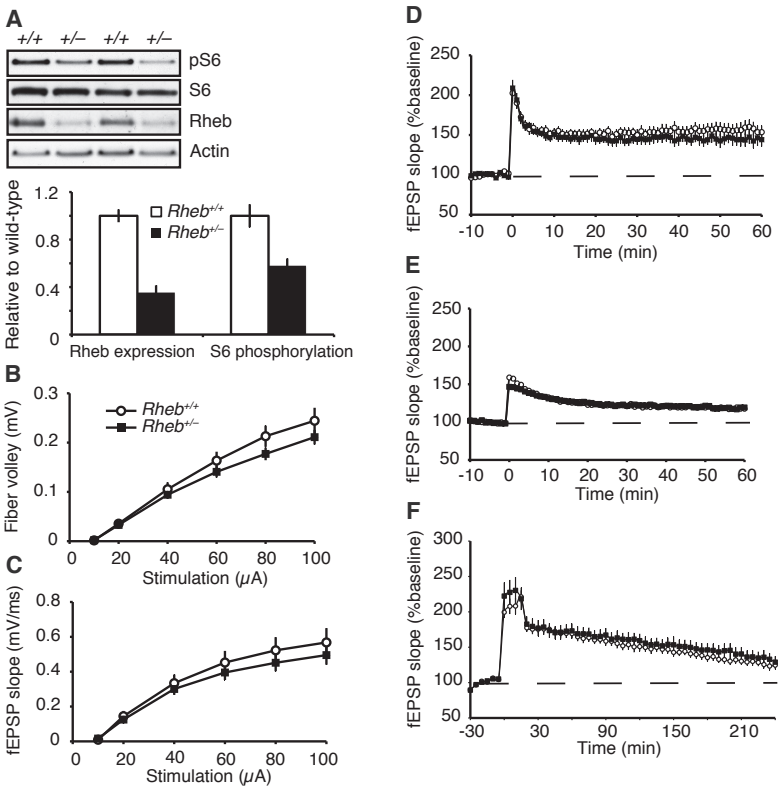

Fig. 1. *Rheb1*$^{+/-}$ mice have decreased TORC1 activity but intact basal transmission and early and late phase LTP. (A) Representative immunoblot of Rheb1 expression and S6 S240/244 phosphorylation, an established marker for TORC1 activity, in hippocampal tissue of *Rheb1*$^{+/+}$ and *Rheb1*$^{+/-}$ mice (upper panel) and the corresponding quantification (lower panel). N = 4 for both genotypes and all samples were probed at least twice. Error bars represent the standard error of the mean (SEM). **(B-C)** Basal synaptic transmission at hippocampal CA3-CA1 synapses is not different in *Rheb1*$^{+/-}$ and wild-type mice. Plots show the presynaptic fiber volley as a function of stimulation strength **(B)** and the field excitatory post synaptic potential (fEPSP) as a function of the stimulation strength **(C)**. 25 slices from 7 *Rheb1*$^{+/+}$ mice and 22 slices from 7 *Rheb1*$^{+/-}$ mice are included for analysis. Error bars represent SEM. **(D-E)** E-LTP when elicited with either a single train 100 Hz protocol **(D)** or a two theta-burst protocol **(E)** was of equal value in both genotypes. For 100 Hz LTP, 10 slices from 4 *Rheb1*$^{+/+}$ mice and 9 slices from 4 *Rheb1*$^{+/-}$ mice were included for analysis; for theta-burst LTP, 12 slices from 5 *Rheb1*$^{+/+}$ mice and 7 slices from 6 *Rheb1*$^{+/-}$ mice were included. Error bars represent SEM. **(F)** A 4 train 100 Hz protocol induces L-LTP with a similar magnitude in *Rheb1*$^{+/-}$ and *Rheb1*$^{+/+}$ mice. Eight slices from 5 *Rheb1*$^{+/+}$ mice and 9 slices from 7 *Rheb1*$^{+/-}$ mice were included for analysis. Error bars represent SEM.

f/- mice with tamoxifen induces translocation of the Cre-ER™ protein to the nucleus to drive recombination of the floxed *Rheb1* allele, conferring a homozygous *Rheb1* null genotype on the mice. Four tamoxifen injections on subsequent days were sufficient to drive nearly complete deletion of *Rheb1* (Fig 3A). Loss of *Rheb1* was lethal and the mice had a median survival time of 18 days after initiating *Rheb1*

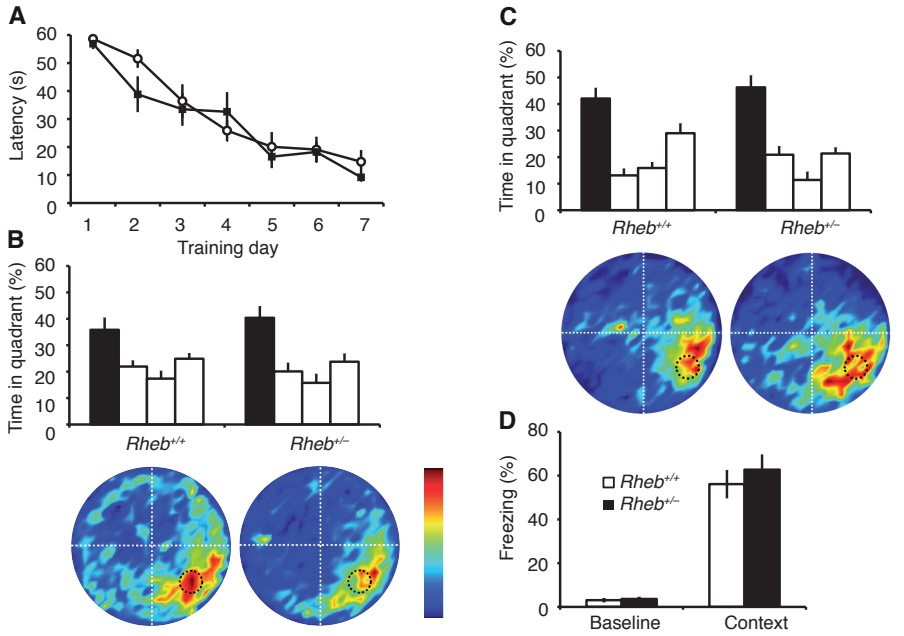

Fig. 2. *Rheb1*$^{+/-}$ mice display intact spatial learning. (A) Latency to reach the hidden platform during training is not different between the two groups (*Rheb1*$^{+/-}$ and *Rheb1*$^{+/+}$). Error bars represent SEM. **(B-C: upper panels)**: quantification of the time spent in each quadrant at a probe trial given at 7 **(B)** or 14 days **(C)** after the last training day. Black bar indicates the target quadrant; white bars indicate adjacent right, opposite, and adjacent left quadrants, respectively. Error bars represent SEM. **(B-C: lower panels)**: visual representations of all the combined swimming tracks of both groups of mice from the probe trial. The color indicates the time spent at a certain location (red is high, blue is low; see color bar). This clearly shows that both groups of mice search specifically for the platform. N=11 for both genotypes. **(D)** *Rheb1*$^{+/-}$ mice are not impaired in contextual fear conditioning. Bars represent the amount of freezing behavior, which is used as an index for recollection of the context. Number of mice tested: *Rheb1*$^{+/+}$: 13 and *Rheb1*$^{+/-}$: 11. Error bars represent SEM.

