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TITLE: Temporal Loss of Tsc1: Neural Development and Brain Disease in Tuberous Sclerosis

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CONTRACTING ORGANIZATION: **Brown University**
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14. ABSTRACT. The purpose of our research proposal is to determine how the deletion of <i>Tsc1</i> and mTOR dysregulation affects thalamus development and function. An addition goal of our research was to use our conditional gene deletion system to test the ability of the mTOR inhibitor rapamycin to ameliorate neurological phenotypes depending upon the time and duration of treatment. During this research period, we further advanced our novel genetic approach to control <i>Tsc1</i> gene deletion concomitant with cell lineage tracing and biochemical analysis to better understand the developmental aspects of Tuberous Sclerosis. A major set of findings is that we identified cellular, molecular, circuitry, and behavioral changes that occur during development and are specific to distinct temporal roles of <i>Tsc1</i> and the mTOR pathway. Specifically, we showed that early embryonic deletion of <i>Tsc1</i> resulted in mTOR dysregulation within 48 hours and this dysregulation persisted throughout the life of the mice; this is the first report of the kinetics of mTOR dysregulation. In addition, we showed that neural circuits that connect the thalamus and cerebral cortex are disrupted by early or late deletion of <i>Tsc1</i> and that the neural circuit abnormality is first observed at the end of embryogenesis (five days after mTOR dysregulation). Thus, specific phenotypes emerge rapidly and others appear over a more prolonged developmental window. We then used biochemistry to show that proteins involved in synaptic architecture are altered by the early deletion of <i>Tsc1</i> . Finally, we show that behavioral alterations are strongly associated with the time of <i>Tsc1</i> function. We initiated studies to address our additional and have begun delineate the most effective method and dose of rapamycin that can support development while at the same time effectively suppressing the mTOR pathway. The findings generated from this funding period are important for Tuberous Sclerosis research because we have determined critical developmental periods affected by <i>Tsc1</i> deletion and important details regarding mTOR inhibition as a strategy to intervene in these early developmental windows.					
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PRE-INTRODUCTION AND RATIONALE FOR EARLY TERMINATION

I want to clarify and provide context for the early termination of award W81XWH-12-1-0187. The reason for terminating the award early is complicated, but I recently went through the tenure process at Brown and received positive votes to approve my tenure from my department (Molecular Biology, Biochemistry, and Cell Biology) and subsequently from the University TPAC (Tenure, Promotion, and Appointments Committee). However, the Provost overturned those positive votes and denied my tenure case. There is no appeal process at Brown and I was fortunate to have numerous faculty contact the Provost on my behalf to voice their concern over the decision including my department Chair and the Chair of Neuroscience. This outreach, coupled with meetings that I had with him and the University President, still resulted in my tenure being denied.

Unfortunately, the primary rationale (really the only reason) was the lack of R01 funding. He acknowledged the work from my lab although frankly he only paid attention to my most recent Neuron paper and not any of the others (including a very nice Development paper). He also acknowledged my DoD funding; however, he said that because these grants were not renewable that they did not hold the same stature as R01 funding. He was also aware of my being named as a Simmons Foundation Autism Research Initiative (SFARI) investigator.

Regardless, the outcome is that I have left Brown University. I was fortunate to have made good connections during my time as a faculty member and I was also presented with a unique opportunity to go to industry and enter at a high level - thus I have taken a position at Amgen where I will be running the *in vivo* Neuroscience (Behavioral Pharmacology) group and investigating therapeutic areas related to Neurological disease.

I appreciate the DoD funding opportunities I have received and I am still deeply committed to Tuberous Sclerosis research. I would love to develop this as a therapeutic area in industry but that may take some time. I have been in contact with Cheryl A. Lowery (Contract Specialist US Army Medical Research Acquisition Activity) and Meropi Athanasiou (Science Officer Congressionally Directed Medical Research Program) and they are aware of the specific details of my situation.

INTRODUCTION

The purpose of our research proposal was to determine how the deletion of *Tsc1* and mTOR dysregulation affects brain development with an emphasis on thalamus development and function. To accomplish the goals of our proposal we used a conditional gene deletion, cell lineage tracing, and genetic neural circuit analysis (Figure 1; Normand et al., 2013). We focused on neural circuitry and synaptic organization of circuits modulated by thalamic activity. Additionally, we proposed to use our conditional gene deletion system to interrogate the ability of the mTOR inhibitor rapamycin to ameliorate neurological phenotypes with a focus on the time and duration of rapamycin treatment. During this research period, we advanced the use of our novel genetic approach to control *Tsc1* gene deletion concomitant with cell lineage tracing, cellular and biochemical analysis, and synaptic architecture analysis using unbiased stereology. We utilized these approaches to better understand the behavioral abnormalities in the mouse model we generated and to delineate the developmental trajectory and mechanisms underpinning Tuberous Sclerosis (TS).

A major set of findings from this funding period is that we linked specific phenotypic changes related to the loss of *Tsc1* and mTOR dysregulation during embryogenesis to long lasting changes in neural circuits and synaptic architecture. During the funding of this award, we

showed that early embryonic deletion of *Tsc1* resulted in mTOR dysregulation within 48 hours and this dysregulation persisted throughout the life of the mice; this is the first report of the kinetics of mTOR dysregulation. In addition, we showed that neural circuits that connect the thalamus and cerebral cortex are disrupted by early or late deletion of *Tsc1* and that the neural circuit abnormality is first observed at the end of embryogenesis (five days after mTOR dysregulation). Thus, some phenotypes emerge rapidly and others appear over a prolonged developmental window. We then used biochemistry to show that proteins involved in synaptic architecture are altered by the early deletion of *Tsc1*. Finally, we show that behavioral alterations are strongly associated with the time of *Tsc1* function. A second set of findings were achieved by initiating studies to identify the most effective delivery route and dose of rapamycin that can support development while effectively suppressing the mTOR pathway. The findings generated from this funding period are important for Tuberous Sclerosis research because we determined critical developmental periods affected by *Tsc1* deletion and obtained advances in mTOR inhibition as a strategy to intervene in these early developmental windows.

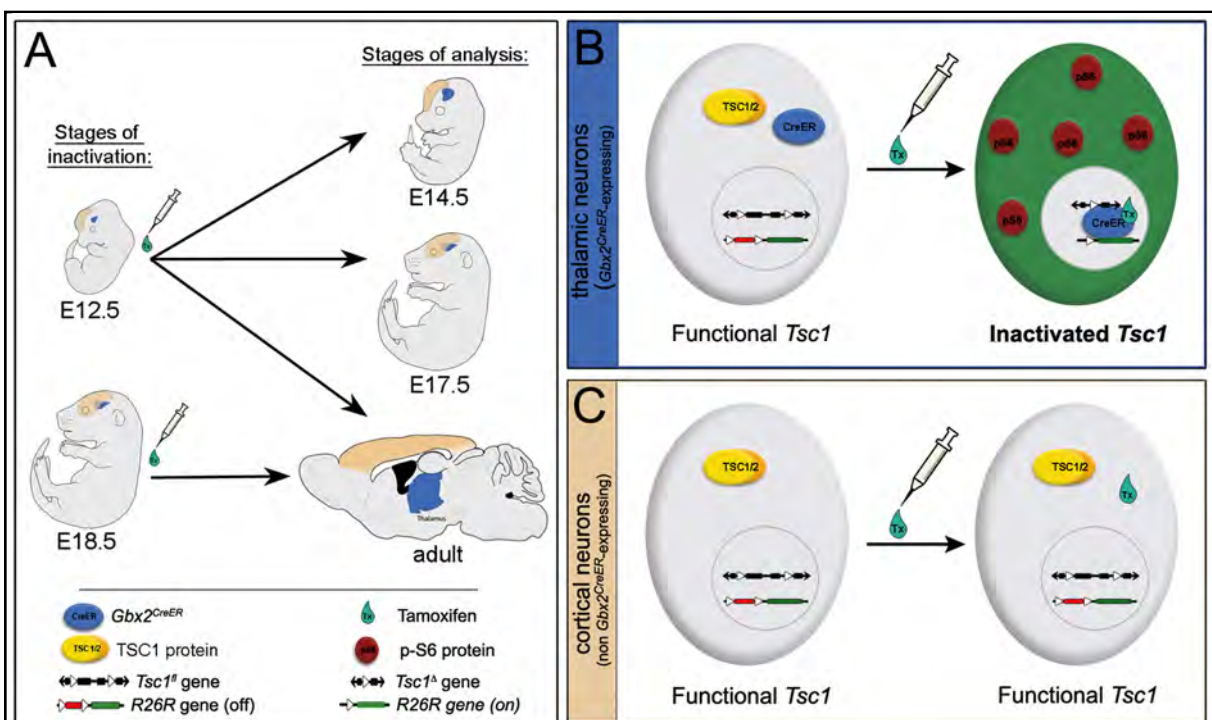


Figure 1. Spatial and temporal control over *Tsc1^{fl}* allele recombination. (A) Experimental approach. Thalamus is shown in blue, cerebral cortex is in tan. (B) Within thalamic *Gbx2^{CreER}*-expressing cells, tamoxifen activates the CreER protein (blue), allowing it to translocate into the nucleus, where it mediates recombination of *loxP* sites (triangles), thereby deleting the *Tsc1^{fl}* allele (black) and activating the reporter allele (green). (C) In cells, such as cortical neurons, that do not express *Gbx2^{CreER}*, the *Tsc1^{fl}* allele is functional and the reporter allele is quiescent, despite being exposed to tamoxifen. Adapted from Normand et al., 2013.

KEYWORDS

Tsc1
Tuberous Sclerosis
Conditional Gene Deletion
Neural circuits and synapses
Rapamycin

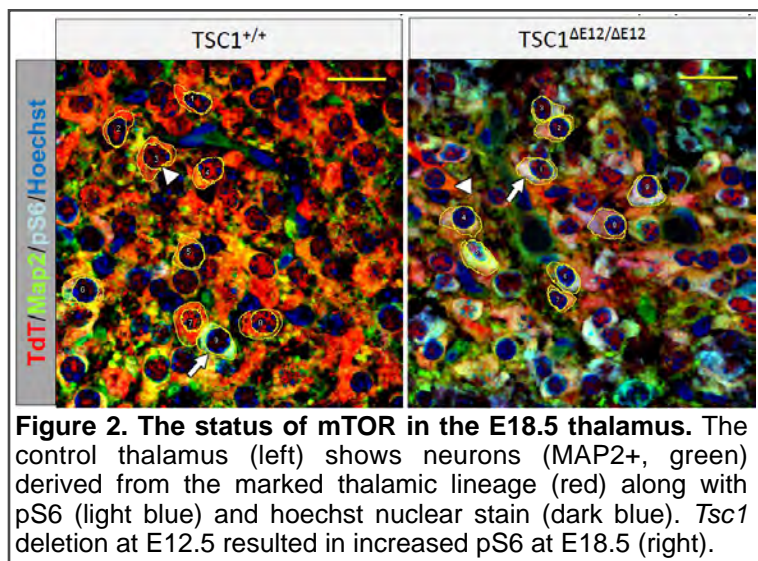
OVERALL PROJECT SUMMARY

Our funded research project “*Temporal loss of Tsc1: Neural development and brain disease in Tuberous Sclerosis*” uses sophisticated genetic approaches in mouse. The information obtained from this project provides important insight of the role of *Tsc1* and the first description, that we are aware of, regarding the early developmental changes that occur in the developing brain as a result of mTOR dysregulation. Our research findings deepen our understanding of mechanisms that cause the diverse array of phenotypes in Tuberous Sclerosis (TS) and functioned as important background information needed to address our second task, which was to design effective therapeutic strategies with translational relevance to human TS. The advances during this funding period of the project may lead to improved patient care by: 1. Pinpointing how specific cell types, neural circuits and neurological disease features arise subsequent to mTOR dysregulation and 2. Determining the timing and delivery route of mTOR inhibitors required to ameliorate early developmental manifestations of the disease. The progress on our aims and tasks from our Statement of Work are described below.

This proposal and current research focuses on the developing thalamus because it is an epicenter that synchronizes information processing and generates rhythmic activity and is related to aberrant electrical activity in the brain underpinning epilepsy and seizures, both of which are prominent in TS. More directly, patients with TS have been shown to have structural changes in the thalamus that are tightly correlated with poor performance on cognitive tasks (Ridler et al., 2001). The thalamus has also been linked to the autism component in human TS (Asano et al., 2001) and is poised to play an important role in brain dysfunction in TS.

We tackled our tasks using CreER/*loxP* technology (Zervas et al., 2004; Ellisor et al., 2009; Brown et al., 2009; Ellisor and Zervas, 2010; Hagan and Zervas, 2012; Yang et al., 2013; Normand et al., 2013) (Figure 1 and see Supplemental Figure 1 in Appendix 1). This approach allowed us to control the temporal deletion of *Tsc1* in a region specific manner (Normand et al., 2013) followed by the analyzing developing neurons and neural circuits as they are being established using a number of approaches (described below).

The dysregulation of mTOR and circuit development. As part of our first aim, we proposed to establish crucial time periods of *Tsc1* function in the developing thalamus through three tasks that involved deleting *Tsc1* at specific time points followed by analysis of subsequent developmental stages. We made significant progress on these tasks. We administered tamoxifen to delete *Tsc1* in the thalamic primordium at E12.5 and analyzed the thalamus at E14.5 and E18.5 for mTOR status (pS6 levels) and also for the connectivity and synapse formation of thalamocortical axons (TCAs) (Figures 2-8). Note that the designation of $\Delta E12/\Delta E12$ in the allele nomenclature indicates that tamoxifen was administered at E12.5 and converted both conditional ‘floxed’ alleles to null (Δ) alleles. We previously reported that control embryos had very low levels



of pS6 while *Tsc1*^{ΔE12/ΔE12} littermates had appreciably higher pS6 specific to the thalamus at E14.5 (First Annual Report; See also Appendix 1; Normand et al., 2013). This finding indicates that mTOR dysregulation occurs rapidly, within forty-eight hours after *Tsc1* deletion. We now report that deleting *Tsc1* at E12.5 resulted in significant increase in pS6 that persisted at E18.5 and would be predicted by mTOR dysregulation (Figure 2).

***Tsc1* controls cell size, neural circuitry, and behavior during critical developmental periods.** As part of aim 1 and our first set of tasks we proposed to compare four parameters subsequent to deleting *Tsc1* either early or late. The parameters were circuitry, cellular analysis, biochemistry, and behavior. We previously reported significant enlargement of mutant thalamic neurons that were pS6+ compared to pS6-thalamic neurons from controls at adulthood (Normand et al., 2013; Appendix I). We also showed that the cell size increase was cell autonomous as nearby non-mutant cells (those without increased pS6) were of normal size. Because mTOR regulation is critical for cell growth and we previously showed enlarged thalamic neurons in adulthood, we analyzed E18.5 embryos because at this time point mutant neurons had substantial increase in pS6 which reflects mTOR pathway. The early *Tsc1* deletion (*Tsc1*^{ΔE12/ΔE12}) did not result in differences in soma, nucleus, or cytoplasm size (Figure 3). Collectively, our recent findings and our published report indicate that *Tsc1* and mTOR pathway function over a limited time window during development (between E12.5 and before E18.5) to control a cell growth program that determines terminal cell size. In addition, the increase in cell size occurs after embryonic development.

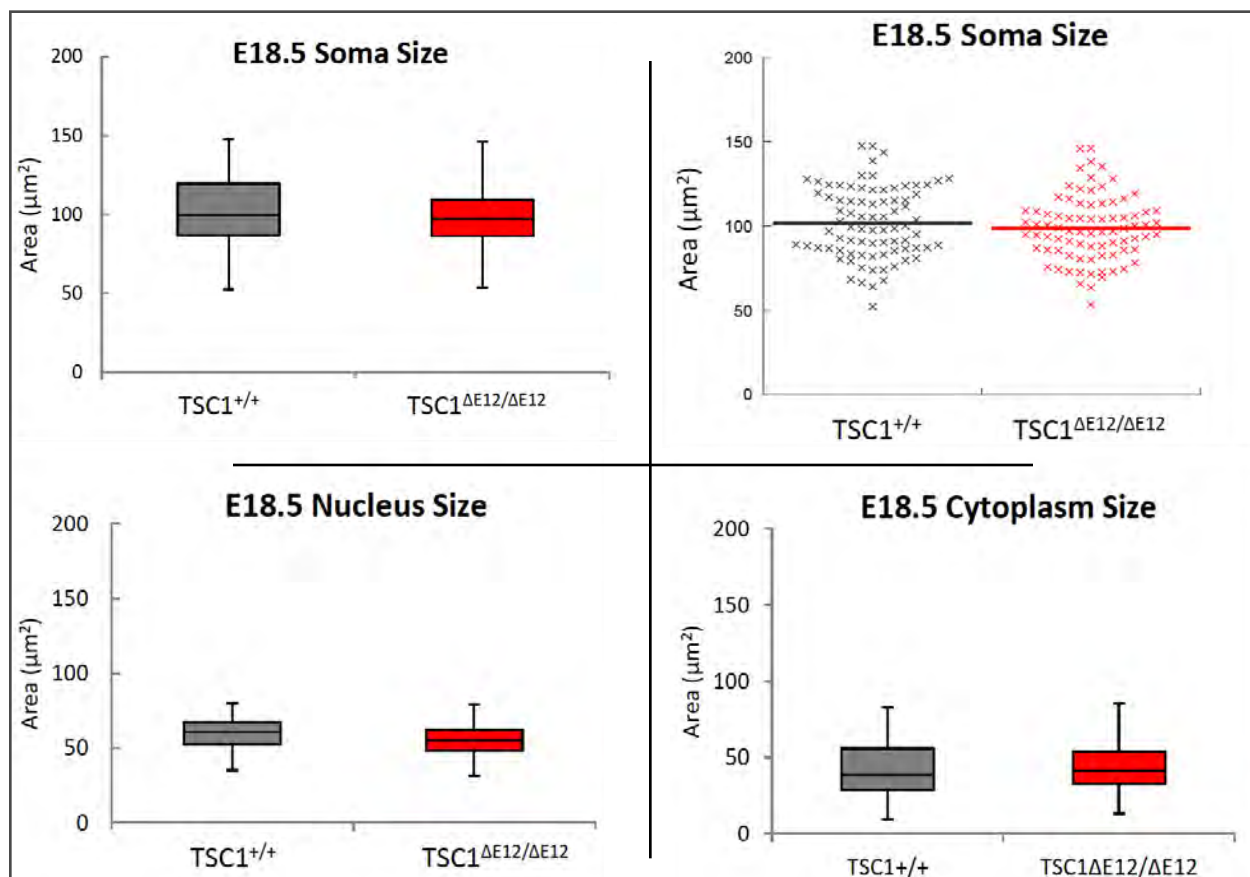
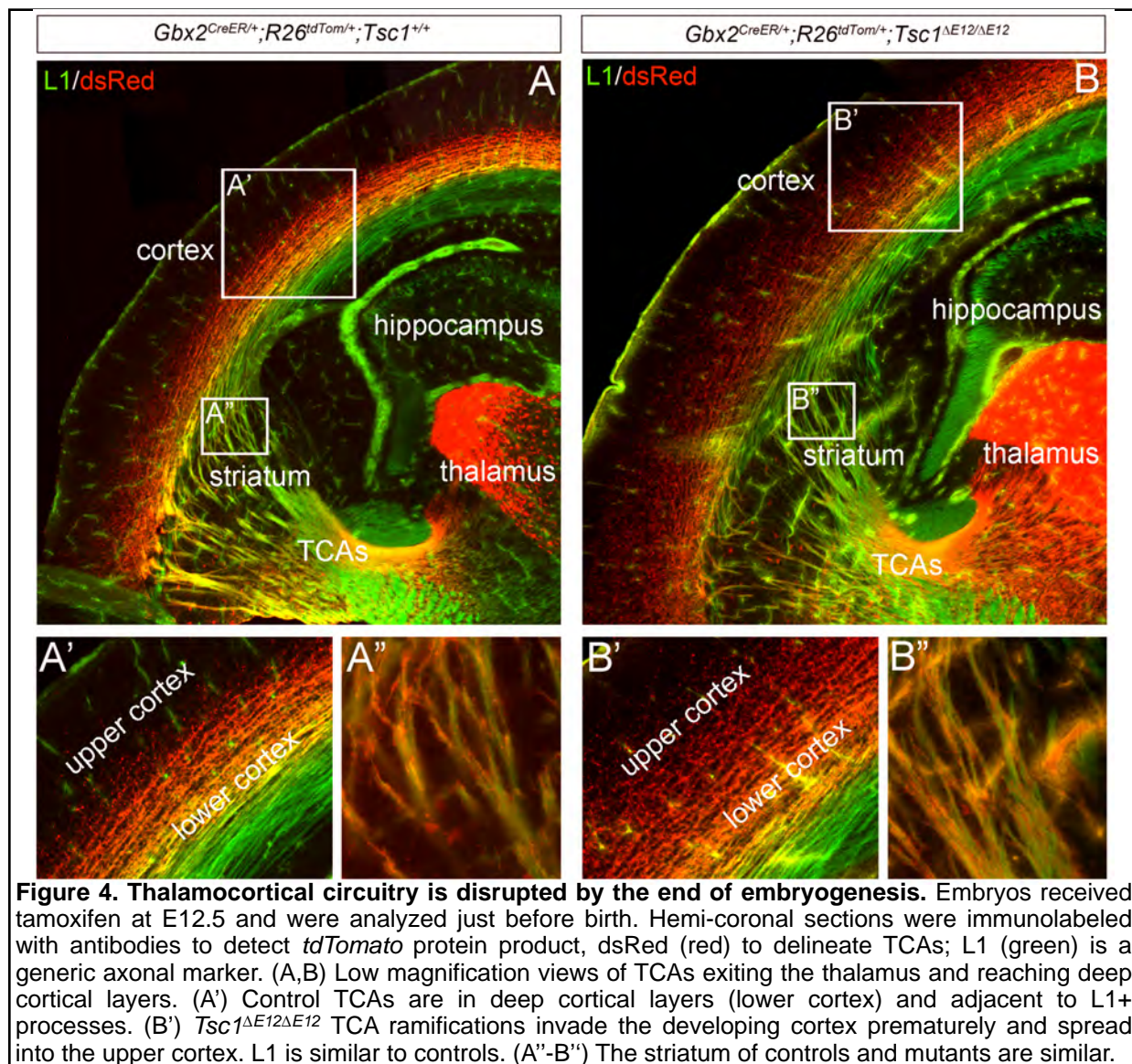
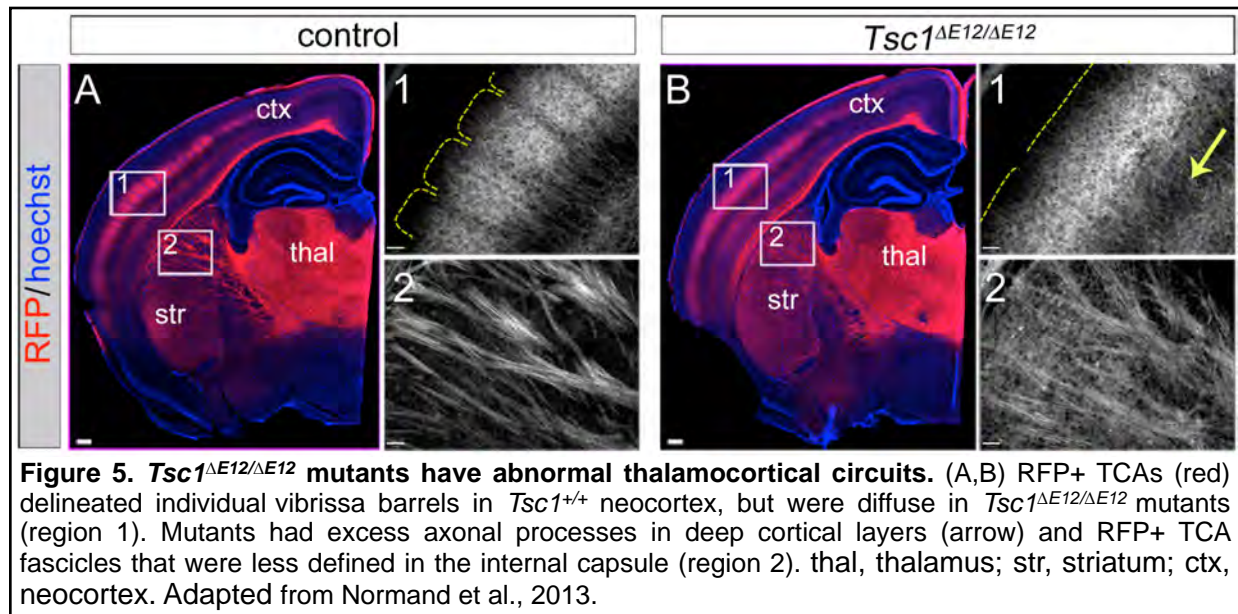


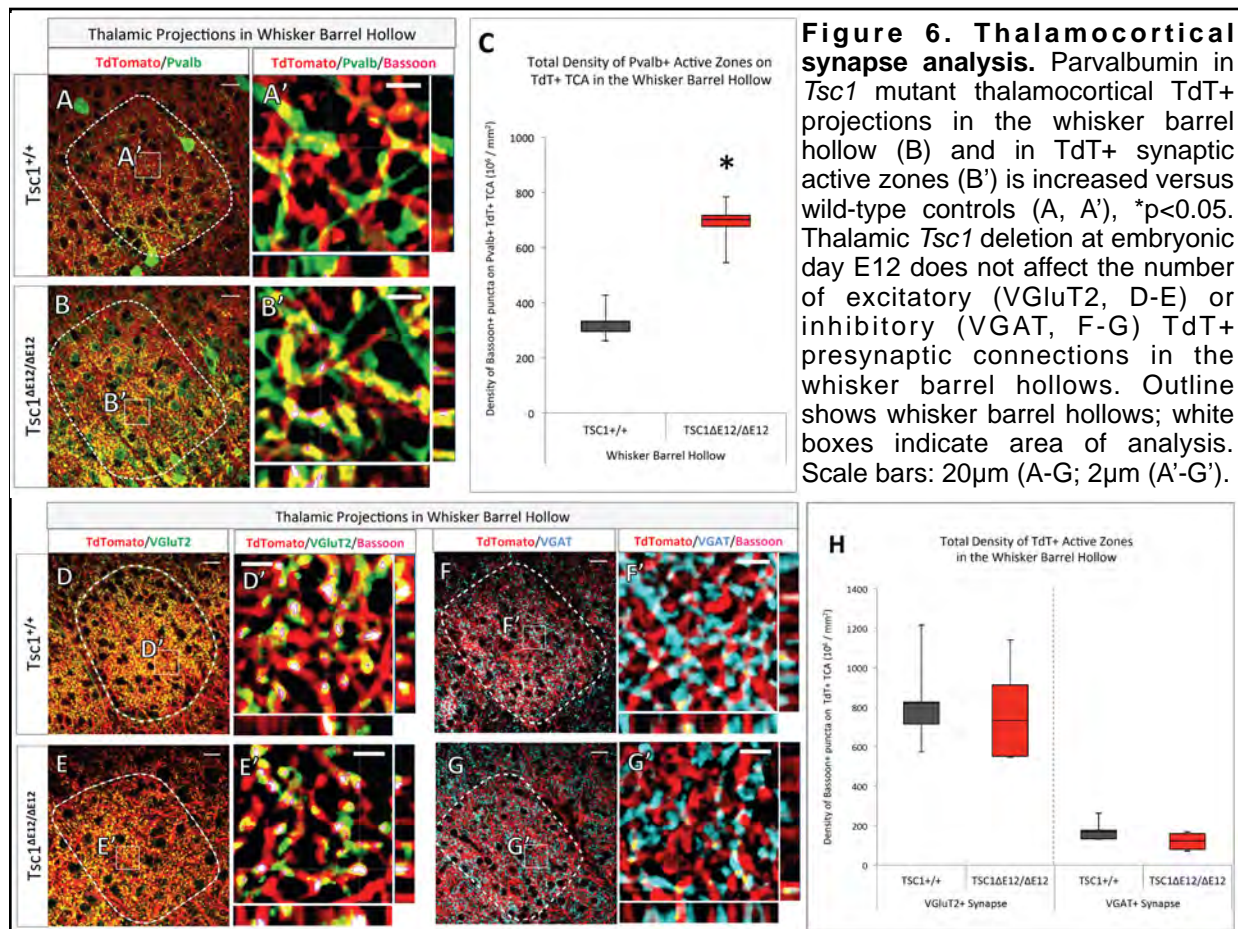
Figure 3. Neuronal size in the developing thalamus. *Tsc1* was deleted at E12.5 and cell sized was analyzed at the end of embryogenesis (E18.5). Wildtype controls (grey) are plotted versus conditional mutants (red). The box and whisker plots show median, minimum, maximum, first and third quartiles. There were no differences in the size of the soma, nucleus, and cytoplasm. The scatter plot shows individual cells in the two cohorts.

