DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Epileptogenesis and Treatment Options in a Tuberous Sclerosis Complex Epilepsy Mouse Model

Cátia Alexandra Pinho Silva

2013

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Epileptogenesis and Treatment Options in a Tuberous Sclerosis Complex Epilepsy Mouse Model

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, com especialização em Neurobiologia, realizada sob a orientação científica do Professor Doutor Ype Elgersma (Centro Médico Universitário de Roterdão) e da Professora Doutora Emília Duarte (Universidade de Coimbra).

Cátia Alexandra Pinho Silva

2013

Acknowledgements

First, I wish to thank my supervisor Ype Elgersma, for accepting me as his Master student and always pushing me forward.

I am most indebted to my wonderful tutor Elisabeth, for her comprehension, support and creative solutions.

It is with immense gratitude that I acknowledge the scientific support and true friendship of Geeske, but also Sara and Jaga.

I thank everyone in the group for the friendly environment: Laura-Anne, Jolet, Iris, Edwin, Martijn, Thijs, Caroline, Sanne, Lana, Rianne, Dorine, Linda, Stefan and Madhi. Also, I am very thankful for the fellowship at the Department of Neuroscience, especially with the Kushner's lab and with Mark Nellist from the Clinical Genetics.

In the name of everyone working in the lab, I express my gratitude towards the unwearyingly technical support of Minetta, Mehrnoush, Nanda, Arshna, Erika and Elize: without them this project would not be possible.

Por fim, estou extremamente grata aos meus pais, pelo constante apoio e motivação, bem como ao João e ao Bruno, pela amizade incondicional com que me premeiam todos os dias.

May the synaptic connections that I have with each
and every one of you last forever.

Contents

Figures and Tables **5**

Abstract **11**

Resumo **13**

1. Introduction **15**

1.1 Molecular Mechanisms Underlying TSC 17

1.2 TSC Patients and Mouse Models 20

1.3 TSC Therapeutic Options 24

1.4 Open questions in TSC 25

1.5 Aim of the study 28

2. Materials and Methods **31**

2.1 Mice generation and breeding 33

2.2 EEG surgical procedure 34

2.3 EEG measurements 35

2.4 Tamoxifen administration 36

2.5 Immunohistochemistry 36

2.6 Western blot 37

2.7 Drug preparation 38

2.8 Statistical analysis 39

3. Results **41**

3.1 Characterization of *Tsc1*-αCaMKII mouse model 43

3.2 αCaMKII-driven acute deletion of *Tsc1* is sufficient to induce lethal epilepsy in adult mice 50

3.3 αCaMKII-driven acute deletion of *Tsc1* in adulthood is accompanied by mTORC1 hyperactivation 55

3.4 Epileptogenesis in *Tsc1*-αCaMKII mice 59

3.5 No obvious brain pathology in *Tsc1*-αCaMKII mice 63

3.6 Testing novel therapeutic options for TSC 65

3.7 Preliminary findings in *Tsc1*-GFAP mouse model 70

4. Discussion **73**

5. Conclusion **83**

References **87**

Figures and Tables

Figure 1. Summarized scheme of the signaling pathway in TSC. In this disorder, the lack of a functional TSC1/2 complex leads to mTORC1 hyperactivation, culminating in uncontrolled cell growth and proliferation, as well as aberrant synapses. **19**

Table I. Molecular changes found in tuberal tissue from TSC patients **21**

Table II. Summary of the *Tsc1/2* conditional mouse models of TSC **23**

Figure 2. Initially (1), the *Tsc1* gene is flanked by loxP sites and the cre is fused to a mutated estrogen receptor (ER) in the cytosol (the grey “pacman”). Tamoxifen administration (2), a potent ligand of the ER, induces the translocation of the ER-cre complex to the nucleus (3), where the cre recombinase recombines the loxP sites, resulting in *Tsc1* gene deletion. If tamoxifen is not administered, or the mouse does not express the cre recombinase, the mouse remains similar to WT, i.e, $Tsc1^{+/+}$. **27**

Figure 3. The EEG surgical procedure takes place with the mouse deeply anesthetized (1). An incision is made in the skin and fascia overlying the skull (2). Stainless steel screws (a) are used as EEG electrodes (3) and implanted in the skull at the approximate coordinates shown in b. In the end, the other end of the electrodes is plugged in a connector (highlighted in orange) and covered with dental cement (4). Scheme in (b) is adapted from George Paxinos and Keith Franklin: The **35**

Mouse Brain in Stereotaxic Coordinates, *Academic Press*, 2001.

Figure 4. *Tsc1*$^{f/f}$ mice were crossed with the cre line carrying the αCaMKII promoter until the *Tsc1*$^{f/f}$-αCaMKII-er$_{T2}$-cre construct, with cre+ and cre- littermates, was obtained. **43**

Figure 5. PCR for *Tsc1* using different brain areas and liver of *Tsc1*-αCaMKII mice. Only cre+ tamoxifen injected mice show partial deletion in cortex and hippocampus. f: floxed band; -: deletion band **44**

Figure 6. TSC1 and pS6/S6 levels in αCaMKII-positive brain regions of *Tsc1*-αCaMKII mice that underwent *Tsc1* deletion: hippocampus (Hc), cortex (Ct) and cerebellum (Cb). All protein levels were normalized to the respective brain area in control mice (1 unit, dark line). All brain areas show decreased TSC1 levels, following *Tsc1* gene deletion. However, only hippocampus and cortex show mTORC1 hyperactivation (increased pS6/S6 levels). n=2 per group (preliminary data). **45**

Figure 7. Sagital sections (5 x magnification) of a control (left) and a mutant *Tsc1*-αCaMKII mouse brain stained with an antibody against pS6. pS6-positive cells are highly present in cortex and hippocampus of the mutant *Tsc1*-αCaMKII mouse, while no differences are found in cerebellum. n=4 per group. **47**

Figure 8. pS6-positive cells (red) highly co-localize with αCaMKII-positive cells (green) in cortex (**A**, 10x; **B**, 20x) and hippocampus (**C**, 5x, **D**, 20x dentate gyrus, **E**, 20x CA3 region) of *Tsc1*-αCaMKII mice. n=5. **48**

Figure 9. pS6-positive cells (red) do not co-localize with PV-positive cells (green) in cortex (**A**, 10x; **B**, 20x) and hippocampus (**C**, 5x, **D**, 20x dentate gyrus, **E**, 20x CA3 region) of *Tsc1*-αCaMKII mice. **49**

Figure 10. Adopted protocol to assess epilepsy in *Tsc1*-αCaMKII mice through EEG recordings. After the EEG surgical procedure the mice are given a minimum of two days of rest. Then, two vehicle injections are administered and baseline measurements are acquired. After one day of rest, gene deletion is induced by tamoxifen administration and EEG measurements are acquired every night. **50**

Figure 11. Examples of the observed types of recordings (**A**, baseline measurement; **B**, increased activity; **C**, seizure; **D**, lethal seizure) and the wireless neurologger device **50**

Figure 12. The epilepsy onset of tamoxifen-injected cre+ mice varies between 9 and 12 days after the first tamoxifen injection in adulthood. Tamoxifen-injected cre- mice don’t show epilepsy. n=10. **51**

Figure 13. Lifespan of tamoxifen-injected cre+ mice varies between 12 and 16 days after the first tamoxifen injection in adulthood. Tamoxifen-injected cre- mice don’t show decreased lifespan. n=4. **52**

Figure 14. Number (per 16h of recording; upper graph) and length of seizures (in seconds; lower graph) of the recorded mice. Each color represents one mouse. n=3. **52**

Figure 15. TSC1, TSC2, pS6/S6 and pAkt/Akt levels in control mice (c; **55**

cre- tamoxifen-injected) and mutant mice (tamoxifen injected cre+) at day 8 (8; pre-epileptic state) and day 13 (13; epileptic state). Significant differences are shown by * (p=0.01, 1-way ANOVA), n=4 per group.

Figure 16. NR1, NR2A and NR2B NMDA subunits and GluR1 AMPA subunit levels in hippocampal total lysates of control (c) and mutant mice at day 8 (8) or day 13 (13). n=4 per group, no significant differences were found (preliminary data). **59**

Figure 17. NR2B, NR2A and NR1 NMDA subunits and GABA$_A$Rα1 subunit levels in cortical synaptosomes of control (c) and mutant mice at day 8 (8) and day 13 (13). n=4 per group, no significant differences were found (preliminary data). **61**

Figure 18 No differences in gross brain morphology are observed in the hippocampus of *Tsc1*-αCaMKII mice (control on the left, mutant on the right). (All perfused brains were screened and no changes were found.) **63**

Figure 19 No pS6-stained giant cells are found in the cortex of *Tsc1*-αCaMKII epileptic mice (10 x magnification on the left, 40 x magnification on the right). **64**

Figure 20. AZD8055 (10 mg/kg) is able to downregulate mTORC1 (pS6/S6 levels) and mTORC2 (pAkt/Akt levels) in the hippocampus of WT mice. n=2 per group (preliminary data). **65**

Figure 21. The effect of GD124 on mTORC1 and mTORC2 in the hippocampus of WT mice is inconclusive. n=2 per group (preliminary **66**

data).

Figure 22. Number (per 16h of recording; upper graph) and length of seizures (in seconds; lower graph) of the recorded mice. Each color represents one mouse: green, vehicle-injected mouse; red and purple: PF-4708671(75 mg/kg)-treated mice. Treatment was administered at the moment indicated by the arrow, i.e, the day following the first night of seizures. (Preliminary data.) **68**

Figure 23. PCR for *Tsc1* in the *Tsc1*-GFAP mice under different conditions. Numbers code for brainstem tissue of adult mice: **1**, 4 tamoxifen injections; **2**, 8 tamoxifen injections. Letters code for samples from a 4 weeks old mouse injected 8 times with tamoxifen: **a**, brainstem; **b**, cortex; **c**, hippocampus; **d**, cerebellum. A weak deletion band is only observed in the brainstem and hippocampus of 4 weeks old mice injected 8 times with tamoxifen. f: floxed band; -: deletion band **70**

Abstract

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder, resulting from inactivating mutations in either *Tsc1* or *Tsc2* genes. It is characterized by multisystemic benign tumors throughout the body, including the brain, together with neurological abnormalities such as epilepsy, autism and mental retardation (Han and Sahin, 2011; Orlova and Crino, 2010).

The TSC1/2 protein complex has been found to play a crucial role in the regulation of cell growth and proliferation via the mTORC1 pathway. In the central nervous system this complex is also responsible for orchestrating finely tuned systems that have distinctive roles under different conditions; such as dendritic arborisation or axonal outgrowth and targeting (Orlova and Crino, 2010).

Given that 80 to 90% of all patients develop epileptic seizures (Han and Sahin, 2011), epileptogenesis is probably the most devastating and therapeutically challenging manifestation of TSC. Therefore, a rational preventive strategy is the targeting of the mTORC1 pathway, for its known contribution to epileptogenesis through protein synthesis and synaptic plasticity (Wong, 2010).

Our lab has previously shown that global *Tsc1* deletion in adult mice (using the CreER/loxP recombination system) results in epilepsy due to mTORC1 hyperactivation (Abs et al., *in press*).

In order to further dissect the molecular mechanisms underlying epileptogenesis in TSC, we now induce *Tsc1* deletion in adult mice, in a brain type cell-specific manner: using the *Tsc1*$^{f/f}$ αCaMKII-er$_{T2}$-cre mouse model. These mice develop lethal epilepsy a few days after gene deletion onset (assessed by EEG measurements). This epilepsy is accompanied by mTORC1 hyperactivation (assessed by western blotting and immunohistochemistry) and no obvious brain pathology (immunohistochemistry).

Moreover, this mouse model is hereby used to test possible treatments for epilepsy in TSC. We use different treatment options, such as dual mTOR inhibitors and a S6K inhibitor (contributing also to the understanding of epileptogenesis in these mice).

Together with other cell-specific *Tsc1* inducible KO mouse models, this project allows us to investigate the contribution of different types of brain cells to epilepsy (particularly in TSC). It will also help unraveling the molecular changes caused by acute mTORC1 hyperactivation (after all the developmental stages have taken place) that induce epileptogenesis. In addition, we are able to test novel treatment options for epilepsy in our mouse model.

This project and the use of complementary mouse models are of great importance to both the scientific and medical fields, since they aim at a better understanding of epilepsy in TSC and the investigation of possible treatments.

Keywords: TSC **|** Epilepsy **|** mTORC1 **|** Treatment

Resumo

O Complexo Esclerose Tuberosa (TSC) é uma doença autossómica dominante, resultante de mutações inativantes em qualquer um dos genes *Tsc1* ou *Tsc2*. A doença é caracterizada por tumores benignos espalhados pelo corpo, incluindo no cérebro, juntamente com anomalias neurológicas tais como: epilepsia, autismo e atraso mental (Han and Sahin, 2011; Orlova and Crino, 2010).

O complexo proteico TSC1/2 desempenha um papel crucial na regulação do crescimento e proliferação celular através da via mTORC1. No SNC este complexo também é responsável por orquestrar sistemas finos com papéis distintos sob diferentes condições como arborização dendrítica ou crescimento e orientação axonal (Orlova and Crino, 2010).

Tendo em conta que 80 a 90% de todos os pacientes desenvolvem convulsões epilépticas (Han and Sahin, 2011), a epileptogénese é provavelmente a manifestação mais devastadora e terapeuticamente a mais desafiante em TSC. Uma estratégia preventiva racional tem como objectivo a modulação da via mTORC1, pela sua conhecida contribuição na epileptogénese através da síntese proteica e plasticidade sináptica (Wong, 2010).

O nosso laboratório demonstrou anteriormente que a eliminação global de *Tsc1* em ratinhos apenas na idade adulta (usando o sistema de recombinação CreER/loxP) resulta no desenvolvimento de epilepsia devido à hiperactivação da via mTORC1 (Abs et al., *in press*).

De forma a dissecar os mecanismos moleculares subjacentes à epileptogénese em TSC, induzimos agora a eliminação de *Tsc1* em ratinhos apenas na idade adulta, mas apenas em células específicas do cérebro: o modelo *Tsc1*$^{f/f}$ αCaMKII-er$_{T2}$-cre. Estes ratinhos desenvolvem epilepsia letal logo após o início da deleção do gene (comprovado por meio de medições de EEG). Esta epilepsia é acompanhada por hiperativação da via mTORC1 (verificado por western blotting e imunohistoquímica) e é livre de patologia cerebral (imunohistoquímica).

Além disso, este modelo de ratinho será aqui usado para testar possíveis tratamentos para a epilepsia em TSC. Temos testado diferentes opções de tratamento tais como: inibidores duais de mTOR e um inibidor da S6K (contribuindo também para a compreensão da epileptogénese nestes ratinhos).

Juntamente com outros modelos de ratinho passíveis de sofrerem eliminação induzida de *Tsc1*, este projecto permite-nos investigar a contribuição de diferentes tipos celulares para a epilepsia (particularmente em TSC). Este protejo também nos ajudará a desvendar as alterações moleculares causadas pela hiperactivação aguda da via mTORC1 (após todas as fases de desenvolvimento terem ocorrido) que induzem epileptogénese. Além disso, poderemos testar novas opções terapêuticas para epilepsia com este mesmo modelo.

Este projecto juntamente com o uso de modelos complementares de ratinho são de grande importância para ambos os domínios: científicos e médicos, uma vez que visam uma melhor compreensão da epilepsia em TSC e investigação de tratamentos emergentes.

Palavras-chave: TSC | Epilepsia | mTORC1 | Tratamento

Chapter 1

Introduction

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder due to heterozygous inactivating mutations of either *Tsc1* or *Tsc2* tumor suppressor genes causing the development of benign tumors, epilepsy and cognitive deficits (Crino et al., 2006).

The prognosis of the patients depends on the severity of the symptoms and is very variable, from doing well and living long productive lives, to suffering from serious disabilities and dying from kidney failure, brain tumor or *status epilepticus* (Crino et al., 2010).

1.1 Molecular Mechanisms underlying TSC

The encoded proteins of the *Tsc1* and *Tsc2* genes are, respectively, TSC1 or hamartin and TSC2 or tuberin (see Figure 1). Together, they build up a complex where hamartin stabilizes and regulates tuberin, which then directly downregulates Rheb (Ras homologue enriched in brain) through its GAP (GTPase-activating protein) activity. Rheb, in its activated form (GTP-bound), is able to promote mTORC1 (mammalian or mechanistic target of rapamycin complex 1) catalytic activity in an amino acid availability-dependent manner (Huang and Manning, 2008).