deletion. However, we analyzed the mice 14 days after the first injection, when nearly all the animals were still healthy, despite significantly reduced Rheb1 protein levels and minimal TORC1 activity, as assessed by pS6 levels ($t_{1,19} = 8.50$; $p < 0.0001$, unpaired t-test; Fig 3A-B). Notwithstanding the significant reduction in TORC1 activity, basal synaptic transmission in the CA3-CA1 subfield of the hippocampus was unaffected (fiber volley: effect of genotype: $F_{1,30} = 0.45$; $p = 0.51$; fEPSP: effect of genotype: $F_{1,30} = 0.49$; $p = 0.49$, two way repeated-measures ANOVA; Fig 3C-D). Surprisingly, L-LTP, which is generally believed to critically rely on proper TORC1 signaling[6], was also unaffected in the *Rheb1* deficient mice (average fEPSP slope in the last 30 minutes: $t_{1,19} = 0.12$; $p = 0.90$, unpaired t-test; Fig 3E). Finally, we observed that the mice were perfectly able to establish a contextual fear memory when trained on day 13 and tested on day 14 after the initiation of *Rheb1* deletion ($t_{1,13} = 0.22$; $p = 0.83$, unpaired t-test; Fig 3F). Therefore, despite a clear reduction in TORC1 activity, no effect was observed on L-LTP and learning.

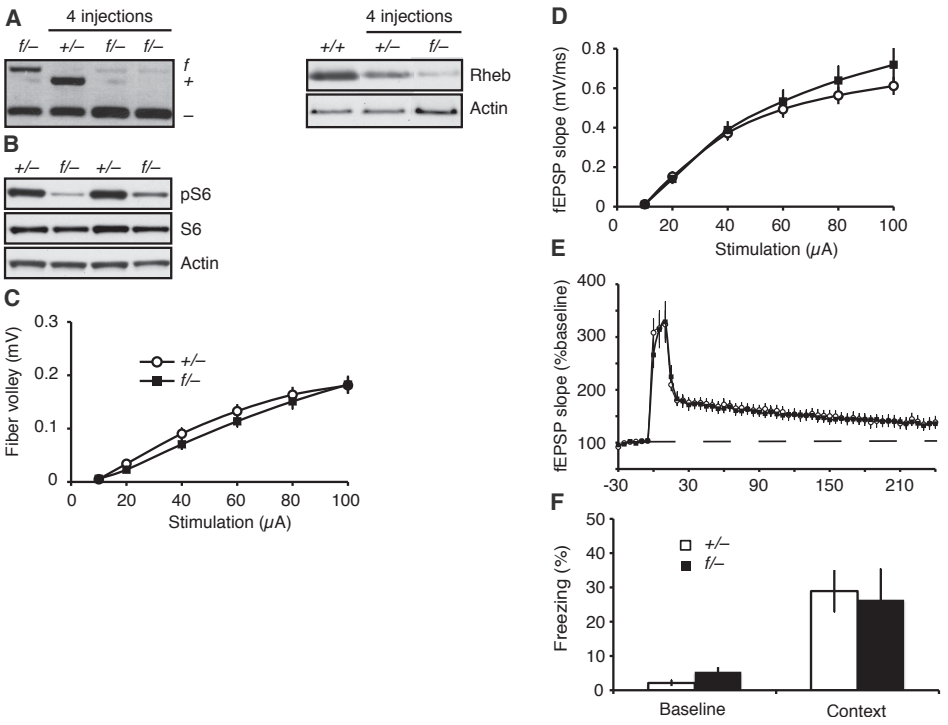

Fig 3. Inducible *Rheb1* knockout mice show intact plasticity and learning. (A) PCR analysis to show that 4 tamoxifen injections are sufficient to lead to recombination of the floxed allele in hippocampal tissue of *Rheb1$^{f/-}$ Cre-ER^{TM}+* (*f/–*) mice. **(B)** Representative immunoblot of Rheb1 expression and S6 S240/244 phosphorylation, an established marker for TORC1 activity, in hippocampal tissue of *Rheb1$^{+/-}$ Cre-ER^{TM}+ (+/–)* and *f/–* mice (left panel) and the corresponding quantification (right panel). *+/–* : n = 3; *f/–* : n=4, samples were probed 3 times. **(C-D)** Basal synaptic transmission at hippocampal CA3-CA1 synapses is not different in f/- mice. Plots show the presynaptic fiber volley as a function of stimulation strength **(C)** and the fEPSP as a function of the stimulation strength **(D)**. Twenty slices from 7 *+/–* mice and 12 slices from 5 *f/-* mice were included for analysis. **(E)** A 4-train 100 Hz protocol induces similar L-LTP in slices of *f/–* and +/– mice. Twelve slices from 6 *+/–* mice and 9 slices from 6 *f/-* mice were included for analysis. For all experiments depicted in **A-E** mice were sacrificed on day 14 after the first injection. Error bars represent SEM. **(F)** *f/–* mice have intact contextual fear conditioning. Bars represent the amount of freezing behavior, which is used as an index for recollection of the context. Number of mice tested: *+/–*: 7 and *f/–*: 8. Error bars represent SEM.