We used genetic circuit tracing (Ellisor et al., 2009; Normand et al., 2013) to analyze developing TCAs and showed that the early deletion (E12.5) of *Tsc1* did not significantly alter TCAs as they exited from the thalamus or acquired their intermediate target site although TCA fascicles appeared broader and wavy proximal to the intermediate target (striatum) as they pause before entering the cerebral cortex (First Annual Report). Thalamic neurons have a distinct progression of neural circuit formation whereby thalamocortical axons arrive at the cerebral cortex by E18.5 and pause at the lower levels of the cortex until early postnatal development (Figure 4). The conditional deletion of *Tsc1* at E12.5 caused over-exuberant axons that entered and spread in the cortex (compare Figure 4B' to Figure 4A') as well as fine axonal ramifications inappropriately located in the striatum (compare Figure 4B'' and Figure 4A''). We tracked TCAs to the adult stage and observed clearly de-fasciculated TCA projection bundles and ectopic fine axonal branches in the striatum and poorly defined SI cortex (Figure 5; Normand et al., 2013; Appendix I). Thus, an ectopic TCA phenotype, but not cell size abnormality, is seen just before birth. The aberrant circuitry persist as poorly organized and ectopic TCAs in adults (Normand et al., 2013).

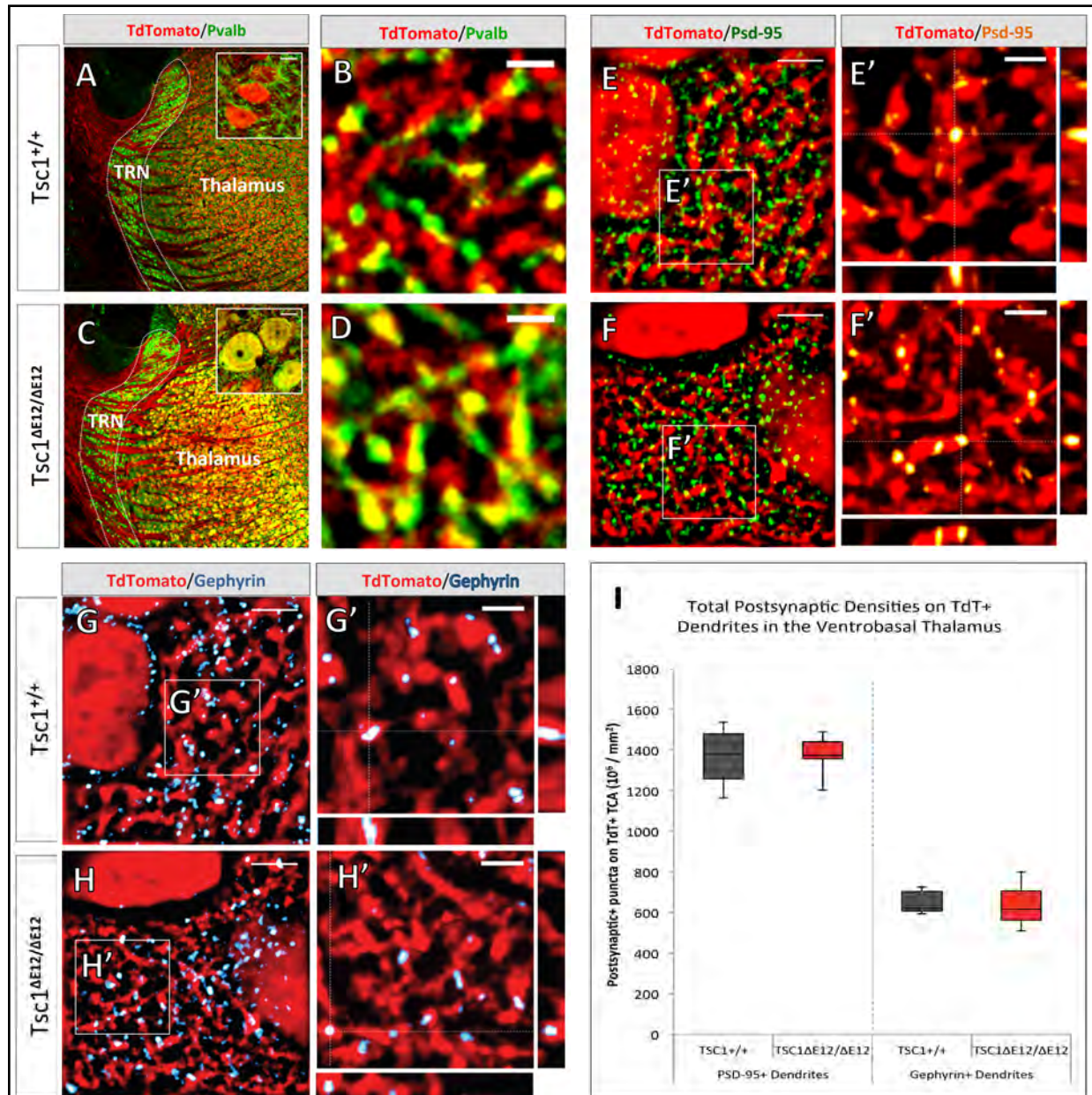




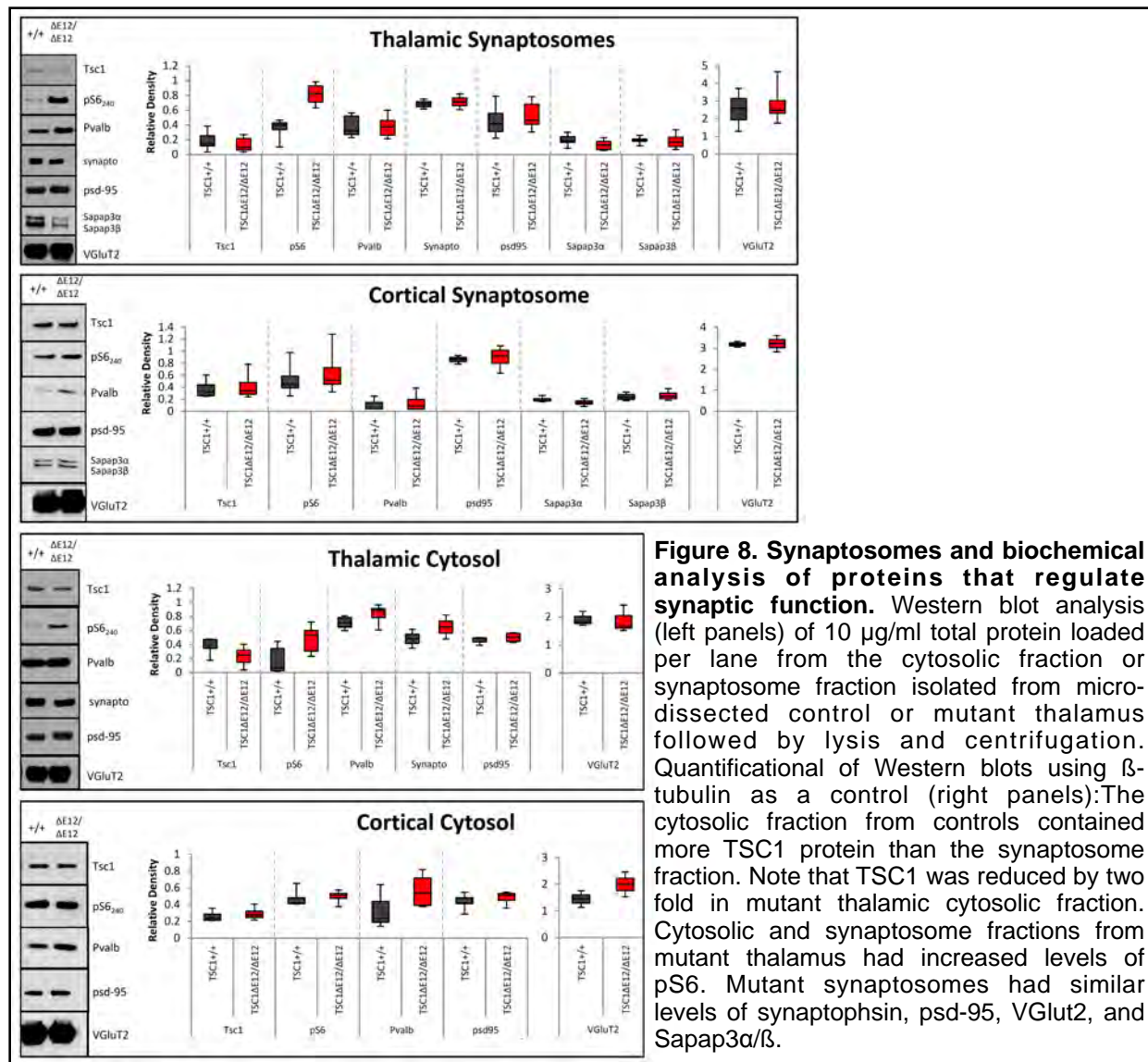
Synapses. The alterations in thalamocortical axons that innervate the cerebral cortex coupled with alterations in the somatosensory barrel organization (Figure 5B-1; Normand et al., 2013) suggested a necessity to analyze synapses that are formed between the axons emanating from thalamic relay neurons, which are marked using genetic circuit tracing (tdTomato+), and neurons in the target site (Figure 6). Analysis of bassoon, a marker for synaptic active zones, and either Parvalbumin (calcium binding protein), vesicular glutamate



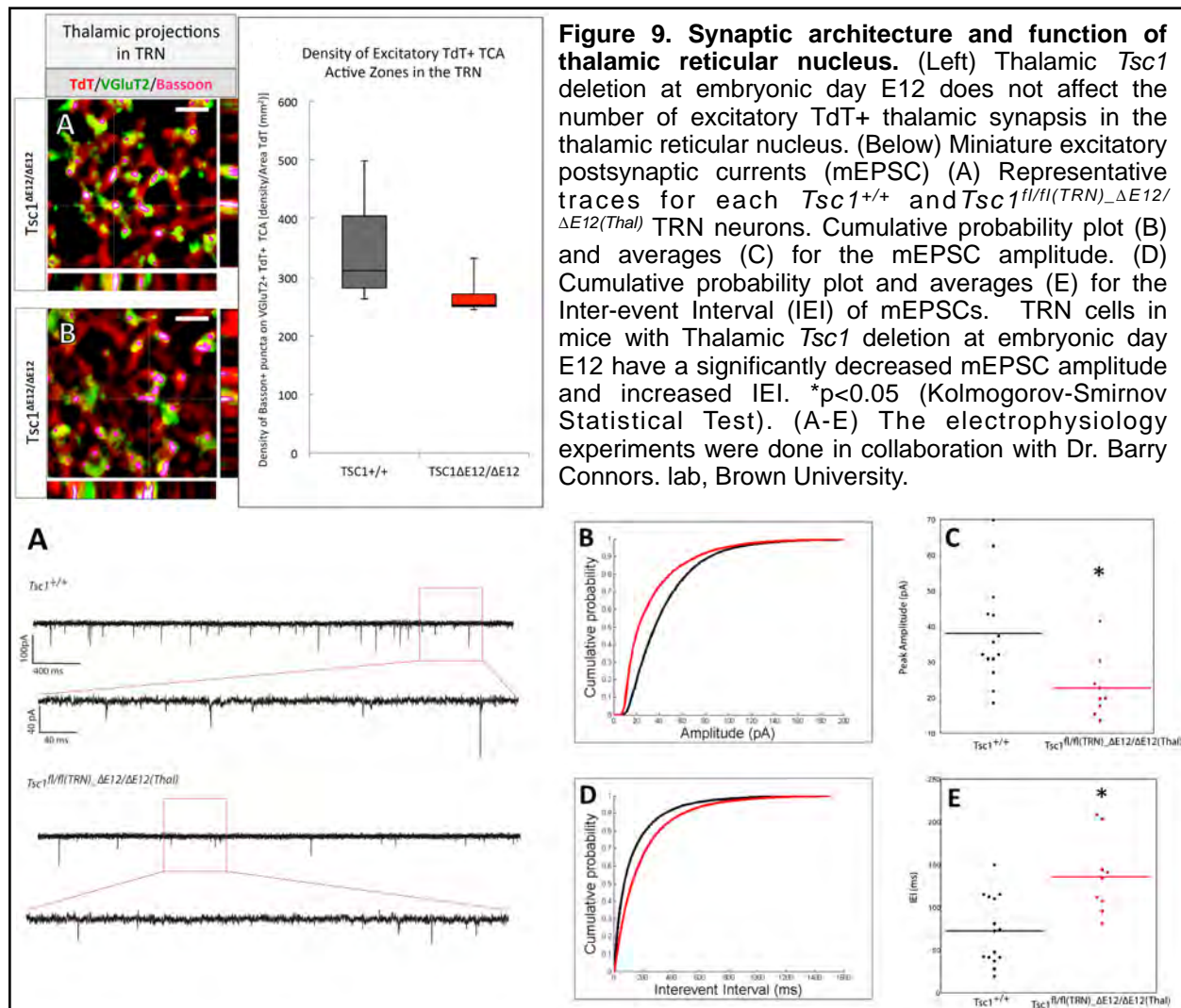
transporter (VGluT2, excitatory neurons), or vesicular GABA transporter (VGAT, inhibitory neurons) revealed a substantial increase of Parvalbumin localized to the adult somatosensory cortex (whisker barrel hollows; Figure 6C). However, there were no differences in the density of excitatory or inhibitory synapses (Figure 6H). In addition to Parvalbumin localized to thalamocortical synapses, we observed Parvalbumin in axons and the cell bodies of thalamic relay neurons (Figure 7A-D). We analyzed the synapses that form of the dendrites on mutant thalamic relay neurons (Figure 7E-H'), which were unchanged compared to controls (Figure 7I).



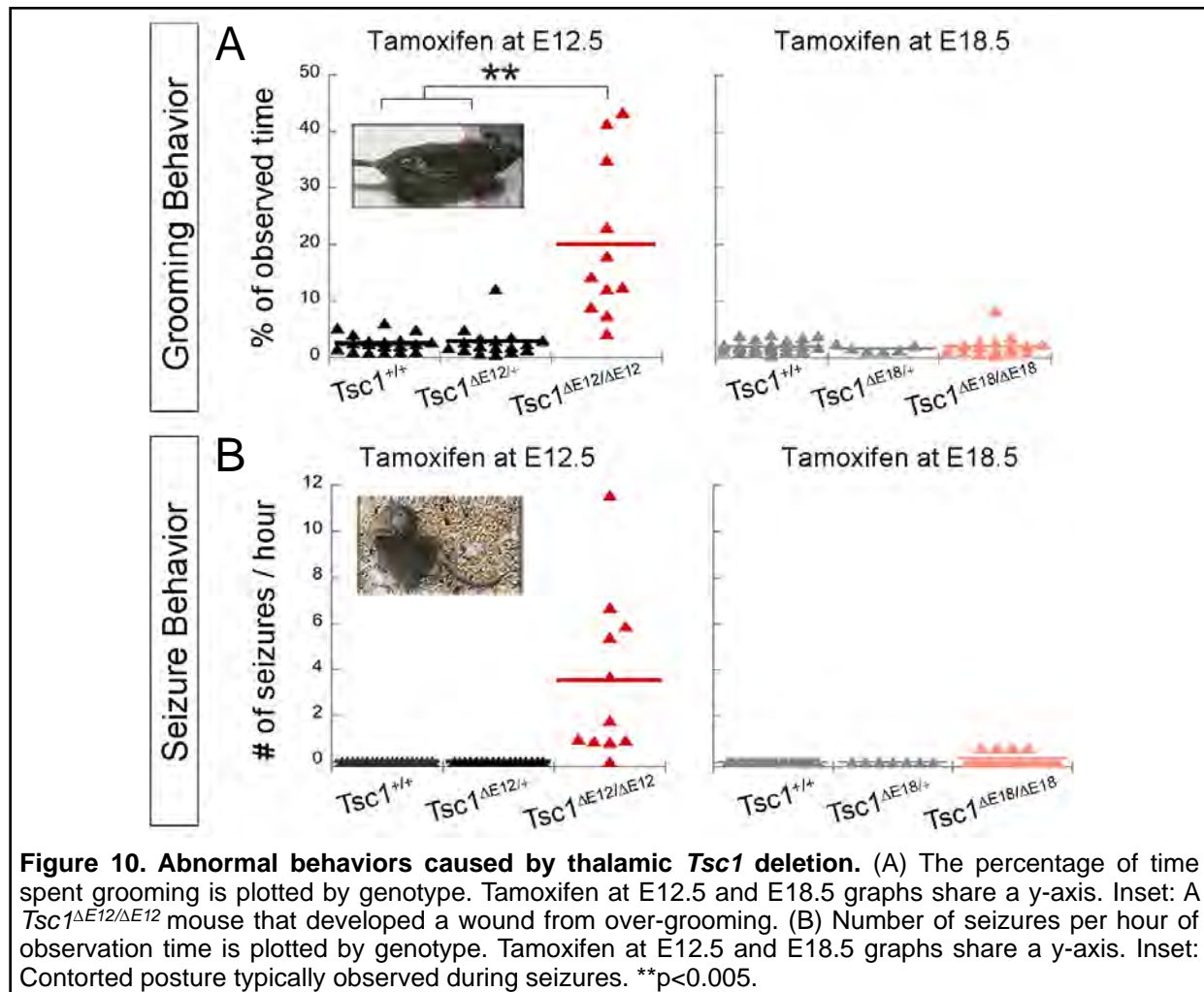
Biochemistry. We used biochemistry to establish a molecular link between the early deletion of *Tsc1* (*Tsc1*^{ΔE12/ΔE12}) and the ensuing enlarged thalamic neurons and altered neural circuits. Western blot analysis showed that the loss of TSC1 protein and mTOR dysregulation (increased pS6) did not result in concomitant changes in proteins that get distributed to synapses with the exception of Parvalbumin. Specifically, we used Thermo Scientific Syn-PER Synaptic Protein Isolation Reagent and centrifugation to fractionate the cytosol and synaptosomes (presynaptic and postsynaptic terminals, which maintain structural and functional integrity). Both the cytosol and to a lesser degree synaptosomes from control thalamus had TSC1 protein (Hamartin), which was reduced in both fraction from mutant thalamus. The cellular readout of mTOR activity (pS6₂₄₀) was increased in mutant fractions (Figure 8). Proteins enriched in synapses were not different between control and mutant synaptosome fractions. These findings indicate that the synaptic micro-architecture is not disrupted by TSC1 loss.



All thalamic axons that exit the thalamus en route to the cortex pass through the thalamic reticular nucleus (TRN) and also have an axon collateral that synapses on TRN neurons. The synaptic architecture of the TRN was unaffected by early *Tsc1* deletion (Figure 9). We took advantage of the well defined TRN physiology and initiated a collaboration between my lab and Dr. Barry Connor's at Brown University (collaborative effort carried out by a shared graduate student who recently received an award to perform the electrophysiology experiments (Figure 9A-E). We showed that the amplitude and inter-event interval of mini excitatory postsynaptic currents (mEPSC) was significantly affected (Figure 9). Thus, the early deletion of *Tsc1* does not apparently alter the synaptic architecture but does diminish synaptic function. Collectively our analyses show how cell size, altered development of circuitry, and changes in synaptic proteins converge to alter behavior. Notably, these are all platforms to study the efficacy on mTOR inhibition. Therefore, one of the product/deliverables that described in our initial Statement of Work that has been provided through this first grant period is the use of our complex allelic line of mice (*Gbx2^{CreER/+};R26^{tdTomato/+};Tsc1^{fl/fl}*) to interrogate how the temporal deletion of *Tsc1* and mTOR dysregulation affects brain development and function. We published a high profile manuscript related to this aspect of the project (Normand et al., 2013, Appendix 1) and have a second manuscript in preparation.



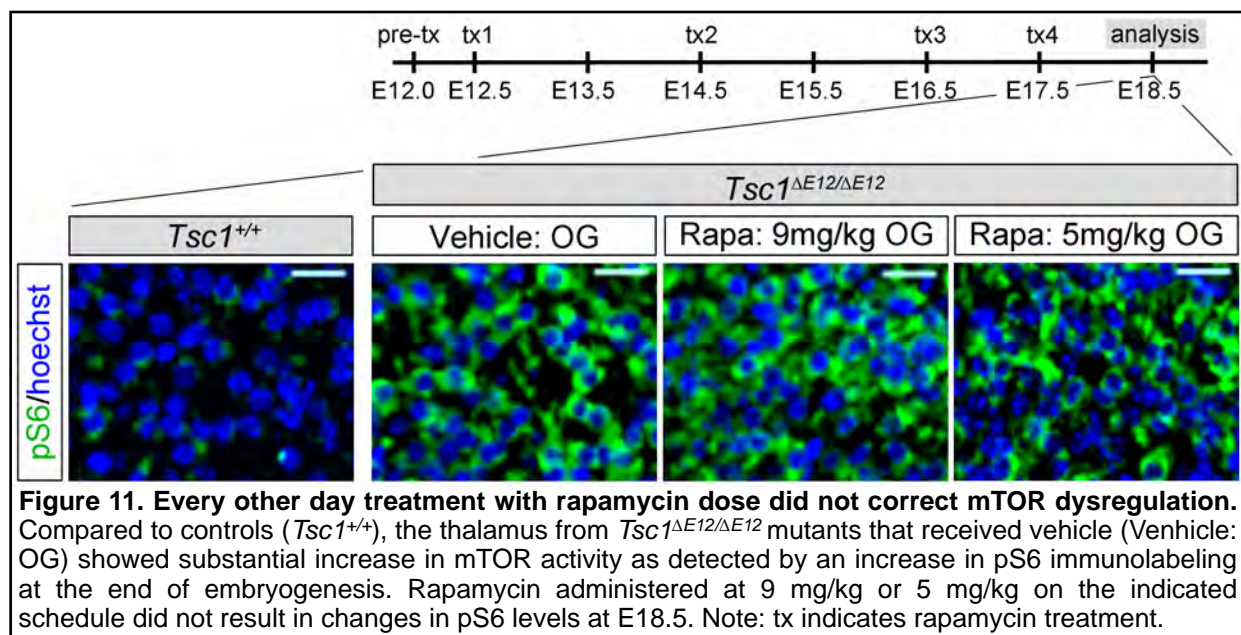
Behavior. The extent of cellular, circuit, and synaptic phenotypes suggested that behavior could be affected by deleting *Tsc1* in the thalamus during embryogenesis. Indeed, mTOR dysregulation had a profound effect on behavior (Figure 10). We observed that adult mice with the early deletion of *Tsc1* ($Tsc1^{\Delta E12/\Delta E12}$; n=11) experienced a prominent repetitive self-grooming defect which was not observed in any mice with the later deletion ($Tsc1^{\Delta E18/\Delta E18}$; n=17) (Figure 10A). Similarly, $Tsc1^{\Delta E12/\Delta E12}$ mice had robust, frequent spontaneous seizures while mice with the later deletion were largely seizure free with only four of seventeen $Tsc1^{\Delta E18/\Delta E18}$ mice showing rare seizures induced only upon handling (Figure 10B).