The mTORC1 is roughly composed by mTOR, the rapamycin-sensitive positive regulator RAPTOR (regulatory-associated protein of mTOR) and the negative regulator DEPTOR (DEP domain-containing mTOR-interacting protein). It controls several pathways that determine the mass or size of the cell, and it is also involved in axon guidance and temporal regulation of cell growth through protein translation (Huang and Manning, 2008). As shown in Figure 1, mTORC1 has three main downstream targets, addressed next:

1. mTORC1 phosphorylates the S6K1 (ribosomal S6 kinase 1) on Thr389, thereby activating it. In turn, S6K1 phosphorylates and activates S6 (40S ribosomal S6 protein) on Ser235/6, enhancing translation of mRNAs (Huang and Manning, 2008).

2. Phosphorylation of 4EBP (eukaryotic translation initiation factor 4E-binding protein) by mTORC1 stimulates cap-dependent translation of mRNAs that encode for proteins associated with the proliferative response and the transition from G1 to S phase in the cell cycle, critical for oncogenesis (Tsukiyama-Kohara et al., 2001).

3. mTORC1 also has a direct negative influence on autophagy (very important for energy homeostasis and essential for normal development), inhibiting its classical initiators Ulk1 and Atg13 (autophagy-related protein 13). Autophagy levels are low in *Tsc2* null cells and this deregulation may probably contribute to the neurologic manifestations of TSC (McMahon et al., 2012).

mTOR can also associate with rapamycin-insensitive Rictor (rapamycin-insensitive companion of mTOR), building up mTORC2 and being affected by rapamycin only under longer exposure times (Huang and Manning, 2008). mTORC2 signals to the actin cytoskeleton, controlling its polarity and thereby determining the shape of the cell. Akt (Protein kinase B) is activated by phosphorylation at Ser473 by mTORC2, in a PI3K (Phosphoinositide 3 kinase) dependent manner, promoting cell survival, growth and proliferation, as well as angiogenesis (Tavazoie et al., 2005).

mTORC1 and 2 regulate each other: mTORC2’s signaling to Akt is a positive regulator of mTORC1. In turn, mTORC1 hyperactivation negatively modulates Akt through phosphorylated S6K mediated feedback loop (see

Figure 1). Although the involvement of mTORC2 in TSC is not entirely understood, there is some evidence of mTORC1 hyperactivation and reduced mTORC2 signaling (Tavazoie et al., 2005).

In summary, the TSC1/2 complex is a key regulator of the mTORC1 signaling pathway, serving as a hub for receiving a wide variety of cellular upstream signals (from growth factors or nutrients to inflammatory and stress signals), playing a major role in controlling protein synthesis, cell growth and proliferation, synaptic plasticity and axon specification and guidance (see scheme in Figure 1).

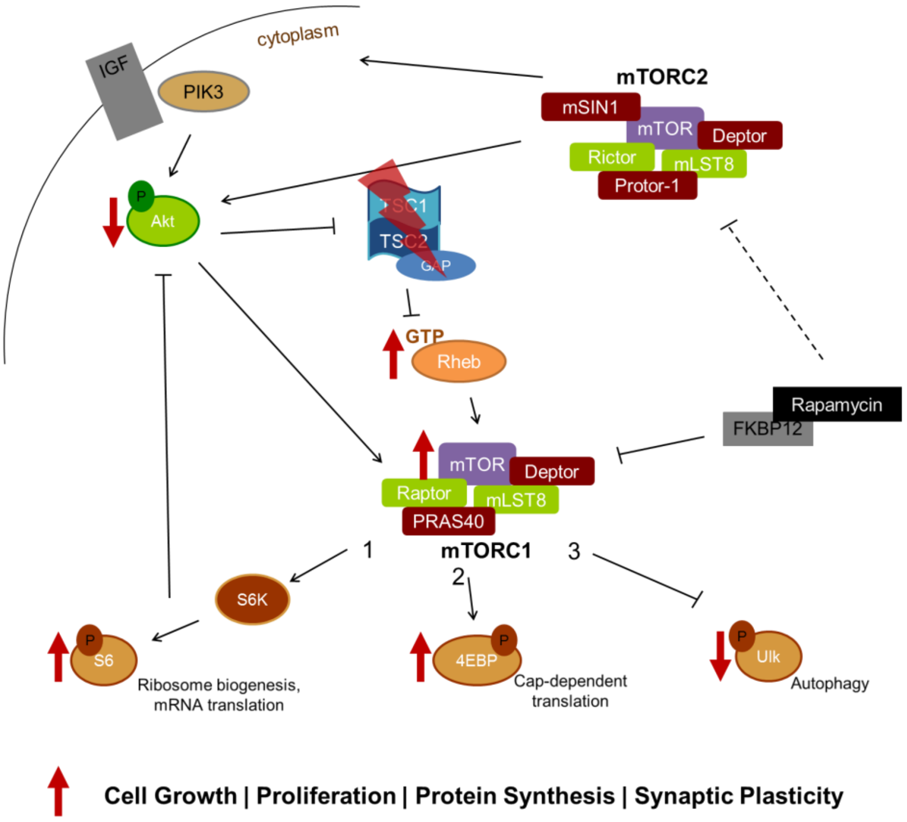

cytoplasm
IGF
PIK3
mTORC2
mSIN1
mTOR
Deptor
Rictor
mLST8
Protor-1
P
Akt
TSC1
TSC2
GAP
GTP
Rheb
Rapamycin
FKBP12
mTOR
Deptor
Raptor
mLST8
PRAS40
1
mTORC1
3
2
S6K
P
S6
Ribosome biogenesis,
mRNA translation
P
4EBP
Cap-dependent
translation
P
Ulk
Autophagy
Cell Growth | Proliferation | Protein Synthesis | Synaptic Plasticity

Figure 1. Summarized scheme of the signaling pathway in TSC. In this disorder, the lack of a functional TSC1/2 complex leads to mTORC1 hyperactivation, culminating in uncontrolled cell growth and proliferation, as well as aberrant synapses.

1.2 TSC Patients and Mouse Models

Several different TSC mouse models (with genetic engineered deletion of either *Tsc1* or *Tsc2* genes) were generated, aiming a better understanding of the different manifestations of the patients. In order to better understand how these models are comparable to TSC patients, I will hereupon discuss them side-by-side.

TSC is the second most common hereditary neurocutaneous tumor syndrome, characterized by the development of tumors throughout the body, such as in skin, kidneys, lung and heart. Accordingly, TSC mouse models with heterozygous mutations in either *Tsc1/2* genes also progressively develop peripheral tumors while aging, but no brain pathology (Onda et al., 1999).

The brain of the patients is as well very affected in this disorder, with the development of giant cell astrocytomas, cortical tubers (focal malformations composed by giant cells with disrupted migration) and subependymal nodules. These may enhance and enlarge over time, becoming subependymal giant cell astrocytomas (SEGAs), potentially inducing obstructive hydrocephalus and death (Crino et al., 2006). Analysis of brain tissue from TSC patients has further taught us that there is unbalanced excitatory versus inhibitory signaling in tubers (Wang et al., 2007). Furthermore, changes in the levels of Glutamate and GABA receptors subunits, growth factors, as well as in ion channels (also involved in familial forms of epilepsy) were found and are summarized in Table I.

Table I. Molecular changes found in tuberal tissue from TSC patients

AMPA-related	↑ GluR1, 4 (↑ Ca^{2+} permeability) [b, c] ↓ GluR2 and 3 [b, c]
NMDA-related	↑ ↓ NR2A (↓ Mg^{2+} sensitivity) [b, c] ↑ NR2B, 2C, 2D, 3A [a, c]
GABAR-related	↓ $GABA_A$ R α_1 (benzodiazepine insensitivity and GABA-mediated excitation) [c]
Ion channels	KCNQ2/3 [d] ↑ NKCC1 (deregulation Cl^- transporter) [c] ↓ KCC2 [c]
Others	↓ BDNF (immature transcription pattern) [c] Synapsin in dysplastic neurons (↑ network excitability) [b]

Legend: [a] (White et al., 2001); [b] (Talos et al., 2008); [c] (Talos et al., 2012) and [d] (Scott and Holmes, 2012)

The majority of the patients also show neurological and neuropsychiatric manifestations such as epilepsy, intellectual disability and autism spectrum disorders. Heterozygous TSC mouse models present cognitive deficits in the Morris water maze and contextual fear conditioning (Ehninger et al., 2008; Ehninger and Silva, 2011; Goorden et al., 2007); social deficits in social approach and nest building tests (Goorden et al., 2007; Sato et al., 2012) and anxiety with increased ultrasonic vocalizations between mother and pup (Ehninger and Silva, 2011; Young et al., 2010). Altogether, heterozygous TSC mouse models provided information concerning the tumors, cognition impairments and even autistic-like features. However, they did not allow the study of epilepsy, which is the most common neurological manifestation in TSC (affecting almost 90% of the patients) and probably also the most challenging and very incapacitating, since it often presents itself in a severe and intractable form, being resistant to available pharmacological intervention (Thiele, 2004).

Besides TSC-related, mTORC1 hyperactivation (following a seizure) was observed in several other models of epilepsy, such as in the kainate model (Wong 2010) or pentylenetetrazole-induced model (Zhang 2012).

Until recently, the neurologic manifestations were directly associated with the brain pathology. However, there are now descriptions of patients suffering from seizures in the absence of brain tumors or following tuberectomy (Major et al., 2009; Orlova and Crino, 2010). Moreover, heterozygous mouse models show no brain pathology, suggesting that both alleles have to be silenced in the brain for the pathology and seizures to fully develop (Goorden et al., 2007).

Homozygous deletion of either *Tsc1* or *Tsc2* genes, both in humans and mice, is embryonically lethal, due to disrupted neuroepithelial growth (Kwiatkowski et al., 2002). Interestingly, tumor tissue from the patients shows loss of heterozygosity (LOH) (Crino et al., 2010), strengthening the hypothesis that homozygous deletion in mice, if possible, should allow further studying of the disease.

As a consequence, conditional mouse models were generated using cell-specific cre expression and Cre-Lox recombination, allowing the generation of TSC mouse models with homozygous deletion of either *Tsc1* or *Tsc2* genes in specific cell types.

As expected due to the cell-specific homozygous KO, some of these mouse models show progressive severe electroencephalographic seizures. Moreover, brain pathology was observed in some models, with giant and ectopic cells, impaired migration and aberrational spine formation. Molecular analysis of conditional mouse models results in the most various conclusions, from changes in ion channels to glutamate receptor subunits shifts (following what has been found in human tissue). These findings are summarized in Table II.

Table II. Summary of the *Tsc1/2* conditional mouse models of TSC

Cell Specificity	**Behavior**	**Brain pathology**	**Molecular analysis**
GFAP [a-d] *(Radial-glia)*	• Epilepsy • Premature death	↑ # astrocytes ↑ Brain size • Neural disorganization	↓ Kir channel ↓ Glt1 ↓ GLAST ↓ Connexin43
Synapsin I [e] *(Post-mitotic neurons at E13)*	↓ Development • Epilepsy • Premature death	↓ Myelination • Aberrant neurons	↑ pS6
Nestin [f] *(Neuroprogenitor cells during early brain development)*	↑ Brain size ↓ Mother-pup interaction • Premature death	(Not investigated)	↓ TSC2 ↓ pAkt ↑ pS6
Emx1 [g, h] (Neuroepithelium stem cells at E10)	• Epilepsy • Premature death	↑ Neuronal cell size ↓ Myelination • Abnormal migration	↓ TSC2 ↓ pAkt ↑ pS6 ↑ pErk ↑ pSTAT3
αCaMKII [i] (Excitatory neurons of the forebrain)	• Epilepsy • Premature death	(Not investigated)	↑ pS6 ↑ pUlk
Dlx5/6 [j] (GABAergic interneurons)	↓ Growth • Decreased seizure threshold • Premature death	↑ # GABA neurons • Impaired migration	↑ pS6

PCP2 [k] or L7 [l] (Purkinje cells in cerebellum)	↓ Motor skills ↓ Social interaction ↑ Anxiety ↑ Grooming	↓ # Purkinje cells ↑ Cell size ↑ Spine density • Abnormal axonal collaterals	↑ pS6

Legend: [a] (Uhlmann et al., 2002); [b] (Wong et al., 2003); [c] (Jansen et al., 2005); [d] (Way et al., 2009); [e] (Meikle et al., 2007); [f] (Anderl et al., 2011); [g] (Magri et al., 2011); [h] (Carson et al., 2012); [i] (McMahon et al., 2012); [j] (Fu et al., 2012); [k] (Reith et al., 2011) and [l] (Tsai et al., 2012).

1.3 TSC Therapeutic Options

Drugs targeting mTORC1 have been helpful in further investigating the disease and helping TSC patients.

Rapamycin (the most widely described mTOR inhibitor) promotes RAPTOR dissociation, therefore down regulating the mTORC1 signaling pathway. This drug has been extensively studied *in vitro*, in animal models and even in TSC patients (clinical trials). In general terms, rapamycin is able to inhibit cell growth, proliferation and survival, therefore reducing hallmark processes of cancer (Seto, 2012). It has been used extensively in clinical trials of TSC patients not only for the tumors, but also for epilepsy and cognition. However, rapamycin's strong immunosuppressant effect in some patients is a major problem (Bissler et al., 2008); so other mTOR inhibitors were generated.

Advances in cancer therapeutics led to the development of ATP-competitive mTOR inhibitors (also called second generation of mTOR inhibitors). These small molecules compete with ATP in the catalytic site of mTOR and down regulate its signaling pathway, therefore blocking the feedback activation of PI3K/Akt signaling. Although this dual action might be of great interest for cancer (Wander et al., 2011), its value for TSC is still uncertain, and more drugs should be developed and tested in preclinical trials.

Epilepsy in TSC is often refractory, meaning that it is resistant to several available therapies. However, there are some fairly successful cases to report. The ketogenic diet, used for years to treat epilepsy in children (including TSC-related), is now described to act through down regulation of the mTOR pathway (McDaniel et al., 2011; Yamada, 2008). Also the anticonvulsives Vigabatrin, Lamotrigine and Clobazam seem to be effective in the treatment of epilepsy and infantile spasms in TSC; however, all these options carry dangerous side-effects (Kessler et al., 2011; Wild et al., 2007), prompting for the discovery of novel options.

Downstream proteins of the mTOR pathway should also be considered as potential therapeutic targets, since they will probably represent fewer side-effects. One good example is the emergent S6K1 inhibitor PF-4708671, shown to strongly downregulate the readout for mTORC1 hyperactivation (pS6) (Di et al., 2012).

1.4 Open questions in TSC

From what was said, it becomes clear that a lot of questions remain to be answered in the study of TSC.

The molecular mechanisms underlying the neurological manifestations are only partially understood. Therefore, this is one of the most important scientific questions. Then again, research should also be focused on the patients, thus the discovery and testing of treatment options is mandatory.

TSC mouse models are liable of being used in order to address both questions. Conditional mouse models allowed us to study epilepsy, but with limitations, because constitutive cre expression does not allow the study of the gene function in later stages of development (compensatory mechanisms occur during development, therefore masking the gene’s function).

Therefore, inducible Cre recombination mouse models, experimentally controlled by tamoxifen administration, may be the answer. In this case, a modified version of Cre recombinase is fused to a mutated human estrogen receptor (ER), allowing the temporal control of Cre-mediated recombination (Feil et al., 1997; Metzger and Chambon, 2001). Peripheral tamoxifen administration (a synthetic ER ligand) induces the nuclear import of Cre-ER and the site-specific recombination of loxP-flanked target genes. In other words, these mice are born with an intact gene and normal protein expression, and exclusively upon peripheral tamoxifen administration the recombination takes place and they undergo *Tsc1* biallelic deletion in a specific cell type (Figure 2). As a consequence, using inducible Cre makes the subsequent mouse breeding process simpler, because the mice do not suffer from developmental complications.