DISCUSSION

TORC1 inhibition holds great promise for clinical interventions. Here, we set out to resolve whether sustained reduction of TORC1 activity affects synaptic plasticity and learning, and also whether further reduction of TORC1 activity affects cognitive function in adult animals. First, we showed that *Rheb1$^{+/-}$* mice have a significant reduction in hippocampal TORC1 activity, as assessed by pS6 levels. This is in marked contrast to the normal TORC1 activity reported in the brains of *Tor$^{+/-}$* mice[13], suggesting that Rheb1 is rate-limiting for TORC1 activity in the brain. The *Rheb1*

inducible knockout mice showed even less TORC1 activity than *Rheb1*$^{+/-}$ mice, consistent with the further reductions in Rheb1 protein levels. Remarkably, despite the marked and sustained reductions in TORC1 activity, both mutants showed intact L-LTP and learning. These results do not match findings obtained with acute TORC1 inhibition by rapamycin (reviewed in[6]). Several reasons could account for the observed differences. First, it is important to note that the rapamycin literature shows inconsistencies between studies and that in some studies considerably higher concentrations of rapamycin (for example 140 mg/kg)[12] were required to impair learning and plasticity in mice, which may have caused off-target effects of rapamycin. Furthermore, the translational relevance of these findings is unclear, as rapamycin treatments that are clearly sub-threshold to impair learning in mice when given systemically (e.g. 8 mg/kg) still result in 10- fold higher blood concentrations of rapamycin (100 ng/ml)[20] compared to human rapamycin treatments (10 ng/ml)[5]. Second, most studies measured the effect of rapamycin within hours of injection, or upon direct application to the slice. The lack of an effect does not imply that prolonged rapamycin treatment would be safe. From a clinical point of view, we believe that our results are most relevant when considering the effects of longer treatments, since extended treatments will probably be required to alleviate conditions such as TSC and Alzheimer's disease. Our data suggest that long-term suppression of TORC1, even in the developing brain, does not necessarily lead to cognitive deficits. Interestingly, our data also support the notion that TORC1-dependent regulation of LTP and learning is much more sensitive to up- than down-regulation, as modest increases in TORC1 activity already result in learning deficits in human and mice[1].

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7

GENERAL DISCUSSION

TSC is a relatively common, genetic multi-organ disorder, characterized by the development of benign malformations and specific neurological and psychiatric symptoms. TSC is associated with a high burden of neurological involvement, for which adequate therapies are currently lacking. Since the identification of the *TSC1* and *TSC2* genes, significant advancements have been made in understanding the pathobiology of TSC that have led to the recognition of TORC1 inhibitors as promising targeted treatments for TSC. Recently, the target of rapamycin complex 1 (TORC1) inhibitor everolimus was approved for the treatment of TSC patients with inoperable subependymal giant cell astrocytomas, and clinical trials have shown benefit for other TSC lesions[1]. However, therapeutic efficacy for neuropsychiatric symptoms has yet to be established[100]. The studies described in this thesis contribute to the preclinical framework on which the current attempts to target the CNS manifestations of TSC and related conditions, using TORC1 inhibitors, are based.

ETIOLOGY OF CNS MANIFESTATIONS IN TSC

In **Chapter 3**, we show that learning and social deficits develop in *Tsc1*$^{+/-}$ mice in the absence of brain lesions and epilepsy[2]. Our findings brought into question the widespread belief that tubers and epilepsy are the underlying causes of cognitive dysfunction in TSC. Instead, our results supported a model in which *TSC1* or *TSC2* haploinsufficiency is sufficient to lead to aberrations in neuronal functioning, resulting in impaired learning and social behavior. Together with other studies[3], our results indicated that targeted drug treatments for these cognitive deficits would be worth exploring.

As a follow-up we initiated a study in which we deleted the intact copy of the *Tsc1* gene in adult *Tsc1*$^{+/-}$ mice (**Chapter 4**). We observed that epilepsy, previously considered to be intimately linked to developmental neuropathology, developed in these mice, despite intact brain development. Although we did not fully explore the underlying mechanisms, we hypothesize that several disease features observed in patient brain tissue and in mouse models may cause epilepsy in the absence of tubers. These include a reduced threshold for the late phase of long-term potentiation, altered expression, function and subunit composition of neurotransmitter receptors, favoring excitatory transmission[4-8], and decreased functioning of astrocyte-specific potassium and glutamate transporters[9-11]. Our inducible model will prove to be a valuable tool in the future since it allows the identification of preceding and secondary features pertinent to the development of seizures.

Notwithstanding the large number of studies performed recently, a number of issues remain to be addressed with regard to the mechanisms that underlie the brain manifestations of TSC.

Is reduced AKT activity involved in the pathogenesis of the brain manifestations associated with TSC?

From a molecular point of view, enhanced TORC1 activity, with decreased AKT and TORC2 activity characterizes brain tissue of TSC mouse models[12]. Treatment with

rapamycin normalizes AKT and TORC1 signaling, yet has minimal effects on target of rapamycin complex 2 (TORC2) activity[12, 13]. Accordingly, any phenotypes present in TSC mouse models that show responsiveness to rapamycin could be inferred to depend on increased TORC1 and/or decreased AKT activity. Most likely, the majority of morphological phenotypes, including macrocephaly, increased cell size, and neuronal polarity defects are caused by increased TORC1 signaling, since these phenotypes are recapitulated in *Phosphatase and tensin homolog* (*Pten)* deficient (AKT and TORC1 hyperactive) mice[14], and subsequently ameliorated by rapamycin[15, 16]. Moreover, mice with significant reductions in expression levels of AKT in the brain have a distinct phenotype of microcephaly with decreased cell size and number[17, 18]. Nevertheless, AKT proteins have a wide spectrum of interaction partners and previous studies have indicated that intact PI3K-AKT signaling is necessary for learning and plasticity in general[19], raising the question of to what extent cognitive and behavioral abnormalities in TSC can be attributed to decreased AKT signaling. Clearly, AKT signaling abnormalities in TSC brain have largely escaped attention and deserve further study. To this end, *Pten Tsc* inter-crosses could be a valuable tool to disentangle AKT and TORC1-dependent brain phenotypes. Somewhat paradoxically these mice could show a rescue of TSC-induced learning and social deficits.

What are the critical TORC1 targets that control differentiation, migration and synaptic plasticity?

Despite the increasing awareness that TORC1 hyperactivity is at the root of many of the disease features associated with TSC, remarkably little is known about the TORC1 targets that may control differentiation, migration and synaptic plasticity, which are all deregulated in TSC. Histological analyses of tuber tissue has revealed inappropiate expression and activation of several proteins that could be involved in differentiation and migration defects, including Double-cortin[20], doublecortin-like[21] and signal transducer and activation of transcription 3 (STAT3)[22]. Double-cortin and doublecortin-like are known to be involved in neuronal migration and differentiation during murine brain development, whereas human Double-cortin mutations are detected in several malformations of cortical development[23], suggesting a role for these proteins in the abnormal differentiation and migration observed in TSC. Interestingly, STAT3, a major regulator of cell proliferation, differentiation and development, was recently observed to be highly phosphorylated and aberrantly localized in the nuclei of neural stem cell-specific *Tsc1*$^{-/-}$ mice, and associated with the precocious differention of precursor cells into neuronal and astroglial cells[24]. In general, histopathological analyses of perituberal tissue have revealed aberrant expression and activation of a wide range of proteins, which deserve follow up studies in TSC animal models. Use of these mouse models allows us to ask questions about the time dependence of these changes and their causality with respect to the development of pathology and epilepsy.