Rapamycin inhibition of mTOR dysregulation. The second aim and related series of tasks in our Statement of Work for this funding period was to begin to determine whether rapamycin administration will ameliorate or prevent specific developmental and neurological deficits dependent upon the time and duration of treatment. We have made progress on two tasks: 1. We tested the dose and delivery method and have begun to determine the time periods when mTOR dysregulation as measured by pS6 (Normand et al., 2013; Appendix 1) can be prevented, ameliorated, or reversed by rapamycin. For this task, pregnant female mice with $Tsc1^{\Delta E12/\Delta E12}$ embryos were treated with rapamycin (stock solution of 20 mg/ml in ethanol diluted in 0.25% Tween 80, 0.25% polyethylene glycol 400) at doses of 1, 3, 5, or 9 mg/kg by oral gavage or intraperitoneal injection until the stage of analysis (end of embryogenesis, See

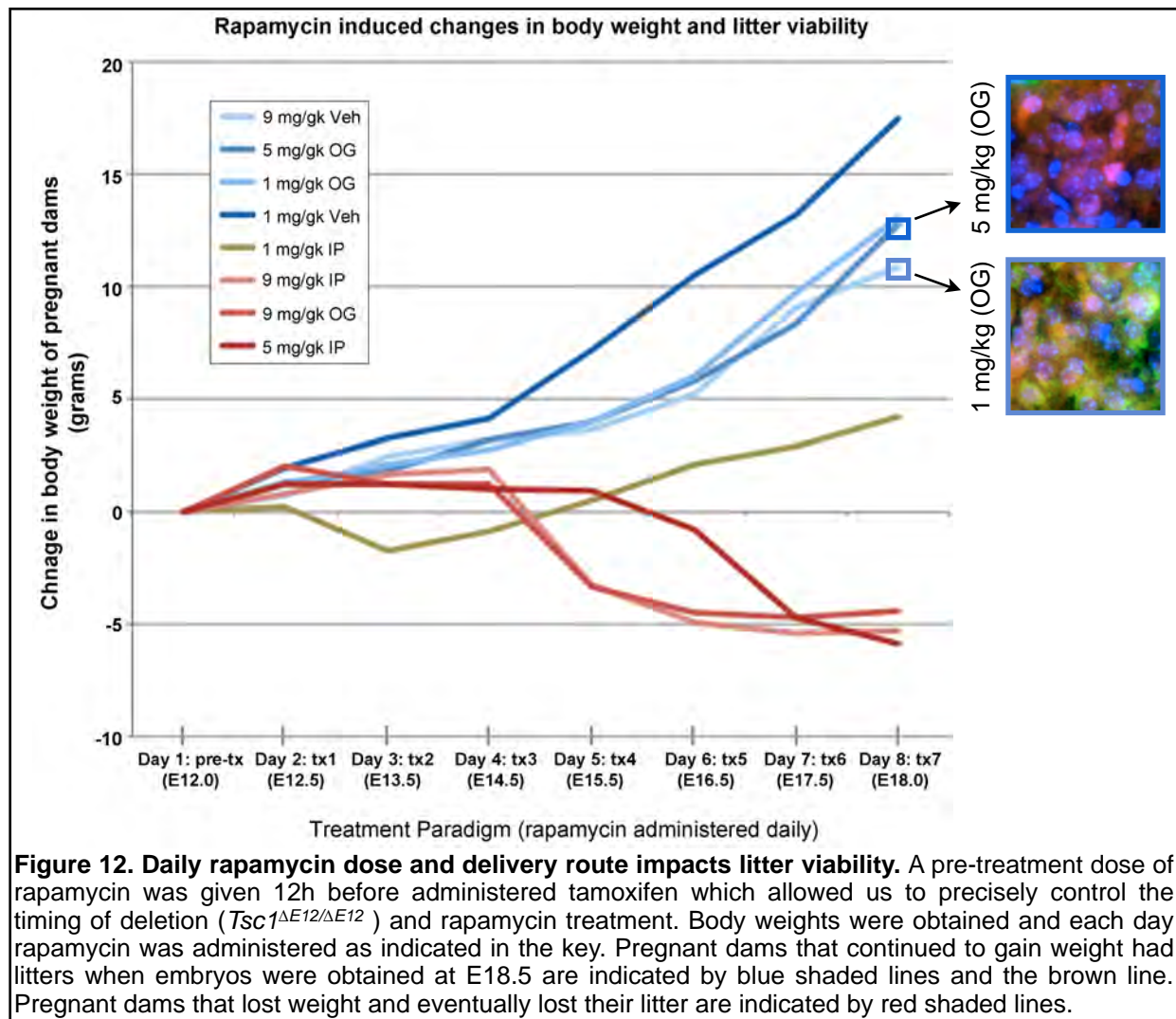
Appendix 2 for detailed information of mice used in initial studies). 2. We also treated adult *Tsc1*^{ΔE12/ΔE12} mutant mice with 9mg/kg rapamycin after the onset of behavioral abnormalities (seizures and repetitive self-grooming. The results are described below in two parts:

The effect of prenatal rapamycin on pS6 levels at the end of embryogenesis. Homozygous loss of *Tsc1* gene caused a predicted up-regulation of the mTOR pathway, which was robust at E18.5 (Figure 10, compare *Tsc1*^{+/+} and *Tsc1*^{ΔE12/ΔE12} Vehicle). We coupled *Tsc1* deletion at E12.5 with a paradigm of administering rapamycin to pregnant dams by oral gavage (OG) every other day over the course of approximately one week (E12.0 through E18.5) at varying dosage concentrations (1, 3, 5, 9 mg/kg; per dams body weight) (Figure 11). Embryos from pregnant dams given rapamycin at E12.5 were analyzed at E18.5 for mTOR pathway activity based on immunolabeling of sections for pS6 (Normand et al., 2013). In vehicle treatment groups pS6 levels were substantially increased in *Tsc1*^{ΔE12/ΔE12} embryos compared to the wildtype embryos (Figure 11). An intermediate dose of 5 mg/kg and the highest concentration of rapamycin (9 mg/kg) administered via OG every other day did not suppress mTOR activity as revealed by high levels of pS6 expression in the mutant embryos (Figure 11). An advantage of our system is that we can control the timing of *Tsc1* deletion and administration of rapamycin. To ensure maximal mTOR inhibition with rapamycin we gave rapamycin 12h prior to inducing *Tsc1* deletion (pre-treatment). These findings suggest that pulses of rapamycin are not sufficient to control dysregulated mTOR pathway in TS and that a single dose of rapamycin will not correct mTOR dysregulation resulting from *Tsc1* mutations. We then tested whether a more consistent (daily) paradigm was required and whether the delivery method impacted pS6.



Delivery route of prenatal rapamycin affects embryo viability. We compared two routes of daily rapamycin administration: oral gavage (OG) versus intraperitoneal (IP) injections and again analyzed litters based on the weight of pregnant Dams determined daily from E12.5 to E18.5 (Figure 12). Dose and the route of rapamycin administration had a profound effect on the overall development of embryos because a daily dose of 9 mg/kg by OG or IP injections resulted in the complete reabsorption of embryos at this highest rapamycin concentration. In contrast, 5 mg/kg given daily by IP did not support viability whereas the same dosage

concentration delivered via OG resulted in fully developed litters (Figure 12). A low dose of rapamycin (1 mg/kg) allowed fully viable litters at E18.5 when administered by either delivery route. We identified a critical threshold exists where 5 mg/kg by OG is acceptable, but 5 mg/kg by IP is not, which may indicate lower bioavailability by oral delivery. Once below the threshold (at 1 mg/kg) either route is acceptable. We then tested the efficacy of correcting pS6 levels around the threshold dosage by analyzing immunolabeled sections. Importantly, the daily 5 mg/kg OG group had substantial reduction of pS6 in stark contrast to the every other day paradigm (compare Figure 12, inset to Figure 11). The 1 mg/kg OG group did not correct pS6 levels (mTOR pathway) (Figure 12, insets).



We tested whether an intermediate dose 3 mg/kg of rapamycin by OG corrected the mTOR pathway (Figures 13-15). The rationale for these experiments was that 5 mg/kg was just at the threshold where deleterious effects could arise if the amount of free drug crossed the threshold. We determined that pregnant Dam's body weight increased similarly to vehicle treated controls suggesting that development proceeded normally which we validated by measuring the embryonic body weight and morphology at E18.5 (Figure 13).

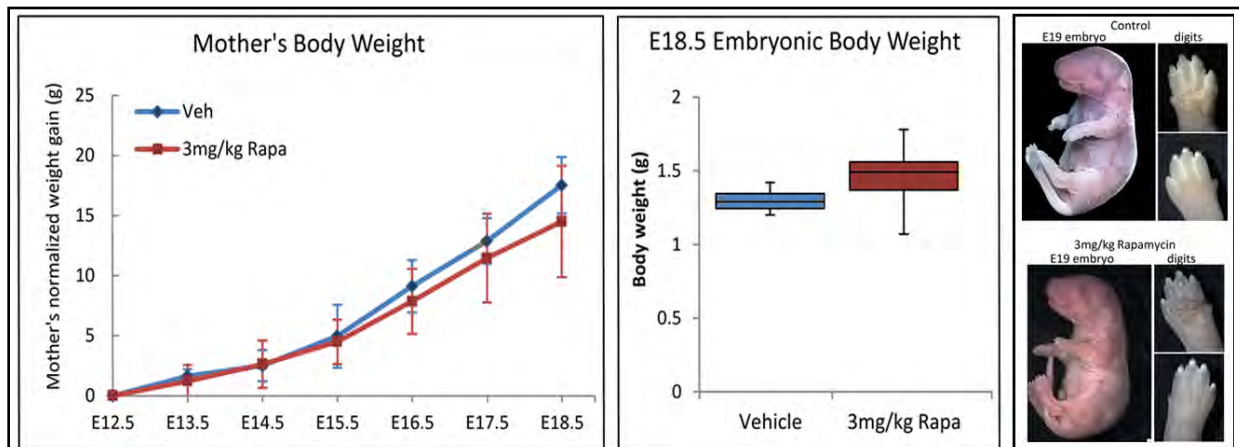


Figure 13. Impact of rapamycin (3 mg/kg) on pregnant Dam and embryonic development. Rapamycin (3 mg/kg) or vehicle was administered daily by oral gavage beginning at E12.0 to pregnant Dams followed by daily weight measurements (Left Graph). Daily weight gain increased across developmental time and was not impacted by rapamycin administration. The weight of embryos was determined at the end of embryogenesis, which also did not change from rapamycin treatment although there was higher variability in the range of weights (E18.5, Center Graph). No obvious differences in whole body morphology or size in E18.5 embryos (Right Panels: Top, Control; Bottom, rapamycin).

Analysis of the mTOR pathway by immunolabeling with antibodies that recognize pS6 confirmed that daily administration of 3 mg/kg of rapamycin to pregnant Dams attenuated mTOR activity, which was increased in *Tsc1 Δ E12/ Δ E12* embryos at E18.5 (Figure 14B,F,D,H). We took advantage of our lineage tracer to validate that recombination (of the reporter allele) occurred in a similar manner in controls versus conditional mutants; the difference being that the conditional mutants had two copies of the *Tsc1^{fl/fl}* alleles which were converted to the deleted

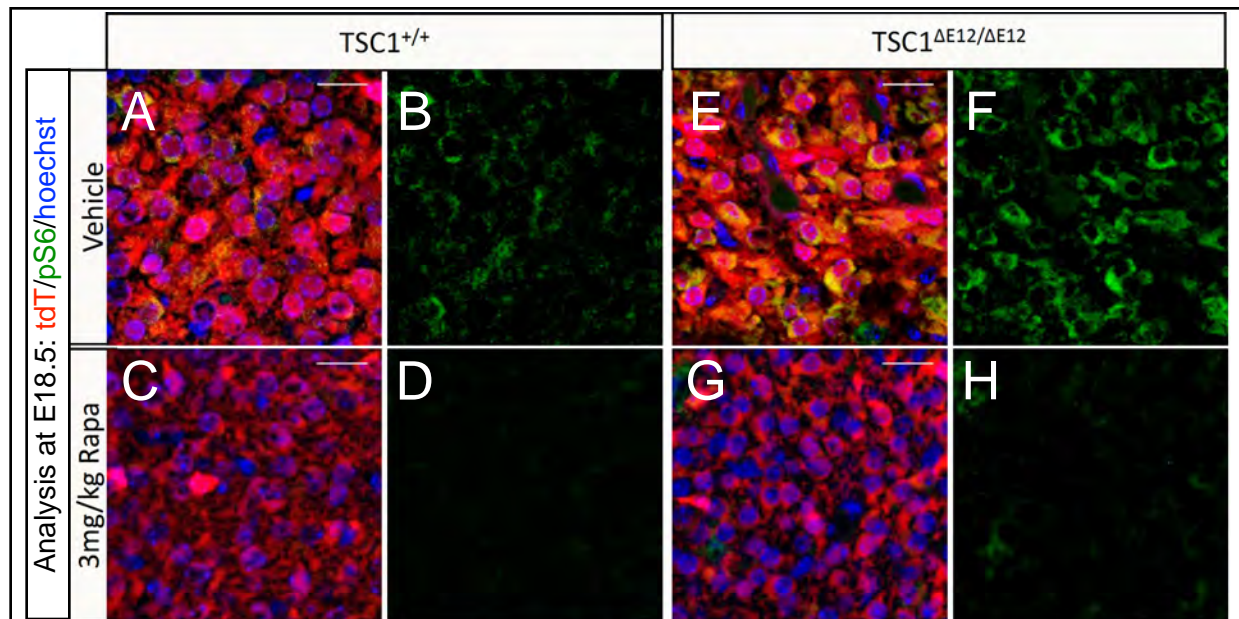
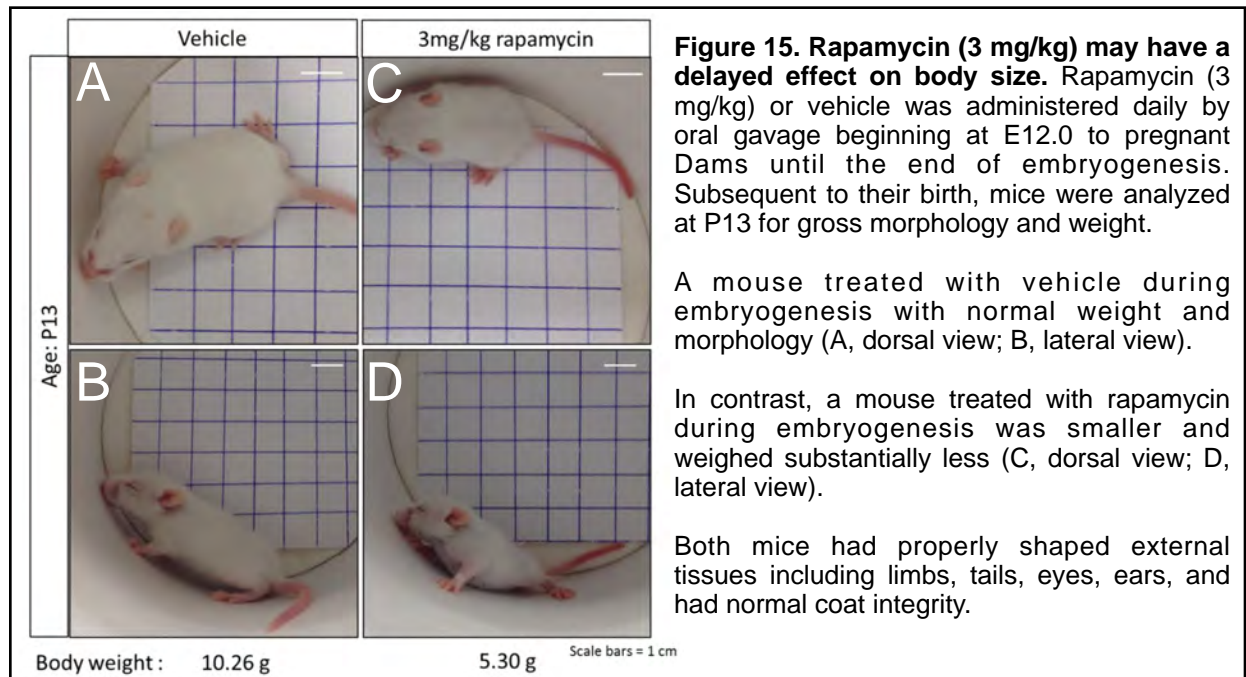


Figure 14. Rapamycin (3 mg/kg) corrects mTOR dysregulation. Rapamycin (3 mg/kg) or vehicle was administered daily by oral gavage beginning at E12.0 to pregnant Dams and embryos were obtained and processed for immunolabeling with antibodies that recognize the lineage tracer (tdTomato, tdT, red) or pS6 as a read out for mTOR activity (green); hoechst nuclear counter stain (blue). The lineage tracer serves as an internal control for recombination and results in cell marking similarly between Wildtype (A,C) and conditional mutant (E,G). Wildtype embryos at E18.5 had low levels of pS6, which were reduced by rapamycin treatment (B,D). *Tsc1 Δ E12/ Δ E12* embryos had substantially higher levels of pSG (F) versus controls (B). Rapamycin diminished pS6 in mutants (H).

ales ($Tsc1^{\Delta E12/\Delta E12}$) (Figure 14A,C,E,G). Subsequent to rapamycin treatment, embryos were allowed to go to be delivered and were analyzed for whole body morphology, size, and weight (Figure 15). Surprisingly, 3 mg/kg rapamycin resulted in smaller mice that weighed less than controls, but had normal shaped external features (e.g., limbs, eyes, ears, tail, coat). Together, these findings demonstrate significant progress on the tasks described in the statement of work and underscore the importance of fully evaluating mTOR inhibition as a therapeutic strategy to ameliorate phenotypes associated with TS.



KEY RESEARCH ACCOMPLISHMENTS

- The mTOR pathway becomes dysregulated within 48 hours after *Tsc1* deletion
- Cell size phenotype does not occur until after embryonic development
- Early but not late *Tsc1* deletion causes persistent changes in neuron size
- There is a delay between mTOR dysregulation and neural circuit abnormalities
- Genetic circuit mapping shows neural circuits are disrupted by the end of embryogenesis
- Genetic circuit mapping shows neural circuit abnormalities persist into adulthood
- Comparative analysis shows early and late requirements for *Tsc1* on neural circuits
- Thalamic neurons have ectopic Parvalbumin
- Thalamocortical synapses on somatosensory cortex have ectopic Parvalbumin
- Distribution of excitatory and inhibitory mutant synapses are similar to controls
- Amplitude and frequency of mEPSC are altered by *Tsc1* loss of function
- Early but not late *Tsc1* deletion is linked to repetitive grooming
- Early *Tsc1* deletion in the thalamus causes severe seizures in mice
- Pulses of rapamycin are ineffective at correcting mTOR dysregulation
- rapamycin dose and method of administration affects embryo viability
- Optimal dose of rapamycin required to correct mTOR dysregulation is daily 3 mg/kg OG

CONCLUSION

Our research proposal takes advantage of an innovative genetics-based approach based on a number of criteria including testing novel ideas, developing new animal model systems, and modifying existing molecular approaches to specifically address hypotheses relevant to altered brain development in TS. We also a novel mouse model of TS as a preclinical model to begin to test a treatment paradigm designed to ameliorate TS disease phenotypes. TS is a multi-systemic disorder that causes epilepsy or seizures in nearly all TS patients and cognitive deficits and autism in a substantial cohort of TS patients. However, a deep understanding of how the TS brain changes during development compared to controls has not been ascertained in TS. We took advantage of genetic approaches in mice allow to test hypotheses that designed to elucidate how mechanisms underpinning TS. Specifically, we are using a sophisticated genetic method that I helped pioneer, which combines spatial and temporal control of gene deletion and cell lineage tracing *in vivo* (Zervas et al., 2004; Brown et al., 2009; Ellisor et al., 2009; Ellisor and Zervas, 2010; Brown et al., 2011; Hagan and Zervas, 2012; Yang et al., 2013; Normand et al., 2013) During this funding period, we showed how early developmental alterations and the developmental progression of TS result in long lasting persistent changes in brain structure, function, and behavior, which had not previously been elucidated. Notably, the animal model we established mimics salient features of TS. We made an innovative advance by addressing developmental mechanisms that impact TS.

We successfully applied our combined temporal gene deletion, lineage marking, and genetic circuit tracing to inactivate *Tsc1* and subsequently mark and track mutant neurons during critical windows of brain development. Thus, innovative conceptual issues we addressed during this funding period are the following: 1. Even though neurons rapidly undergo mTOR dysregulation subsequent to *Tsc1* deletion (within 48 hours), the aberrant cell size does not occur until much later. 2. There is also a delay between the time of mTOR dysregulation and when deficits in neural circuitry are first observed. 3. We show for the first time that neural circuits are disrupted by the end of embryogenesis and persist into adulthood. 4. *Tsc1* functions over a longer time period for the control of neural circuit formation than for the control of neuron size. 5. Global synaptic architecture of thalamic circuits is unaffected by *TSc1* deletion although abnormal calcium binding protein Parvalbumin is expressed abnormally in thalamic axons and synapses. 6. Spontaneous physiological activity is disrupted in thalamic circuits. 7. The route (IP versus OG) and the dosage of rapamycin administration affects litter viability and the ability to correct the mTOR pathway. 8. Daily administration of rapamycin (3 mg/kg by OG) to pregnant Dams allows for embryonic viability and pS6 correction at the end of embryogenesis, but may impact postnatal development.

This project advanced the use of our novel paradigm in which specific neural circuits affected in TS are followed over developmental time using genetic circuit tracing, which is advantageous because we tracked mutant neurons, defined by genetic lineage, at distinct time points in development. Our analysis represent more than an incremental understanding of TS by delineating how brain regions and neurons relevant to TS change during critical developmental windows. In addition, the combination of mouse lines we use are beneficial to determine the efficacy of mTOR inhibitors such as rapamycin to treat specific cellular phenotypes, neural circuit deficits, and behavioral changes in TS. Finally, the cellular and circuit changes are accompanied by behavioral abnormalities including robust and frequent seizures and repetitive self-grooming that are correlated with distinct temporal windows of *Tsc1* function. The highly innovative nature of our approach is exemplified by providing the first detailed analysis of brain development including neural circuitry, synaptic architecture, and physiology in a mouse model with salient features of TS. There are no significant changes that are required to

better tackle the problems that we are addressing. Notably, we took advantage of newly gained knowledge of rapamycin administration to refine the optimal dose, time, and duration of treatment to achieve the most likely successful rescue of behavioral deficits. This is not a departure from our initially proposed work, but rather an evolution using multiple approaches that we advanced during this research period, consistent with our Statement of Work.

This project is clinically relevant to TS because the gene that we are deleting (*Tsc1*), which encodes TSC1 protein, which forms a heterodimeric complex with TSC2 protein. Together these proteins regulate mTOR, a central hub of intracellular signaling, vital for cellular processes including cell growth, axon guidance, and transcriptional regulation (Crino, 2004; Crino et al., 2010; Ess, 2006; de Vries and Howe, 2007; Swiech et al., 2008). Signal transduction through mTOR culminates in the phosphorylation of the ribosomal protein S6 (pS6), which is elevated to high levels when mTOR signaling is dysregulated. We have begun to elucidate how the loss of *Tsc1* and mTOR dysregulation causes changes in brain development including the disruption of early neural circuit formation that persists to the adult stage and underlies behavioral deficits. Notably, the time of deletion has a significant impact on the behavioral phenotype indicating that we identified critical periods of development that are impacted in TS. A profound clinical implication is that the mTOR pathway can be suppressed pharmacologically with the drug rapamycin. However, a significant, clinically relevant problem to treating TS patients with rapamycin is the manner, dose, speed and extent that specific brain regions or cell types respond to *Tsc1* deletion and mTOR dysregulation and how mTOR inhibition may ameliorate these changes. We have now begun to determine effective time points to administer rapamycin, which has not previously been tested, in particular how rapamycin impacts embryonic development. Given the importance of mTOR in regulating developmental processes (Hentges et al., 2010), our ability to control the timing of *Tsc1* deletion/mTOR dysregulation and our determination of how rapidly and robustly neurons respond to rapamycin provided essential guidance when considering this approach as a therapeutic paradigm for human TS.

This project has made original and important contributions to advancing TS related research. First, we isolated critical time periods that *Tsc1* deletion is most pathogenic and showed how the deletion affected early brain development - specifically how neural circuit formation is disrupted *in vivo*. Second, we showed how quickly neurons respond to *Tsc1* deletion and that cellular and circuit phenotypes occur in a sequential pathogenic cascade. Third, we identified how dosage and delivery method impact on mTOR dysregulation. This project will positively affect TS research and possibly patient care by taking advantage of novel animal model that allows for testing therapeutic approaches in a cell-type specific manner. Indeed, this strategy has revealed that pulses or rapamycin or a single dose is unlikely to correct neurological phenotypes in TS and that a daily precise dose is required to support development while correcting the mTOR pathway. This approach is laying the foundation for understanding whether rapamycin is a viable treatment strategy during early development to ameliorates specific features of TS. My background in using animal models of neurological developmental brain disorders and therapeutic intervention in Niemann-Pick Disease Type C (NPC) led directly to human clinical trials to NPC. Thus, we have a track record of successfully conducting innovative approaches to understand brain diseases and show that our potential gains to uncover novel aspects of the developmental mechanisms underpinning TS greatly outweighs the perceived risk of using a complex genetic strategy. We have begun to establish a correlation between gene inactivation, changes in neural circuit structure, and physiology. Our animal model system is allowing us to test the feasibility of pharmacological treatment strategies in ameliorating features of TS with an emphasis on how specific brain regions (thalamus) respond to rapamycin administration.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Publications and Manuscripts (in chronological order)

Normand EA, Crandall SR, Thorn CA, Murphy EM, Voelcker B, Browning C, Machan JT, Moore CI, Connors BW, **Zervas M** (2013) Temporal and mosaic *Tsc1* deletion in the developing thalamus disrupts thalamocortical circuitry, neural function, and behavior. *Neuron* 78:895-909.

Note: This paper was selected for F1000Prime as being of special significance in its field (<http://f1000.com/prime/718021981?bd=1&ui=21604>). It was also featured by the DoD CDMRP (http://cdmrp.army.mil/tscrp/research_highlights/13zervas_highlight.shtml) and by SFARI (<https://sfari.org/news-and-opinion/news/2013/mouse-model-mimics-mosaic-mutation-in-tuberous-sclerosis>).

Normand E, Browning C, Hagan N, **Zervas M** (2014) Genetic marking of neural circuits. *Gene Exp Patterns*, Manuscript# MODGEP1128, Under Revision.

Smith J, Martinez Garcia R, Connors B, **Zervas M** (2014) Temporal deletion of *Tsc1* during embryonic development alters synaptic architecture and spontaneous activity. *J. Neurosci.*, in preparation.

Abstracts During Award Period (in chronological order)

Normand E, Browning C, **Zervas M** (2012) Cellular and behavioral consequences of mTOR pathway dysregulation within a population of subcortical neurons during mouse embryogenesis. Keystone Symposia: *Synapses and Circuits: From Formation to Disease*, Apr 1-6 2012, Steamboat Springs, Colorado. (* student travel award recipient).

Normand E, Browning C, **Zervas M** (2012) Seizures and compulsive grooming behaviors resulting from thalamus-specific *Tsc1* gene inactivation. *Gordon Research Conference: Fragile X and Autism-related Disorders*. June 10-15, 2012, Stonehill College, MA.

Normand E, Browning C, Machan JT, Voelcker B, **Zervas M** (2012) The deletion of *Tsc1* at specific developmental stages and in distinct regions of the thalamus disrupts neural circuit architecture and causes unique behavioral abnormalities. *Gordon Research Conference: Neural Development*. August 12-17, 2012, Salve Regina, RI.

Normand E, Smith J, **Zervas M** (2013) A dynamic developmental requirement for *Tsc1* in the embryonic mouse thalamus. International TSC Research Conference: Summit for Drug Discovery in TSC and Related Disorders. June 20-23, 2013, Washington DC.

Voelcker B, Martinez Garcia R, **Zervas M**, Connors B (2014) Cellular phenotypes of early deletion in thalamocortical neurons. Brown University summer research symposium, Providence RI, August 2014.

Smith J, Martinez Garcia R, Connors B, **Zervas M** (2014) Embryonic deletion of thalamic *Tsc1*, seizure behaviors, and synaptic architecture. Second biennial NRI Symposium and Workshop: When Epilepsy is Catastrophic: Molecular Causes and Their Consequences, Houston TX, April 2014.

Invited Seminars During Award Period (in chronological order)

Zervas M. "Genetic Approaches in Mouse to Interrogate Brain Development and Disease". University of Massachusetts, Amherst, February 2012.

Zervas M. “Temporal and mosaic disruption of *Tsc1* causes abnormal thalamocortical circuitry and complex behaviors in murine Tuberous Sclerosis”. Brandeis University, Neurobiology Journal Club, Waltham MA, September 2012.

Zervas M. “Temporal and mosaic disruption of *Tsc1* causes abnormal thalamocortical circuitry and complex behaviors in murine Tuberous Sclerosis”. U. Conn Health Center, Department of Neuroscience Seminar Series, Farmington CT, September 2012.

Zervas M. “Delineating a temporal window of *Tsc1* requirement for proper thalamus development and function”. Harvard University Medical School, Departmental Seminar Series, Cambridge MA. November 2012.

Zervas M. “*Tsc1* deletion with genetic mosaicism alters neural circuits, neuronal physiology, and behaviors”. International Research Conference on TSC and Related Disorders: Molecules to Medicines, Washington DC, June 2013.