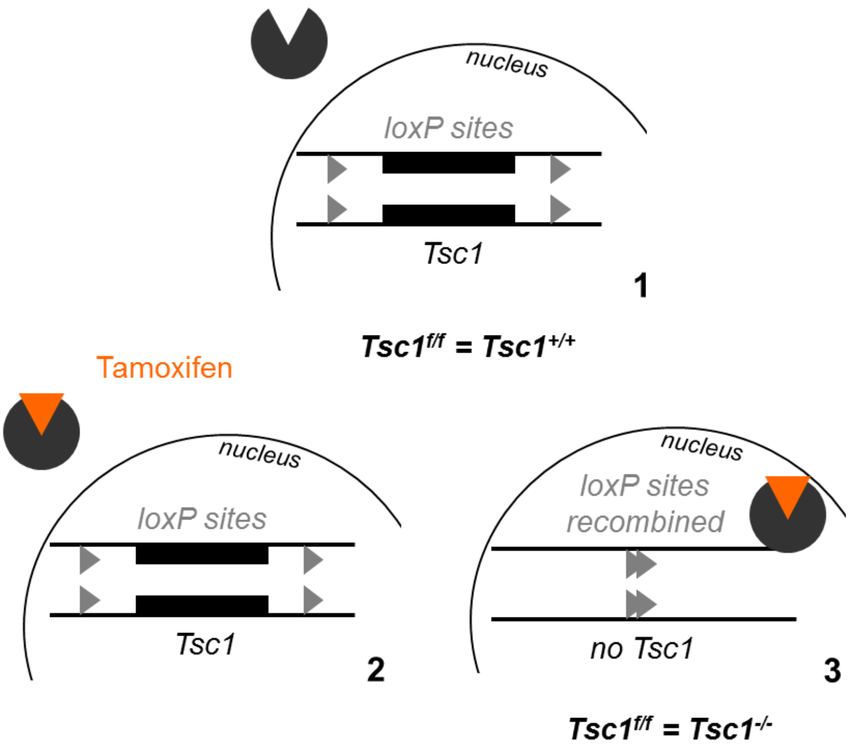

nucleus
loxP sites
Tsc1
1
Tsc1f/f = Tsc1+/+
Tamoxifen
nucleus
loxP sites
Tsc1
2
nucleus
loxP sites
recombined
no Tsc1
3
Tsc1f/f = Tsc1-/-

Figure 2. Initially (1), the *Tsc1* gene is flanked by loxP sites and the cre is fused to a mutated estrogen receptor (ER) in the cytosol (the grey “pacman”). Tamoxifen administration (2), a potent ligand of the ER, induces the translocation of the ER-cre complex to the nucleus (3), where the cre recombinase recombines the loxP sites, resulting in *Tsc1* gene deletion. If tamoxifen is not administered, or the mouse does not express the cre recombinase, the mouse remains similar to WT, i.e, *Tsc1*$^{+/+}$.

In these models, it will be very important to test if the cre recombinase is activated only upon tamoxifen administration, i.e., if it is not leaky. Also, optimal conditions to achieve maximal deletion rates with minimum tamoxifen administration must be found: tamoxifen dose and route of administration, age, gender and genetic background of the mice are probably variants to take into account.

Following these ideas, our lab previously crossed a *Tsc1*$^{f/f}$ mouse line with a cag-ert-cre mouse line, resulting in the *Tsc1*$^{f/f}$–cag-ert-cre (henceforward referred to as *Tsc1*-cag) mouse model. This is a global inducible *Tsc1* KO mouse model that may undergo *Tsc1* deletion in all cells of the body upon tamoxifen administration during adulthood. Therefore, this mouse suffers acute mTORC1 hyperactivation, with no developmental complications. We reported that this mouse develops epilepsy and suffers premature death, with no obvious brain pathology, only a few days after gene deletion onset in adulthood (Abs et al., *in press*). In line with emerging evidence in the literature (showing increased mTORC1 in acquired epilepsy and the antiepileptogenic properties of mTOR inhibitors (McDaniel and Wong, 2011)) we concluded that mTORC1 has a direct signaling role in epilepsy. However, further investigation of the molecular mechanisms underlying the epilepsy in this mouse model (*Tsc1*-cag) was not possible, due to the shortened lifespan, so complementary mouse models are required.

1.5 Aim of the study

To further investigate the role of mTORC1 hyperactivation in TSC-related epilepsy, we now crossed the original *Tsc1*$^{f/f}$ mouse line with inducible cre lines (again, the cre recombinase is fused to a tamoxifen-sensitive estrogen receptor ER$_{T2}$) driven by different brain cells-specific promoters. That way, we have now three different mouse models available that are able to undergo acute biallelic loss of *Tsc1* in adulthood in a cell-specific manner: *Tsc1*$^{f/f}$-αCaMKII-er$_{T2}$-cre, *Tsc1*$^{f/f}$-GFAP-er$_{T2}$-cre and *Tsc1*$^{f/f}$-GAD2-er$_{T2}$-cre.

The *Tsc1*$^{f/f}$-αCaMKII-er$_{T2}$-cre mouse (henceforward referred to as *Tsc1*-αCaMKII) may suffer *Tsc1* deletion upon tamoxifen administration in αCaMKII-positive cells, which roughly comprises the pyramidal neurons of the forebrain (cortex and hippocampus), key brain structures in epileptogenesis, but also Purkinje cells in the cerebellum. This mouse will give insight on the αCaMKII cell-dependent mechanism contributing to epileptogenesis, disregarding of their role during development.

The second mouse model, *Tsc1*$^{f/f}$-GFAP-er$_{T2}$-cre (*Tsc1*-GFAP), can suffer *Tsc1* deletion in GFAP-positive cells, i.e., mature astrocytes.

Third, the inhibitory neuron contribution to epileptogenesis will be addressed by inducing *Tsc1* deletion in these cells, through tamoxifen administration in *Tsc1*$^{f/f}$-GAD2-er$_{T2}$-cre (*Tsc1*-GAD2) mice.

We want to study epilepsy and, hopefully, further dissect the molecular mechanisms of epileptogenesis in each mouse model; assessing the different networks contribution to this devastating neurologic manifestation. We propose to address epilepsy using EEG recordings, and molecular and electrophysiological techniques to investigate the mechanisms underlying epileptogenesis in these mice.

Moreover, by collaborating closely with small drug discovery companies, we also aim to test novel therapeutic options, therefore providing mechanistic data for the use of original drugs targeting different partners of the mTORC1 molecular pathway.

Overall, this project will investigate the contribution of different cell types to epilepsy (particularly in TSC) by acute mTORC1 upregulation and unraveling the molecular changes caused by mTORC1 hyperactivation that induce epileptogenesis. In addition, we are able to test novel treatment options for epilepsy in our mouse model, providing novel information concerning these drugs' action.

Chapter 2

Materials and Methods

2.1 Mice generation and breeding

Tsc1$^{f/f}$ mice (Kwiatkowski et al., 2002) obtained from the Jackson Laboratory were crossed with transgenic mice carrying the cre-gene under the αCaMKII promoter (Erdmann et al., 2007) or GFAP promoter (Hirrlinger et al., 2006). From these crossings, *Tsc1*$^{f/f}$ αCaMKII-er$_{T2}$-cre mice (*Tsc1*-aCaMKII) and *Tsc1*$^{f/f}$ GFAP-er$_{T2}$-cre mice (*Tsc1*-GFAP) were obtained, respectively. From the litters, only homozygous ($^{f/f}$) mice were used, both carrying the cre gene (cre+, mutants) and not carrying it (cre-, control littermates).

The mice were kept in IVC (individually ventilated cages) until adulthood and then collected to mouse experimental stables. Animals were kept in a 12-hour light/dark cycle with *ad libitum* access to food and water. Genotyping of the mice short after birth (toe) and after death (ear) was made by specialized technicians using the following primers 5'-GTCACGACCGTAGGAGAAGC-3', '5'-GAATCAACCCCACAGAGCAT-3' and 5'-AGGAGGCCTCTTCTGCTACC-3' for *Tsc1*; 5'-GGTTCTCCGTTTGCACTCAGGA-3', 5'-CTGCATGCACGGGACAGCTCT-3' and 5'-GCTTGCAGGTACAGGAGGTAGT-3' for the cre in the *Tsc1*-aCaMKII mice; and 5'-CCTGGAAAATGCTTCTGTCCG-3' and 5'-CAGGGTGTTATAAGCAATCCC-3' for the cre in *Tsc1*-GFAP.

For electroencephalography (EEG) recordings, western blot analysis and immunohistochemistry, independent groups of mice were used (numbers specified throughout the text). All animal experiments were approved by the Local Animal Experimentation Ethical Committee, in accordance with Institutional Animal Care and Use Committee guidelines. The experimenter remained blind to the genotype during data collection and initial analysis.

2.2 EEG surgical procedure

Adult *Tsc1*-αCaMKII mice were deeply anesthetized with isoflurane (~1.5%) and injected with Rimadyl (Pfizer 1 µl/g body weight). Stainless steel screws (0.5 mm diameter) were used as EEG electrodes (lowered 1-1.5 mm below the skull to touch the dura mater). These were placed both on left and right sides above motor cortex (approximately 1.0 mm with reference to Bregma and midline), sensory cortex (approximately 2.0 mm with reference to Bregma and midline) and cerebellar cortex (approximately 1.0 mm with reference to midline). Before implantation, each screw was soldered to a soft insulated copper wire and, the other end of the wire to a pin (the screw to be used on the right side of the cerebellar cortex was soldered to two wires with two pins, being one the ground). All wires were connected to one common 7-pin connector compatible with the logging device and the whole structure was covered with dental cement (Kemdent, Swindon, UK) in a way suitable with the carrying of the apparatus. At the end of surgery, mice received 150 µl of morphine and were afterwards kept single caged (in a 12-hour light/dark cycle and *ad libitum* access to food and water). Figure 3 shows a scheme of the EEG surgical procedure.

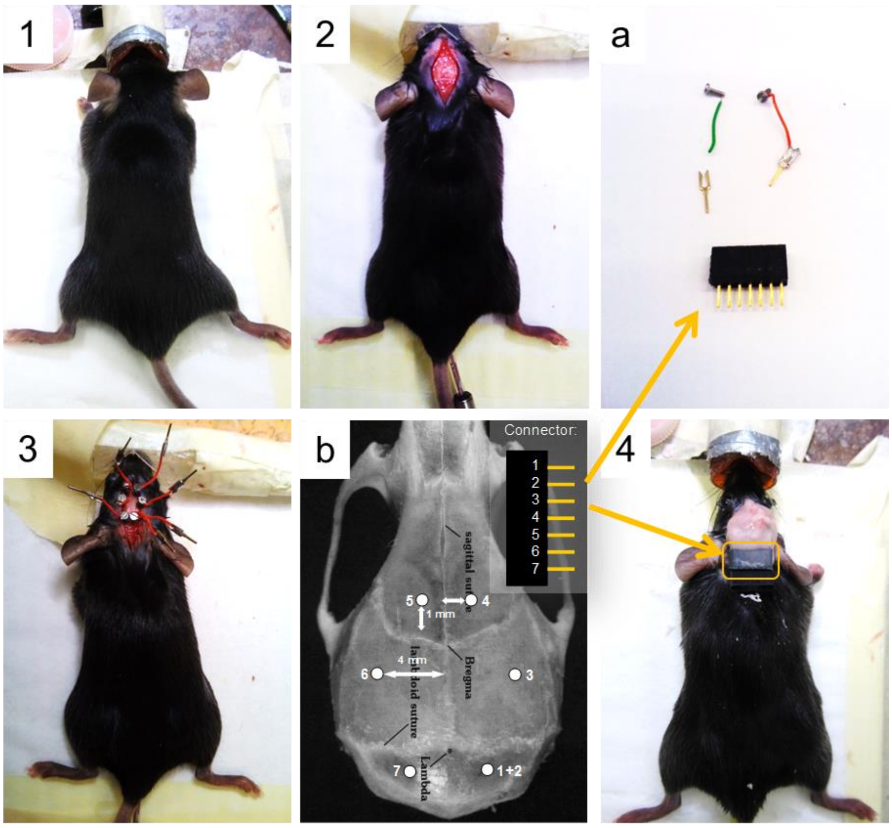

1
2
a
3
b
Connector:
1
2
3
4
5
6
7
sagittal suture
5
4
1 mm
4 mm
6
3
Bregma
lambdoid suture
7
1+2
Lambda
4

Figure 3. The EEG surgical procedure takes place with the mouse deeply anesthetized (1). An incision is made in the skin and fascia overlying the skull (2). Stainless steel screws (a) are used as EEG electrodes (3) and implanted in the skull at the approximate coordinates shown in b. In the end, the other end of the electrodes is plugged in a connector (highlighted in orange) and covered with dental cement (4). Scheme in (b) is adapted from George Paxinos and Keith Franklin: The Mouse Brain in Stereotaxic Coordinates, *Academic Press*, 2001.

2.3 EEG measurements

Shortly before the start of the subsequent dark phase of the day, a wireless EEG recorder data logger (NewBehavior AG, Zurich, Switzerland), hereupon called neurologger, was connected to the mice and 16 hours recordings were obtained every day. EEG analysis was made by scoring number and duration of seizures for all recordings.

2.4 Tamoxifen administration

Tamoxifen (Sigma) was dissolved in sunflower oil in a concentration of 20 mg/ml by vortex and sonication. To induce *Tsc1* gene deletion, *Tsc1*-αCaMKII adult mice (at least 8 weeks old) were given 4 intraperitoneal injections of 0.1 mg/g of body weight of the tamoxifen emulsion.

2.5 Immunohistochemistry

After deep anesthesia with 0.15 ml Pentobarbital, mice were perfused with 4% paraformaldehyde; the brains were post-fixed for 2 h and left overnight in a 30% sucrose solution. The brains were sagittally sliced in 40 µm thick slices using a cryotome and transferred into 0.05 M Phosphate Buffer (PB). Free floating slices were rinsed in Phosphate Buffer Saline (PBS), blocked in a PBS solution with 10% normal horse serum (NHS) and 0.5% Triton X-100 and incubated with primary antibody in a PBS solution with 2% NHS and 0.5% Triton X-100, overnight. After rinsing, the incubation with the secondary antibody (Jackson Immunoresearch Europe Ltd, 1:200) took place for 1-2 h (depending on antibody). In the end, slices were rinsed several times in PB 0.05 M and mounted on glass with chromaluin. For immunohistochemistry, slices were also blocked for blood as first step in a PBS solution with 10% H_2O_2

and after the secondary antibody the slices went through the standard avidin-biotin-immunoperoxidase complex method (ABC, Vector Laboratories) with diaminobenzidine (DAB) as the chromogen.

αCaMKII stainings require an antibody solution with 5% Triton X-100, 2% NHS and 1% BSA in PBS, without blocking step.

To assess gross brain morphology sections were mounted on glass and stained with thionin. Visual examination of pS6 stained slices was done in order to identify enlarged cells- included when they were 4 times larger than neighboring cells.

DAB and thionin stained slices were observed under an Olympus microscope (type CX 41), while fluorescent stained slices were observed under a Zeiss LSM700 confocal microscope.

Primary antibodies against αCaMKII (Novus Biologicals NB100-1983, 1:500 for fluorescence), Parvalbumin (SWANT, 1:9000) and $pS6_{S235/6}$ (CST #2211, 1:1000) were used.

2.6 Western blot

Lysates were prepared after quick dissection of the different brain areas (hippocampus, cortex and cerebellum) followed by homogenization in lysis buffer (0.1 M Tris HCl pH 6.8, 2.5% SDS for total lysates, 10 mM Hepes pH 7.4 with 0.32 M sucrose, 1 mM $NaHCO_3$ and 1 mM $MgCl_2$ for synaptosomes) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich).

The concentration of all lysates was adjusted to 1 mg/ml and 15 µl were loaded on a 4-12% gradient SDS-PAGE, running at 150 V for 1.5 h, followed by a semi-dry transfer for 1.5 h at 15 V, or a wet transfer for 40 min at 100 V (depending on the molecular weight of the proteins of interest). The blot was incubated with the primary antibodies with 2% milk in Tris-buffered saline-Tween 20 0.1% (TBST) overnight, after blocking with 5% milk. After washing with TBST, the incubation with the secondary antibody took place for 1 h (Odyssey, 1:5000). The Li-Cor Odyssey fluorescence imaging system was used for the membrane development and the LiCor Odyssey 2.1 software was used for quantification. Primary antibodies against TSC1 (#4906, 1:1000), TSC2 (#3612, 1:1000), $pS6_{S235/6}$ (#2211, 1:2000), S6 (#2217, 1:3000), $pAkt_{S473}$ (#4060, 1:2000), Akt (#2920, 1:8000), NR1 (#5704, 1:1000), NR2A (#4205, 1:1000), NR2B (#5580, 1:1000), GluR1 (#8850, 1:1000) all from Cell Signaling Technology, Danvers, MA and $GABA_AR$ α1 (Milipore #06-868, 1:1000) and actin (MAB1501R; Chemicon, Billerica, MA, 1:20000) were used. All samples were run in at least two independent experiments.

2.7 Drug preparation

The AZD8055 dual mTOR inhibitor, a kind gift from Griffin Discoveries, was aliquoted in DMSO in a concentration of 50 mg/ml and kept at $-80^{o}C$. For the testing of the compound in wild-type mice, fresh syringes were prepared after vortexing of the aliquots and administration of 10 mg/kg.

The novel dual mTOR inhibitor GD124, also a kind gift from Griffin Discoveries, was aliquoted in DMSO in a solution of 10 mg/ml and kept at $-80^{o}C$. For the testing of the compound in wild-type mice, fresh syringes were prepared after vortexing of the aliquots and administration of 15 mg/kg.