Studies performed in cultured neurons have shown that Brain derived neurotrophic factor (BDNF) application and NMDA receptor activation can induce local protein synthesis in a TORC1 dependent manner[25, 26]. Specific mRNAs show up-regulation of translation, including several that encode proteins implicated in neuronal plasticity, such as Calcium/Calmodulin-dependent protein kinase II

(CaMKII), activity-regulated cytoskeleton-associated protein (Arc), Synapsin, and Glur1[25, 26]. However, whether these proteins are more highly expressed in the brains of TSC mouse models remains to be clarified. Follow up studies using specific inhibitors of these proteins, or genetic manipulation, should help assess the roles of these proteins in TSC pathophysiology.

Is there more than AKT-TORC1 signaling in the TSC brain?

Studies in TSC mouse models have shown that pathogenic changes to astrocytes and oligodendrocytes can be reversed by postnatal rapamycin treatment. In contrast, however, other neuronal features including neuronal dysplasia, impaired cortical lamination and organization are not corrected[12, 27]. Such findings can be expected from postnatal treatment because the generation of neurons from neural progenitor cells takes place during early embryogenesis, followed by a switch to astrocyte production and then, in the postnatal period, to oligodendrocytes. Therefore, it can be hypothesized that prenatal rapamycin treatment could rescue neuronal phenotypes[28]. Alternatively, it may be that these neuronal phenotypes reflect TORC1-independent mechanisms. In non-neuronal cells, TORC2, a target of the TSC1-TSC2 complex, is implicated in the regulation of actin cytoskeleton dynamics and cell motility via its interaction with the small GTPase Rho[29]. Moreover, the TSC1-TSC2 complex has been directly associated with regulation of Rho through its interaction with ezrin-radixin-moesin proteins *in vitro*.[30-33]. Therefore, altered Rho signaling in neurons and radial glial cells might contribute to the observed defects in cortical lamination and organization in TSC. Further research could involve assessing the activation states of Rho and its targets in different developmental stages in the embryonic TSC brain, and manipulating Rho activation in neuronal migration assays using *Tsc1* and *Tsc2* mutant neurons.

Etiology of phenotypic variability in TSC

Besides the incomplete knowledge of the general mechanisms controlling neurological dysfunction in TSC, we also lack insight into the mechanisms causing phenotypic variability between patients. Specifically, the CNS manifestations amongst TSC patients are very variable, with a subgroup of profoundly affected patients in which cognitive and neuropsychiatric symptoms cluster, and a larger, less severely affected group many of whom are able to lead productive and fulfilling lives despite specific cognitive deficits[34].

Genotype-phenotype studies show that *TSC1* mutations[35-38] and some *TSC2* missense mutations[39-43] are associated with less severe TSC disease, indicating that the nature of the mutation may explain some of the phenotypic variability. Interestingly, mouse studies show that *Tsc2* mutations intrinsically cause a more severe neurological phenotype, related to higher levels of TORC1 activation[44]. Possibly, TSC2 exerts additional independent functions from TSC1. Alternatively, residual TSC2-GAP activity may persist in the absence of TSC1.

Nevertheless, within families and even between monozygotic twins, TSC individuals carrying identical mutations can differ significantly in CNS phenotype[45]. Probably, modifier genes influence disease severity, as has been shown for kidney angiomyolipomas[46, 47]. These modifier genes could include the *TSC1* and *TSC2* genes

themselves, since allele-specific variation in *TSC1* and *TSC2* mRNA expression has been recently observed in healthy volunteers[48]. Furthermore, environmental factors such as gestational immune activation may confer an increased risk for some traits[49]. In addition, some of the variability could be due to the stochastic nature of second-hit events occurring during brain development and maturation. These events are associated with the different lesion types found in the brains of individuals with TSC, like tubers, subependymal nodules and subependymal giant cell astrocytomas[50-52].

TSC patients can have remarkably high tuber numbers, sometimes exceeding 50. This suggests the timing and/or frequency of second hit mutations during brain development may be critical for determining the tuber load. Interestingly, emergent clues point to a mechanism whereby TSC patients could have a higher than average intrinsic tendency to accumulate mutations in any gene, including *TSC1* and *TSC2*, resulting in a large number of brain lesions. Evidence comes from a series of studies in which TSC2 was found to regulate expression levels of 8-oxoG-DNA glycosylase (OGG1), a DNA repair enzyme involved in the repair of oxidative DNA damage[53, 54]. Accordingly, OGG1 levels were found to be decreased in kidney lesions of the Eker rat and TSC patients, accompanied by an accumulation of 8-oxodG species[53]. This finding remains to be confirmed in brain tissue. Notably, mice lacking *Ogg1* in the brain display an increased mutation frequency when prenatally exposed to low doses of radiation[55]. It would be interesting to investigate whether *Tsc1* and *Tsc2* heterozygous knock-out mice exhibit enhanced mutation rates when challenged with low doses of radiation during the prenatal period[55].

Somatic mutations that occur during late brain development could also result in additional sub-radiological aberrations in brain morphology[56]. These *TSC1* or *TSC2* deficient spots can be expected to have similar epileptogenic characteristics to the *TSC1* and *TSC2* deficient neurons in TSC brain lesions and may also disturb the networks involved in cognitive function (see Figure 1). Thus, these spots may significantly contribute to brain dysfunction, especially in severely affected individuals with minimal tuber loads.