Zervas M. “*Tsc1* deletion with genetic mosaicism alters neural circuits, neuronal physiology, and behaviors”. Lieber Institute for Brain Development Departmental Seminar Series, Baltimore MD. October 2013.

Zervas M. “Temporal and mosaic *Tsc1* deletion in the developing thalamus disrupts thalamocortical circuitry, neural function, and behavior”. Amgen Invited Seminar, Amgen, Cambridge MA, October 2013.

Zervas M. “*Tsc1* deletion with genetic mosaicism during brain development alters neural circuits, neuronal physiology, and behaviors”. University of Rhode Island’s College of Pharmacy Seminar Series, Kingston RI, November 2013.

Zervas M. “Mosaicism, thalamic dysfunction, and seizures in a novel mouse model of Tuberous Sclerosis”. Second biennial NRI Symposium and Workshop: When Epilepsy is Catastrophic: Molecular Causes and Their Consequences, Houston TX, April 2014. Invited, but did not attend due to change in research institutes.

Zervas M. Invited panel member for NIH, Department of Defense, TS Alliance organized TSC conference to update the Research Plan for TSC; Neurocognition working group. Upcoming: March 2015.

INVENTIONS, PATENTS, AND LICENCES

Nothing to report

REPORTABLE OUTCOMES

Pre-clinical research model of TS (spatially and temporally controlled):

Gbx2^{CreER/+}; *R26*^{tdTomato/+}; *Tsc1*^{fl/fl} converted to *Gbx2*^{CreER/+}; *R26*^{tdTomato/+}; *Tsc1*^{ΔE12/ΔE12}

Genetic circuit tracing

OTHER ACHIEVEMENTS

Funding applied for based on work supported by this award (in chronological order)

SFARI ID#: 275701 (PI: **Zervas, M**)

Simons Foundation Autism Research Initiative Annual RFA (2013)

Linking genetic mosaicism, neural circuit abnormalities, and behavior.

Dates: September 01, 2013-August 31, 2015

Role: Principal Investigator; Total Award: \$250,000 (direct costs)

The major goals of this project are to determine how mosaicism affects behavioral and to determine whether mutant neurons recruit genetically unaffected neurons into dysfunctional circuits and amplify mutant phenotypes in a cell non-autonomous manner.

Status: Awarded

R01 Accession # 3459040 (PI: **Zervas M**)

NIH R01

Dates: Dec 01, 2012-November 30, 2017

Subcortical brain structures and neurological disease in Tuberous Sclerosis

Role: Principal Investigator; Total Award: \$1,250,000

The major goals of this project are to conditionally delete *Tsc1* in the striatum during embryonic development and ascertain how FMRP phosphorylation and SAPAP3 protein expression are affected to link a molecular pathway to repetitive behaviors in a mouse model of Tuberous Sclerosis.

Scored: 39 Percentile

TS130073 (PI: **Zervas, M**)

DOD-CDMRP Idea Development Award-Optional Nested Postdoctoral Traineeship

Timing of Mosaic Gene Deletion in Mouse Blastocysts and Multitissue Disease Development in Tuberous Sclerosis.

Dates: September 2014-August 2017

Role: Principal Investigator; Total Award: \$575,000 (direct costs)

The major goals of this project are to mutate *Tsc1* in single blastomeres during early embryonic development in mice to induce mosaicism with temporal control and conduct detailed cross-tissue clonal analysis in individual mice to identify how the same genetic insult (bi-allelic *Tsc1* loss of function) affects different cell types and tissues. Our mosaic deletion strategy is precisely controlled temporally and allows us to mimic human TS mosaicism and human TS disease phenotypes in a way that has not yet been achieved.

Status: Awarded

Employment received based on experience/training supported by this award.

I was presented with a unique opportunity to go to industry and enter at a high level and obtained a position at Amgen where I will am running the *in vivo* Neuroscience (Behavioral Pharmacology) group and investigating therapeutic areas related to Neurological disease.

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TRAINING OR FELLOWSHIP AWARDS

Training supported by this award

Elizabeth Normand, Graduate Student in the Brown Neuroscience Graduate Program has been supported in part by this grant and has conducted the experiments described in this report. Elizabeth is also the first author on our recently *Neuron* publication and new manuscript currently under review. She has presented her findings at three meetings and has received travel awards because of the high quality data and impact of her work. Elizabeth successfully defended her Ph.D. thesis (Ph.D. earned in 2013).

Bettina Voelcker, Brown University undergraduate, Tina conducted independent studies with this award providing funding for supplies to carry out the work. Her project was "The

differential expression of mTOR signaling components during brain development and establishment of thalamocortical circuitry as a result of *Tsc1* deletion and mTOR dysregulation in the developing thalamus” . Tina is an author on our recent *Neuron* publication (2012-2013).

Jeannie Smith, Ph.D., Postdoctoral Fellow was supported to conduct experiments that determined how *Tsc1* deletion affects synaptic architecture and how molecular components of synapses changed in Tuberous Sclerosis brain (mouse). Jeannie also conducted the mTOR inhibitions studies described in this report which helped to determine efficacy of rapamycin in ameliorating neurologic phenotypes in our novel mouse model of Tuberous Sclerosis (2012-2014).

Rosa Martinez Garcia, Graduate Student in MCB and Neuroscience. Rosa’s project “Thalamic neuron subtype specification in brain development and the role of TRN-thalamus microcircuits in Tuberous Sclerosis” was the foundation data and concept for a recently awarded student NSF Fellowship that proposed to understand the synaptic function and physiological processing in thalamic circuits in Tuberous Sclerosis. Co-mentor with Dr. Barry Connors (2013-2014).

Fellowship obtained as a result of research supported this award

Rosa Martinez Garcia, Graduate Student in MCB and Neuroscience
NSF Graduate Research Fellowship Awarded (2014)

Temporal and Mosaic *Tsc1* Deletion in the Developing Thalamus Disrupts Thalamocortical Circuitry, Neural Function, and Behavior

Elizabeth A. Normand,¹ Shane R. Crandall,¹ Catherine A. Thorn,¹ Emily M. Murphy,¹ Bettina Voelcker,² Catherine Browning,² Jason T. Machan,³ Christopher I. Moore,¹ Barry W. Connors,¹ and Mark Zervas^{2,*}

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SUMMARY

Tuberous sclerosis is a developmental genetic disorder caused by mutations in *TSC1*, which results in epilepsy, autism, and intellectual disability. The cause of these neurological deficits remains unresolved. Imaging studies suggest that the thalamus may be affected in tuberous sclerosis patients, but this has not been experimentally interrogated. We hypothesized that thalamic deletion of *Tsc1* at distinct stages of mouse brain development would produce differential phenotypes. We show that mosaic *Tsc1* deletion within thalamic precursors at embryonic day (E) 12.5 disrupts thalamic circuitry and alters neuronal physiology. *Tsc1* deletion at this early stage is unique in causing both seizures and compulsive grooming in adult mice. In contrast, only a subset of these phenotypes occurs when thalamic *Tsc1* is deleted at a later embryonic stage. Our findings demonstrate that abnormalities in a discrete population of neurons can cause global brain dysfunction and that phenotype severity depends on developmental timing and degree of genetic mosaicism.

INTRODUCTION

Tuberous sclerosis (TS) is a complex mosaic genetic disorder that affects one in 6,000 children and commonly presents in infancy or early childhood, suggesting an early developmental basis for the disease. TS is characterized by benign hamartomas in multiple organs, but neurological involvement is common and debilitating. Patients may experience seizures (70%–90%), intellectual disability (50%), autism (25%–50%), and sleep disturbances (McClintock, 2002). Hamartomas in the brain were thought to cause neurological symptoms, but the extent of hamartomas does not necessarily correlate with the severity of neurological impairment (Wong and Khong, 2006). This suggests

that subtle aspects of brain development or function are perturbed in TS.

Genetically, TS is caused by mutations in either of two tumor suppressor genes, *TSC1* or *TSC2*, and is inherited in an autosomal dominant manner. In addition to the inherited mutation, a somatic mutation in the remaining functional allele results in loss of heterozygosity and gives rise to isolated TSC null cells that proliferate and contribute to the formation of hamartomas (Au et al., 1999). This “two-hit” mechanism results in a mosaic population of cells in a patient’s organs: a discrete population that has undergone a second hit to become null for *TSC1* or *TSC2* and surrounding heterozygous cells. However, it is unclear whether this two-hit mechanism underlies neurocognitive aspects of TS (Crino et al., 2010). To experimentally emulate this mosaic state within the brain and to test whether targeted disruption of *Tsc1* in a focal manner can disrupt global brain function, we employed an inducible CreER/*loxP*-based method of gene inactivation in mice, which produces a spatially restricted, mosaic population of *Tsc1* mutant cells surrounded by genetically unaffected cells.

The TSC1 and TSC2 proteins form a heterodimer that negatively regulates the mTOR pathway, which in turn modulates a wide array of cellular processes (Hay and Sonenberg, 2004). The multifaceted nature of the mTOR pathway raises the possibility that the effects of TSC loss of function vary depending on a cell’s identity, functional role, or developmental state at the time of *TSC* mutation. During brain development, cell fate specification, cell growth, differentiation, and axonal connectivity are tightly regulated to establish proper brain architecture and function. Thus, spatially and temporally controlling *Tsc1* deletion in targeted cell types and comparing the resulting phenotypes will be instructive to our understanding of this complex disease. Because our CreER/*loxP* experimental system is temporally inducible, we are able to target *Tsc1* inactivation at distinct stages of brain development.

Numerous studies have evaluated how *Tsc1/2* deletion affects the cerebral cortex. Subcortical regions have not been extensively evaluated thus far, although one such structure that warrants investigation based on previous findings is the thalamus. MRI-imaging studies of TS patients show that changes in

thalamic gray matter volume correlate with poor cognitive performance (Ridler et al., 2007). Thalamic involvement in TS is relevant because the thalamus provides specific, information-carrying afferents to the cerebral cortex and plays a crucial role in higher-order cognitive processes (Saalman and Kastner, 2011). The thalamus also projects robustly to the striatum, a pathway implicated in attentional orientation (Smith et al., 2004). Notably, dysfunction of the thalamus and striatum are implicated in obsessive compulsive disorder and autism (Hardan et al., 2008; Fitzgerald et al., 2011). The relay cells of the thalamus receive extensive excitatory feedback from the neocortex and inhibitory inputs from the thalamic reticular nucleus (TRN). Due, in part, to this extensive reciprocal connectivity, the thalamus plays a key role in oscillatory neocortical dynamics and in the generation of low-frequency rhythms, which are prominent in specific forms of epileptic activity (Blumenfeld, 2003). We have used spatially and temporally controlled *Tsc1* gene deletion to address how altered thalamic development has the potential to perturb widespread neural function and behavior.

RESULTS

Spatiotemporal Contribution of the *Gbx2* Lineage to Adult Thalamic Neurons

To temporally and spatially control *Tsc1* gene deletion, we combined three genetically modified mouse alleles (see Figure S1A available online): (1) *Gbx2*^{CreER}, which targets CreER expression to thalamic cells (Chen et al., 2009); (2) *Tsc1*^{fl}, which is converted into a null allele (*Tsc1*^d) by Cre-mediated recombination (Kwiatkowski et al., 2002); and (3) either *R26*^{LacZ} (Soriano, 1999) or *R26*^{tdTomato} (Madisen et al., 2010), which produce β -galactosidase (β -gal) or red fluorescent protein (RFP), respectively, upon Cre-mediated recombination. CreER remains quiescent until it is transiently activated by tamoxifen. Subsequently, the *Tsc1*^{fl} gene is permanently converted to *Tsc1*^d and the conditional reporter genes are permanently activated in the thalamus (Figures S1B and S1C). *Gbx2*^{CreER} expression has been reported in the spinal cord (Luu et al., 2011) but, within the brain, regions outside of the thalamus had only very sparse recombination with tamoxifen at E12.5 (Figure S1). We validated the fidelity of *Tsc1*^{fl} recombination in the thalamus compared to the neocortex (Figures S1D and S1E). Operationally, we use *Tsc1*^{dE12/dE12} to indicate mutant animals that received tamoxifen on embryonic day (E) 12.5 and *Tsc1*^{dE18/dE18} to indicate mutants that received tamoxifen on E18.5. We first performed genetic inducible fate mapping on *Gbx2*^{CreER}; *R26*^{LacZ} animals to characterize the extent, spatial distribution, and molecular identity of recombined cells (Figure 1). We administered tamoxifen to pregnant females carrying *Gbx2*^{CreER}; *R26*^{LacZ} embryos at E12.5 or E18.5 and determined the long-term lineage contribution to the thalamus. Postnatal brain sections were analyzed by immunohistochemistry (IHC) for β -gal expression from the activated *R26*^{LacZ} allele. E12.5 fate-mapped cells (green) were distributed widely throughout the full medial-lateral extent of the thalamus (Figures 1A–1F). In animals that received tamoxifen at E18.5, the spatial extent of recombination was reduced (Figures 1G–1L). Regions that underwent recombination at both E12.5 and E18.5 include the anteromedial and mediodorsal nuclei. The ventrolateral,

ventromedial, ventrobasal, laterodorsal, and the lateral geniculate nuclei underwent recombination at E12.5 but were not marked at E18.5. Nuclei that underwent extensive recombination early (E12.5) and moderate mosaic recombination later (E18.5) include the posterior nucleus and the medial geniculate nucleus. We investigated whether recombination occurred in a particular cell type by IHC for β -gal in combination with parvalbumin (PV, red, Figures 1A–1C and 1G–1I) or calbindin (Calb, red, Figures 1D–1F and 1J–1L). Within relay nuclei, β -gal+ cells contributed to both Calb– and Calb+ cells at both E12.5 and E18.5 (Figures 1D–1F and 1J–1L, arrowheads). Although most excitatory relay neurons did not express any PV+ within their soma, there were a few examples of neurons with low PV+ levels that also expressed β -gal at E12.5 (Figures 1A–1C, arrowheads). Notably, the highly PV+ inhibitory thalamic reticular nucleus (TRN) did not undergo recombination at either stage.

mTOR Pathway Dysregulation Occurs Rapidly after *Tsc1* Recombination

We used the inducible nature of our system to control the timing of *Tsc1* gene deletion and determine how rapidly mTOR dysregulation occurs. We administered tamoxifen to E12.5 embryos with *Gbx2*^{CreER} and either *Tsc1*^{+/+} or *Tsc1*^{fl/fl}. E12.5 is a stage when thalamic neurons have differentiated and are beginning to extend axonal projections toward the cortex (Molnár et al., 1998). We compared mTOR activity in the *Tsc1*^{+/+} and *Tsc1*^{dE12/dE12} thalamus at E14.5 by IHC for the S6 protein phosphorylated at Ser240/244 (pS6), which is a reliable readout of mTOR pathway activity. We observed basal pS6 expression in the E14.5 *Tsc1*^{+/+} brain (Figure 2A), consistent with the requirement for mTOR activity during early development (Hentges et al., 2001). Nevertheless, in the E14.5 *Tsc1*^{dE12/dE12} thalamus, there was an increase in thalamic pS6 levels over controls (Figure 2B). In E17.5 *Tsc1*^{dE12/dE12} embryos, thalamic levels of pS6 were also dramatically increased compared to controls (Figures 2C and 2D). These experiments show how rapidly neurons respond to *Tsc1* gene inactivation in vivo during embryogenesis. mTOR dysregulation persisted in the postnatal *Tsc1*^{dE12/dE12} thalamus but was negligible in the *Tsc1*^{+/+} and *Tsc1*^{+/dE12} controls (Figures 2E–2G). *R26*^{LacZ} reporter activation (β -gal, green) validated that all genotypes had a similar extent of CreER-mediated recombination. Similar results were seen with IHC for pS6(Ser235/236), another mTOR-dependent S6 phosphorylation site (data not shown).

E12.5 *Tsc1* Deletion Alters Morphology and Circuitry in Mature Thalamic Neurons

To determine whether mTOR dysregulation affected the morphology of adult thalamic neurons, we quantified soma size based on the somatodendritic marker microtubule-associated protein 2 (MAP2). Sections were also stained for pS6 (red). CreER-mediated recombination produced mTOR dysregulation in 70% of thalamic neurons in *Tsc1*^{dE12/dE12} mice (621 out of 878 MAP2+ neurons). We took advantage of this mosaicism and sorted neurons into two populations: dysregulated *Tsc1*^{dE12/dE12} neurons (pS6+, filled arrowheads) and unaffected neurons (pS6–, open arrowheads, Figure 3B). The geometric mean soma area of pS6+ *Tsc1*^{dE12/dE12} neurons was 403 μ m, which was

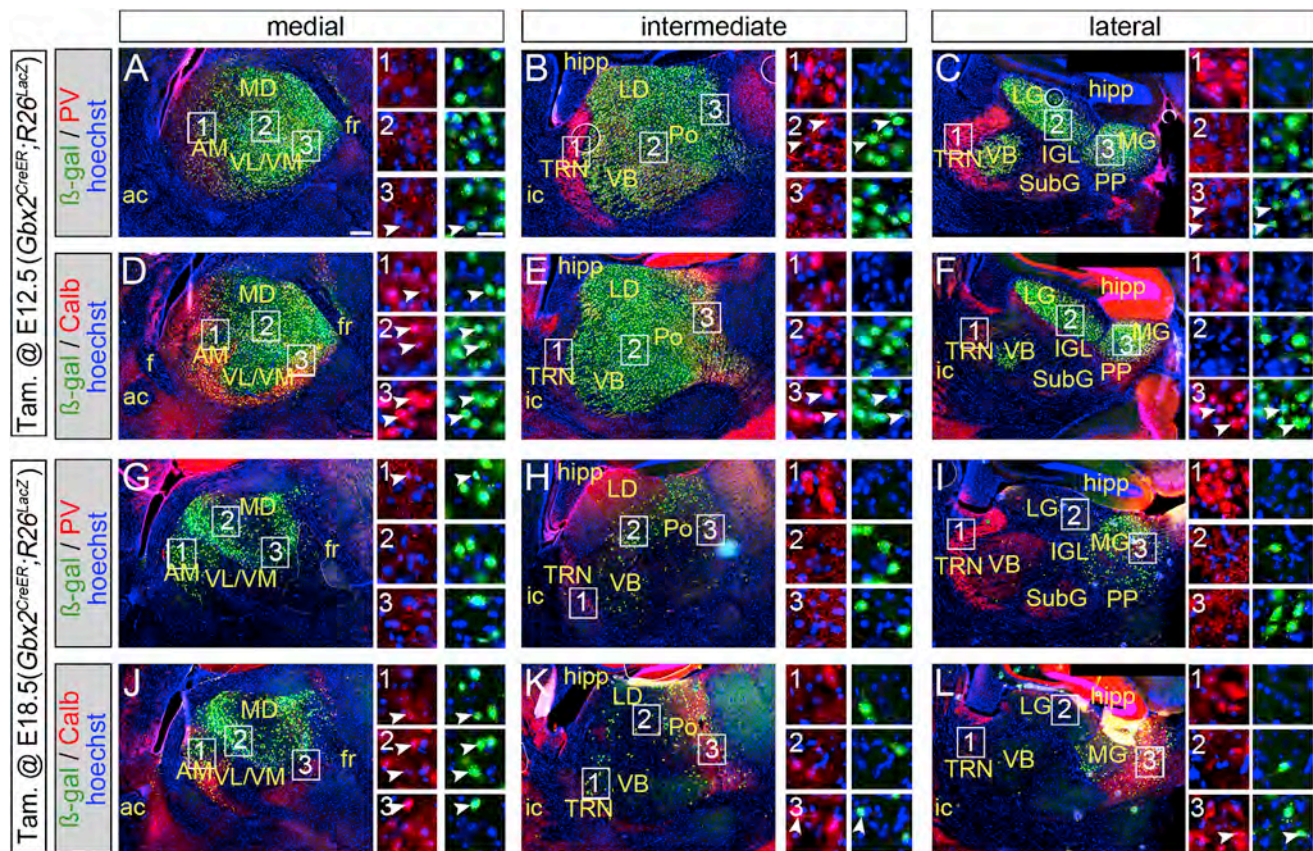


Figure 1. *Gbx2*^{CreER}-Mediated Recombination in Thalamic Neurons

(A–F) Tamoxifen at E12.5. Expression of β-gal (green) in medial (A and D), Intermediate (B and E), and lateral (C and F) sagittal sections of adult thalamus. Colocalization with parvalbumin (PV, red; A, B, and C) or calbindin (Calb, red; D, E, and F) is indicated by arrowheads.

(G–L) Tamoxifen at E18.5. Recombination in medial (G and J), Intermediate (H and K), and lateral (I and L) sagittal section of adult thalamus showing recombined cells (β-gal, green) and PV+ (G–I) or Calb+ (J–L) neurons. Scale bar in (A) (270 μm) applies to low-magnification panels; scale bar in (A3) (30 μm) applies to high-magnification panels. Thalamic nuclei: AM, anteromedial; LD, laterodorsal; LG, lateral geniculate; MD, mediodorsal; MG, medial geniculate; Po, posterior; PP, peripeduncular; SubG, subgeniculate; TRN, thalamic reticular; VB, ventrobasal; VL, ventrolateral; VM, ventromedial; a.c., anterior commissure; f, fornix; fr, fasciculus retroflexus; hipp, hippocampus; i.c., internal capsule; IGL, intergeniculate leaflet. See also Figure S1.

significantly larger than *Tsc1*^{+/+} (220 μm²), *Tsc1*^{ΔE12/+} (209 μm²), and pS6–*Tsc1*^{ΔE12/ΔE12} (203 μm²) neurons ($p = 0.003$, $n = 3$ mice per genotype, Figure 3B, see Table S1 for variability estimates). Because normal-sized pS6– cells neighbored enlarged pS6+ cells, we conclude that neuron overgrowth occurs in a cell-autonomous manner. We also detected substantial PV expression in fibers within the internal capsule of *Tsc1*^{ΔE12/ΔE12} brains (Figures 3E and 3E'), which was absent in controls (Figures 3C and 3C'). Because corticothalamic and thalamocortical axons (TCAs) intermingle in the internal capsule, we assayed for *R26*^{tdTomato} expression. Comparison of RFP/PV colocalization in the fibers (Figures 3C, 3C' and 3E, 3E') and cell bodies of thalamic relay neurons (Figures 3D, 3D', 3F, and 3F') confirmed that the PV+ signal was from the *Tsc1*^{ΔE12/ΔE12} relay neurons and their TCAs. Because previous TS mouse models have described myelination defects and astrogliosis (Meikle et al., 2008; Way et al., 2009; Carson et al., 2012), we assayed for myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP). Control mice had clear MBP labeling throughout the brain,

including within the thalamus and the internal capsule, and this did not differ between mutants and controls (Figure S2). Only sporadic GFAP+ cells were observed in the thalamus of both mutants and controls (Figure S2). Because the enlarged *Tsc1*^{ΔE12/ΔE12} thalamic neurons were reminiscent of dysmorphic neurons in neuronal storage disorders, we assayed for GM2 ganglioside, which accumulates in these disorders (Zervas et al., 2001). GM2 was not detected in *Tsc1*^{+/+} or *Tsc1*^{ΔE12/ΔE12} thalamic neurons (data not shown).

We next investigated whether deleting *Tsc1* at E12.5 affected thalamocortical circuit development. We took advantage of the highly organized and stereotyped projections from the thalamic ventrobasal nuclear complex (VB) to the vibrissa barrels in layer IV of primary somatosensory cortex (SI) (Woolsey and Van der Loos, 1970). We used *R26*^{tdTomato} to label thalamic projections for neural circuit analysis. In control animals (adults), TCAs innervated layer IV of somatosensory cortex in discrete clusters corresponding to individual vibrissae (Figure 4A, region 1), similar to descriptions using nongenetic labeling (Wimmer et al., 2010). In

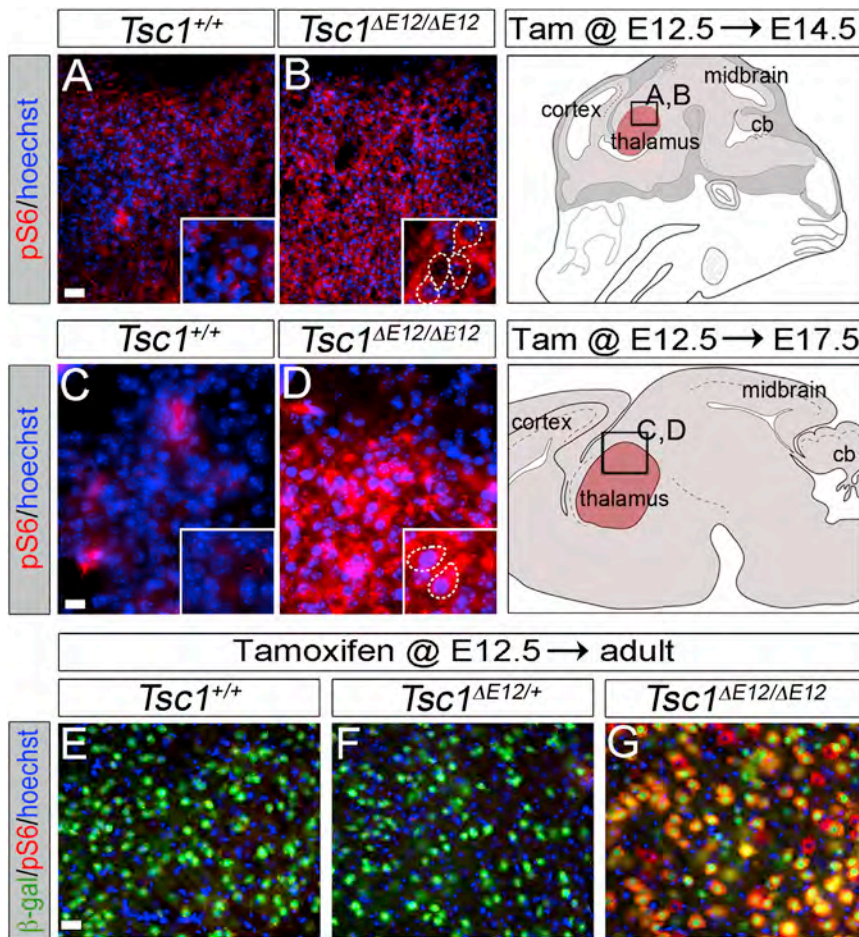


Figure 2. Conditional Deletion of *Tsc1* in the Thalamus Causes Rapid mTOR Dysregulation

(A and B) pS6 (red) immunolabeling in E14.5 *Tsc1*^{ΔE12/ΔE12} embryos.