The S6K1 inhibitor PF-4708671 (Pfizer) was kept as a powder at room temperature. For the treatment experiment in our mutant mice, fresh syringes were prepared with vigorous vortex after sonication of the drug in a solution of 5% Tween-80 (v/v) in saline and the mice were injected with 75 mg/kg.

2.8 Statistical analysis

GraphPad Prism data analysis software (San Diego, CA) was used for statistical analysis. Mean values and SEM are shown as error bars and $p<0.05$ was considered significant (being identified in the graphs by an asterisk (*)).

Chapter 3

Results

3.1 Characterization of the *Tsc1*-αCaMKII mouse model

To investigate whether acute biallelic loss of *Tsc1* in a cell-specific manner is sufficient to induce epilepsy in adult mice and further dissect the molecular mechanisms of epileptogenesis, we generated inducible *Tsc1* KO mice using the cre/LoxP gene-targeting strategy with the cre fused to a tamoxifen-sensitive mutated estrogen receptor. Firstly, we used the cre-line driven by the αCaMKII promoter, originating *Tsc1*-αCaMKII mice (Figure 4). For this study, cre+ and cre- littermates are used.

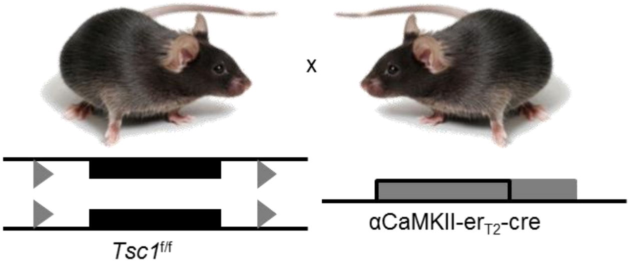


Figure 4. $Tsc1^{f/f}$ mice were crossed with the cre line carrying the αCaMKII promoter until the $Tsc1^{f/f}$-αCaMKII-er_{T2}-cre construct, with cre+ and cre- littermates, was obtained.

This mouse model, upon peripheral administration of tamoxifen, undergoes biallelic *Tsc1* deletion in αCaMKII-positive cells, which are widely expressed in pyramidal excitatory neurons of the forebrain, not being present in any peripheral organ. Injection of *Tsc1*-αCaMKII cre+ mice with tamoxifen induces translocation of the $creER_{T2}$ protein to the nucleus to drive recombination of the floxed *Tsc1* allele only in αCaMKII-positive cells, resulting in cell-specific *Tsc1* null mice. On the other hand, tamoxifen injection to *Tsc1*-

αCaMKII cre- mice does not result in recombination; since the cre is not present (see Figure 2). To confirm the specificity of this mouse model and its cre line, DNA was isolated of different brain areas and peripheral tissue from these mice after 4 tamoxifen injections. A PCR was made to assess *Tsc1* gene deletion (primers used described in chapter 2, Figure 5). As expected, we observed partial *Tsc1* deletion in both cortex and hippocampus (where αCaMKII is widely expressed), with no deletion in cerebellum (due to the lower number of αCaMKII-positive cells) or liver (a very reliable negative control, due to the brain-specificity of this cre-line) of cre+ *Tsc1*-αCaMKII mice. Vehicle-injected mutant mice (cre+) show no recombination, proving that the cre line is not leaky. Control mice (cre-) are also injected with tamoxifen throughout the study, to control for any adverse effect of the drug (accordingly, these mice show no recombination).

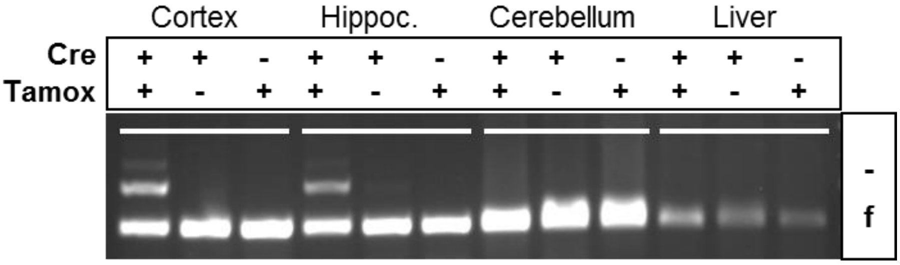


Figure 5. PCR for *Tsc1* using different brain areas and liver of *Tsc1*-αCaMKII mice. Only cre+ tamoxifen injected mice show partial deletion in cortex and hippocampus. f: floxed band; -: deletion band

To confirm this result on the protein level, as well as validating the *Tsc1 deletion* effect on mTORC1 hyperactivation (using pS6/S6 levels as readout), we addressed TSC1 and pS6 levels in these brain areas by western blotting

(Figure 6). TSC1 levels dropped in hippocampus, cortex and cerebellum, however, only the first two show mTORC1 hyperactivation (increased pS6/S6 levels), so we believe the cerebellum will not play a major role in the phenotype of our mouse model. Henceforward, the brain areas of our focus will be the cortex and hippocampus.

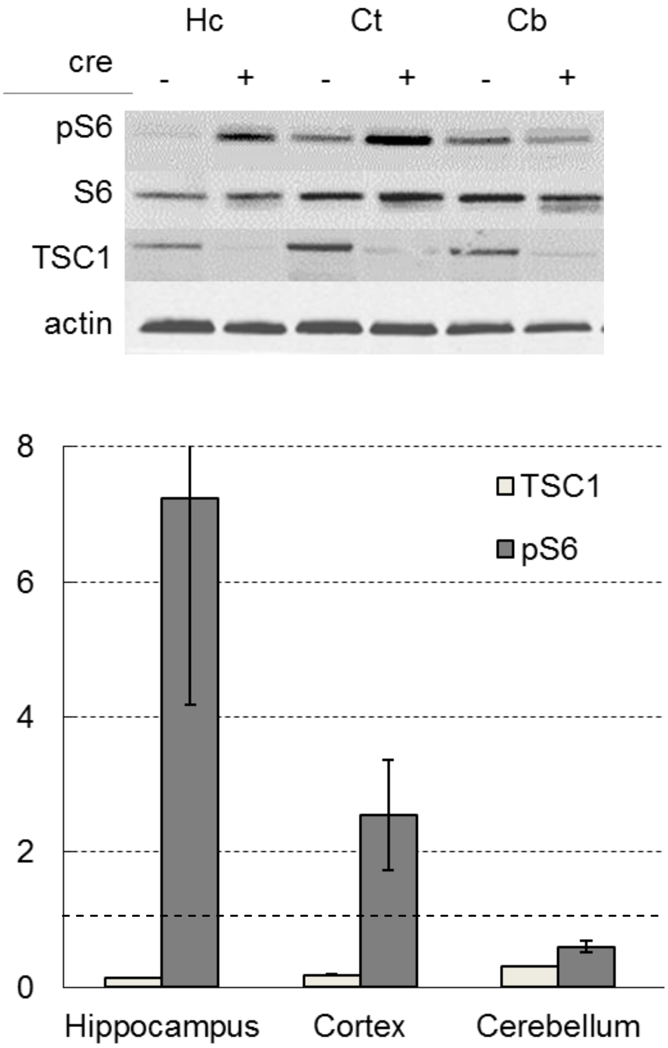


Figure 6. TSC1 and pS6/S6 levels in αCaMKII-positive brain regions of *Tsc1*-αCaMKII mice that underwent *Tsc1* deletion: hippocampus

(Hc), cortex (Ct) and cerebellum (Cb). All protein levels were normalized to the respective brain area in control mice (1 unit, dark line). All brain areas show decreased TSC1 levels, following *Tsc1* gene deletion. However, only hippocampus and cortex show mTORC1 hyperactivation (increased pS6/S6 levels). n=2 per group (preliminary data).

As widely described in literature, pS6 increased levels are used as readouts for mTORC1 hyperactivation. In the case of our mouse model, mTORC1 hyperactivation is due to *Tsc1* deletion. Since no TSC1 antibody is available to be used for immunohistochemistry, we will use pS6 stainings as readout for *Tsc1* deletion (Figure 7). In accordance with our previous result, pS6 increased levels are only observed in the hippocampus and cortex of mutant mice, with no differences in cerebellum

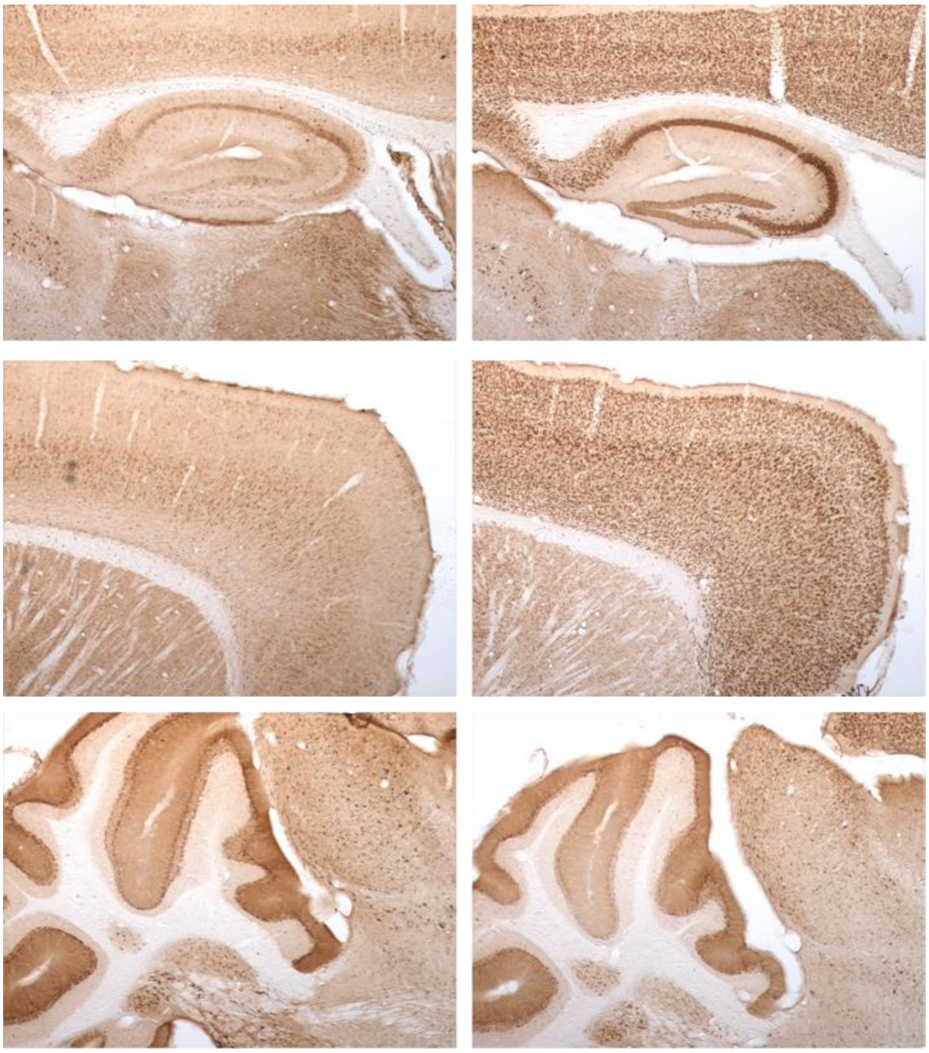


Figure 7. Sagital sections (5 x magnification) of a control (left) and a mutant *Tsc1*-αCaMKII mouse brain stained with an antibody against pS6. pS6-positive cells are highly present in cortex and hippocampus of the mutant *Tsc1*-αCaMKII mouse, while no differences are found in cerebellum. n=4 per group.

To investigate whether the *Tsc1* deletion was specific for αCaMKII-positive cells, we performed co-stainings for pS6 (hereby used as readout for *Tsc1* deletion) and αCaMKII (cell-type that is undergoing *Tsc1* deletion). In our mouse model, pS6-positive cells show high co-localization with αCaMKII-positive cells (Figure 8), but not with Parvalbumin-positive cells (PV is a marker for GABAergic interneurons) (Figure 9).

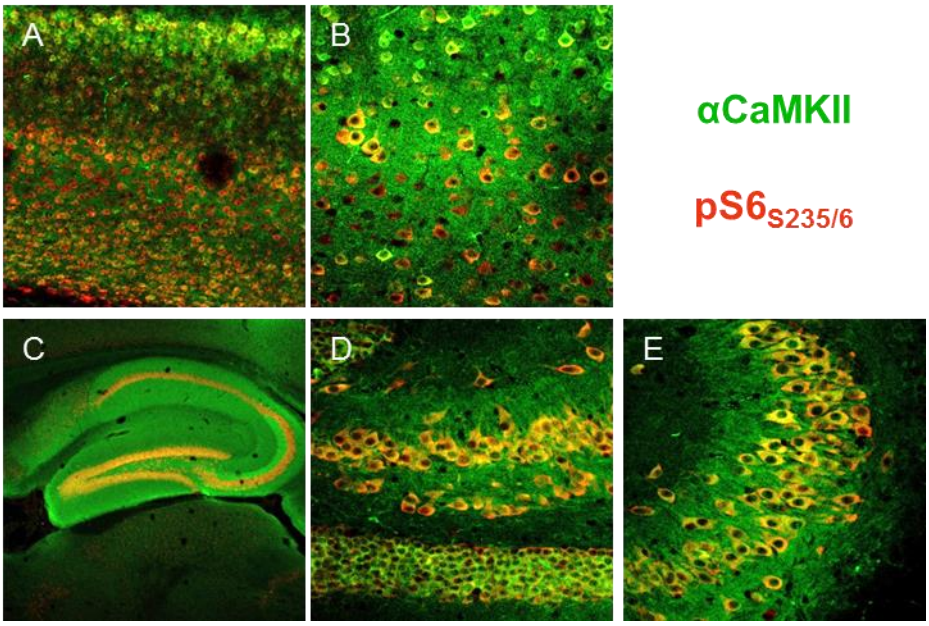


Figure 8. pS6-positive cells (red) highly co-localize with αCaMKII-positive cells (green) in cortex (**A**, 10x; **B**, 20x) and hippocampus (**C**, 5x, **D**, 20x dentate gyrus, **E**, 20x CA3 region) of *Tsc1*-αCaMKII mice. n=5.

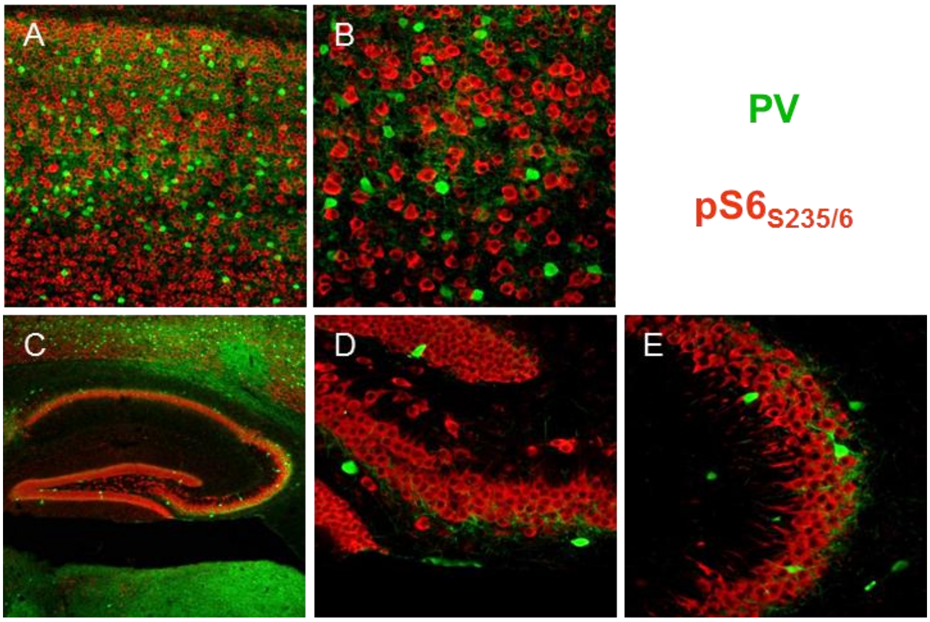


Figure 9. pS6-positive cells (red) do not co-localize with PV-positive cells (green) in cortex (**A**, 10x; **B**, 20x) and hippocampus (**C**, 5x, **D**, 20x dentate gyrus, **E**, 20x CA3 region) of *Tsc1*-αCaMKII mice.

At this point, we are able to claim that our mouse model is able to undergo *Tsc1* gene deletion in αCaMKII-positive cells when injected 4 times with tamoxifen in adulthood. This induced *Tsc1* deletion results in mTORC1 hyperactivation in hippocampus and cortex, crucial brain structures of the forebrain involved in epilepsy.