To further investigate this hypothesis, postmortem brains of TSC patients, instead of only tuber tissue, could be investigated for the presence of small regions containing pS6 positive cells that have distinct morphology to the surrounding tissue. Using a similar approach to that employed recently by Crino *et al*[57], these regions could be microdissected and analysed for somatic *TSC1* and *TSC2* mutations. Possibly, these regions may be visualized using advanced MRI techniques. Subsequently, it should be determined whether there are correlations between the number and location of these regions (visible on MRI) and the severity of neurological symptoms, or whether specific regions (visible on MRI) would correlate with epileptiform activity. This mechanism could also be studied in mouse models. We observed that a small percentage of the inducible *Tsc1*$^{-/-}$ mice described in **Chapter 4** developed epilepsy before they received tamoxifen injections. Perhaps, these mice have lost the second copy of the *Tsc1* allele in some cells during brain development due to leakiness of the Cre system. By undertaking histological analysis of the entire brains of these mice, it can be tested whether these epileptic mice indeed show focal malformations and whether an increased number of such foci

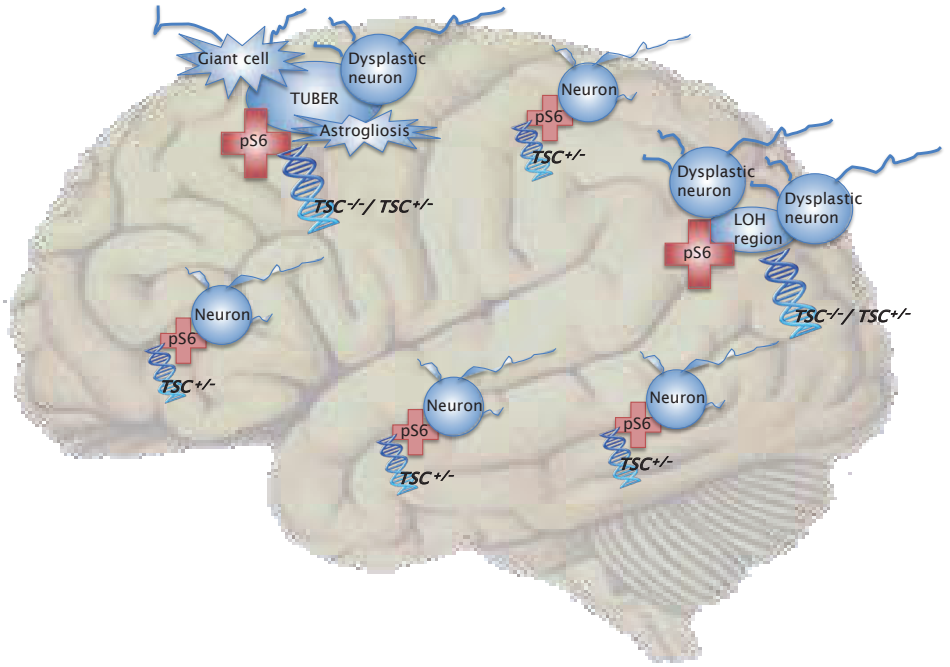

Figure 1. Heterozygosity and homozygosity in the TSC brain. Schematic of the different cell populations and structural abnormalities that could contribute to neuropsychiatric symptoms in TSC. Tubers are thought to develop when early progenitor cells undergo LOH. Only a fraction of the cells within a tuber appear to have lost the second allele (pS6 positive giant cells[50]), indicating that these cells could exert non-autonomous effects on their neighbouring cells. Somatic mutations that occur during late brain development could also result in additional sub-radiological aberrations in brain morphology (depicted here as LOH regions). In addition, *TSC1*$^{+/-}$ or *TSC2*$^{+/-}$ neurons probably also display abnormalities in axon guidance and synaptic plasticity, as is shown for mouse neurons. Neuropsychiatric symptomatology in TSC probably results from a combination of these mechanisms.

predispose individual mice to epilepsy. Focal loss of the remaining *Tsc1* allele could possibly be stimulated by injecting the mice with suboptimal doses of tamoxifen. In conclusion, this is an interesting new area of research that should be explored further and that may provide us with an explanation for the broad spectrum of cognitive function observed in TSC patients and also for how epilepsy can develop in the absence of any obvious, macroscopic brain lesions.

TSC-TOR SIGNALING

In **Chapter 5** we investigated the role of Rheb1, the downstream target of the TSC1-TSC2 complex, in development. Similar to genetic deletion of other components of the TORC1 signaling branch, homozygous germ-line deletion of *Rheb1* resulted in embryonic lethality[61, 62]. These embryos died later than *mTor*$^{-/-}$ embryos, most likely due to residual TORC1 activity and intact TORC2 activity[61-64]. Importantly, *Rheb2* is

not essential for embryonic survival and TORC1 activation *in vivo*, and *Rheb1* has been shown to be dispensable for early brain development, but not for postnatal myelination[61]. Furthermore, we found that *Rheb1* heterozygosity extended the life span of *Tsc1*$^{-/-}$ embryos, indicating that there is a genetic interaction between the *Tsc1* and *Rheb1* genes in mouse.

It is not yet clear how Rheb1 regulates TORC1 function[13]. It is unlikely that Rheb1 regulates TORC1 activity by direct binding alone, since the strength of the interaction between Rheb1 and TORC1 is independent of Rheb1 nucleotide loading status, and inactive *Rheb1* mutants still retain the ability to bind but not to activate TORC1[65]. Two additional mechanisms have been proposed by which Rheb1-GTP can activate TORC1. First, Bai *et al* reported the existence of FK506 binding protein38 (FKBP38), an endogenous inhibitor of TORC1 that acts in a similar manner to the FKBP12–rapamycin complex[66]. Active Rheb1 was found to interact directly with FKBP38, thereby preventing its association with TORC1[66]. However, FKBP38 is unlikely to be the much-sought link by which Rheb1 controls TORC1 activity because the Rheb1-FKBP38 interaction can only be detected under specific conditions[67, 68]. Second, Rheb1 has been found to control TORC1 activity via production of the second messenger phospatidic acid that positively interacts with TORs rapamycin-binding domain[69, 70]. Clearly, future studies are needed to determine the contributions of these different effectors to TORC1 activation and to come up with a comprehensive picture of Rheb1-dependent mTORC1 regulation[65]. *Rheb1*$^{-/-}$ mouse embryo fibroblasts may be useful for the identification of these effectors. Proteins that bind competitively with Rheb1 and TORC1 should be enriched in TORC1, in the absence of Rheb1.

Typically, small G-proteins like Rheb1 are negatively regulated by GTPase activating proteins (GAPs) and positively regulated by guanine exchange factors (GEFs). For Rheb1, the TSC1-TSC2 complex is, thus far, the only established GAP counteracting Rheb1 activation[59]. Moreover, despite *in vitro* observations implicating a relative intracellular excess of Rheb1 GEFs compared to Rheb1 inhibitors[71], a specific Rheb1 GEF remains elusive[68, 72, 73]. It can be anticipated that the mechanisms controlling Rheb1 activity and the Rheb1-TORC1 interaction will be revealed in the near future. Since these proteins function in close proximity to the TSC1-TSC2 complex in regulating TORC1 activity, they could be employed as potential drug targets for treating TSC.