(C and D) E17.5 *Tsc1*^{ΔE12/ΔE12} embryos had a robust increase in pS6 (red) compared to controls. (E–G) Adult *Tsc1*^{ΔE12/ΔE12} mutants had high pS6 levels (red). *R26*^{LacZ} (β-gal, green) independently showed similar recombination efficiency across genotypes. Control and mutant sections were imaged with identical exposure settings. *n* ≥ 3 animals per genotype per stage. Scale bars represent 30 μm in (A), (B), and (E)–(G) and 15 μm in (C) and (D).

indistinct in the *Tsc1*^{ΔE12/ΔE12} cortex (Figures 4D and 4H, gray regions), which was a phenotype reminiscent of that described in *mGluR5* knockout mice (She et al., 2009). To quantitatively assess the large barrels (Figures 4D and 4H, orange regions), we outlined the limits of the SI vibrissa region and the individual barrels based on CO staining in a genotype-blinded manner. The average barrel size was larger in mutants (58 mm²) compared to controls (37 mm², *p* < 0.001, *n* ≥ 72 barrels across 3 mice per genotype, two-sample two-tailed *t* test; Figure 4K). Quantification of the septal proportion of the barrel region based on CO staining showed no significant difference between *Tsc1*^{ΔE12/ΔE12}

contrast, *Tsc1*^{ΔE12/ΔE12} mice (adults) had a diffuse pattern of cortical innervation: individual barrels were indistinguishable in layer IV (Figure 4B, region 1) and projections were overabundant in the deep layers (arrow). Within the internal capsule, TCA fascicles appeared less sharply defined compared to controls (Figures 4A and 4B, region 2). We confirmed these findings by stereotaxic injection of lentiviral-GFP into VB in control and mutant animals (Cruikshank et al., 2010), which filled infected neurons with GFP, including their axons and terminal projections (Figure S3).

To assess the effect of the disorganized TCAs on genetically normal cortical targets, we used cytochrome oxidase (CO) staining, which is enriched in the dendritic mitochondria of layer IV spiny stellate barrel neurons (Wong-Riley and Welt, 1980) and nicely delineates the barrel hollow structures (Figures 4C–4J). In controls, RFP+ TCAs were enriched in the CO+ barrel hollows and largely excluded from the surrounding septa (Figure 4E, asterisks and arrowheads, respectively). In *Tsc1*^{ΔE12/ΔE12} mutants, the TCAs were not only localized to barrel hollows (Figure 4I, asterisks) but were also heavily distributed throughout the septal regions (arrowheads). The CO staining pattern was also altered in *Tsc1*^{ΔE12/ΔE12} brains, suggesting that the cortical barrels were improperly patterned (Figure 4, compare 4C and 4D to 4G and 4H). The small vibrissa barrels were particularly

(21%) and controls (25%, *p* = 0.16, *n* = 3 mice per genotype, two-sample two-tailed *t* test; Figure 4L). To determine whether the organization of the cortical cell bodies was altered, we combined NeuN antibody labeling with CO staining to quantify cell density in the barrel hollows (outer limit of the CO+ barrel hollow is indicated by the dashed lines in Figures 4F and 4J) and the surrounding barrel wall region (indicated by the solid lines in Figures 4F and 4J) (Narboux-Nême et al., 2012). Mutants had lower neuron density in the barrel wall region (3.7 neurons/mm²) than controls (Figure 4M; 4.5 neurons/mm²). This same trend applied to the barrel hollow region (*Tsc1*^{ΔE12/ΔE12} 3.2 neurons/mm²; *Tsc1*^{+/+} 3.5 neurons/mm², *p*_{wall} < 0.001, *p*_{hollow} = 0.020, *n* ≥ 20 nonadjacent barrels across 3 animals per genotype, two-sample two-tailed *t* test; Figure 4M). Together, these experiments confirmed that thalamic *Tsc1* inactivation causes mTOR dysregulation, cell overgrowth, aberrant PV expression, and altered thalamocortical projections that affect the genetically normal neocortex.

Later Deletion of *Tsc1* Causes More Subtle Cellular Changes than Those Arising from Early Inactivation

We administered tamoxifen at E18.5 to compare the effects of thalamic *Tsc1* inactivation at a later developmental stage. By E18.5, thalamic neurons have fully differentiated, their axonal

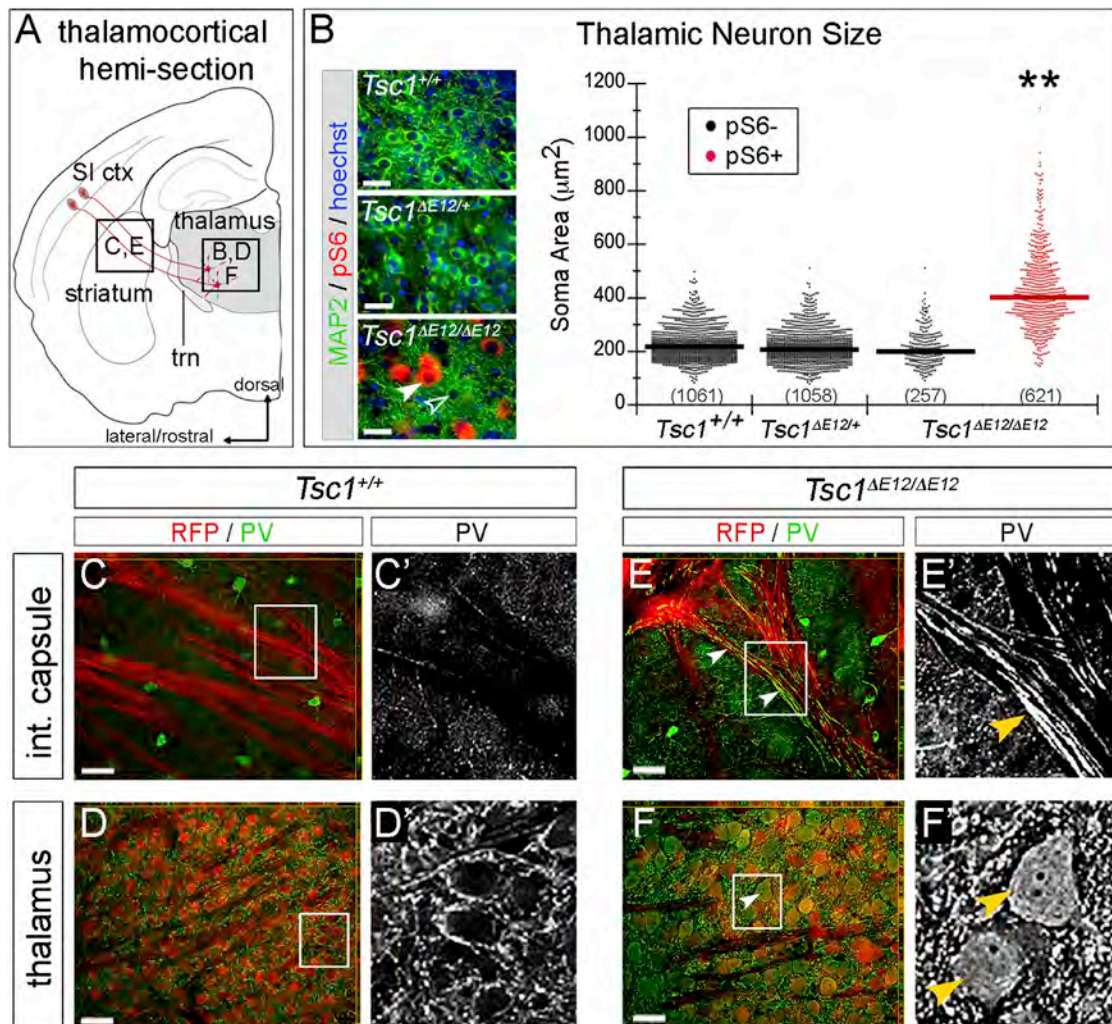


Figure 3. Cellular Phenotypes Caused by *Tsc1* Deletion in Thalamus at E12.5

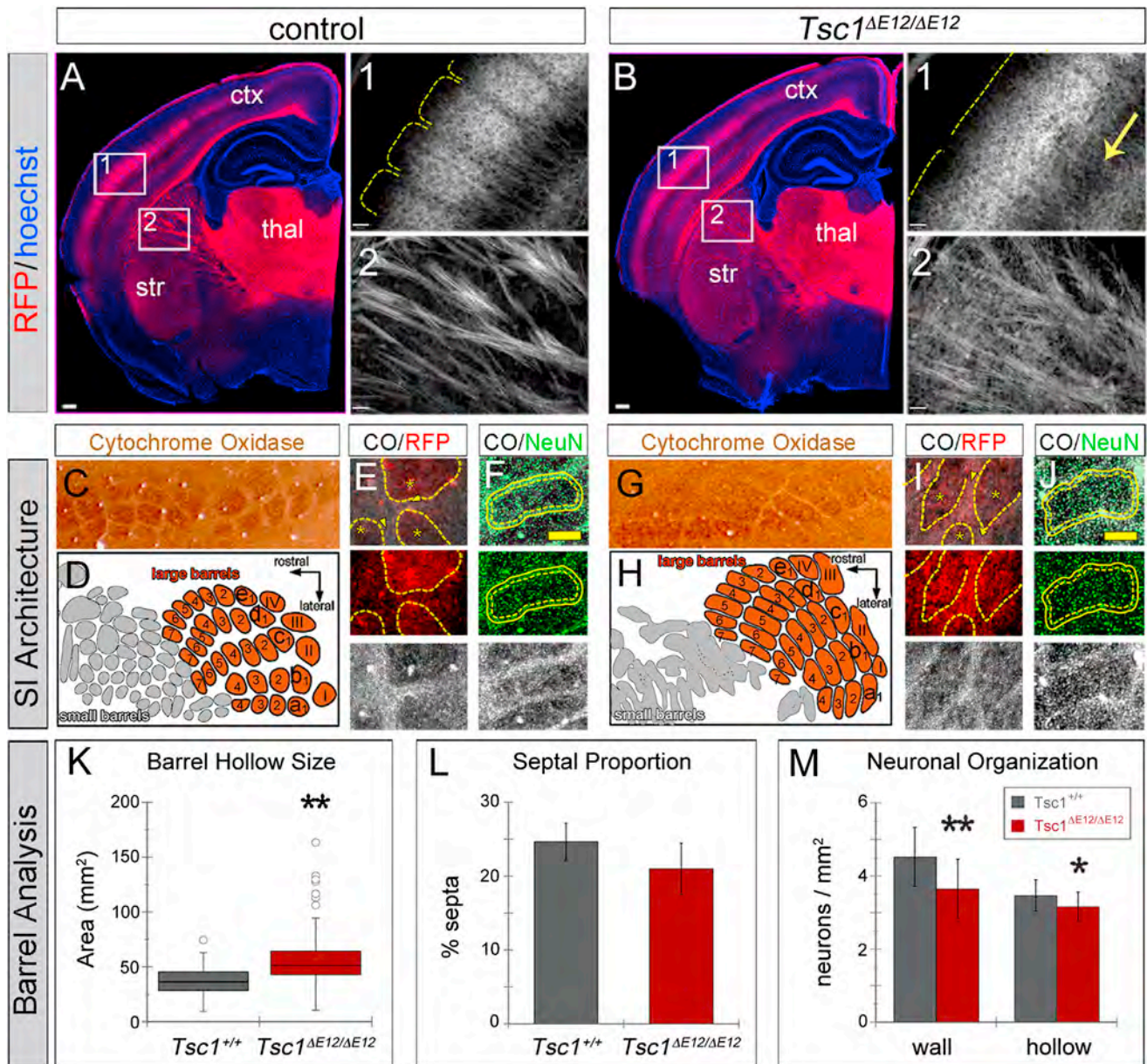
(A) Thalamocortical regions of interest.

(B) Sections from adult mice were immunolabeled for MAP2 (green), pS6 (red), and counterstained with hoechst (blue). Thalamic neurons of $Tsc1^{+/+}$ and $Tsc1^{\Delta E12/+}$ mice were pS6⁻. Recombination produced a mosaic thalamus of unaffected (pS6⁻, open arrowhead) and affected (pS6⁺, filled arrowhead) neurons. Soma area is plotted by genotype and pS6 status. Numbers of neurons are listed and geometric means are indicated by horizontal lines.

(C–F') Analysis of PV (green) and RFP (red) revealed PV⁺ fibers in the internal capsule of $Tsc1^{\Delta E12/\Delta E12}$ mice (E and E', arrowheads), but not in controls (C and C'). Soma of $Tsc1^{\Delta E12/\Delta E12}$ RFP⁺ neurons were also PV⁺ (F and F', arrowheads, which was not seen in controls (D and D')). $n = 3$ animals per genotype. Scale bars represent 32 μm in (B) and 48 μm in (C)–(F). ** $p < 0.005$. See also Figure S2.

projections have accumulated in the subplate of their cortical target regions, and they are beginning to invade the cortical layers (Molnár et al., 1998). Upon reaching adulthood, $Tsc1^{\Delta E18/\Delta E18}$ brains were analyzed for mTOR activity and cell size (Figure 5A). mTOR was dysregulated in 29% of neurons (221 out of 542 MAP2⁺ cells) in the $Tsc1^{\Delta E18/\Delta E18}$ thalamus, as evidenced by increased pS6 (Figure 5A). We analyzed cell size as described in Figure 3. Although some pS6⁺ $Tsc1^{\Delta E18/\Delta E18}$ neurons skewed toward larger cell sizes than pS6⁻ neurons, on average, pS6⁺ $Tsc1^{\Delta E18/\Delta E18}$ neurons (359 μm^2) were not significantly larger than pS6⁻ $Tsc1^{\Delta E18/\Delta E18}$ (246 μm^2), $Tsc1^{\Delta E18/+}$ (242 μm^2), or $Tsc1^{+/+}$ (253 μm^2) cells ($p = 0.11$; Figure 5A). We observed rare pS6⁺ neurons in the

$Tsc1^{+/+}$ (2 out of 632 cells, average size 304 μm^2 , data not shown) and $Tsc1^{\Delta E18/+}$ (8 out of 1,069 cells, average size: 277 μm^2) thalamus, which were not graphed for clarity. Unlike the E12.5 findings, aberrant PV expression was not apparent in either axons or cell bodies of $Tsc1^{\Delta E18/\Delta E18}$ thalamic neurons (Figures 5B and 5C, region 3, data not shown). $Tsc1^{\Delta E18/\Delta E18}$ thalamocortical projections appeared coarse within the internal capsule and overabundant within deep cortical layers (Figures 5B and 5C, arrows), similar to the E12.5 findings. Because of the different recombination pattern, the vibrissa barrel-projecting neurons in VB did not undergo substantial recombination and thus were not labeled by the $R26^{tdTomato}$ reporter. For this reason, TCA innervation of the vibrissa barrels could not be



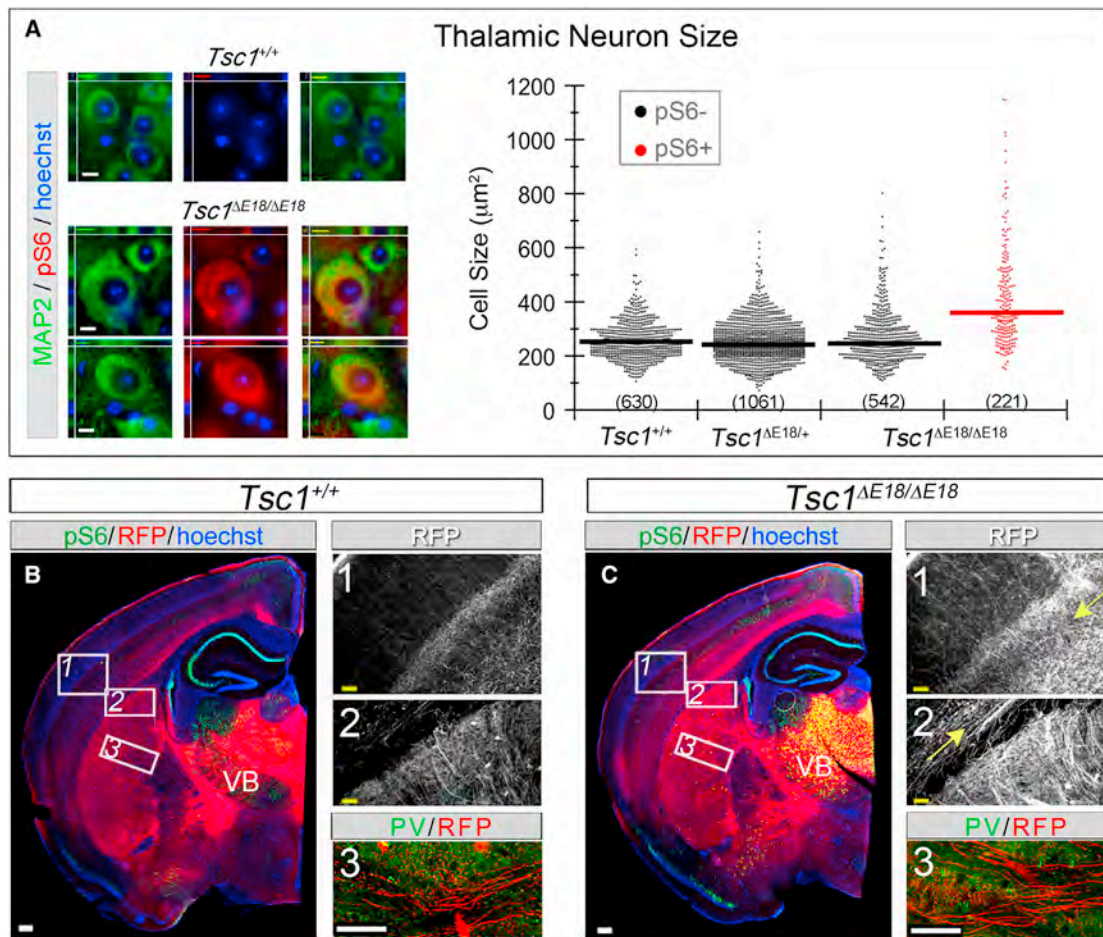


Figure 5. *Tsc1* Deletion at E18.5 in the Thalamus Causes Excessive Thalamic Axons

(A) Control and *Tsc1*^{ΔE18/ΔE18} sections from adults were immunostained for MAP2 (green) and pS6 (red). Soma size was graphed by genotype and pS6 expression and showed no significant difference. Note that pS6+ neurons were rarely observed in *Tsc1*^{+/+} (two cells) and *Tsc1*^{ΔE18/+} brains (eight cells) and were not graphed for clarity.

(B and C) *Tsc1*^{+/+} (B) and *Tsc1*^{ΔE18/ΔE18} (C) sections were immunolabeled for pS6 (green) and RFP (red). *Tsc1*^{ΔE18/ΔE18} TCAs were superfluous and disorganized in deep cortical layers (region 1, arrow) and internal capsule (region 2, arrow). PV (region 3, green, from adjacent sections) was absent from *Tsc1*^{ΔE18/ΔE18} and *Tsc1*^{+/+} TCAs (red). Scale bars represent 8 μm in (A), 240 μm in (B) and (C), 61 μm in (B1), (B2), (C1), and (C2), and 57 μm in (B3) and (C3). See also Figure S4.

visualized by RFP expression. Nevertheless, we assessed vibrissa barrel formation using CO staining, which showed that the *Tsc1*^{ΔE18/ΔE18} somatosensory cortex did not have any patterning disruptions (Figure S4).

Intrinsic Physiology Is Abnormal in *Tsc1*^{ΔE12/ΔE12}, but Not *Tsc1*^{ΔE18/ΔE18} Thalamic Neurons

To interrogate the functional effects of *Tsc1* deletion at E12.5 versus E18.5 on individual cells, we performed whole-cell patch-clamp recordings on thalamic VB neurons in mature thalamocortical slices (Figure 6). (For all data in this section, see Table S1 for variability estimates, nonsignificant means, and p values.) We recorded from VB because it is easily identifiable and its relay neurons exhibit stereotyped, well-characterized physiological properties (Landisman and Connors, 2007). We used RFP fluorescence from the *R26*^{tdTomato} reporter allele to target our recordings to recombined neurons. Biocytin was

added to the recording pipette to identify neurons post hoc, reconstruct their morphology, and confirm mTOR dysregulation in mutant neurons (Figure 6A). We characterized the intrinsic membrane properties of *Tsc1*^{ΔE12/ΔE12} and *Tsc1*^{ΔE18/ΔE18} VB neurons compared to neurons from their respective *Tsc1*^{+/+} littermates. *Tsc1*^{ΔE12/ΔE12} VB neurons had significantly lower input resistance than neurons in *Tsc1*^{+/+} littermates (72.6 MΩ versus 137.2 MΩ, *p* = 0.001; Figure 6B). In addition, *Tsc1*^{ΔE12/ΔE12} VB neurons had a higher capacitance than *Tsc1*^{+/+} neurons (417.6 pF versus 219.7 pF, *p* = 0.004, Figure 6B). In contrast, *Tsc1*^{ΔE18/ΔE18} neurons did not differ from their controls in either resistance or capacitance (Figure 6B). The membrane time constant was unchanged in *Tsc1*^{ΔE12/ΔE12} and *Tsc1*^{ΔE18/ΔE18} compared to controls (Figure 6B), because the decrease in resistance offset the increase in capacitance.

We also analyzed the properties and dynamics of action potentials in VB neurons (Figure 6C). Action potential thresholds

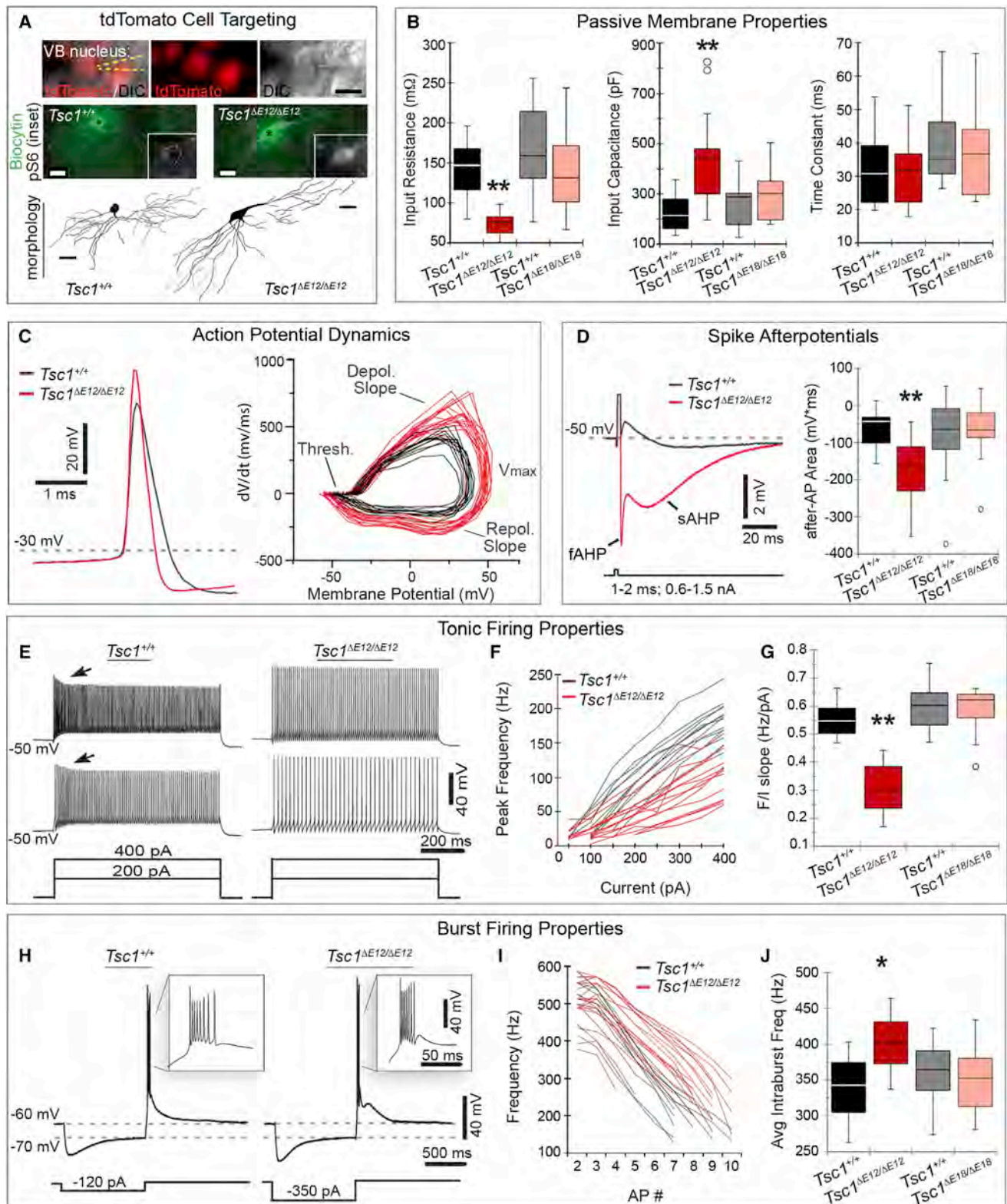


Figure 6. *Tsc1*^{ΔE12/ΔE12} Thalamic Neurons Have Altered Electrophysiological Properties

(A) DIC/fluorescence shows electrode (yellow dashed lines) targeted to a RFP+ (red) VB neuron. Neurons were filled with biocytin (green) and immunostained for pS6 (white, insets). Morphology was reconstructed as shown below each filled neuron.

(legend continued on next page)

in $Tsc1^{\Delta E12/\Delta E12}$ neurons were similar to those of $Tsc1^{+/+}$. However, $Tsc1^{\Delta E12/\Delta E12}$ neurons, when compared to $Tsc1^{+/+}$ neurons, had significantly larger spike amplitude (82 mV versus 70 mV, $p = 0.0002$) and faster rates of depolarization (618 mV/ms versus 423 mV/ms, $p = 0.0001$) and repolarization (-263 mV/ms versus -151 mV/ms, $p < 0.0001$) (Figure 6C). $Tsc1^{\Delta E18/\Delta E18}$ spikes did not differ significantly from those of $Tsc1^{+/+}$ neurons in terms of amplitude, depolarization rate, or repolarization rate (Figure S5). VB action potentials are typically followed by fast and slow afterhyperpolarizations (AHPs) and an afterdepolarization (ADP) of intermediate duration (Figure 6D, black trace). To compare these events, we summed the total area under the postaction potential trajectory, which revealed that the $Tsc1^{\Delta E12/\Delta E12}$ neurons had significantly more negative afterpotentials compared to controls (-177 mV \cdot ms versus -64 mV \cdot ms, $p = 0.0026$; Figure 6D). The $Tsc1^{\Delta E18/\Delta E18}$ afterpotentials did not differ significantly from controls (Table S1).