Following the conditional deletion in this same cell type (see Table II, (McMahon et al., 2012)) and the severe epilepsy developed by our global inducible mouse model (*Tsc1*-cag; (Abs et al., *in press*)); we hypothesize that this mouse will develop epilepsy and allow the study of mTORC1-dependent epileptogenesis.

3.2 αCaMKII-driven acute deletion of *Tsc1* is sufficient to induce lethal epilepsy in adult mice

To assess epilepsy in these mice, surface electrodes were implanted in the brain through the EEG surgical procedure (explained in detail in chapter 2, Figure 3). EEG recordings were performed using the neurologger device (protocol in Figure 10, examples of recordings and device in Figure 11).

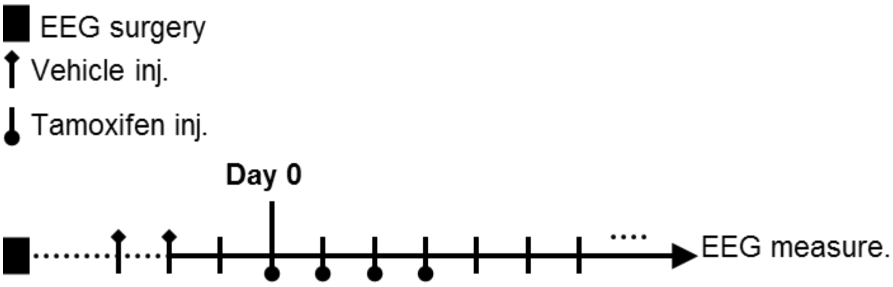


Figure 10. Adopted protocol to assess epilepsy in *Tsc1*-αCaMKII mice through EEG recordings. After the EEG surgical procedure the mice are given a minimum of two days of rest. Then, two vehicle injections are administered and baseline measurements are acquired. After one day of rest, gene deletion is induced by tamoxifen administration and EEG measurements are acquired every night.

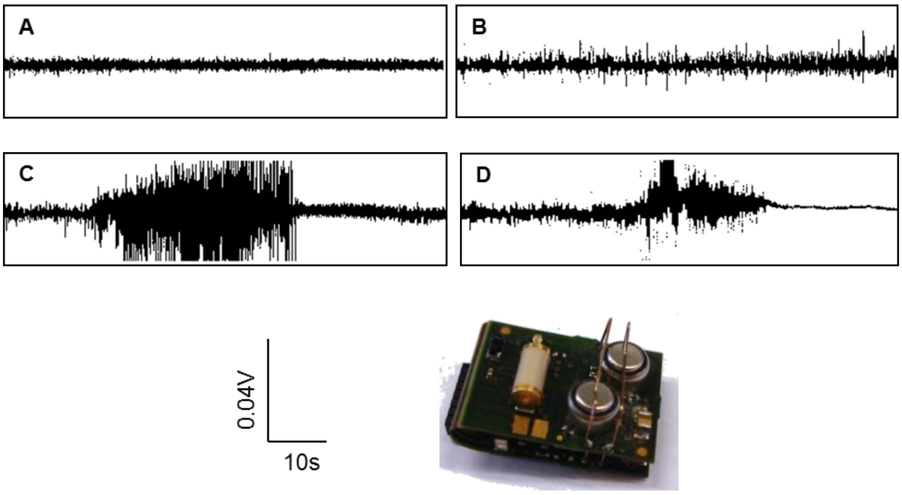


Figure 11. Examples of the observed types of recordings (**A**, baseline measurement; **B**, increased activity; **C**, seizure; **D**, lethal seizure) and the wireless neurologger device.

Here we show that αCaMKII-driven *Tsc1* deletion in adulthood led to the development of spontaneous or handling-induced seizures. Epilepsy onset occurred as soon as 9 days after gene deletion onset (Figure 12) and was lethal, with the cre+ mice having a median survival of 15 days after the first tamoxifen injection, i.e., after gene deletion onset (Figure 13). Epilepsy in these mice is tonic-clonic, followed by a period of loss of upright posture and generalized clonus of all limbs

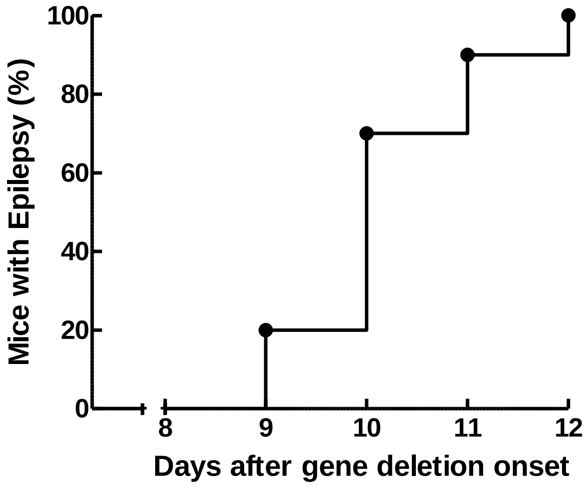


Figure 12. The epilepsy onset of tamoxifen-injected cre+ mice varies between 9 and 12 days after the first tamoxifen injection in adulthood. Tamoxifen-injected cre- mice don’t show epilepsy. n=10.

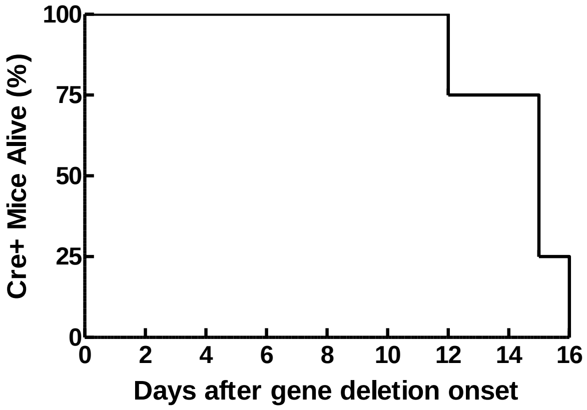
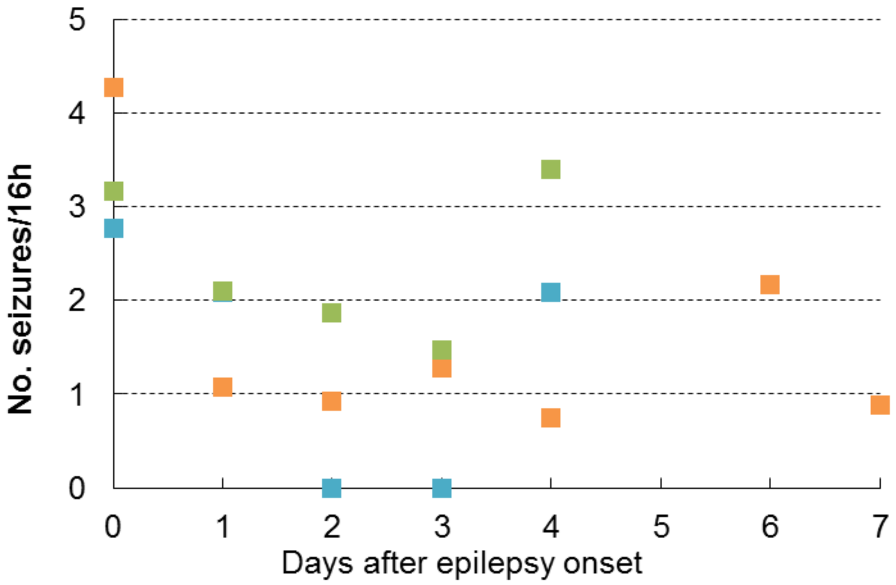


Figure 13. Lifespan of tamoxifen-injected cre+ mice varies between 12 and 16 days after the first tamoxifen injection in adulthood. Tamoxifen-injected cre- mice don't show decreased lifespan. n=4.

The number and length of all recorded seizures are presented in Figure 14. These mice show, in average, 2 seizures of 30 seconds per 16 h of recording and the number of seizures seems to decrease with the epilepsy development over time.



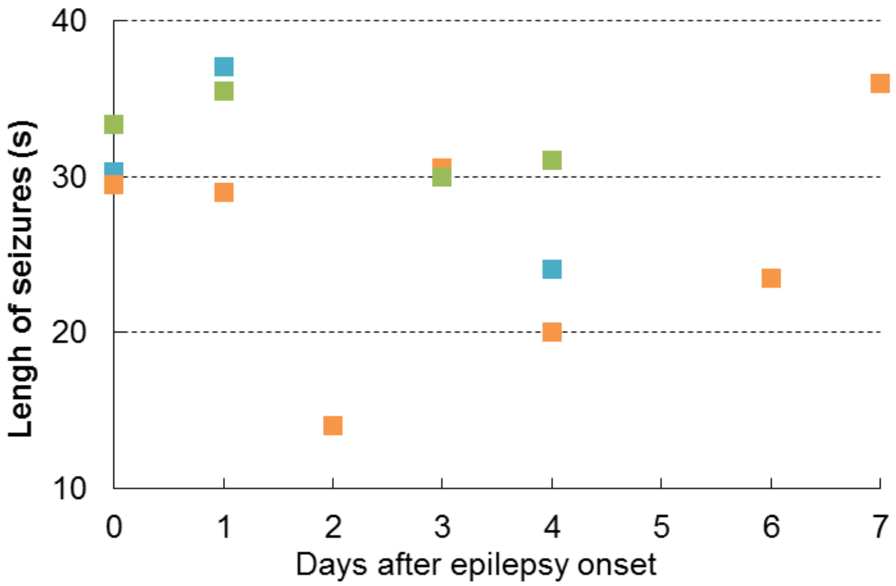


Figure 14. Number (per 16h of recording; upper graph) and length of seizures (in seconds; lower graph) of the recorded mice. Each color represents one mouse. n=3.

As a conclusion, *Tsc1*-αCaMKII mice develop epilepsy only 9 days after gene deletion onset and die a few days after. In contrast to the *Tsc1*-cag mice (Abs et al., *in press*), the *Tsc1*-αCaMKII mice always die after a seizure, whereas the first do not (their death is most probably related to kidney failure). Therefore, a brain-specific *Tsc1* deletion, in excitatory neurons of the forebrain, reveals itself as a more suitable tool to study mTORC1-dependent epilepsy (and epilepsy in general). Moreover, the cell-specific KO shows less variability in epilepsy onset than the global KO (*Tsc1*-cag mice develop epilepsy 2 to 9 days after gene deletion onset). Yet, epilepsy appears to be equally severe, leaving only a small window to study the molecular mechanisms underlying epileptogenesis.

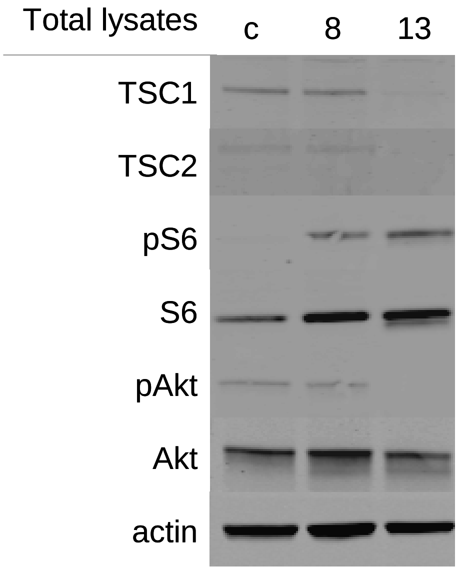
In summary, *Tsc1*-αCaMKII mice develop epilepsy around 10 days after gene deletion onset and die approximately 15 days after, following a seizure.

The number of seizures per period of EEG recording seems to decrease over time, probably due to acute *Tsc1* deletion followed by some compensatory mechanisms taking place afterwards. Moreover, it prompts us to hypothesize that molecular changes take place in a time-dependent manner.

These results allow us to use day 8 as a pre-epileptic time-point and day 13 as an epileptic state of the mice, and address the molecular changes in these two moments.

3.3 αCaMKII-driven acute deletion of *Tsc1* in adulthood is accompanied by mTORC1 hyperactivation

To address mTORC1 hyperactivation in these mice, we measured TSC1 and TSC2 levels, as well as pS6 (readout for mTORC1) and pAkt levels (readout for mTORC2) in hippocampal tissue (Figure 15), 8 and 13 days after gene deletion onset. Accordingly, TSC1 protein levels decrease in a time-dependent manner (due to gene deletion) and TSC2 levels follow the first, as previously described (Benvenuto et al., 2000). mTORC1 hyperactivation is evident by the 3 fold increase in pS6 levels in mutant mice and mTORC2 down regulation, as assessed by decreased pAkt levels in mutant mice.



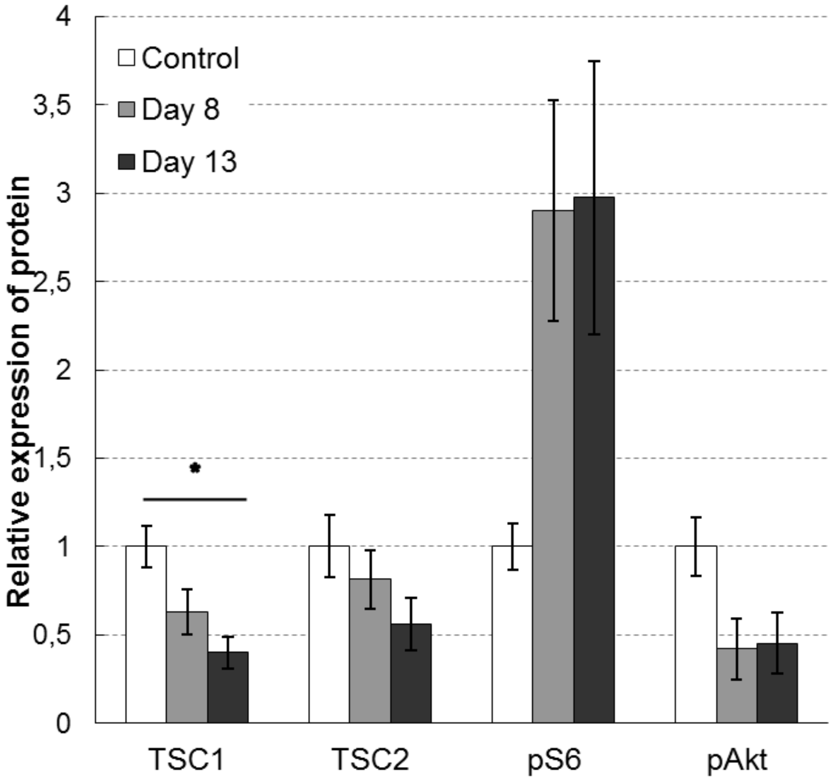


Figure 15. TSC1, TSC2, pS6/S6 and pAkt/Akt levels in control mice (c; cre- tamoxifen-injected) and mutant mice (tamoxifen injected cre+) at day 8 (8; pre-epileptic state) and day 13 (13; epileptic state). Significant differences are shown by * (p=0.01, 1-way ANOVA), n=4 per group.

3.3 Ongoing work

As we could observe, after gene deletion onset TSC1 levels drop in a time-dependent manner, followed by mTORC1 hyperactivation (pS6/S6 levels increase). However, mTORC1 hyperactivation is also triggered by epilepsy, including non-TSC related. For example, the kainate-induced epilepsy model in rats shows increased levels of pS6 in several different time points (Wong, 2010).

To distinguish between mTORC1 hyperactivation due to *Tsc1* loss and the hyperactivation caused by the seizures themselves we will perform the following additional experiments.

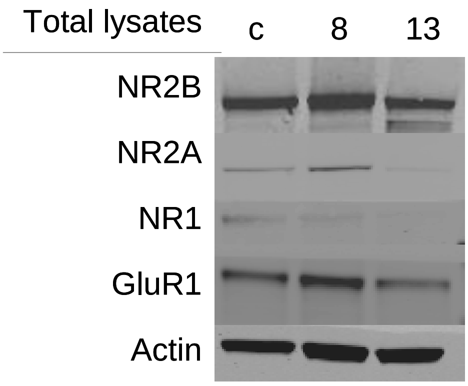
Firstly, we will address mTORC1 hyperactivation at the exact moment where half the animals show epilepsy and the other half does not. That way, we can observe in the group without epilepsy the mTORC1 hyperactivation only due to reduced TSC1 protein levels. The group with epilepsy should have similar amounts of TSC1 left at that time point, but probably shows a further increased mTORC1 activity due to the presence of seizures. To test this we will stain brains of tamoxifen injected cre+ mutant mice that, at the same time-point after the initiation of gene deletion, show or do not show epilepsy.