Targeting TORC1

In **Chapter 6** we observed intact learning and plasticity in *Rheb1*$^{+/-}$ and inducible *Rheb1*$^{-/-}$ mutant mice, despite significant reductions in TORC1 activity. Previous studies using acute rapamycin treatments implicated a role for TORC1 signaling in long-term memory formation and its presumed cellular correlate, L-LTP[74]. However, it is important to note that these studies show inconsistencies in that in some cases very high concentrations of rapamycin (for example 140 mg/kg)[3] were required to impair learning and plasticity in mice. This may have caused off-target effects. Therefore, the translational relevance of these earlier findings to rapalog treatments in humans is questionable. From our study we can conclude that TORC1 hypo-activity does not necessary lead to plasticity and learning deficits in mice.

In the future it should be tested whether long-term (weeks to months) rapamycin treatment in wild-type mice yields similar results to our genetic approach. This would provide important information about possible cognitive side-effects of TORC1 inhibitor therapy.

CONCLUSION

Our studies showed that neurological and cognitive dysfunction in TSC can develop in the absence of neuropathology, indicating that these symptoms may be sensitive to treatment initiated later in life. Furthermore, we found that Rheb1 is an essential activator of TORC1 and is indispensable for embryonic development. In contrast, deleting *Rheb1* and thereby reducing Rheb1-TORC1 signaling in adult life has minimal effect on learning and synaptic plasticity. These latter results suggest that TORC1 inhibitor therapy will not necessarily result in cognitive side-effects.

Since the cloning of the *TSC1* and *TSC2* genes, and the identification of their role in the TORC1 signaling network, a large research effort has been dedicated to unraveling the cellular and molecular mechanisms of the CNS manifestations associated with TSC. Our studies, amongst many others, contribute to the preclinical framework on which current attempts to target CNS manifestations using TORC1 inhibitors are based.

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SUMMARY
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SUMMARY

Tuberous Sclerosis Complex (TSC) is an autosomal dominant multi-organ disease, characterized by the development of benign lesions affecting the skin, kidney, heart and brain, and by specific neurological and neuropsychiatric symptoms. The neurological and neuropsychiatric symptoms are diverse and include epilepsy, intellectual impairments, autistic spectrum disorders, attention-deficit hyperactivity disorder, mood and anxiety disorders and specific neuropsychological deficits. Strikingly, it has been estimated that only 10% of TSC patients do not experience significant impairments in daily life because of cognitive and psychiatric symptoms.

TSC is caused by mutations to the *TSC1* and *TSC2* genes. The *TSC1* and *TSC2* gene products form a protein complex, the TSC1-TSC2 complex, that functions as an inhibitor of the small GTPase Rheb in the TORC1 signaling pathway. The identification of the physiological role of the TSC1-TSC2 complex and the development of several TSC mouse models have significantly accelerated study into the pathophysiology of the disease. The overall objectives of this thesis were to investigate the causal relations between brain lesions, epilepsy, and cognitive and behavioral symptoms in TSC, and to gain more insight into the function of Rheb, the main target of the TSC1-TSC2 complex.

In **Chapter 2** we summarize current knowledge on the pathophysiology underlying the neuropsychiatric symptoms of TSC and related neurocutaneous syndromes. In the subsequent years an increasing number of disease have been associated with TORC1 hyperactivity. Collectively these are referred to as TORCopathies. Therefore, TSC can be viewed as a model system with relevance to many disorders.

It has been a long-prevailing belief that tubers (a typical TSC brain lesion) and epilepsy represent the correlate for the neuropsychiatric symptoms associated with TSC. Yet, studies assessing the relationships between tuber load and intellectual disabilities or autism have often yielded contradictory and confusing results. Tuber load and epilepsy explain only part of the variability in cognitive ability. In **Chapter 3**, we reinvestigated the relationships between cerebral lesions, epilepsy and cognitive function using *Tsc1*$^{+/-}$ mice. We observed no spontaneous seizures or cerebral lesions in the brains of these mice. In addition, giant dysmorphic cells were absent, and spine number and dendritic branching appeared to be normal. Nevertheless, *Tsc1*$^{+/-}$ mice showed impaired learning in hippocampus-dependent learning tasks and showed impaired social behavior. These findings support a model in which *TSC1* or *TSC2* haploinsufficiency leads to aberrations in neuronal functioning, resulting in impaired learning and social behavior.

Epilepsy in TSC appears to be related to the presence of tubers as epileptiform activity often correlates to the location of a tuber. Furthermore, surgical removal of a dominant epileptogenic tuber zone frequently has beneficial effects with respect to seizure control. However, the precise mechanisms underlying epilepsy in TSC are still not fully understood. For instance, up to 10% of individuals with TSC suffering from intractable epilepsy have normal brain MRIs, with no evidence of cortical tubers, while other patients with a high tuber load do not develop epilepsy, indicating that there may be other processes contributing to epilepsy development in TSC. In addition, even when a causative tuber can be identified, it is not clear

which components of these complex structures trigger the epilepsy. Possibly, the neuropathological disturbance of the local circuitry is essential for the development of epilepsy. Alternatively, aberrant *TSC1* or *TSC2* deficient neurons in these lesions could experience molecular abnormalities that instigate seizures. In **Chapter 4** we took a reductionist approach to this question and tested whether acute, global homozygous deletion of the *Tsc1* gene in adult mice is sufficient to trigger seizure onset in the absence of any developmental neuropathology. We observed that mice developed seizures and abnormal interictal EEG patterns upon loss of the *Tsc1* gene. In addition, we observed biochemical alterations, with enhanced TORC1 and ERK activity. Finally, we found that acute deletion of *Tsc1* resulted in a decreased threshold for protein-synthesis-dependent synaptic potentiation. Taken together, these data indicate that intact levels of TSC1 protein are required to prevent unrestrained synaptic potentiation and epilepsy. These findings provide novel evidence that loss of the TSC1-TSC2 complex can directly contribute to seizure development in TSC patients.

The TSC1-TSC2 complex functions as an inhibitor of the small GTPase Rheb in the TORC1 signaling pathway. To gain further insights into the TSC1-TSC2 complex-Rheb-TORC1 signaling axis, we inactivated the *Rheb1* gene in mice (**Chapter 5**). We observed that *Rheb1*$^{-/-}$ embryos died around midgestation, most likely due to impaired development of the cardiovascular system. *Rheb1*$^{-/-}$ embryonic fibroblasts showed decreased TORC1 activity, were smaller and showed impaired proliferation. Furthermore, we found that *Rheb1* heterozygosity extended the life span of *Tsc1*$^{-/-}$ embryos, indicating that there is a genetic interaction between the *Tsc1* and *Rheb1* genes in mouse.