Thalamic relay neurons fire in both tonic and bursting modes, depending on the state of the resting membrane potential. We characterized tonic firing by holding the membrane potential at -50 mV and applying steps of depolarizing current. While the amplitudes of $Tsc1^{+/+}$ action potentials declined over the first 100 ms of spiking (adaptation), the amplitudes of $Tsc1^{\Delta E12/\Delta E12}$ action potentials remained constant (Figure 6E, arrows). The relationship between firing frequency and stimulus current was roughly linear for both $Tsc1^{+/+}$ and $Tsc1^{\Delta E12/\Delta E12}$ cells (Figure 6F). The average slope of the frequency/current relationship for $Tsc1^{\Delta E12/\Delta E12}$ cells (0.27 Hz/pA) was significantly lower than that of $Tsc1^{+/+}$ cells from littermate controls (0.53 Hz/pA, $p < 0.001$, $n \geq 11$ cells recorded from $n \geq 3$ animals per group; Figure 6G). Frequency/current relationships of $Tsc1^{\Delta E18/\Delta E18}$ cells did not differ from those of littermate controls (Figures 6G and S5). We next characterized the cells' burst firing by holding membrane potentials initially at -60 mV, then injecting a 1 s step of current sufficient to bring the membrane to -70 mV. Upon release of the current, VB neurons fired a single burst of spikes (Figure 6H). Each burst comprised a similar number of action potentials that did not vary by genotype; however, the mean duration of the $Tsc1^{\Delta E12/\Delta E12}$ bursts were shorter. Figure 6I plots the intraburst firing frequency as a function of spike number within the bursts; $Tsc1^{\Delta E12/\Delta E12}$ neurons had a significantly higher mean spiking frequency throughout the burst (401 Hz) compared to $Tsc1^{+/+}$ littermate controls (mean of 339 Hz, $p = 0.026$). $Tsc1^{\Delta E18/\Delta E18}$ neurons were not significantly different from neu-

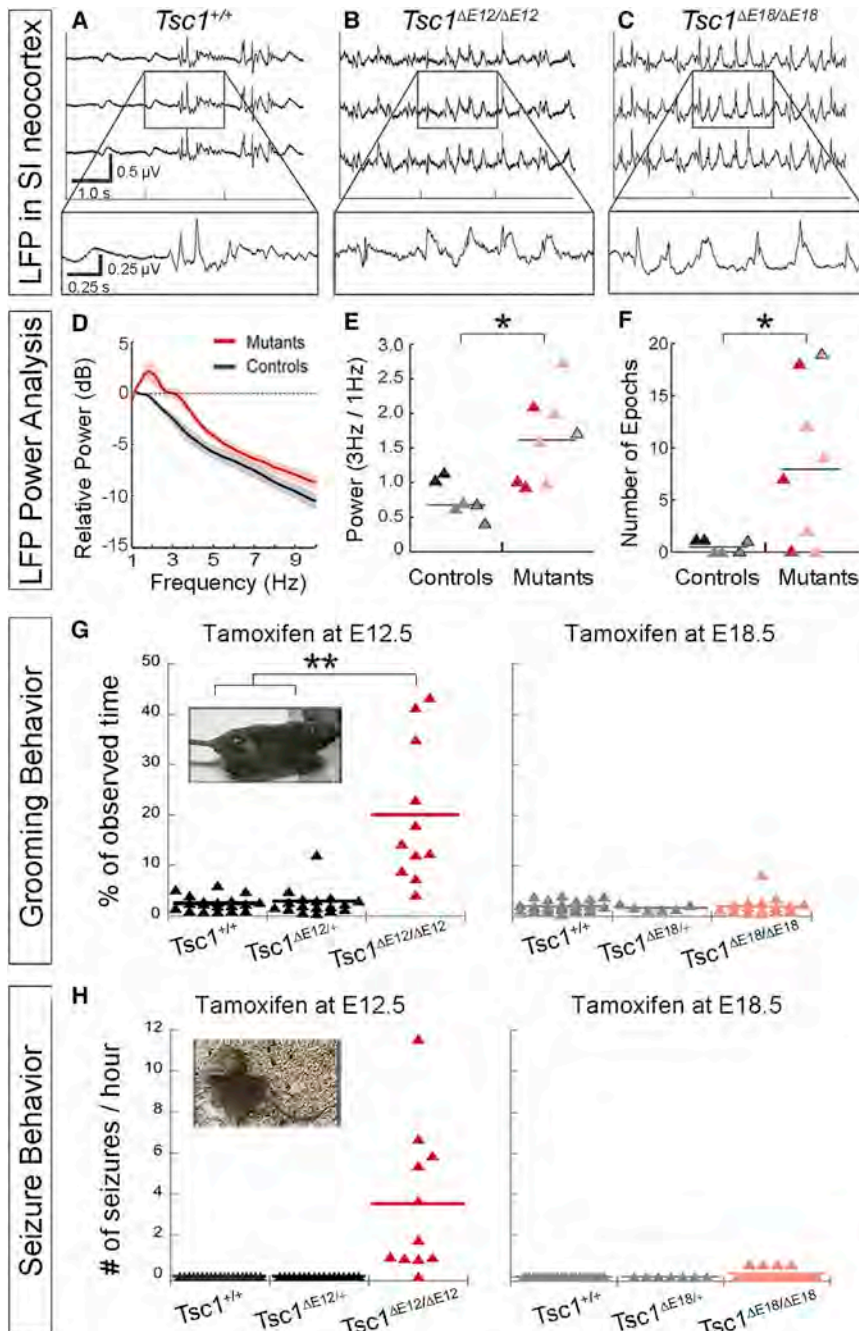
rons of $Tsc1^{+/+}$ littermates (Figures 6J and S5). These experiments revealed that the enlarged $Tsc1^{\Delta E12/\Delta E12}$ neurons require stronger input currents to modify their membrane potentials, have larger, faster action potentials, and have altered firing properties in both tonic and bursting mode, compared to wild-type VB neurons, whereas $Tsc1^{\Delta E18/\Delta E18}$ neurons were unaltered.

Thalamic Tsc1 Deletion at E12.5 and E18.5 Causes Abnormal Neural Activity and Behavior

To determine whether the changes in thalamic development and physiology impact neocortical physiology, we recorded local field potentials (LFPs) in the vibrissa representation of primary SI of adult anesthetized mice. We chose SI because it receives robust input from VB, where we detected changes in circuit organization and whole-cell physiology. We confirmed targeting to barrel cortex by stimulating vibrissae to drive sensory-evoked responses (data not shown). We observed prominent low-frequency oscillations in both $Tsc1^{\Delta E12/\Delta E12}$ and $Tsc1^{\Delta E18/\Delta E18}$ mice (Figures 7A–7C, $n = 6$ $Tsc1^{+/+}$, $n = 3$ $Tsc1^{\Delta E12/\Delta E12}$, $n = 5$ $Tsc1^{\Delta E18/\Delta E18}$ mice). Quantitative analysis of LFP activity showed that mutants had higher power across multiple frequencies, particularly in the 3 Hz range (Figure 7D). This is a frequency associated with spike-and-wave epileptiform activity, which is related to altered thalamic dynamics (Blumenfeld, 2003). Mutants had significantly higher 3 Hz power than controls ($p = 0.008$, Figure 7E), which was evident in the comparison across all individuals (controls in black/gray, mutants in red/pink triangles). Further, the number of epochs of high-power 3 Hz activity lasting ≥ 20 s was significantly higher in $Tsc1^{\Delta E12/\Delta E12}$ (red triangles) and $Tsc1^{\Delta E18/\Delta E18}$ (pink triangles) mutant animals compared to controls ($p = 0.028$, Figure 7F). Older (>8 months) $Tsc1^{\Delta E18/\Delta E18}$ animals and controls were also assessed to account for possible age-related differences in brain activity. These data points are differentiated by black outlines in Figures 7E and 7F.

We addressed whether there were any behavioral ramifications of this altered brain activity. At 2 months of age, $Tsc1^{\Delta E12/\Delta E12}$ mice seemed to groom more frequently than control littermates and developed severe skin lesions (Figure 7G, inset). Because control littermates never developed lesions but were housed in the same cage as affected mice, we hypothesized that the lesions were due to the excessive self-grooming, rather than environmental factors, fighting, or allogrooming. Importantly, overgrooming was apparent before wounds

- (B) $Tsc1^{\Delta E12/\Delta E12}$ neurons (red) had lower membrane input resistance and higher input capacitance but unchanged time constants compared to littermate controls (black). Note that $Tsc1^{\Delta E18/\Delta E18}$ mutants (pink) and their controls (gray) are also plotted.
- (C) Representative traces from control and $Tsc1^{\Delta E12/\Delta E12}$ neurons (left) show that $Tsc1^{\Delta E12/\Delta E12}$ action potentials were faster and larger. $Tsc1^{\Delta E12/\Delta E12}$ action potential dynamics (right) were significantly different with respect to depolarization rate, maximum amplitude, and repolarization rate.
- (D) $Tsc1^{\Delta E12/\Delta E12}$ spike afterpotentials (red) were more negative during the fast (fAHP) and during the slow phase (sAHP) compared to controls (black). Total postspike membrane potential was integrated over time and quantified by integrating the voltage signal over 280 ms (right).
- (E) Representative tonic voltage response of a $Tsc1^{+/+}$ and $Tsc1^{\Delta E12/\Delta E12}$ neuron to current injections (400 pA, top and 200 pA, bottom).
- (F) Peak firing frequency per current step (F/I) is plotted for $Tsc1^{+/+}$ (black, $n = 12$) and $Tsc1^{\Delta E12/\Delta E12}$ (red, $n = 17$) neurons.
- (G) Linear slopes of the F/I curves are quantified.
- (H) Representative voltage response of a $Tsc1^{+/+}$ and a $Tsc1^{\Delta E12/\Delta E12}$ thalamic neuron to hyperpolarizing current step. Insets show rebound bursts.
- (I) Intraburst firing frequency as a function of spike number within each burst is plotted for $Tsc1^{+/+}$ (black, $n = 11$) and $Tsc1^{\Delta E12/\Delta E12}$ (red, $n = 18$) neurons.
- (J) Mean intraburst firing frequencies are quantified. Note that $Tsc1^{\Delta E18/\Delta E18}$ mutants (pink) did not significantly differ from their controls (gray; B, D, G, and J). Box plots represent minimum, first quartile (Q1), median, Q3, and maximum. Outliers (open circles) were $>Q3 + 1.5 \cdot IQR$ or $<Q1 - 1.5 \cdot IQR$. Scale bars in (A) represent 20 μ m (DIC) and 30 μ m (biocytin/morphology). * $p < 0.05$, ** $p < 0.005$. See also Figure S5 and Table S1.



and did not develop wounds or groom more often than *Tsc1*^{+/+} or *Tsc1*^{ΔE18/+} littermates, regardless of age ($n = 25$ and $n = 6$ respectively, Figure 7G).

Tsc1^{ΔE12/ΔE12} mice also exhibited spontaneous seizures beginning around 2 months of age, consistent with the increase in 3 Hz LFP activity. The seizure events were highly stereotyped and began with prolonged grooming of the hindlimb, followed by loss of upright posture, then a tonic-clonic state during which the body entered into a convulsive, twisted posture typically lasting 10 s (Figure 7H, inset; Movie S1). An observer blinded to genotype quantified the frequency and duration of seizures. The *Tsc1*^{ΔE12/ΔE12} mice averaged 3.7 seizures/hr (CI_{95} : 2.0–6.9 seizures/hr), while control littermates never exhibited seizures (Figure 7H). Ninety-one percent of

the *Tsc1*^{ΔE12/ΔE12} mice (10/11) that were analyzed experienced convulsive seizures as described above during the observation periods. While the remaining mouse did not have overt seizures, it did display abnormal behavior in that it remained in a motionless, sleep-like state for minutes at a time, which may have been absence seizures. In contrast, *Tsc1*^{ΔE18/ΔE18} mice did not exhibit seizures at 2 months of age. However, by 8 months of age, four of the 17 *Tsc1*^{ΔE18/ΔE18} mice had experienced a seizure (Figure 7H, Movie S2), but these rare seizure events only occurred upon handling. Thus, we conclude that 100% of *Tsc1*^{ΔE12/ΔE12} mice and 24% of *Tsc1*^{ΔE18/ΔE18} mice displayed

developed, indicating that the wound was not the trigger for the grooming but rather a result of it. To confirm this, animals were videotaped for 8 min periods twice a week in their homecage before wounds appeared. An observer scored the amount of time spent grooming by each mouse in a genotype-blinded manner. *Tsc1*^{ΔE12/ΔE12} mice spent significantly more of their time grooming (24.1%, 95% confidence interval (CI_{95}): 21.8%–26.5%) than *Tsc1*^{+/+} (3.0%, CI_{95} : 2.4%–3.9%) and *Tsc1*^{ΔE12/+} (3.8%, CI_{95} : 3.0%–4.9%) mice ($p < 0.0001$, $n \geq 11$ mice per genotype; Figure 7G). In contrast, *Tsc1*^{ΔE18/ΔE18} mice displayed no overt phenotypes by 3 months of age ($n = 17$)

abnormal behavior, with some variation in form and severity. Notably, the severity of the grooming and the seizure phenotypes was not correlated within individuals.

Because *Gbx2*^{CreER} mediates recombination in the spinal cord at E12.5 (Luu et al., 2011), we tested peripheral sensory and motor function (Figure S6). We did not detect a significant difference in tactile sensitivity (von Frey filament test, $p = 0.315$) or motor function (wire hang assay, $p = 0.134$) between control and *Tsc1*^{ΔE12/ΔE12} animals. We also showed that thermal pain sensitivity was unaffected in *Tsc1*^{ΔE12/ΔE12} mutants (hot plate test, $p = 0.188$). Because *Gbx2*^{CreER} is no longer expressed in the spinal cord after E14.5 (John et al., 2005), we did not perform similar tests on *Tsc1*^{ΔE18/ΔE18} animals. Taken together, our collective analysis of thalamocortical circuitry, neuronal physiology, and neocortical local field potentials strongly suggest that the primary drive of these *Tsc1*^{ΔE12/ΔE12} or *Tsc1*^{ΔE18/ΔE18} phenotypes is mTOR dysregulation in the thalamus.

DISCUSSION

TS is a developmental mosaic genetic disorder caused by disrupting the TSC/mTOR pathway. In this study, we tested the hypothesis that disrupting the mTOR pathway elicits different phenotypes depending on the identity and developmental state of cells in which *Tsc1* is deleted and mTOR is dysregulated.

Genetic circuit tracing showed that *Tsc1*^{ΔE12/ΔE12} thalamic projections are disorganized and have excessive processes that innervate layer IV septal regions of the somatosensory barrel cortex. This phenotype may result from the lack of activity-dependent pruning or excess axonal ramifications filling intra-barrel spaces. Our observations are consistent with previous reports describing abnormal axonal targeting of retinal projections in both the *Drosophila* and mouse brain, in which *Tsc1* mutant axons overshoot their target and have branches that terminate outside the normal target regions (Knox et al., 2007; Nie et al., 2010). It is probable that other cortical areas receive similarly disorganized *Tsc1*^{ΔE12/ΔE12} thalamic inputs. We also analyzed *Tsc1*^{ΔE18/ΔE18} TCA projections as they traversed the striatum and entered the cortex. Similar to *Tsc1*^{ΔE12/ΔE12}, there was a qualitative excess of RFP+ *Tsc1*^{ΔE18/ΔE18} TCA projections within the deep cortical layers. However, a direct comparison of *Tsc1*^{ΔE18/ΔE18} and *Tsc1*^{ΔE12/ΔE12} vibrissa barrel innervation was precluded because of their different recombination patterns. Regardless, these thalamocortical projection phenotypes in deep layers are consistent with disrupted neuronal processes in response to mTOR dysregulation (Choi et al., 2008).

We uncovered multiple electrophysiological alterations upon early deletion of *Tsc1*. The increased input capacitance and reduced input resistance are both consistent with increased membrane area as a result of cell growth. Notably, action potential dynamics were also altered, yet spike threshold potentials were unaffected. The altered action potentials of *Tsc1*^{ΔE12/ΔE12} neurons may partially compensate for the changes in passive properties. As the input resistance of a neuron falls, larger synaptic currents are required to modify membrane voltage. Mutant *Tsc1*^{ΔE12/ΔE12} neurons also have larger amplitude, briefer action potentials with normal thresholds, and rates of rise and fall that are considerably faster than normal. The maximum rate-of-rise of an action po-

tential is proportional to peak inward sodium current in many neurons (Cohen et al., 1981). Therefore, these changes in spike kinetics strongly suggest that voltage-gated sodium and potassium channels are altered in the mutant cells. The spike shapes are consistent with either higher membrane channel densities or altered single-channel properties, such as subunit composition or phosphorylation, that affect conductance and gating dynamics. In support of these possibilities, the mTOR pathway has been reported to control expression levels and subunit composition of some voltage-gated ion channels (Raab-Graham et al., 2006). Multiple ion channel involvement is further suggested by changes in both the tonic and burst firing modes of mutant cells. The reduced slope of the tonic frequency/current relationship in mutant cells is most easily explained as a consequence of their lower input resistance, while more rapid intraburst spiking is likely due to changes in ion channels. In addition to altered spike-related sodium and potassium channels, it is possible that the rapid intraburst spiking in *Tsc1*^{ΔE12/ΔE12} cells is caused by altered density or kinetics of low-threshold calcium channels. Additionally, the ectopic production of PV, a protein that acts as a slow Ca²⁺ buffer, in *Tsc1*^{ΔE12/ΔE12} thalamic relay neurons may disrupt internal Ca²⁺ dynamics, which can affect gene transcription, synaptic function, and membrane potential and could contribute to some of the physiological changes we describe (Schwaller, 2010).

Importantly, our data show that the effects of early mutation spread well beyond the cells with the *Tsc1* deletion. Individually mutated neurons ensnare the neocortex into hyperexcitable networks, as evidenced by abnormal LFPs in SI. Thus, disruption of an anatomically distinct but functionally connected node within a circuit can propagate the disease phenotype. Comparing the effects of early and late *Tsc1* deletion is informative. We did not detect abnormal physiological properties of *Tsc1*^{ΔE18/ΔE18} VB neurons, which indicates that, at least for VB neurons, there is a critical window of *Tsc1*/mTOR required to establish proper intrinsic excitability properties. Nevertheless, a striking finding is that neocortical (SI) LFP activity was altered in some E18.5 deletion animals. The most likely reason for the global abnormalities is that feedback loops involving multiple thalamic nuclei have altered physiology, which is propagated both locally and to other brain regions. The sources of altered feedback may involve thalamic nuclei that undergo substantial recombination at E18.5 (such as Po) and that subsequently disrupt the reticulo-thalamic or the corticothalamic loops. By comparing the early versus later deletion of *Tsc1*, we are able to discern that abnormalities, even in a small proportion of cells, can cause reverberating global changes in neural activity.

Comparison of our thalamic *Tsc1* mutant phenotypes to other mouse models can be informative in considering the contribution of individual brain regions to global neural dysfunction. Behaviorally, *Tsc1*^{ΔE12/ΔE12} animals groomed excessively, to the extent that they gave themselves severe lesions. A similar overgrooming phenotype has been described in genetic mouse models of autism and obsessive compulsive disorder in which *Slitrk5*, *Shank3*, or *Sapap3* is deleted (Welch et al., 2007; Shmelkov et al., 2010; Peça et al., 2011). Because striatum-specific gene rescue can ameliorate the phenotype, these groups implicate the corticostriatal circuit in causing abnormal repetitive behaviors. The thalamus projects both directly and indirectly, via

neocortex, to the striatum (Smith et al., 2004), suggesting that abnormal thalamic modulation of the striatum in our mice contributes to the repetitive grooming phenotype. However, it is possible that sparse recombination in other subcortical brain structures, such as the striatum and hindbrain, may also contribute to the behavioral changes. *Tsc1* or *Tsc2* knockout in Purkinje cells of the cerebellum also causes repetitive grooming (Tsai et al., 2012; Reith et al., 2013), possibly by disrupting signals from the cerebellum to the motor cortex, which are relayed by the ventrolateral thalamus. In addition, all *Tsc1*^{ΔE12/ΔE12} and some *Tsc1*^{ΔE18/ΔE18} mice experience seizures and abnormal neural activity with epileptiform features. Seizures are a common feature of TS clinically. *Tsc1* knockout in forebrain neurons leads to seizures in 10% of mice (Meikle et al., 2007), while *Tsc1* deletion in astrocytes (and likely neurons as well; Casper and McCarthy, 2006) causes frequent seizures and premature death (Uhlmann et al., 2002). Widespread deletion of *Tsc1* in neural progenitors has also been shown to cause spontaneous seizures in adult mice (Goto et al., 2011). Ours, however, is a conditional *Tsc1* knockout that causes both seizures and overgrooming. Although one may presume that this is simply because the thalamus is a central structure and its dysregulation therefore compromises multiple functional circuits, the explanation cannot be that simple; in the Meikle et al. and Goto et al. studies, *Tsc1* recombination occurs in the thalamus as well as the rest of the forebrain. The fact that more comprehensive *Tsc1* knockouts do not produce similar overgrooming suggests that perturbing a single node of a neural network has the potential to be more deleterious than disrupting the entire network, perhaps because global homeostatic mechanisms are not invoked when only part of a highly interconnected and integrative system is dysregulated. This is an important consideration for brain structures, such as the thalamus, which feature complex feedback loops and widespread reciprocal connectivity that could amplify and spread the effects of a slight functional imbalance. This concept is particularly relevant given the mosaic nature of TS in humans, in which subsets of cells undergo biallelic *TSC1/2* mutations, leading to discrete cohorts of mutant cells (Crino et al., 2010). It is important to note, however, that while thalamic *Tsc1* knockout replicates salient features of TS, we are not implying that TS is a disease of the thalamus. Rather, our findings suggest that the thalamus and other subcortical regions warrant further investigation and that the complex nature of disorders like TS involve multiple brain regions that may respond differentially to the same genetic insult.

The phenotypes related to E12.5 versus E18.5 *Tsc1* inactivation suggest three contributing factors: the spatial pattern of recombination, the overall number of affected cells, and the developmental timing of *Tsc1* inactivation. The spatial pattern of recombination is clearly important and experimentally arises from the dynamic expression of the *Gbx2* gene regulatory elements that drive *CreER* expression (Chen et al., 2009). The dynamic recombination pattern causes the MD, MG, and AM nuclei to undergo recombination at both E12.5 and E18.5. In contrast, the Pf and VB nuclei are largely spared by recombination at E18.5. This differential involvement of nuclei probably leads to distinct consequences. The Pf and VB nuclei project, either directly or indirectly, to the dorsolateral striatum (Pan et al., 2010), which is a central component in a circuit that regu-

lates a syntactic chain of grooming behaviors (Cromwell and Berridge, 1996). Disruption of this circuit at E12.5, but not at E18.5, could underlie the compulsive grooming behavior in *Tsc1*^{ΔE12/ΔE12}, but not *Tsc1*^{ΔE18/ΔE18}, mutant animals. Alternatively, there may be a threshold extent of mosaicism that can be tolerated and compensated for by the brain, above which compensatory mechanisms become ineffective. In this regard, the lower overall number of recombined cells in the *Tsc1*^{ΔE18/ΔE18} thalamus might place the system near the tolerance threshold, resulting in abnormal neural activity but with only a subset of animals experiencing overt seizures and only upon external stimulation. In contrast, the extensive recombination within the *Tsc1*^{ΔE12/ΔE12} thalamus may be above the tolerance threshold, resulting in unmitigated disruption of thalamic development and function. Finally, because mTOR regulates many developmental cellular programs including proliferation, cell growth, axon formation, and synapse formation and maintenance, it is also possible that the later deletion of *Tsc1* results in a diminished phenotype simply because there is a critical period during which thalamic neurons require functional *Tsc1*. By E18.5, thalamic neurons have already extended their axons to their cortical target regions, so this developmental event would be spared when *Tsc1* inactivation occurs at E18.5 but may be affected by earlier *Tsc1* inactivation. This idea is consistent with the fact that, at the single-cell level, recombined VB neurons display aberrant protein expression and altered electrophysiological properties when recombination occurs at E12.5, while VB neurons are apparently unaffected when recombination occurs at E18.5.

It is likely that all three of these factors—the specific cells that suffer the genetic insult, the number of cells that are affected, and the developmental stage at which the genetic hit occurs—contribute to the distinct E12.5 and E18.5 phenotypes to some degree. Although this complex interplay of multiple factors precludes making simple conclusions about mechanisms, it does nicely mimic the complex nature of mosaic disorders such as TS. Mosaic genetic diseases can have extremely variable penetrance, expressivity, and severity. The factors that can contribute to this disease variability, similar to those in our mouse model, include (1) when during development the initial genetic mutation occurs, (2) in which cell that mutation happens (and how the gene functions in that cell type), and (3) how extensively that initial cell's lineage contributes to the final organism (Hall, 1988). Our temporally and spatially controllable mouse model of TS allows us to manipulate where and when the *Tsc1* gene is deleted, which is instructive in understanding the consequences of mosaic genetic insults at distinct stages of development. Future studies that further parse the contributions of these factors will be instrumental for understanding the developmental underpinnings and mechanisms that contribute to tuberous sclerosis and to mosaic diseases in general.

EXPERIMENTAL PROCEDURES

Mice, Tissue Processing, and Cellular Analysis

Tsc1^{fl}, *Rosa26*^{loxP-STOP-loxP-LacZ} (*R26*^{LacZ}), *R26*^{loxP-STOP-loxP-tdTomato} (*R26*^{tdTomato}), and *Gbx2*^{CreER-IRES-eGFP} (*Gbx2*^{CreER}) mice were described

previously (Soriano, 1999; Kwiatkowski et al., 2002; Chen et al., 2009; Madisen et al., 2010). Mice were housed and handled in accordance with Brown University Institutional Animal Care and Use Committee guidelines. Genotyping, tamoxifen, immunohistochemistry (IHC), antibodies, and cytochrome oxidase (CO) staining are described in Brown et al. (2009) and Ellis et al. (2009) and Supplemental Experimental Procedures. Identical exposure settings were used when comparing labeling intensity across the three genotypes. For neuron density analysis, a barrel outline was created based on CO+ staining ("barrel hollow") and a perimeter was made 15 μm outside the inner outline ("barrel wall"). The area and the number of NeuN-positive objects in the barrel hollow and wall regions were determined and analyzed for significance by Student's *t* test. For cell size analysis, five thalamic regions from five medial-to-lateral brain sections were assessed. The measure function (VoloCity) was used to calculate the perimeter and area of all outlined cell bodies. Generalized estimating equations (log-normal generalized model) were used to compare genotypes with regards to neuronal size. Pairwise comparisons were made using orthogonal contrast statements, with *p* values adjusted using the Holm test to maintain family-wise alpha at 0.05. Statistical and experimental details are provided in the Supplemental Experimental Procedures.

Whole-Cell Recordings

Brain slice preparation, solutions, and recording conditions (Agmon and Connors, 1991; Cruikshank et al., 2010, 2012) are provided in detail in the Supplemental Experimental Procedures. Data were collected with Clampex 10.0 and analyses were performed post hoc using Clampfit 10.0. Resting membrane potentials (R_m), input resistances (R_{in}), membrane time constants (τ_m), and input capacitances (C_{in}) were determined as described in the Supplemental Experimental Procedures. Burst properties were characterized by holding the soma at a membrane potential of -60 mV with intracellular current and subsequently injecting large negative currents. Tonic and single action potential properties were characterized by holding the soma at a membrane potential of -50 mV with intracellular current and injecting suprathreshold positive current. Single action potential data were obtained by injecting the minimum current needed to elicit an action potential. Afterhyperpolarizations were evoked by injecting a 2 ms suprathreshold positive current. Generalized hierarchical linear modeling was used to test for differential effects of gene deletion. Comparisons by genotype were made using orthogonal linear comparisons.