Secondly, we aim to discriminate between the effect of chronic and acute epilepsy. For that, we will sacrifice a group of animals, again at day 13 (mice already suffering from epilepsy), shortly after inducing a seizure by gentle fixation. That way, all the mice will have been through an epileptic state shortly before our molecular analysis, possibly showing differential levels of mTORC1 hyperactivation (similar to the non-TSC related models of epilepsy, where increased pS6 is observed a couple of hours after seizure induction) and therefore allowing us to further dissect its role in epilepsy. Moreover, this approach will, hopefully, allow us to amplify the molecular changes that follow seizures, further adding understanding to the molecular mechanisms following epileptogenesis.

Altogether, this data will allow us to draw more conclusions regarding the role of mTORC1 hyperactivation in epilepsy, as well as provide some clues concerning the molecular changes underlying epileptogenesis (addressed in detail in the following subchapter).

3.4 Epileptogenesis in *Tsc1*-αCaMKII mice

To investigate the molecular changes potentially leading to epileptogenesis, we decided to explore the molecular changes found in tuberal tissue from patients (see Table I), also shown is some conditional mouse models (see Table II). Again we used three different groups of mice: the control mice, that do not undergo *Tsc1* deletion even though they are tamoxifen-injected; mice sacrificed at day 8, i.e., that do not show epilepsy, but may demonstrate already some molecular changes that underlie epilepsy development and mice at day 13, that by now show epilepsy for at least 2 days. To assess changes in the amount of AMPA and NMDA receptors subunits, firstly in hippocampal total lysates (Figure 16), we run western blots with antibodies against NR1, NR2A and NR2B (NMDAR subunits) and GluR1 (AMPAR subunit).



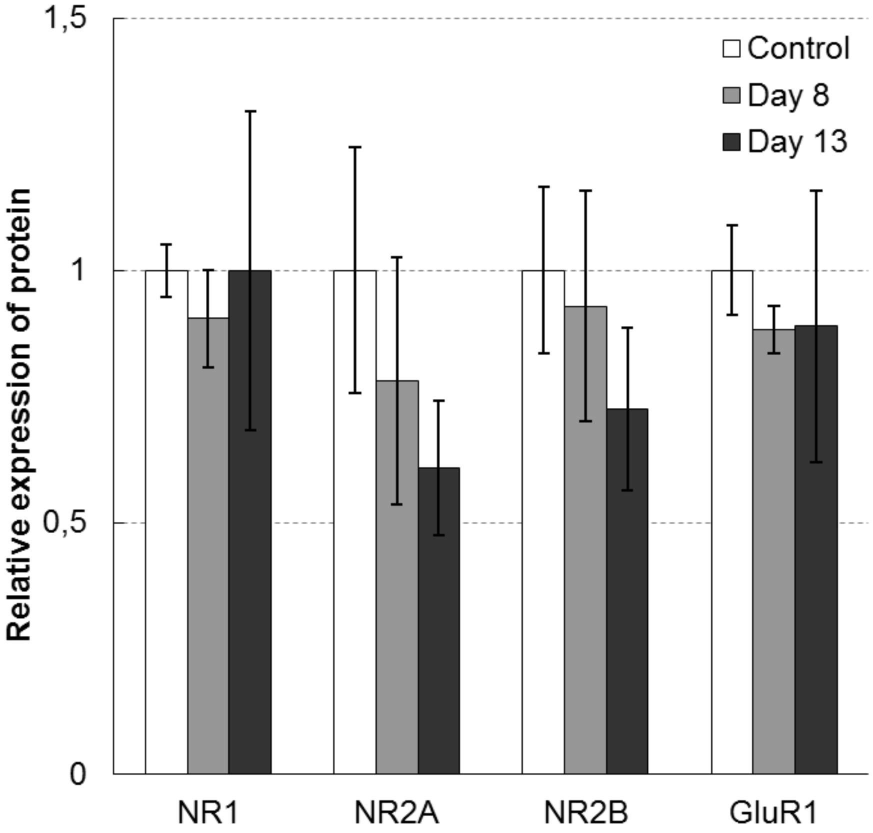


Figure 16. NR1, NR2A and NR2B NMDA subunits and GluR1 AMPA subunit levels in hippocampal total lysates of control (c) and mutant mice at day 8 (8) or day 13 (13). n=4 per group, no significant differences were found (preliminary data).

Unfortunately, no differences between the groups were found, so we questioned ourselves whether it is not the total amount of subunits hat it is changed, but the location of these subunits. In other words, we speculated whether changes in these receptors subunits might be due to trafficking, and not protein amount. To address this question, we isolated synaptosomes of cortical tissue from mice at the same time-points and investigated NMDAR and GABAR subunits levels (Figure 17). Again, no differences were found.

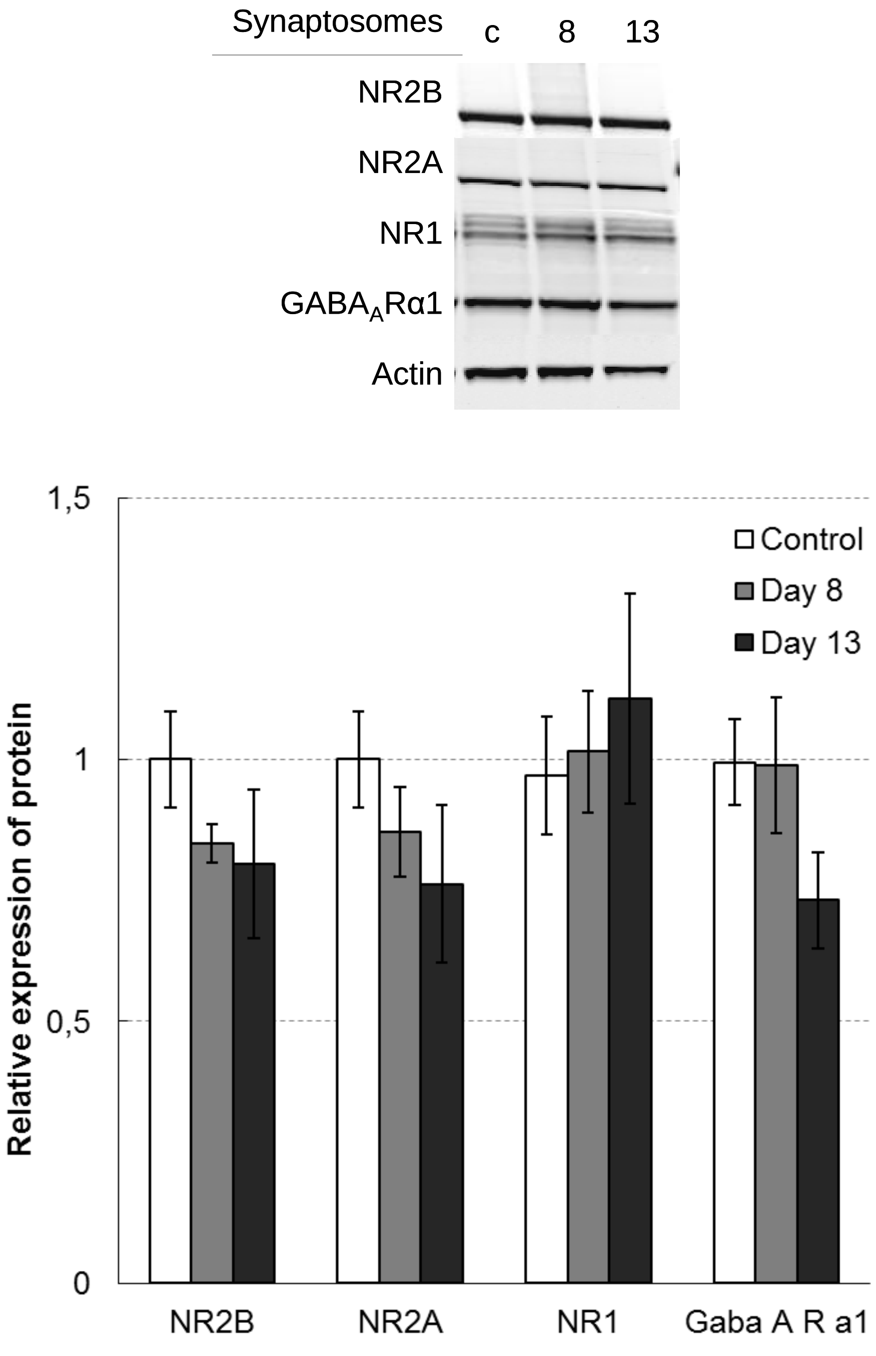


Figure 17. NR2B, NR2A and NR1 NMDA subunits and GABA$_A$Rα1 subunit levels in cortical synaptosomes of control (c) and mutant mice at day 8 (8) and day 13 (13). n=4 per group, no significant differences were found (preliminary data).

3.4 Ongoing work

In order to investigate the synaptic mechanisms that may be underlying epilepsy in these mice, possibly through mTORC1 signaling, we will focus on exploring the molecular changes found in the patients' brain tissue (see Table I). At the moment, we are still measuring protein levels of AMPAR and NMDAR subunits in the synaptosomes preparations and GABAAR subunits in the total lysates. Changes in synapsin, an important protein that regulates neurotransmitter release at synapses (shown to be increased in brain tissue from TSC patients - see Table I), will also be investigated. Lysates with enriched post synaptic density (PSD) proteins are also being addressed for all these receptors subunits. That way, we will measure protein amount in different cell compartments, obtaining indirect evidence of trafficking of these receptors.

Altogether, we are confident that this data will allow us to draw some conclusions concerning the network-dependent molecular changes in these mice. In other words, we might be able to understand mTORC1 hyperactivation involvement in amount or location of different Glutamate and GABA receptors subunits that may be underlying epileptogenesis in our mouse model. Moreover, pinpointing one or another subunit will have a major impact in how we look at these synapses from the electrophysiological point of view (in the future electrophysiology experiments).

3.5 No obvious brain pathology in *Tsc1*-αCaMKII mice

In line with the observations in the *Tsc1*-cag mouse, adult deletion of *Tsc1* only in αCaMKII-positive cells does not result in changes in gross brain morphology as assessed by thionin stainings (Figure 18).

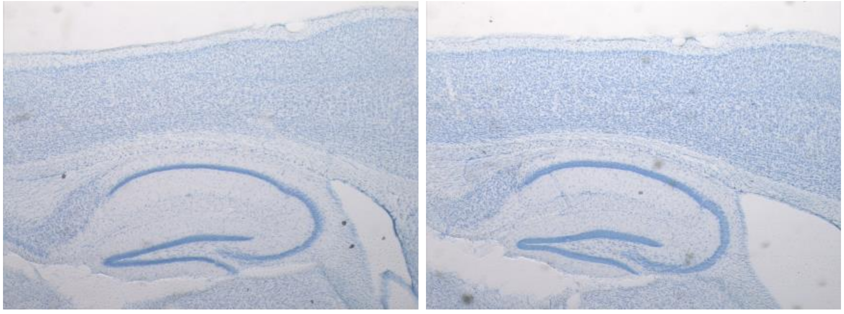


Figure 18 No differences in gross brain morphology are observed in the hippocampus of *Tsc1*-αCaMKII mice (control on the left, mutant on the right). (All perfused brains were screened and no changes were found.)

Also, it does not lead to the development of giant cells (widely described as developmental brain pathology in some conditional mouse models, see Table II), as assessed by screening for pS6 positive cells that are 4 times bigger than the surrounding cells in the cortex of epileptic mice (Figure 19).

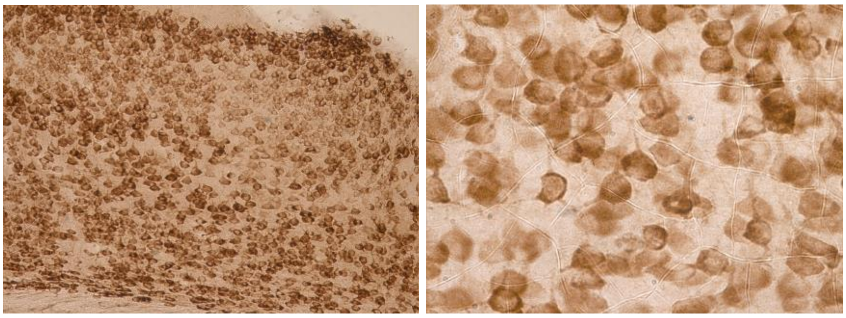


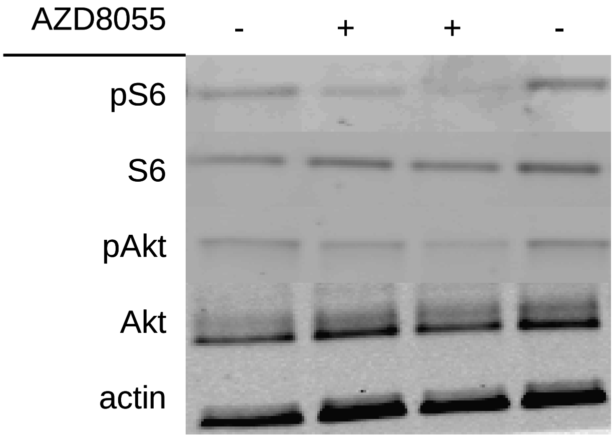
Figure 19 No pS6-stained giant cells are found in the cortex of *Tsc1*-αCaMKII epileptic mice (10 x magnification on the left, 40 x magnification on the right).

Although experiments are still ongoing, it is clear that this mouse model is a valuable tool to study epileptogenesis in TSC, independent of brain pathology. Therefore, we asked ourselves if we would be able to treat this epilepsy (by extending lifespan or reduce seizure burden), while providing initial mechanistic data for novel drugs.

3.6 Testing novel therapeutic options for TSC

In order to investigate the therapeutic value of novel dual mTOR inhibitors in TSC, we initially validated these drugs ability to cross the blood brain barrier and to have an effect on mTORC1 and mTORC2 signaling in the brain of wild-type (WT) mice.

First, we tested the AZD8055 compound, which was described as an antitumor drug, strongly down regulating mTORC1 (decreasing pS6/S6 and p4EBP1/4EBP1 and inducing autophagy) as well as mTORC2 (pAkt/Akt levels) signaling in xenographs of mouse models of cancer (Chresta et al., 2010). We injected mice two times with either AZD8055 (10 mg/kg) or vehicle (DMSO), with an inter-injection interval of 16 h. The mice were sacrificed 3 h after the last injection. 10 mg/kg of AZD8055 were able to decrease both mTORC1 and mTORC2 readouts (pS6/S6 and pAkt/Akt, respectively) in the brain of WT mice (Figure 20).



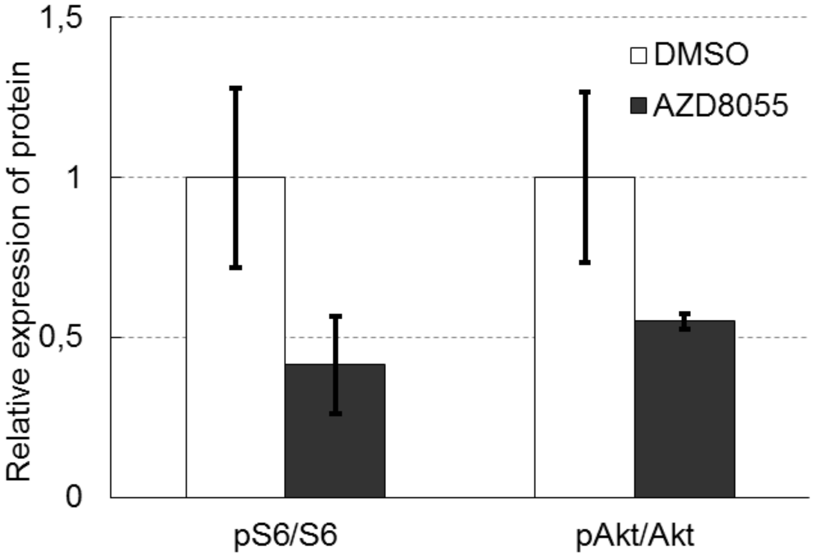
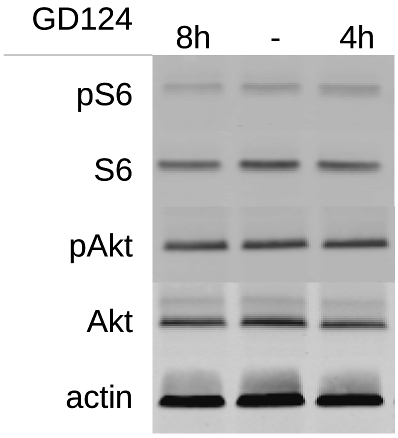


Figure 20. AZD8055 (10 mg/kg) is able to downregulate mTORC1 (pS6/S6 levels) and mTORC2 (pAkt/Akt levels) in the hippocampus of WT mice. n=2 per group (preliminary data).