TORC1 inhibitor therapy holds great promise for treating a number of diseases, not only genetic syndromes like TSC but also sporadic cancers, aging-related disorders and epilepsy. However, such treatments are likely to be long lasting and could involve young children. Therefore it is vital that the effects of sustained TORC1 inhibition on cognitive function are determined. In **Chapter 6** we addressed this by using heterozygous *Rheb1* knock-out mice which show a significant reduction of hippocampal TORC1 activity, but develop normally and show intact synaptic plasticity and learning. Reducing TORC1 activity further in inducible homozygous *Rheb1* knock-out mice also had no effect on these measures. Our findings indicate that sustained TORC1 hypo-activity does not necessarily lead to impaired cognitive function, in mice.

Since the identification of the *TSC1* and *TSC2* genes, and the subsequent placement of the TSC1-TSC2 complex within the TORC1 signaling axis, a large research effort has been dedicated to unraveling the pathogenetic cellular and molecular mechanisms underlying the CNS manifestations associated with TSC. Our studies, amongst many others, contribute to the preclinical framework on which current attempts to target CNS manifestations using TORC1 inhibitors are based.

SAMENVATTING

Tubereuze Sclerose Complex (TSC) is een autosomaal-dominant overervende ziekte die meerdere organen aantast. De ziekte wordt gekenmerkt door de ontwikkeling van goedaardige tumoren aan de huid, de nieren, het hart en het brein en door het optreden van specifieke neurologische en neuropsychiatrische symptomen. Deze symptomen zijn divers en kunnen bestaan uit epilepsie, verstandelijke handicap, autisme spectrum stoornissen, aandachts-tekort-stoornis met hyperactiviteit, stemmings- en angststoornissen en neuropsychologische functiestoornissen. Slechts 10% van de TSC patiënten ondervindt in het dagelijkse leven geen hinder van hun neuropsychiatrische problematiek.

TSC wordt veroorzaakt door inactiverende mutaties in de *TSC1* en *TSC2* genen. De TSC1 en TSC2 eiwitten vormen een eiwit complex, het TSC1-TSC2 complex, dat het kleine GTPase eiwit Rheb remt als onderdeel van de TORC1 signaalroute. Het achterhalen van de functie van het TSC-TSC2 complex en de ontwikkeling van verschillende TSC muismodellen heeft het onderzoek naar de pathofysiologie van de ziekte in een stroomversnelling gebracht. De doelstellingen van dit proefschrift zijn om de oorzakelijke verbanden tussen hersentumoren, epilepsie en neuropsychiatrische symptomen bij TSC op te helderen en om meer inzicht te krijgen in de functie van Rheb, het doeleiwit van het TSC1-TSC2 complex.

In **Hoofdstuk 2** geven we een overzicht van de aanwezige kennis over de pathofysiologie van de neuropsychiatrische symptomen optredend bij TSC en verwante neurocutane syndromen. In de daaropvolgende jaren zijn een groot aantal ziektes geassocieerd met overactiviteit van de TORC1 signaalroute. Deze worden geschaard onder de nieuwe term: TORCopathies. Hierdoor kan TSC worden beschouwd als een modelziekte die relevantie heeft voor vele verwante ziektes.

Het is lang een dogma geweest dat tubers (een goedaardige hersentumor die kenmerkend is voor TSC) en epilepsie de oorzaken zijn voor de neuropsychiatrische problematiek die gezien wordt bij TSC. Echter, studies die het oorzakelijk verband tussen tubers en verstandelijke handicap of autisme hebben onderzocht, hebben inconsistente resultaten opgeleverd. Tubers en epilepsie verklaren ook maar een gedeelte van de variantie in IQ waardes. In **Hoofdstuk 3** hebben we de oorzakelijke verbanden tussen tubers, epilepsie en cognitief functioneren opnieuw onderzocht, ditmaal in *Tsc1*$^{+/-}$ muizen, die een vergelijkbare mutatie hebben als TSC patiënten. We hebben geen tubers aangetroffen in de hersenen van deze muizen en ook geen subtiele morfologische afwijkingen, zoals dysmorfe neuronen of verschillen in vertakkingen en contactpunten. Daarnaast ontwikkelen deze muizen geen spontane epileptische aanvallen. Desondanks namen we waar dat deze *Tsc1*$^{+/-}$ muizen minder goed presteren in spatiële leertaken en in een test voor sociaal gedrag. Deze bevindingen wijzen erop dat een heterozygote mutatie in het *TSC1* of *TSC2* gen afwijkingen veroorzaakt in het functioneren van neuronen, resulterend in verminderd cognitief en sociaal functioneren.

Epilepsie bij TSC patiënten heeft te maken met de aanwezigheid van tubers, aangezien epileptiforme activiteit vaak correleert met de locatie van een tuber. Daarnaast leidt het verwijderen van een tuber frequent tot een vermindering van de epilepsie. Echter, hoe precies epilepsie ontstaat bij TSC patiënten is onvolledig bekend. Bij sommige patiënten met ernstige epilepsie worden geen tubers gezien

op MRI, terwijl andere patiënten die veel tubers hebben, geen epilepsie ontwikkelen. Waarschijnlijk zijn er andere processen in werking die bijdragen aan het ontstaan van epilepsie bij TSC patiënten. Zelfs wanneer er een tuber kan worden geïdentificeerd die de epilepsie veroorzaakt, is het niet bekend welke componenten van de tuber precies epilepsie veroorzaken. Het is mogelijk dat de neuropathologie ter plaatste het neuronale circuit ontregelt wat tot epilepsie leidt. Een andere mogelijkheid is dat neuronen, die geen functioneel TSC1-TSC2 complex hebben, epileptogeen worden. In **Hoofdstuk 4** hebben we dit onderzocht door middel van een reductionistisch systeem: we hebben het *Tsc1* gen acuut uitgeschakeld in volwassen muizen. Snel na het uitschakelen van het *Tsc1* gen begonnen de muizen epilepsie te ontwikkelen. Daarnaast hebben we biochemische veranderingen waargenomen in de vorm van overactiviteit van de ERK en TORC1 signaalroutes. Tenslotte hebben we gevonden dat de grens voor het induceren van een eiwit-synthese afhankelijke vorm van synaptische plasticiteit verlaagd is in deze muizen. Deze bevindingen wijzen erop dat een intact TSC1-TSC2 complex noodzakelijk is om ongeremde synaptische potentiatie en epilepsie te voorkomen.