LFP Recordings

Surgical procedures, recordings, and analysis are described in the Supplemental Experimental Procedures. NeuroNexus probes were used for recording sessions. LFP signals were sampled, filtered, and recorded using a Cheetah Data Acquisition System (Neuralynx). The probe was lowered 1,600 μm and responses to vibrissa deflections confirmed electrode placement in SI. Ten minutes of pre- and postbaseline activity and a stimulus period were recorded. Stimuli periods had a mean period of 5 s. For each animal, a single SI recording session was selected for LFP analysis using the layer IV contact. Recorded signals were low-pass filtered, downsampled, and clipping artifacts were removed. Data were analyzed using MATLAB. The power spectral density (PSD) for 20 s nonoverlapping time windows was estimated using Welch's method with a 4,096 point FFT, normalized by dividing by the sum of the PSD across all frequencies and smoothed using a 5 pt moving average filter. Relative power at 3 Hz was calculated as the ratio of the normalized PSD at 3 Hz by the value at 1 Hz for each time window, averaged across the session. The number of 20 s epochs that exceeded 97.5th percentile of normalized 3 Hz power was counted. Two-tailed two-sample *t* tests were performed by grouping all controls versus all mutants (significance level, α of 0.05).

Behavioral Analysis

An independent observer assessed videos to score seizures and overgrooming as detailed in the Supplemental Experimental Procedures. Generalized estimating equations were used to compare genotypes with regards to percent minutes grooming (binomial generalized model grooming/total minutes) and seizure frequency (negative-binomial generalized model offset by log total hours). Pairwise comparisons were made using orthogonal

contrast statements, with *p* values adjusted using the Holm test to maintain family-wise alpha at 0.05. Sensorimotor testing details are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, Supplemental Experimental Procedures, and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2013.03.030>.

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SUPPLEMENTAL FIGURES and LEGENDS

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Supplemental Information

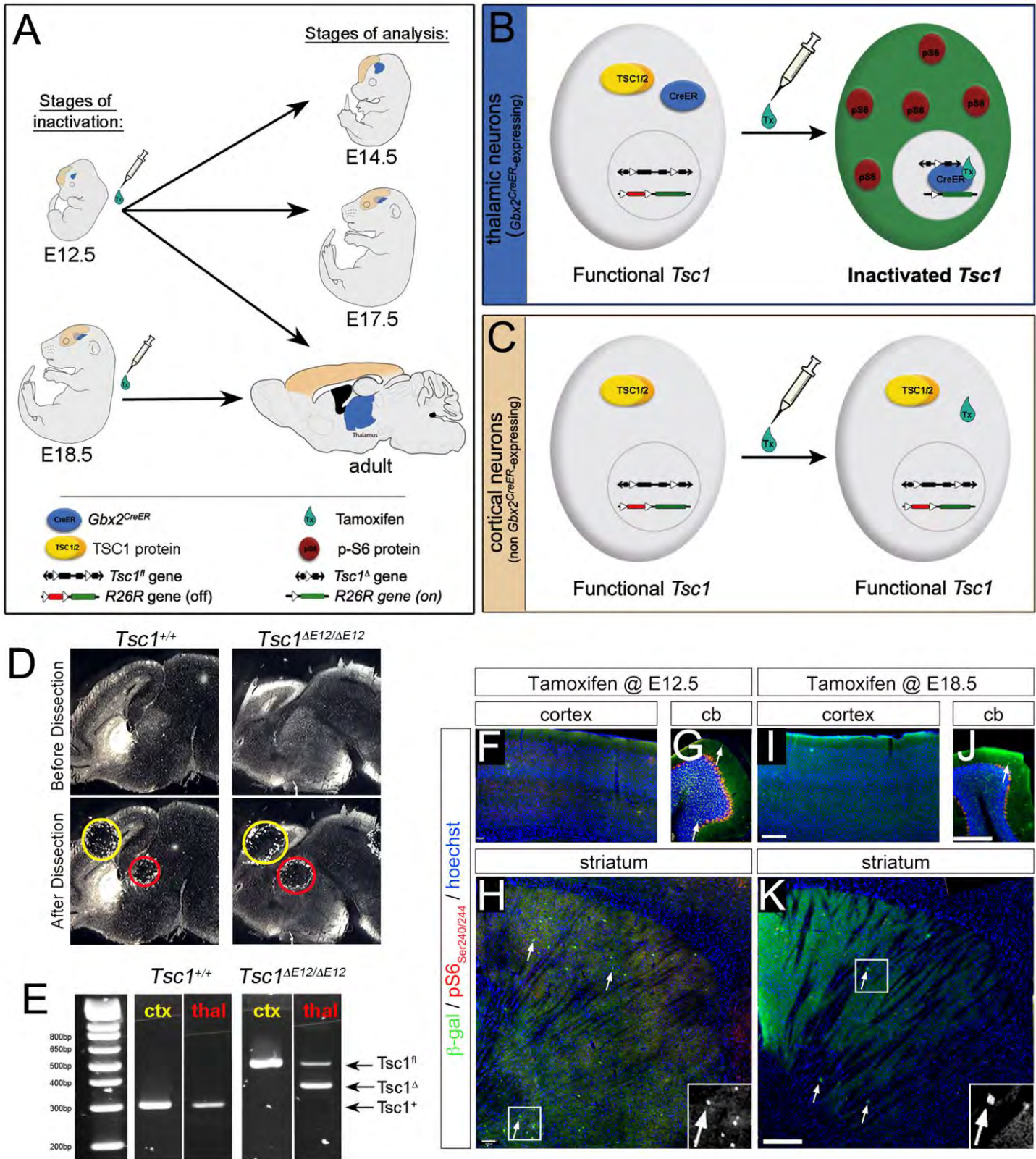
Temporal and Mosaic Tsc1 Deletion

in the Developing Thalamus Disrupts Thalamocortical

Circuitry, Neural Function, and Behavior

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SUPPLEMENTAL FIGURES and LEGENDS



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Figure S1, related to Figure 1. Spatial control over *Tsc1^{fl}* allele recombination. (A) Experimental approach. Tamoxifen is administered at E12.5 or E18.5 and mice are analyzed at E14.5, E17.5, or postnatally. Thalamus is shown in blue, cerebral cortex is in tan. (B) Within thalamic *Gbx2^{CreER}*-expressing cells, tamoxifen activates the CreER protein (blue), allowing it to translocate into the nucleus, where it has access to the genome and mediates recombination of *loxP* sites (triangles), thereby deleting the *Tsc1^{fl}* allele (black) and activating the reporter allele (green). (C) In cells, such as cortical neurons, that do not express *Gbx2^{CreER}*, the *Tsc1^{fl}* allele remains functional and the reporter allele remains quiescent, despite being exposed to tamoxifen. (D) Controls (*Tsc1^{+/+}*) and mutants (*Tsc1^{ΔE12/ΔE12}*) were harvested at E17.5. Sagittal brain sections (12 μm) were manually microdissected to collect thalamic tissue (red circle) as well as control tissue from the cerebral cortex (yellow circle). (E) Tissue was lysed and PCR was performed to detect three alleles of *Tsc1*: *Tsc1⁺* (295bp), *Tsc1^{fl}* (486bp), or *Tsc1^Δ* (368bp). Conversion of the *Tsc1^{fl}* allele into the *Tsc1^Δ* allele is seen only in the thalamic tissue where *Gbx2^{CreER}* is expressed, but not in the cortical tissue, where there is no CreER expression. *Tsc1⁺* is unaffected in both the cortical and thalamic tissue samples, as expected. (F-H) IHC was performed on *Gbx2^{CreER};R26^{LacZ};Tsc1^{+/+}* animals that received tamoxifen at E12.5 (F-H) or E18.5 (I-K). β-gal labeling (green) indicates sparse recombination within the cerebellum (arrows) and striatum and a lack of any recombination in the cortex. Purkinje cells of the cerebellum express high basal levels of p-S6.

SUPPLEMENTAL FIGURES and LEGENDS

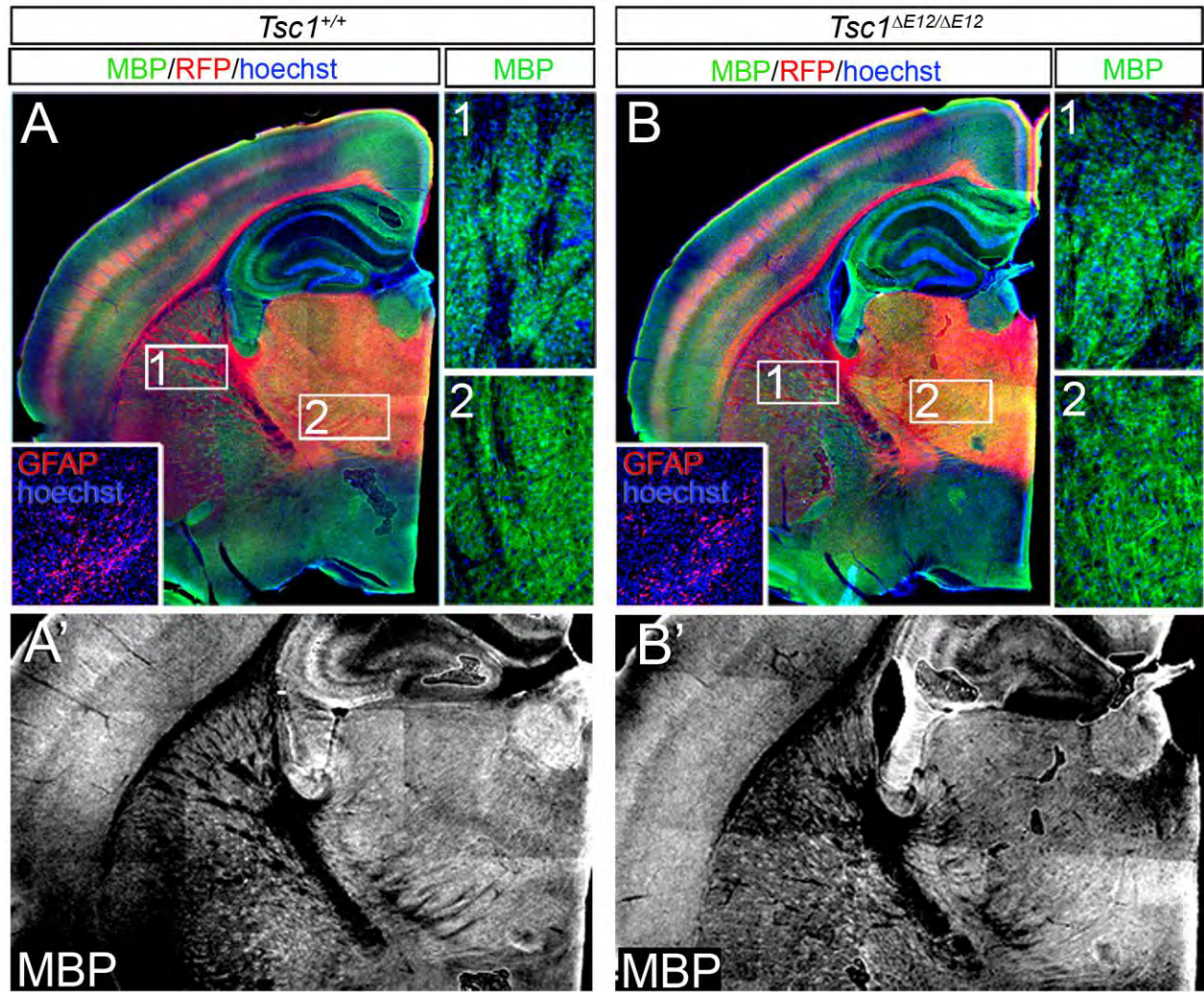


Figure S2, related to Figure 3. Myelination and astrocytes are unaffected in *Tsc1*^{ΔE12/ΔE12} mutants. *Tsc1*^{+/+} (A and A') and *Tsc1*^{ΔE12/ΔE12} (B and B') adult brain sections were stained for myelin basic protein (MBP, green) and RFP (red). MBP staining was present throughout the brain, as expected, and there were no apparent differences between control and mutant staining patterns. High magnification panels show details of MBP labeling (green) within the internal capsule (region 1) and thalamus (region 2). Insets: IHC for GFAP (red), an astrocyte marker, was also performed on thalamic sections to determine if gliosis occurred as a result of early *Tsc1* deletion. GFAP⁺ astrocytes were sparse in the thalamus, and no differences in staining were observed between control and *Tsc1*^{ΔE12/ΔE12} thalamus. MBP is isolated and shown in A' and B'.

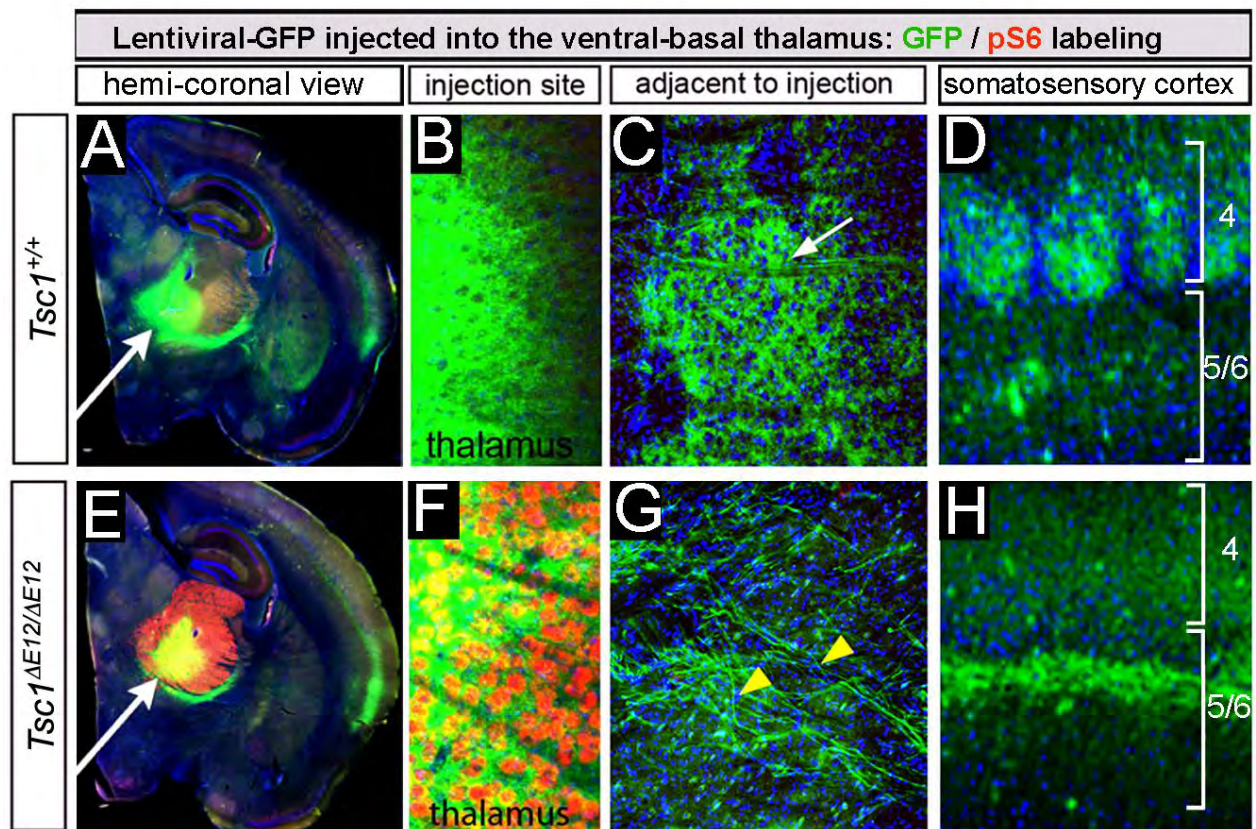


Figure S3, related to Figure 4. Altered distribution of thalamic projections in the internal capsule and cerebral cortex of *Tsc1*^{ΔE12/ΔE12} mutants. Lentiviral-GFP was stereotactically injected into the ventrobasal region of the thalamus. After waiting two weeks for expression, the brains were harvested, sectioned, and immunostained for GFP (green) and pS6 (red). GFP+ thalamic axons can be seen exiting the control (A and B) or mutant thalamus (E and F), traversing the striatum (C and G), and entering the cerebral cortex (D and H). Characteristic whisker barrels of the somatosensory cortex can be clearly delineated by the preferential thalamocortical innervation in control brains (D), whereas this barrel pattern is much less apparent in the *Tsc1*^{ΔE12/ΔE12} brain (H) and the GFP+ projections instead stratify in deeper cortical layers.

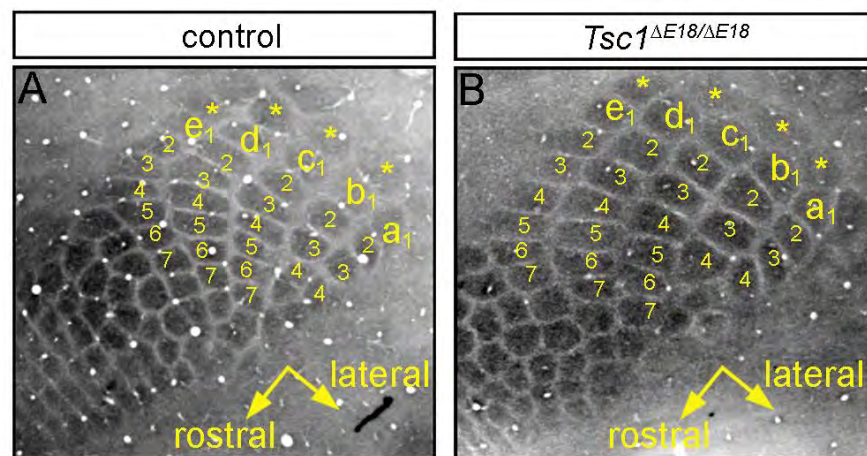


Figure S4, related to Figure 5. *Tsc1*^{ΔE18/ΔE18} animals have normal whisker barrel structure. Tangential sections through layer IV somatosensory cortex stained for cytochrome oxidase (black) showed well-organized barrel fields in both control (A) and *Tsc1*^{ΔE18/ΔE18} (B) mutant brains. Conventional vibrissae identifiers are indicated.

E18.5 whole-cell patch clamp results

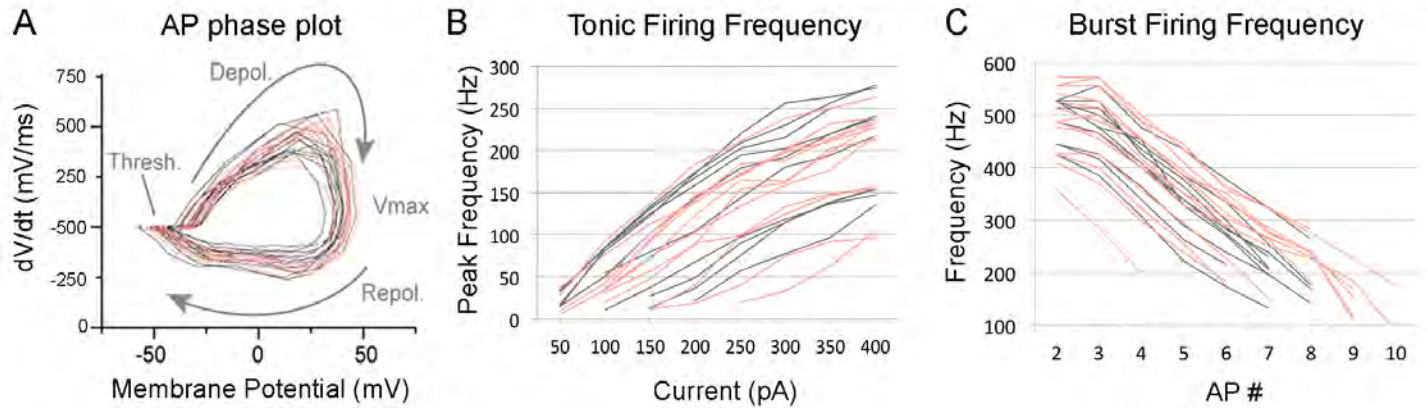


Figure S5, related to Figure 6. Whole-cell physiology of *Tsc1*^{ΔE18/ΔE18} mutant thalamic neurons. (A) AP dynamics are shown in a phase plot for *Tsc1*^{+/+} and *Tsc1*^{ΔE18/ΔE18}, similar to Figure 6C. There was no difference in any of the AP properties analyzed. (B) Peak firing frequency per current injection was plotted, similar to Figure 6F. *Tsc1*^{ΔE18/ΔE18} VB neurons (pink lines) have firing frequency similar to *Tsc1*^{+/+} neurons (gray lines). (C) Firing frequency per AP within a post-hyperpolarization rebound burst is plotted, similar to Figure 6I. There was no difference between the *Tsc1*^{ΔE18/ΔE18} neurons and the *Tsc1*^{+/+} neurons. See Figure 6 and Table S1 for means and statistics.

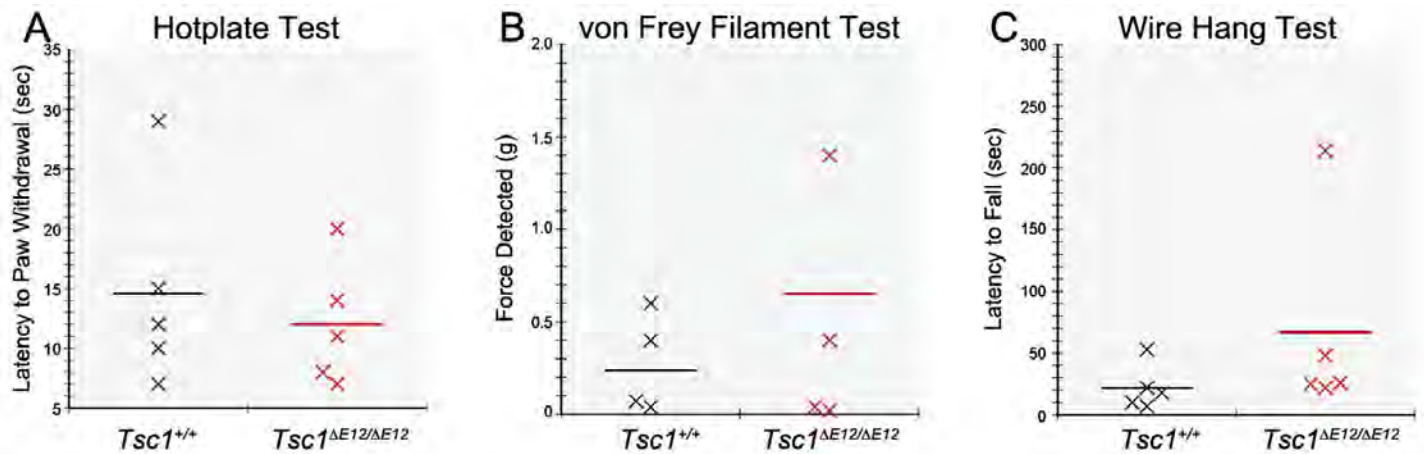


Figure S6, related to Figure 7. Sensory and motor function is unaffected in *Tsc1*^{ΔE12/ΔE12} mice. Sensory perception and motor function in *Tsc1*^{ΔE12/ΔE12} mice and their littermate controls were compared using the hotplate test (A), von Frey filaments (B), and a wire hang assay (C) to test thermal pain sensitivity, tactile sensitivity, and motor function, respectively. *Tsc1*^{ΔE12/ΔE12} (n=5) did not differ significantly from control animals (n=5) in any of the assays (two-tailed t-tests, p>0.05), indicating that their sensorimotor functions are not compromised. Data points indicate performance level for individual mice. Horizontal lines indicate population means.

Table S1. Mean Values, Sample Sizes, and Statistical Analysis of Cellular Properties