Secondly, we tested the novel compound GD124. This drug was designed to be a potent dual mTOR inhibitor and shown to inhibit mTORC1 activity (pS6 levels) in an *in vitro* essay. For this experiment, we injected WT mice with either GD124 (15 mg/kg) or vehicle (DMSO) and sacrificed them 4 and 8 h after a single injection (to also investigate the drug's half-life). However, we failed to find any effect on pS6 or pAkt (Figure 21).



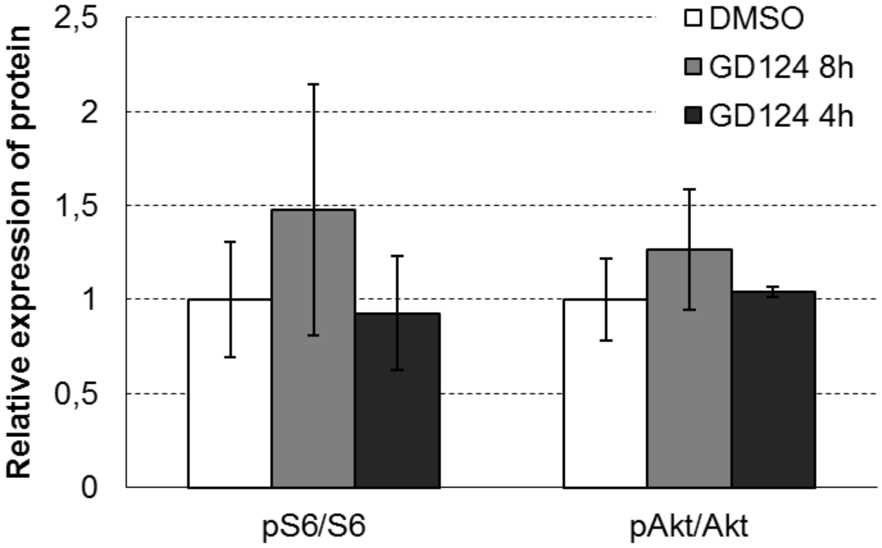
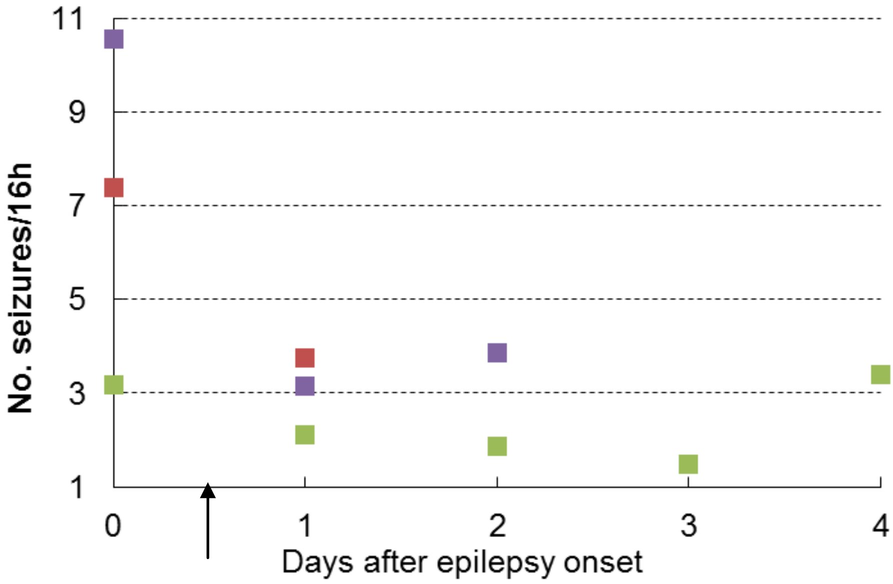


Figure 21. The effect of GD124 on mTORC1 and mTORC2 in the hippocampus of WT mice is inconclusive. n=2 per group (preliminary data).

In addition, we tested the S6K1 inhibitor, PF-4708671; previously used in a mouse model of cardiac infarction and able to reduce pS6 levels in this organ (Di et al., 2012).

Following validation of this drug's effect in decreasing S6K activity by a collaborating group, we decided to test this drug as a therapeutic approach in our mouse model. We wonder if inhibiting only the S6K downstream arm of the mTORC1 pathway, especially because it is also involved in the crosstalk with mTORC2 (see Figure 1), would be enough for the treatment of epilepsy. For that, we started with a pilot study with only 3 mutant mice. We induced *Tsc1* deletion in adulthood (as previously) and we assessed epilepsy onset: these 3 mice showed epilepsy onset either at day 10 or 11, fitting our previous findings (see Figure 12). During the day of the subsequent EEG recording that showed epileptic seizures, we administered the S6K inhibitor in a concentration of 75 mg/kg in two of the mice and only vehicle in the third mouse (as a control). Unfortunately, this pilot study was not very successful, since the two treated mice died 1 and 2 days after treatment onset. We could speculate that the used concentration was too low, or that the treatment was initiated too late (after epilepsy onset) or even that, in fact, inhibiting only this arm of the mTORC1 pathway is not enough to treat epilepsy.

In Figure 22, we show number of seizures per 16 h of recording and length of seizures of these 3 mice, being the vehicle-injected mouse the one in green. We can see that the two mice used for the treatment had a high number of seizures during the first night (roughly, 7 and 10 compared to 3), as well as a higher average of length of seizures, so that might suggest that the seizure burden was too high to be treated with this drug.



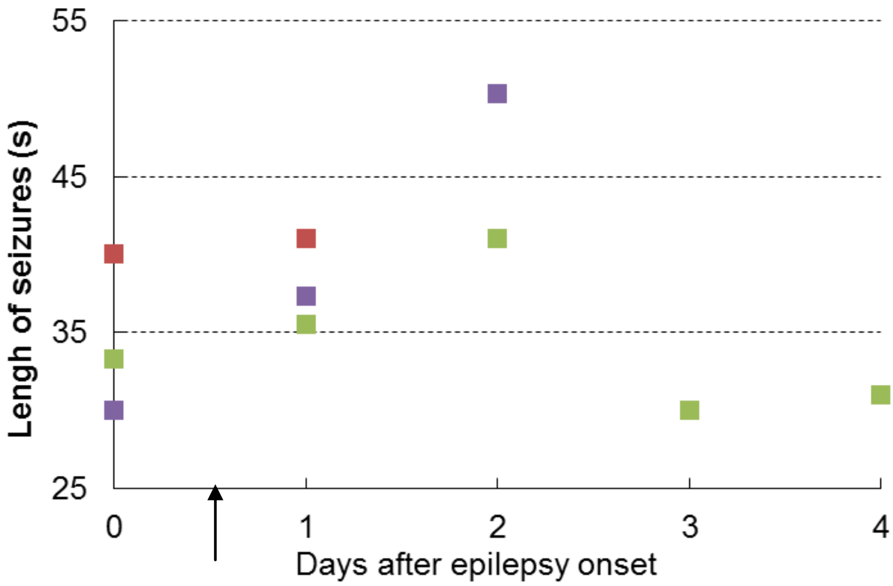


Figure 22. Number (per 16h of recording; upper graph) and length of seizures (in seconds; lower graph) of the recorded mice. Each color represents one mouse: green, vehicle-injected mouse; red and purple: PF-4708671(75 mg/kg)-treated mice. Treatment was administered at the moment indicated by the arrow, i.e, the day following the first night of seizures. (Preliminary data.)

3.6 Ongoing work

At the moment, we are further testing these drugs in their ability as antiepileptic treatments in our mouse model, i.e., their ability to alleviate seizure severity or reduce epilepsy progression as well as increase lifespan.

3.7 Preliminary findings in the *Tsc1*-GFAP mouse model

As explained in chapter 1, our overall interest is investigating the molecular mechanisms underlying epilepsy in TSC and, for that, we generated different mouse models. The next one to be addressed is the *Tsc1*$^{f/f}$-GFAP-er$_{T2}$-cre, in which tamoxifen administration results in *Tsc1* deletion in GFAP-positive cells.

As a first approach, we injected adult mice with 4 and 8 tamoxifen injections. Due to the failure in inducing cre-recombination with this treatment scheme, we then injected 4 weeks old mice for 8 consecutive days (as described in (Hirrlinger et al., 2006)). However we again failed to achieve *Tsc1* gene deletion (Figure 23).

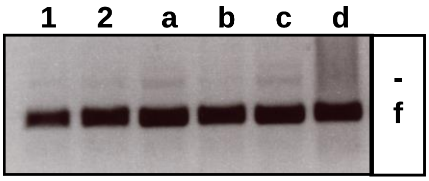


Figure 23. PCR for *Tsc1* in the *Tsc1*-GFAP mice under different conditions. Numbers code for brainstem tissue of adult mice: **1**, 4 tamoxifen injections; **2**, 8 tamoxifen injections. Letters code for samples from a 4 weeks old mouse injected 8 times with tamoxifen: **a**, brainstem; **b**, cortex; **c**, hippocampus; **d**, cerebellum. A weak deletion band is only observed in the brainstem and hippocampus of 4 weeks old mice injected 8 times with tamoxifen. f: floxed band; -: deletion band

Due to these difficulties, we are now setting up a new breeding of these mice, in which we will try different approaches, from earlier tamoxifen-injection, to changes in the used dose.

Chapter 4

Discussion

TSC is a complex disease with an underlying molecular pathway up until now not fully understood. Moreover, targeted treatments have been under investigation but none has been successful for all aspects of this multifaceted disease (they either have an effect on the tumors, or partially control the seizures, etc). Therefore, research in TSC is very challenging, but mandatory for the patients.

Epilepsy is probably the most challenging neurologic manifestation of TSC and, so far, it has only been possible of being observed in conditional mouse models (where biallelic *Tsc1* or *Tsc2* deletion occurs only in a specific subset of cells). As a result, our lab previously studied a global inducible *Tsc1* KO, that could undergo *Tsc1* deletion only in adulthood: *Tsc1*-cag-esr-cre (Abs et al., *in press*). Similar to developmental *Tsc1* deletion, deletion in adulthood is lethal, probably due to kidney failure (which is a major cause of death also among the patients), but only after epilepsy development. However, the time window to study epileptogenesis was too narrow in this mouse model, prompting the generation of cell-specific inducible mouse models.

We now crossed the *Tsc1*$^{f/f}$ mouse line with the transgenic cre-lines driven by the αCaMKII, GFAP or GAD2 promoters, in order to obtain *Tsc1*-αCaMKII, *Tsc1*-GFAP and *Tsc1*-GAD2 mouse models, respectively. These mice may undergo biallelic *Tsc1* deletion in excitatory neurons, astrocytes or inhibitory neurons, only upon tamoxifen administration. In other words, we now have available three TSC mouse models that will, most likely, not show peripheral complications (due to the brain-specific gene deletion), and may allow the study of the mTORC1 hyperactivation role in epilepsy, probably in a more focused way (no developmental or peripheral complications will mask the possible epilepsy development and the molecular or electrophysiological changes that accompanied it).

In this thesis we make use of the first mouse model, *Tsc1*-αCaMKII, to investigate the molecular mechanisms underlying epileptogenesis and the ability of novel drugs to treat epilepsy in TSC.

In accordance to the high αCaMKII expression throughout the forebrain, a key structure in epileptogenesis, the *Tsc1*-αCaMKII mouse develops severe and lethal epilepsy in just 9 to 12 days after gene deletion onset, with mTORC1 hyperactivation and no developmental brain pathology. In line with the brain-specific driven deletion, no peripheral abnormalities were observed; and, as far as our investigation went, epilepsy was the only symptom of these mice. Therefore, we just provided to the scientific community a very useful mTORC1-dependent epilepsy mouse model that will allow us to study the molecular mechanisms underlying epileptogenesis. Moreover, novel drugs targeting mTORC1/2 were here tested in their ability to target the brain, as well as an S6K inhibitor, that is now being tested as an antiepileptic drug in our mouse model. Altogether, this project represents a major first step in investigating mTORC1 hyperactivation-dependent epileptogenesis, a feature also present in non-TSC related forms of epilepsy.

Gene deletion magnitude is dependent on tamoxifen amount. Here we report that four tamoxifen injections are enough to induce mTORC1 hyperactivation in the majority of αCaMKII-positive cells, due to *Tsc1* gene deletion. Further analysis of this statement will be done by crossing our mouse model with a Tomato- reporter line, therefore allowing us to investigate the activated cre in a cell-specific manner.

Tsc1 gene deletion is followed by mTORC1 hyperactivation. In order to correctly investigate mTORC1 hyperactivation, the three signaling arms should be analyzed: pS6, p4EBP1 and pUlk (see Figure 1).

First, in our mouse model, phosphorylated S6 was shown to be increased in a time-dependent manner after gene deletion and possibly in a differential manner between epileptic and non-epileptic mice at the same time-point (ongoing work).

Secondly, p4EBP1 would represent a step further in understanding the cap-translational mechanisms, shown to be rapamycin treatment insensitive (Thoreen and Sabatini, 2009) and most likely playing a major role in epileptogenesis in our mouse model. However, we failed to assess its levels in brain (due to the low levels together with inadequate antibody condition). The p4EBP2 isoform is brain-specific, possibly overcoming the protein availably issue, but previous reports also document difficulties in assessing this protein levels (Bidinosti et al., 2010). The third option would be to look at the different isoforms of the 4EBP1/2 proteins, although no differences were found in the *Tsc1*-cag mouse model. Nevertheless, one of these options will most certainly be used in the future to try and understand how this arm of the mTORC1 signaling is involved in epilepsy in our mouse model.

Third, the autophagy arm, suggested to contribute to epileptogenesis (McMahon et al., 2012), was also not addressed in our mouse model, but we aim to do it in the close future.

Tsc1 gene deletion is also followed by decreased levels of pAkt. Although this decrease is widely accepted as meaning mTORC2 down regulation, some topics are still under discussion. Therefore, other readouts for mTORC2, such as pNDRG1, should also be addressed. That way, it will be possible to distinguish between mTORC2 down regulation or direct pAkt inhibition due to S6K feedback loop, representing also a step further in the understanding of the mTOR complexes crosstalk in TSC. We planned to address pNDRG1 in our mouse model in the short run.

The still ongoing experiments concerning the epilepsy characterization will also shed light in some remaining questions, such as mTORC1 hyperactivity role in epilepsy.

The epileptogenesis investigation is, without a doubt, the most important question in this project and further study on this topic will be addressed in a multidisciplinary way.

First, further testing for changes in expression and localization of glutamate receptors subunits, as well as GABAR subunits and ion channels, also in other brain areas, will be taken care of, aiming to understand if brain tissue of this mouse model is comparable to the tubers from the patients.

Secondly, as mentioned in the ongoing work of subchapter 3.4, the molecular investigation of the glutamate signaling in this mouse model will also serve as an indication of the questions to be addressed by electrophysiology experiments: the use of specific blockers will allow us to investigate changes in receptor expression, in addition to the usual questions, such as excitability, mEPSCs, mIPSCs, etc; contributing to the understanding of the excitability pattern of the cell and its network connectivity. In addition, high deletion efficiency is of major importance for electrophysiology experiments, where a homogenous cell population should be present. That way, the neuron is representative for the changes suffered by a pyramidal cell that went through complete *Tsc1* loss, thereby reducing variability.

Third, we need to understand what the molecular cause of death in these mice is. Differently from the global KO (*Tsc1*-cag), which dies from a peripheral complication, the mouse hereby investigated dies after a seizure. This phenomenon is widely known as *status epilepticus* (a major cause of death among TSC patients), and the molecular causes are still under investigation, but somehow meet the molecular changes found in tuberal tissue from patients: Glutamate and GABA receptors subunits. In other words, a lethal seizure happens probably due to a failure in the mechanisms that terminate seizures, such as persistent excitation and failed inhibition signaling. Therefore, dynamic changes in the receptors, instead of presence or trafficking of different subunits (as we address in subchapter 3.4), could be possibly happening, and complementary electrophysiological technical approaches should be considered.

Epilepsy onset in our mouse model is less variable than in the global KO (Abs et al., *in press*). Speculations about this observation encourage us to pursuit these cell-specific mouse models, because they will, as hypothesized, allow the dissection of the different features of complex disorders into narrower and narrower windows and less interference factors. Therefore, for all of these questions, the three cell-specific *Tsc1* mouse models that we have now in our hands could provide us with further insight into the network and cell-dependent mechanisms.