Het TSC1-TSC2 complex remt het kleine GTPase eiwit Rheb in de TORC1 signaalroute. Om meer inzicht te krijgen in de TSC1-TSC2 complex-Rheb-TORC1 signaalroute hebben we een *Rheb1* knock-out muis gecreëerd (**Hoofdstuk 5**). *Rheb1*$^{-/-}$ embryos gaan dood in het midden van de zwangerschapsperiode, waarschijnlijk door onvolledige ontwikkeling van het cardiovasculaire systeem. *Rheb1*$^{-/-}$ embryonale fibroblasten hebben verminderde activiteit van de TORC1 signaalroute en delen minder goed. Tenslotte hebben we waargenomen dat de *Rheb1*$^{+/-}$ mutatie het leven van *Tsc1*$^{-/-}$ embryos verlengt. Dit wijst erop dat er een genetische interactie is tussen de *Tsc1* en *Rheb* genen in de muis.

Voor veel verschillende ziektes lijkt behandeling met TORC1 remmers een succesvolle strategie te zijn. Niet alleen voor TSC, maar ook voor verschillende vormen van kanker, epilepsie en verouderingsziektes. Deze therapieën zijn waarschijnlijk chronisch en zullen al op kinderleeftijd moeten aanvangen. Daarom is het noodzakelijk dat de effecten van langdurige TORC1 inhibitie op cognitief functioneren worden onderzocht. In **Hoofdstuk 6** hebben we dit bestudeerd, gebruikmakend van *Rheb1*$^{+/-}$ muizen die een verlaging van TORC1 activiteit hebben. We vonden dat deze muizen geen stoornissen vertoonden in leren of in synaptische plasticiteit. Wanneer we de TORC1 activiteit nog verder verlagen door gebruik te maken van induceerbare *Rheb1*$^{-/-}$ muizen, nemen we ook geen stoornissen waar. Onze bevindingen wijzen erop dat het verlagen van TORC1 activiteit niet noodzakelijkerwijs leidt tot stoornissen in cognitief functioneren in de muis.

De identificatie van de *TSC* genen en de plaatsing van het TSC1-TSC2 complex in de TORC1 signaalroute heeft het onderzoek naar de cellulaire en moleculaire mechanismen van de neuropsychiatrische problematiek die optreedt bij TSC, geïnspireerd. Ons onderzoek, samen met dat van vele anderen, heeft bijgedragen aan een fundamentele kennisbasis waarop voortgebouwd kan worden bij de behandeling van neuropsychiatrische problematiek van TSC patiënten met TORC1 remmers.

LIST OF PUBLICATIONS

Susanna M.I. Goorden, Geeske M. van Woerden, Louise van der Weerd, Jeremy P. Cheadle, and Ype Elgersma. Cognitive deficits in *Tsc1*$^{+/-}$ mice in the absence of cerebral lesions and seizures. Ann Neurol (2007) 62 (6): 648-655.

Susanna M.I. Goorden*, Lianne C. Krab*, and Ype Elgersma. Oncogenes on my mind: ERK and MTOR signaling in cognitive diseases. Trends Genet (2008) 24 (10): 498-510.
*These authors contributed equally

Susanna M.I. Goorden, Marianne Hoogeveen-Westerveld, Caroline Cheng, Geeske M. van Woerden, Melika Mozaffari, Laura Post, Henricus J. Duckers, Mark Nellist, and Ype Elgersma. Rheb is essential for murine development. Molecular and Cellular Biology (2011) 31 (8): 1672-1678.

Susanna M.I. Goorden, and Ype Elgersma. Rheb: Enrichment beyond the brain. Cell Cycle (2011) 10 (15): 2412-2413.

Susanna M.I Goorden, Marianne Hoogeveen-Westerveld, Caroline F. Bruinsma, Elvedin Aganović, Elisabeth Abs, Nils Z. Borgesius, Mark Nellist, and Ype Elgersma. Deletion of the *Tsc1* gene in adult mice is sufficient to cause epilepsy. In revision for Annals of Neurology: new experiments planned for 2012.

Susanna M.I Goorden*, Elisabeth Abs*, Caroline F. Bruinsma, Ype Elgersma.
Intact plasticity and learning in *Rheb1* mutant mice: implications for rapamycin treatments. Manuscript in preparation.
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EDUCATION

Honors exchange Master program in Neuroscience
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Doctoraal (drs) in Medicine
2002-2005
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Gymnasium
1995-2001
Gertudiscollege, Roosendaal

WORK EXPERIENCE

Clinical chemist in training
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Medial Diagnostic Center, Haarlem&Hoofddorp

PhD student in the Department of Neuroscience
Sept 2007-Febr 2012
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PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student:	Susanna Goorden
Erasmus MC Department:	Neurosciences
Research School:	ONWAR
PhD period:	September 2007-February 2012
Promotor:	Prof. dr. Ype Elgersma

1. PHD TRAINING	YEAR	WORKLOAD
General courses		
» Laboratory animal science	2007	120 hours
» Classical methods for Data-analysis	2007	160 hours
Specific courses		
» SNPs and human disease	2010	40 hours
» Adobe and Photoshop course	2011	10 hours
(Inter)national conferences		
» 7th Dutch ENP meeting, Doorwerth, The Netherlands (poster)	2008	
» Rare disorders of the MAPK pathway, Barcelona, Spain	2008	
» TSC International Research Conference, Brighton, UK (plenary lecture)	2008	
» 8th Dutch ENP meeting, Doorwerth, The Netherlands	2009	
» 15th annual ONWAR PhD meeting, Zeist, The Netherlands (poster)	2009	
» TSC International Research Conference, Bloomingdale, USA (plenary lecture)	2009	
» 7th FENS Forum of European Neurosciences, Amsterdam, The Netherlands (poster)	2010	

2. TEACHING ACTIVITIES	YEAR	WORKLOAD
Lecturing		
» Teaching assistant for several anatomy courses for medical students	2007-2011	150 hours
» Workshops for Msc Neuroscience students	2009-2011	10 hours
Supervising		
» *High school thesis* Saskia Smits/Dieuwertje Bogart: 'Werking van het geheugen'.	2008	40 hours
» *Bachelor thesis* Laura Post: 'Het ontwerpen en toepassen van gedragstesten voor leren, geheugen en autistisch gedrag in muizen'.	2008-2009	6 months
» *Bachelor thesis* Melika Mozzafari: 'Inhibition of the mTOR signaling pathway through blockage of amino acid transporters'.	2009-2010	9 months
» *Bachelor thesis* Elvedin Aganović: 'Moleculaire en cellulaire mechanismen die ten grondslag liggen aan het geheugen en mentale retardatie'.	2010-2011	9 months
» *Master thesis* Elisabeth Abs: 'Exploring reversibility and treatment options for the cognitive deficits in Tuberous Sclerosis Complex'.	2010-2011	18 months

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