In contrast to the other cell-specific TSC mouse models, our inducible mouse model is free of developmental brain pathology and does not seem to show changes in overall brain morphology (no giant cells or gross brain morphology changes). This further strengthens the hypothesis that possibly tubers or tumors are not epileptogenic foci *per se*, but either the homozygous cells within them, that might somehow fail to maintain proper synaptic contact, membrane excitability or regular receptor trafficking; forming aberrant connections and network hyper excitability. Even tough, a closer look at cell morphology using, for example, the Gene gun approach that specifically stains isolated cells in a slice, would either show us different results or further strength the just-mentioned hypothesis.

From the two dual mTOR inhibitors that we tested, AZD8055 and GD124, only the first seems to be able to downregulate both mTOR complexes (decreasing pS6 and pAkt levels) in the brain of WT mice. On the other hand, the novel GD124 compound’s ability to have an effect on mTOR complexes 1 and 2 in the brain of WT mice seems inconclusive and we are now focused on further addressing this question e.g. by testing a higher dose and at different time points as well as additional testing in *in vitro* essays (a good collaboration with Mark Nellist from the *Clinical Genetics Department*). Moreover, these drugs will be further tested in our mouse model, i.e., in their ability of abolishing seizures and increase lifespan, as an antiepileptic treatment. Altogether, this investigation will help us understand how these drugs may or not be valuable in mTORC1-related epilepsy.

The S6K inhibitor showed promising results in its ability of decreasing pS6/S6 levels in the brain of WT mice (unpublished data by Eric Klann group);

therefore, we tested it as an antiepileptic treatment for our mouse model. This pilot study revealed itself as a disappointment and the speculation can go to the most various justifications. Was the dose used not high enough? Did the treatment come too late? Is it just absurd to consider that inhibition of only one mTORC1 downstream pathway could be efficient in treating such severe epilepsy?... A new approach will be defined to try to use this drug as a treatment: a higher dose or administration before the seizure onset, is a possibility to consider. If evidenced that this drug does not work as an anti-epileptic treatment, the S6K inhibitor also represents a major tool in understanding epileptogenesis in TSC by dissecting the role of the mTORC1-S6K arm in epilepsy.

During this project we were also able to have the first preliminary results concerning the second cell-specific mouse model: *Tsc1*$^{f/f}$-GFAP. We attempted the induction of gene deletion by testing different protocols of tamoxifen administration, but the obtained deletion band does not seem to reach the published data on the original transgenic mouse (Hirrlinger et al., 2006). Therefore, optimal conditions to obtain gene deletion will have to be further tested, before using the model in e.g. behavioral experiments. Developmental deletion of *Tsc1* in GFAP-positive cells was widely studied (see Table II) and it is consistent in resulting in epilepsy and brain pathology, so we are looking forward to investigate epilepsy and lifespan when *Tsc1* is deleted only in mature astrocytes. Moreover, the phenotype of this mouse will give us further insight onto the cell-specific contribution to epileptogenesis in the patients.

Soon, the third mouse model, *Tsc1*-GAD2, will be investigated and the same questions will be addressed: epilepsy, lifespan, molecular changes, etc.

We can already speculate that this deletion will not be enough to the development of epilepsy, since the developmental deletion of *Tsc1* in inhibitory neurons (driven by the Dlx5/6 promoter, summarized in Table II) only results in decreased seizure threshold. However, a lot of questions are still to answer concerning the role of mTORC1 hyperactivation in inhibitory neurons.

Chapter 5

Conclusion

Altogether, our results confirm that acute mTORC1 upregulation in only αCaMKII-positive neurons is sufficient to cause epilepsy, which confirms the major role of this cell-type in epileptogenesis in TSC, including in adulthood. Moreover, the cause of lethality in these mice is epilepsy, motivating us to investigate the molecular mechanisms underlying the source of death in this mouse model.

The study of this mouse opens novel questions that will be soon addressed by using the complementary cell-specific mouse models (*Tsc1*-GFAP and *Tsc1*-GAD2) and further studies in other systems. When the story is complete, the cell type (excitatory and inhibitory neurons as well as astrocytes) dependent molecular contribution to epileptogenesis in TSC will be drafted and, together with previous studies, a bigger picture of the cellular and molecular mechanisms, together with the network properties underlying epileptogenesis will be drawn.

In conclusion, this project is the first step on a wide field that will most certainly help us understand epilepsy (not only TSC-related), contributing to the understanding and possibly helping to find therapeutic options for such dramatic neurologic manifestation that incapacitates half a million people worldwide.

References

Abs, E., Goorden, S., Schreiber, J., Overwater, I., Hoogeveen-Westerveld, M., Bruinsma, C., Aganovic, E., Borgesius, N., Nellist, M., and Elgersma, Y. (*in press*). TORC1-dependent epilepsy caused by acute biallelic Tsc1 deletion in adult mice. Annals of neurology.

Anderl, S., Freeland, M., Kwiatkowski, D.J., and Goto, J. (2011). Therapeutic value of prenatal rapamycin treatment in a mouse brain model of tuberous sclerosis complex. Human molecular genetics *20*, 4597-4604.

Benvenuto, G., Li, S., Brown, S.J., Braverman, R., Vass, W.C., Cheadle, J.P., Halley, D.J., Sampson, J.R., Wienecke, R., and DeClue, J.E. (2000). The tuberous sclerosis-1 (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination. Oncogene *19*, 6306-6316.

Bidinosti, M., Ran, I., Sanchez-Carbente, M.R., Martineau, Y., Gingras, A.C., Gkogkas, C., Raught, B., Bramham, C.R., Sossin, W.S., Costa-Mattioli, M.*, et al.* (2010). Postnatal deamidation of 4E-BP2 in brain enhances its association with raptor and alters kinetics of excitatory synaptic transmission. Molecular cell *37*, 797-808.

Bissler, J.J., McCormack, F.X., Young, L.R., Elwing, J.M., Chuck, G., Leonard, J.M., Schmithorst, V.J., Laor, T., Brody, A.S., Bean, J.*, et al.* (2008). Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. N Engl J Med *358*, 140-151.

Carson, R.P., Van Nielen, D.L., Winzenburger, P.A., and Ess, K.C. (2012). Neuronal and glia abnormalities in Tsc1-deficient forebrain and partial rescue by rapamycin. Neurobiology of disease *45*, 369-380.

Chresta, C.M., Davies, B.R., Hickson, I., Harding, T., Cosulich, S., Critchlow, S.E., Vincent, J.P., Ellston, R., Jones, D., Sini, P.*, et al.* (2010). AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity. Cancer research *70*, 288-298.

Crino, P.B., Aronica, E., Baltuch, G., and Nathanson, K.L. (2010). Biallelic TSC gene inactivation in tuberous sclerosis complex. Neurology *74*, 1716-1723.

Crino, P.B., Nathanson, K.L., and Henske, E.P. (2006). The tuberous sclerosis complex. N Engl J Med *355*, 1345-1356.

Di, R., Wu, X., Chang, Z., Zhao, X., Feng, Q., Lu, S., Luan, Q., Hemmings, B.A., Li, X., and Yang, Z. (2012). S6K inhibition renders cardiac protection against myocardial infarction through PDK1 phosphorylation of Akt. The Biochemical journal *441*, 199-207.

Ehninger, D., Han, S., Shilyansky, C., Zhou, Y., Li, W., Kwiatkowski, D.J., Ramesh, V., and Silva, A.J. (2008). Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. Nature medicine *14*, 843-848.

Ehninger, D., and Silva, A.J. (2011). Increased levels of anxiety-related behaviors in a Tsc2 dominant negative transgenic mouse model of tuberous sclerosis. Behavior genetics *41*, 357-363.

Erdmann, G., Schutz, G., and Berger, S. (2007). Inducible gene inactivation in neurons of the adult mouse forebrain. BMC Neurosci *8*, 63.

Feil, R., Wagner, J., Metzger, D., and Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun *237*, 752-757.

Fu, C., Cawthon, B., Clinkscales, W., Bruce, A., Winzenburger, P., and Ess, K.C. (2012). GABAergic interneuron development and function is modulated by the Tsc1 gene. Cerebral cortex (New York, NY : 1991) *22*, 2111-2119.

Goorden, S.M., van Woerden, G.M., van der Weerd, L., Cheadle, J.P., and Elgersma, Y. (2007). Cognitive deficits in Tsc1+/- mice in the absence of cerebral lesions and seizures. Annals of neurology *62*, 648-655.

Han, J.M., and Sahin, M. (2011). TSC1/TSC2 signaling in the CNS. FEBS letters *585*, 973-980.

Hirrlinger, P.G., Scheller, A., Braun, C., Hirrlinger, J., and Kirchhoff, F. (2006). Temporal control of gene recombination in astrocytes by transgenic expression of the tamoxifen-inducible DNA recombinase variant CreERT2. Glia *54*, 11-20.

Huang, J., and Manning, B.D. (2008). The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. The Biochemical journal *412*, 179-190.

Jansen, L.A., Uhlmann, E.J., Crino, P.B., Gutmann, D.H., and Wong, M. (2005). Epileptogenesis and reduced inward rectifier potassium current in tuberous sclerosis complex-1-deficient astrocytes. Epilepsia *46*, 1871-1880.

Kessler, S.K., Gallagher, P.R., Shellhaas, R.A., Clancy, R.R., and Bergqvist, A.G. (2011). Early EEG improvement after ketogenic diet initiation. Epilepsy research *94*, 94-101.

Kwiatkowski, D.J., Zhang, H., Bandura, J.L., Heiberger, K.M., Glogauer, M., el-Hashemite, N., and Onda, H. (2002). A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. Human molecular genetics *11*, 525-534.

Magri, L., Cambiaghi, M., Cominelli, M., Alfaro-Cervello, C., Cursi, M., Pala, M., Bulfone, A., Garcia-Verdugo, J.M., Leocani, L., Minicucci, F.*, et al.* (2011). Sustained activation of mTOR pathway in embryonic neural stem cells leads to development of tuberous sclerosis complex-associated lesions. Cell stem cell *9*, 447-462.

Major, P., Rakowski, S., Simon, M.V., Cheng, M.L., Eskandar, E., Baron, J., Leeman, B.A., Frosch, M.P., and Thiele, E.A. (2009). Are cortical tubers epileptogenic? Evidence from electrocorticography. Epilepsia *50*, 147-154.

McDaniel, S.S., Rensing, N.R., Thio, L.L., Yamada, K.A., and Wong, M. (2011). The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway. Epilepsia *52*, e7-11.

McDaniel, S.S., and Wong, M. (2011). Therapeutic role of mammalian target of rapamycin (mTOR) inhibition in preventing epileptogenesis. Neurosci Lett *497*, 231-239.

McMahon, J., Huang, X., Yang, J., Komatsu, M., Yue, Z., Qian, J., Zhu, X., and Huang, Y. (2012). Impaired autophagy in neurons after disinhibition of mammalian target of rapamycin and its contribution to epileptogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 15704-15714.

Meikle, L., Talos, D.M., Onda, H., Pollizzi, K., Rotenberg, A., Sahin, M., Jensen, F.E., and Kwiatkowski, D.J. (2007). A mouse model of tuberous sclerosis: neuronal loss of Tsc1 causes dysplastic and ectopic neurons, reduced myelination, seizure activity, and limited survival. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 5546-5558.

Metzger, D., and Chambon, P. (2001). Site- and time-specific gene targeting in the mouse. Methods *24*, 71-80.

Onda, H., Lueck, A., Marks, P.W., Warren, H.B., and Kwiatkowski, D.J. (1999). Tsc2(+/-) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. The Journal of clinical investigation *104*, 687-695.

Orlova, K.A., and Crino, P.B. (2010). The tuberous sclerosis complex. Annals of the New York Academy of Sciences *1184*, 87-105.

Reith, R.M., Way, S., McKenna, J., 3rd, Haines, K., and Gambello, M.J. (2011). Loss of the tuberous sclerosis complex protein tuberin causes Purkinje cell degeneration. Neurobiology of disease *43*, 113-122.

Sato, A., Kasai, S., Kobayashi, T., Takamatsu, Y., Hino, O., Ikeda, K., and Mizuguchi, M. (2012). Rapamycin reverses impaired social interaction in mouse models of tuberous sclerosis complex. Nature communications *3*, 1292.

Scott, R.C., and Holmes, G.L. (2012). Before epilepsy unfolds: opening up the potassium door in neonatal seizures. Nature medicine *18*, 1624-1625.

Seto, B. (2012). Rapamycin and mTOR: a serendipitous discovery and implications for breast cancer. Clin Transl Med *1*, 29.

Talos, D.M., Kwiatkowski, D.J., Cordero, K., Black, P.M., and Jensen, F.E. (2008). Cell-specific alterations of glutamate receptor expression in tuberous sclerosis complex cortical tubers. Annals of neurology *63*, 454-465.

Talos, D.M., Sun, H., Kosaras, B., Joseph, A., Folkerth, R.D., Poduri, A., Madsen, J.R., Black, P.M., and Jensen, F.E. (2012). Altered inhibition in tuberous sclerosis and type IIb cortical dysplasia. Annals of neurology *71*, 539-551.

Tavazoie, S.F., Alvarez, V.A., Ridenour, D.A., Kwiatkowski, D.J., and Sabatini, B.L. (2005). Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. Nature neuroscience *8*, 1727-1734.

Thiele, E.A. (2004). Managing epilepsy in tuberous sclerosis complex. J Child Neurol *19*, 680-686.

Thoreen, C.C., and Sabatini, D.M. (2009). Rapamycin inhibits mTORC1, but not completely. Autophagy *5*, 725-726.

Tsai, V., Parker, W.E., Orlova, K.A., Baybis, M., Chi, A.W., Berg, B.D., Birnbaum, J.F., Estevez, J., Okochi, K., Sarnat, H.B.*, et al.* (2012). Fetal Brain mTOR Signaling Activation in Tuberous Sclerosis Complex. Cerebral cortex (New York, NY : 1991).

Tsukiyama-Kohara, K., Poulin, F., Kohara, M., DeMaria, C.T., Cheng, A., Wu, Z., Gingras, A.C., Katsume, A., Elchebly, M., Spiegelman, B.M.*, et al.* (2001). Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. Nature medicine *7*, 1128-1132.

Uhlmann, E.J., Wong, M., Baldwin, R.L., Bajenaru, M.L., Onda, H., Kwiatkowski, D.J., Yamada, K., and Gutmann, D.H. (2002). Astrocyte-specific

TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures. Annals of neurology *52*, 285-296.

Wander, S.A., Hennessy, B.T., and Slingerland, J.M. (2011). Next-generation mTOR inhibitors in clinical oncology: how pathway complexity informs therapeutic strategy. The Journal of clinical investigation *121*, 1231-1241.

Wang, Y., Greenwood, J.S., Calcagnotto, M.E., Kirsch, H.E., Barbaro, N.M., and Baraban, S.C. (2007). Neocortical hyperexcitability in a human case of tuberous sclerosis complex and mice lacking neuronal expression of TSC1. Annals of neurology *61*, 139-152.

Way, S.W., McKenna, J., 3rd, Mietzsch, U., Reith, R.M., Wu, H.C., and Gambello, M.J. (2009). Loss of Tsc2 in radial glia models the brain pathology of tuberous sclerosis complex in the mouse. Human molecular genetics *18*, 1252-1265.

White, R., Hua, Y., Scheithauer, B., Lynch, D.R., Henske, E.P., and Crino, P.B. (2001). Selective alterations in glutamate and GABA receptor subunit mRNA expression in dysplastic neurons and giant cells of cortical tubers. Annals of neurology *49*, 67-78.

Wild, J.M., Ahn, H.S., Baulac, M., Bursztyn, J., Chiron, C., Gandolfo, E., Safran, A.B., Schiefer, U., and Perucca, E. (2007). Vigabatrin and epilepsy: lessons learned. Epilepsia *48*, 1318-1327.

Wong, M. (2010). Mammalian target of rapamycin (mTOR) inhibition as a potential antiepileptogenic therapy: From tuberous sclerosis to common acquired epilepsies. Epilepsia *51*, 27-36.

Wong, M., Ess, K.C., Uhlmann, E.J., Jansen, L.A., Li, W., Crino, P.B., Mennerick, S., Yamada, K.A., and Gutmann, D.H. (2003). Impaired glial glutamate transport in a mouse tuberous sclerosis epilepsy model. Annals of neurology *54*, 251-256.

Yamada, K.A. (2008). Calorie restriction and glucose regulation. Epilepsia *49 Suppl 8*, 94-96.

Young, D.M., Schenk, A.K., Yang, S.B., Jan, Y.N., and Jan, L.Y. (2010). Altered ultrasonic vocalizations in a tuberous sclerosis mouse model of autism. Proceedings of the National Academy of Sciences of the United States of America *107*, 11074-11079.