Property	Tamoxifen	Genotype	number of cells	mean	geometric mean	s.e.m. (lower)	s.e.m. (upper)	95% CI (lower)	95% CI (upper)	p value (if adjusted, with Holm)
Soma area (μm^2)	E12.5	Tsc1+/+ (pS6-)	1061		220.02	-1.96	1.97	211.73	228.62	0.181 (adj.)
	E12.5	Tsc1ΔE12/+ (pS6-)	1058		209.47	-1.86	1.88	201.58	217.69	
	E12.5	Tsc1ΔE12/ΔE12, (pS6-)	257		203.73	-3.66	3.73	188.45	220.26	
	E12.5	Tsc1ΔE12/ΔE12, (pS6+)	621		403.51	-4.67	4.73	383.77	424.24	
	E18.5	Tsc1+/+ (pS6-)	630		253.36	-16.53	17.67	204.42	314.00	n.d.
	E18.5	Tsc1+/+ (pS6+)	2		304.81	n.d.	n.d.	n.d.	n.d.	
	E18.5	Tsc1ΔE18/+ (pS6-)	1061		242.38	-17	18.28	192.30	305.45	0.142 (adj.)
	E18.5	Tsc1ΔE18/+ (pS6+)	8		277.30	-33.1	37.58	185.03	415.59	
	E18.5	Tsc1ΔE18/ΔE18, (pS6-)	542		246.73	-16.09	17.22	199.06	305.82	0.110 (adj.)
	E18.5	Tsc1ΔE18/ΔE18, (pS6+)	221		358.53	-33.87	37.39	358.52	491.67	
Resting Membrane Potential (mV)	E12.5	Tsc1+/+	12	-61.81		-0.43	0.43	-62.81	-60.82	0.006
	E12.5	Tsc1ΔE12/ΔE12	18	-58.06		-0.92	0.92	-60.17	-55.94	
	E18.5	Tsc1+/+	12	-61.86		-0.94	0.94	-64.03	-59.70	0.155
	E18.5	Tsc1ΔE18/ΔE18	13	-60.14		-0.57	0.57	-61.46	-58.81	
Input Resistance (mΩ)	E12.5	Tsc1+/+	12		137.20	-15.648	17.662	103.76	181.15	0.001
	E12.5	Tsc1ΔE12/ΔE12	18		72.59	-3.098	3.236	65.64	80.27	
	E18.5	Tsc1+/+	12		157.75	-15.14	16.748	125.01	199.06	0.318
	E18.5	Tsc1ΔE18/ΔE18	13		132.22	-16.292	18.583	97.64	179.06	
Input Capacitance (pF)	E12.5	Tsc1+/+	12		219.66	-28.066	32.178	160.28	301.09	0.004
	E12.5	Tsc1ΔE12/ΔE12	18		417.63	-35.789	39.143	339.71	513.47	
	E18.5	Tsc1+/+	12		237.75	-30.397	34.853	173.45	325.40	0.439
	E18.5	Tsc1ΔE18/ΔE18	13		267.47	-12.303	12.896	239.94	298.15	
Membrane Time Constant (msec)	E12.5	Tsc1+/+	12		30.14	-4.252	4.951	21.22	42.80	0.958
	E12.5	Tsc1ΔE12/ΔE12	18		30.43	-2.787	3.068	24.39	37.98	
	E18.5	Tsc1+/+	12		37.51	-1.322	1.37	34.53	40.74	0.647
	E18.5	Tsc1ΔE18/ΔE18	13		35.03	-4.54	5.217	25.43	48.26	
Max AP Rise (mV/msec)	E12.5	Tsc1+/+	12		423.41	-11.86	11.86	396.07	450.76	<0.001
	E12.5	Tsc1ΔE12/ΔE12	18		618.44	-26.36	26.36	557.64	679.25	
	E18.5	Tsc1+/+	12		451.44	-19.06	19.06	407.50	495.38	0.478
	E18.5	Tsc1ΔE18/ΔE18	12		472.56	-21.01	21.01	424.10	521.02	
Max AP Fall (mV/msec)	E12.5	Tsc1+/+	12		-151.54	-12.69	12.69	-180.81	-122.27	<0.001
	E12.5	Tsc1ΔE12/ΔE12	18		-262.75	-8.39	8.39	-282.10	-243.40	
	E18.5	Tsc1+/+	12		-175.72	-5.36	5.36	-188.09	-163.35	0.603
	E18.5	Tsc1ΔE18/ΔE18	12		-182.88	-12.07	12.07	-210.71	-155.05	
AP amplitude (mV)	E12.5	Tsc1+/+	12		70.39	-1.72	1.72	66.43	74.36	<0.001
	E12.5	Tsc1ΔE12/ΔE12	18		82.40	-0.74	0.74	80.71	84.10	
	E18.5	Tsc1+/+	12		71.69	-0.66	0.66	70.17	73.21	0.135
	E18.5	Tsc1ΔE18/ΔE18	12		74.10	-1.29	1.29	71.13	77.06	
AP Half-width (msec)	E12.5	Tsc1+/+	12	0.43		-0.0234	0.0256	0.39	0.49	0.002
	E12.5	Tsc1ΔE12/ΔE12	18	0.32		-0.008	0.008	0.31	0.34	
	E18.5	Tsc1+/+	12	0.41		-0.016	0.016	0.37	0.44	0.955
	E18.5	Tsc1ΔE18/ΔE18	12	0.40		-0.022	0.024	0.35	0.46	
Peak fAHP Potential (mV)	E12.5	Tsc1+/+	11	-0.10		0.98	0.98	-2.37	2.17	0.005
	E12.5	Tsc1ΔE12/ΔE12	17	-4.24		0.45	0.45	-5.28	-3.21	
	E18.5	Tsc1+/+	11	-0.11		0.96	0.96	-2.31	2.10	0.871
	E18.5	Tsc1ΔE18/ΔE18	12	0.12		0.92	0.92	-2.00	2.23	
ADP+AHP area (mV)	E12.5	Tsc1+/+	11	-63.72		14.73	14.73	-97.70	-29.75	0.003
	E12.5	Tsc1ΔE12/ΔE12	17	-177.15		21.89	21.89	-227.63	-126.68	
	E18.5	Tsc1+/+	11	-81.95		9.60	9.60	-104.08	-59.82	0.623
	E18.5	Tsc1ΔE18/ΔE18	12	-69.86		21.60	21.60	-119.66	-20.06	
Tonic F/I slope (Hz*pA)	E12.5	Tsc1+/+	12	0.53		0.01	0.01	0.50	0.56	<0.001
	E12.5	Tsc1ΔE12/ΔE12	16	0.27		0.03	0.03	0.21	0.33	
	E18.5	Tsc1+/+	10	0.59		0.04	0.04	0.50	0.69	0.278
	E18.5	Tsc1ΔE18/ΔE18	12	0.52		0.04	0.04	0.42	0.62	
Peak Tonic Firing Frequency @ 400pA (Hz)	E12.5	Tsc1+/+	12	188.30		2.15	2.15	183.34	193.25	0.001
	E12.5	Tsc1ΔE12/ΔE12	16	120.74		12.41	12.41	92.13	149.35	
	E18.5	Tsc1+/+	10	210.30		17.02	17.02	171.05	249.55	0.557
	E18.5	Tsc1ΔE18/ΔE18	12	195.35		17.50	17.50	154.99	235.71	
Mean Intraburst Spike Frequency (Hz)	E12.5	Tsc1+/+	12	339.27		21.43	21.43	289.85	388.70	0.026
	E12.5	Tsc1ΔE12/ΔE12	18	400.89		7.03	7.03	384.68	417.09	
	E18.5	Tsc1+/+	10	355.99		12.16	12.16	327.95	384.03	0.585
	E18.5	Tsc1ΔE18/ΔE18	13	346.78		10.70	10.70	322.09	371.46	
# of spikes per burst	E12.5	Tsc1+/+	12		7.38	-0.704	0.778	5.86	9.30	0.409
	E12.5	Tsc1ΔE12/ΔE12	18		8.28	-0.682	0.744	6.79	10.10	
	E18.5	Tsc1+/+	10		7.27	-0.079	0.08	7.09	7.46	0.738
	E18.5	Tsc1ΔE18/ΔE18	13		7.43	-0.454	0.484	6.43	8.60	
Peak Burst Firing Frequency (Hz)	E12.5	Tsc1+/+	12		496.21	-29.0023	29.0023	429.33	563.09	0.514
	E12.5	Tsc1ΔE12/ΔE12	18		519.79	-18.681	18.681	476.71	562.87	
	E18.5	Tsc1+/+	10		493.73	-15.6677	15.6677	457.60	529.86	0.719
	E18.5	Tsc1ΔE18/ΔE18	13		485.54	-15.3921	15.3921	450.04	521.03	

Supplemental Experimental Procedures

Mice

Tsc1^{fl} (Kwiatkowski et al., 2002) and *R26^{loxP-STOP-loxP-tdTomato}* (*R26^{tdTomato}*) (Madisen et al., 2010) mice were obtained from Jackson laboratories (stock # 005680 and #007905, respectively). *Gbx2^{CreER}* mice (*Gbx2^{CreER}*) (Chen et al., 2009) and *Rosa26^{loxP-STOP-loxP-LacZ}* reporter (*R26^{LacZ}*) mice (Soriano, 1999) were generously provided by J. Li (UConn Health Center) and P. Soriano (Mt. Sinai School of Medicine), respectively. *Gbx2^{CreER}* mice were bred with *Tsc1^{fl/fl}* mice and either *R26^{LacZ}* or *R26^{tdTomato}* mice to maintain a compound line. Genotyping was performed as previously described for the *CreER* and *R26^{LacZ}* alleles (Ellisor et al., 2009). Genotyping for the *R26^{tdTomato}* allele was performed as described on the jax.org website. Genotyping for the *Tsc1⁺*, *Tsc1^{fl}*, and *Tsc1^Δ* allele was performed as previously described (Figure S1) (Kwiatkowski et al., 2002). *Tsc1^{fl}* inactivation experiments were conducted by crossing *Gbx2^{CreER};R26^{LacZ};Tsc1^{fl/+}* or *Gbx2^{CreER};R26^{tdTomato};Tsc1^{fl/+}* males with *Tsc1^{fl/+}* females. The morning (0900) of the day a vaginal plug was detected was designated as embryonic day (E)0.5. 4mg of tamoxifen (20mg/mL in corn oil) was administered by oral gavage (Brown et al., 2009) to the pregnant females harboring embryos at embryonic stage (E)12.5 or E18.5 to simultaneously activate the *R26^{LacZ}* or *R26^{tdTomato}* allele and induce recombination of the *Tsc1^{fl}* allele into the *Tsc1^Δ* allele within the embryos (Figure S1). Mice were housed and handled in accordance with Brown University Institutional Animal Care and Use Committee guidelines.

Tissue Processing, Immunohistochemistry (IHC), and cytochrome oxidase staining

For embryonic analysis, timed-pregnant females harboring embryos at the desired pregnancy stage (E14.5 or E17.5; n≥3 each stage and genotype) were sacrificed at 0900, the uterine chain was dissected out, embryos were genotyped, immersion-fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose, frozen in Optimal Cutting Temperature (OCT, Fisher), and sectioned on a Leica cryostat as previously described (Ellisor et al., 2009). For postnatal tissue analysis, animals were deeply anesthetized with 195mg/kg Beuthanasia-D (Schering-Plough Animal Health Corp.) and intracardially perfused. Craniotomies were performed as previously described (Brown et al., 2009). Brains were sectioned at a sagittal angle (40 μm) or a thalamocortical angle (60 μm) (Agmon and Connors, 1991) using a Leica vibratome. Embryonic sections and adult free-floating sections were matched based on morphology and processed for IHC by standard methods (Ellisor et al., 2009) using primary antibodies raised against the following antigens: phosphorylated S6 ribosomal protein at Ser240/244 (pS6, rabbit, 1:800, Cell Signaling), phosphorylated S6 ribosomal protein at Ser235/236 (1:100, rabbit, Cell Signaling), β-galactosidase (1:500, goat, Biogenesis or 1:500, chicken, Abcam), GFP (1:500, rat, Nacalai Tesque or 1:600, rabbit, Invitrogen), RFP (1:1000, chicken, VWR), MAP-2 (1:500, mouse, Sigma), calbindin D-28K (1:1000, rabbit, Swant), parvalbumin (mouse, 1:1000, Sigma), myelin basic protein (MBP, rabbit, 1:500, Millipore), glial fibrillary acidic protein (GFAP, rabbit, 1:500, Millipore), Neuronal Nuclei (NeuN, mouse, 1:500, Millipore). The following secondary antibodies from Invitrogen were used at a 1:500 dilution in 1% normal donkey serum/PBS-Triton X-100: Alexa 488 donkey anti-rabbit IgG, Alexa 555 donkey anti-rabbit IgG, Alexa 488 donkey anti-rat IgG, Alexa 555 donkey anti-goat IgG, and Alexa 488 donkey anti-mouse IgG (Molecular Probes) and DyLight 549 donkey anti-chicken IgG (Jackson ImmunoResearch). All sections were counterstained using the Hoechst 33342 (Molecular Probes) and mounted on ColorFrost Plus Slides (Fisher) using Fluoromount-G mounting media (Southern Biotech).

For cytochrome oxidase (CO) staining of vibrissa barrels, mice were deeply anesthetized and intracardially perfused with 4% paraformaldehyde/1% glutaraldehyde in PBS. The brains were obtained and the two hemispheres were separated by a longitudinal cut along the midline. The cerebellum, brainstem, thalamus, hippocampus, olfactory bulb, temporal lobe and most of the subcortical white matter were removed. The two hemispheres were flattened between two glass slides in fix solution (4% PFA, 1% glutaraldehyde in PBS) for 1 hour at 4°C. The flattened hemispheres were cryoprotected in 30% sucrose solution at 4°C, frozen in OCT, and sectioned on a Leica cryostat at 50 μm (*Tsc1^{ΔE12/ΔE12}* samples) or 100 μm (*Tsc1^{ΔE18/ΔE18}* samples). The sections were incubated with 5 mg DAB (Sigma), 2mg Cytochrome C (Sigma), and 0.4g sucrose in 10mL PBS at 37°C in the dark for 1-3 hours, washed with PBS, and mounted on slides with Fluoromount-G. Adobe Illustrator was used for outlining the barrels stained with CO on different sections. The outlines from all of the

barrel-containing sections were co-registered based on morphological landmarks and/or blood vessel locations, and collapsed to form a representative map of the full barrel field.

For neuron density analysis, a barrel outline was created based on CO+ staining ("barrel hollow") and a perimeter was made 15 μm outside the inner outline ("barrel wall") using Adobe Illustrator's offset path function. The area and the number of NeuN-positive objects in the barrel hollow and wall regions were determined using the automated "measure area" and "find points" function in Volocity software (Improvision). Quantative barrel analysis was analyzed for significance by Student's t-test.

Microscopy and Cell Size Analysis

Sections were imaged on a Leica DM6000B epifluorescent microscope with Volocity 5.2 imaging software (Improvision). Red, green, and blue channels were imaged separately and pseudocolored as part of the acquisition palettes. Identical exposure settings were used across the three genotypes to allow for direct comparison of labeling intensity. Post-acquisition image processing was performed in Adobe Photoshop, with control and experimental data processed identically. For cell size analysis, free-floating adult sagittal sections from *Tsc1*^{+/+}, *Tsc1* ^{$\Delta E12$ +}, and *Tsc1* ^{$\Delta E12/\Delta E12$} animals (n=3 each genotype) were processed for IHC using primary antibodies to MAP-2 and p-S6_{Ser240/244}, as described above. Five thalamic regions (dorsal, ventral, anterior, posterior, and center) from five medial-to-lateral brain levels were imaged at 40x magnification. After the red channel was cloaked to blind the observer to p-S6 levels, the green MAP-2 signal was used to manually outline the edges of clearly labeled neuronal cell bodies using Volocity's Freehand Tool. The Measure function was used to calculate the perimeter and area of all outlined cell bodies, which were exported to Microsoft Excel for data analysis. After analysis, the red p-S6 channel was unmasked in order to sort cells into "p-S6 positive" and "p-S6 negative" cohorts, based on p-S6 immunolabeling intensity. Numbers of measured cells per cohort are indicated in Figures 3 and 5. Generalized Estimating Equations (log-normal generalized model) were used to compare genotypes with regards to neuronal size. Each mouse had multiple cells, which were treated as having correlated error. Cells were divided into those expressing pS6 and those not expressing pS6. Comparison between sizes of pS6-positive and pS6-negative cells within the knock-outs was a within-subjects comparison, while those between genotypes were between-subjects comparisons. Pair-wise comparisons were made using orthogonal contrast statements, with p-values adjusted using the Holm test to maintain family-wise alpha at 0.05.

Whole-cell Recordings

Slice Preparation: Brain sections were prepared from young mice (postnatal age: 20-23 days) of either sex as previously described (Agmon and Connors, 1991; Cruikshank et al., 2010; Cruikshank et al., 2012). Briefly, mice were deeply anesthetized with isoflurane, then decapitated. The brains were quickly removed and placed in cold (4°C) oxygenated (5% CO₂, 95% O₂) slicing solution containing (in mM): 3.0 KCl, 1.25 NaH₂PO₄, 10.0 MgSO₄, 0.5 CaCl₂, 26.0 NaHCO₃, 10.0 Glucose, and 234.0 sucrose. Brains were then mounted, using a cyanoacrylate adhesive, onto the stage of a vibrating tissue slicer and horizontal brain slices (275-300 μm) containing the VB nucleus were obtained. Slices were immediately transferred to a holding chamber containing oxygenated, physiological saline solution maintained at 32 \pm 1 °C. The oxygenated physiological solution (5% CO₂, 95% O₂) contained (in mM): 126.0 NaCl, 3.0 KCl, 1.25 NaH₂PO₄, 2.0 MgSO₄, 2.0 CaCl₂, 26.0 NaHCO₃, and 10.0 glucose. After 15-20 min, the temperature was reduced to room temperature and the slices were allowed to incubate for an additional 60 min.

Whole-Cell Recording Procedure: Individual brain slices were placed in a submersion-type recording chamber maintained at 32 \pm 1 °C and continuously superfused (2.5-3 ml/min) with oxygenated physiological saline. VB neurons were visualized using a Zeiss Axioskop fixed-stage microscope equipped with IR-DIC optics and a water-immersion objective (40X, 0.75 NA, Zeiss). All but 6 mutant neurons were identified visually by expression of *R26*^{tdTomato}. Electrophysiological data were acquired using an Axoclamp-2B amplifier, filtered at 10 kHz and digitized at 20 kHz using a Digidata 1322A digitizer in combination with pClamp10 software (Molecular Devices). For whole-cell recordings, patch pipettes had tip resistances of 3-6 M Ω when filled with a potassium-based internal solution containing (in mM): 130.0 K-gluconate, 4.0 KCl, 2.0 NaCl, 10 HEPES, 0.2

EGTA, 4.0 ATP-Mg, and 0.3 GTP-Tris, 14.0 phosphocreatine-K (pH 7.25, ~290 mOsm). During all recordings the pipette capacitance was neutralized and access resistance was continually monitored. Membrane potentials were not corrected for liquid junction potentials.

Data Analyses: Data were collected with protocols made with Clampex 10.0 and analyses were performed post-hoc using Clampfit 10.0. Resting membrane potentials (R_m) were measured within 2 min of break-in. Input resistances (R_{in}) were estimated as the slope of the voltage-current relationship obtained with current pulses (-50 to +50 pA, 25-50 pA increments, 800 ms duration). Membrane time constants (τ_m) were calculated from voltage responses to small negative current injections (3-5 pA, 500 ms duration). For τ_m , the voltage responses were fitted with a single exponential to the initial 150 ms of the responses. Input capacitances (C_{in}) were calculated as τ_m / R_{in} . Burst properties were characterized by holding the soma at a membrane potential of -60 mV with intracellular current and subsequently injecting large negative currents (400-1000 pA, 40-100 pA increments, 800 ms duration). When comparing low-threshold bursts, only trials in which the steady-state potential reached -70 ± 2 mV were used. Tonic and single action potential properties were characterized by holding the soma at a membrane potential of -50 mV with intracellular current and injecting suprathreshold positive current. Frequency-current relationships were obtained using large positive current injections (50-400 pA, 50 pA increments, 800 ms duration). Single action potential data were obtained by injecting the minimum current needed to elicit an action potential (10-200 pA, 10-15 pA increments, 800 ms duration). Action potential thresholds were calculated as the voltage difference between the steady-state potential and the point at which the rate of rise was greater than 15 mV/ms. Action potential amplitudes and half-widths were measured relative to threshold potential. After-hyperpolarizations (AHPs) were evoked by injecting a 2 ms suprathreshold positive current (600-1500 pA).

Generalized Hierarchical Linear Modeling was used to test for differential effects of *Tsc1* gene deletion at E12.5 and E18.5, while appropriately accounting for nested measurement of multiple cells within-mouse (up to 6 cells sampled in 12 mice for a total of 55). The choice of distribution on which the statistical model was based was chosen based on model diagnostic residual visualizations. Cell genotype and stage of tamoxifen were treated as fixed effects with cell genotype also treated as a random effect with a compound symmetry variance-covariance structure. Any model misspecification was adjusted for using classical sandwich estimation. Individual comparisons by cell genotype within tamoxifen time-points (i.e. *Tsc1* ^{$\Delta E12/\Delta E12$} mutants versus *Tsc1*^{+/+} littermates) were made using orthogonal linear comparisons. The interaction effect of the omnibus represented the comparison of these effects.

***In vivo* Extracellular Recordings**

Head-post Surgery and Craniotomy: Animals were anesthetized under 3.0% isoflurane (Isothesia, Butler Schein, Dublin OH) in O₂ within a plastic induction chamber and fitted into a stereotaxic apparatus (David Kopf Instruments, Tujunga CA). Throughout surgery, animals received 0.5-2.0% isoflurane in 1.0% O₂; levels were controlled with the use of an Isotec vaporizer (SurgiVet, Waukesha WI). Body temperature was maintained at 36-38°C with a heating pad (Cara, Inc., Warwick RI) during both surgery and recording sessions. Animals received 0.05 mL intraperitoneal injections of atropine sulfate (0.54 mg/mL, Med-Pharmex, Pomona CA) and buprenorphine hydrochloride (.03 mg/mL, Reckitt-Benckiser, Richmond VA), and a 0.025 mL intraperitoneal injection of dexamethasone (2 mg/mL, VEDCO, St. Joseph MO).

The dorsal surface of the head was shaved with a standard razor, and any residual fur was removed using a depilatory agent (Nair). Skull was exposed under aseptic conditions, and the center of the planned craniotomy was marked (AP: -1.2, L: 3.5). A custom-designed titanium head-post was affixed to the skull with C&B metabond (Parkell Inc., Edgewood NY) perpendicular to the sagittal plane. Posts can be clamped for quick and consistent head-fixing. Dental cement (Lang Dental, Wheeling IL) was used to form a surface within the head-post interior for a saline well. The tissue surrounding the head-post was reattached to the head-post exterior edge using superglue (Loctite instant adhesive 454, Rocky Hill CT). An air-powered drill (Midwest Tradition Highspeed Handpiece, Dentsply Professional, Des Plaines IL) outfitted with a 0.5 mm regular carbide bur (Shank Type FGSS, Dentsply Professional) was used to clean away cement at the craniotomy site and thin the

skull. The bone was removed, and the exposed brain was covered with saline. All recording equipment was secured onto a vibration isolation table (Technical Manufacturing Corporation, Peabody MA) to minimize noise and artifact. Animals were head-fixed, and anesthesia was maintained through infusion of 0.5-2.0% isoflurane through a nose cone; isoflurane levels were gradually lowered until the animal was just above the threshold at which there existed a paw pinch response.

NeuroNexus probes were used for all recording sessions. In some cases, bad contacts were present on probes, and these data were discarded. Local field potential (LFP) signals were sampled (30303 Hz), filtered (0.9 to 9000 Hz), and recorded using a Cheetah Data Acquisition System (Neuralynx, Bozeman, MT). A four-axis micromanipulator (Siskiyou, Grants Pass OR) was used to clamp the probe, which was manually lowered to the brain surface. Prior to thalamic recordings, the probe angle was adjusted to approximately 25°. The probe was grounded on the head-post mount, and a reference wire was placed within the saline well. The probe was lowered at a controlled rate to depths of 1600 μm or 2500 μm for cortical and thalamic recordings, respectively. An air puffer, gated by a solenoid, was positioned above contralateral vibrissae and used to test for response to vibrissa deflection: application of such deflections was used to confirm electrode placement in SI and to ensure consistent quality of recording. After validation of probe location, ten minutes of baseline activity was recorded. A stimulus period consisting of 500 air puff trials followed; inter-trial periods were of randomly selected lengths between 2 and 8 seconds long, with a mean period of 5 seconds. At the end of the stimulus period, a 10 minute post-stimulus baseline period was recorded. Following recordings, the saline well was filled with a silicone elastomer (KwikCast, World Precision Instruments, Sarasota FL) to cap and protect the craniotomy between recording sessions. At the start of subsequent recording sessions, KwikCast was removed and the craniotomy area was inspected for bleeding, inflammation, and bone growth. Recordings then proceeded as previously described.

Recording Analysis: For each animal, a single SI recording session was selected for LFP analysis. The session chosen was that which exhibited the least clipping artifact and the highest amplitude responses following vibrissa deflection across the 16 probe channels. Within each chosen session, the contact exhibiting the largest amplitude and shortest duration responses following vibrissa deflection was identified as a putative layer IV contact and selected for analysis. The recorded signal from this contact was then low-pass filtered (0-150 Hz), downsampled (to 505.05 Hz), and clipping artifacts were removed.

Data from the entire recording session, including baseline (no stimulation) and vibrissa-stimulation periods, were analyzed using Matlab (MathWorks, Natick, MA). Each record was divided into 20-second non-overlapping time windows, and any trailing samples not included in these windows were discarded. The power spectral density (PSD) for each window was estimated using Welch's method (Matlab's pwelch command) with a 4096-point FFT, normalized by dividing by the sum of the PSD across all frequencies, and smoothed using a 5-pt moving average filter. For each animal, the average normalized PSD across the entire recording session was computed by taking the mean of the normalized PSDs across all the 20-second windows. Relative power at 3 Hz was calculated for each 20-second window by dividing the value of the normalized PSD at 3 Hz by the value at 1 Hz, and the average for each animal was taken to be the mean of this ratio across windows. For each animal, the number of 20-second windows in which normalized power at 3 Hz exceeded a threshold was then counted. This threshold was determined as the 97.5th percentile of normalized 3 Hz power across all animals. To test for significant differences between control and mutant subjects, two-tailed two-sample t-tests were performed by grouping all $Tsc1^{+/+}$ control animals (tamoxifen delivered at E12.5 and E18.5) and all mutant animals ($Tsc1^{\Delta E12/\Delta E12}$ and $Tsc1^{\Delta E18/\Delta E18}$), with a significance level, α , of 0.05.

Behavioral Analysis

Seizures and over-grooming: $Tsc1^{\Delta E12/\Delta E12}$ mice (n=11) and their littermate controls ($Tsc1^{+/+}$ or $Tsc1^{fl/fl}$, n=19; $Tsc1^{+/+/\Delta E12}$ n=17) were videotaped in their home cage using a digital camera for 8-minute epochs 2-3 times per week between 2 months of age and 8 months of age. Two $Tsc1^{\Delta E12/\Delta E12}$ mice died prematurely of unknown causes and were not included in the behavioral analysis. $Tsc1^{\Delta E18/\Delta E18}$ mice (n=17) and their littermate controls ($Tsc1^{+/+}$ or $Tsc1^{fl/fl}$, n=25; $Tsc1^{+/+/\Delta E18}$, n=6) were observed once per week for 8 minutes, beginning at 2 months of age and continuing through 8 months of age. Videos were analyzed by an observer who was blinded to animal genotypes. Number and duration of all seizures and self-grooming behaviors were manually tallied

(grooming events lasting less than 1 second were rounded up to 1 second duration). An independent observer tallied a subset of videos, and the two observers' tallies had a high level of concordance, confirming the reproducibility of the manual data analysis approach. Animals were euthanized for humane reasons by intracardiac perfusion if they developed severe grooming lesions. Data was analyzed in Excel (Microsoft) and plotted using KaleidaGraph (Synergy). Generalized Estimating Equations were used to compare genotypes with regards to percent minutes grooming (binomial generalized model grooming/total minutes) and seizure event rates (negative-binomial generalized model offset by log total hours). Comparisons represented between-subjects comparisons, with multiple observations within an animal modeled as having correlated error. Pair-wise comparisons were made using orthogonal contrast statements, with p-values adjusted using the Holm test to maintain family-wise alpha at 0.05.

Von Frey Filament test: Withdrawal thresholds from mechanical stimuli of von Frey filaments of ascending bending force (from 0.008 g to 300 g of force) were applied five times to the plantar surface of the bilateral hind paws. A positive response was defined as withdrawal from the von Frey filament on at least 3 of the 5 contacts. Confirmation of threshold was then tested by examining the filament above and below the withdrawal response. Significance was assessed by a one-tailed two-sample t-test, $\alpha=0.05$.

Hot plate test: The test was based on that described by Eddy and Leimbach (1953). A glass cylinder (16 cm high, 16 cm diameter) was used to keep the mice on the heated surface of the plate, which was kept at a temperature of 53°C \pm 0.2°C (Ugo Basile model 7280). The first of two nociceptive thresholds were evaluated: licking of the hind paw or sustained (> 1 s) lifting of the hind paw from the surface. The cut-off for a response was 40 s at which point the trial was terminated. Significance was assessed by a one-tailed two-sample t-test, $\alpha=0.05$.

Wire Hang test: Mice were placed on a wire cage lid, which was then inverted gently 180° so that the mouse gripped the wire at a distance of ~16 cm above the floor of an empty cage. Latency to fall was recorded, with a cut-off time of 300 s. Animals were provided with 4 opportunities to perform on this task and the longest duration to fall was collected. Significance was assessed by a one-tailed two-sample t-test, $\alpha=0.05$.

Mating info:

Dams	Birthday	Genotype	Sire	Sire Genotype	Setup	Plug	Sac Date
JS227	3/9/2013	Tsc1 fl/+	JS164	Gbx;TdT;Tsc1 fl/+	4/23/13	4/26/13	5/14/13
JS228	3/9/2013	Tsc1 fl/+	JS45	Gbx;TdT;Tsc1 fl/+	4/30/13	5/01/13	5/19/13
JS229	3/9/2013	Tsc1 fl/+	JS164	Gbx;TdT;Tsc1 fl/+	4/23/13	4/26/13	5/14/13
JS230	3/9/2013	Tsc1 fl/+	JS41	Gbx;TdT;Tsc1 fl/+	4/23/13	4/27/13	5/15/13
JS231	3/9/2013	Tsc1 fl/+	JS45	Gbx;TdT;Tsc1 fl/+	4/16/13	04/19/13	5/7/13
JS232	3/9/2013	Tsc1 fl/+	JS45	Gbx;TdT;Tsc1 fl/+	4/16/13	04/19/13	5/7/13
JS233	3/9/2013	Tsc1 fl/+	JS164	Gbx;TdT;Tsc1 fl/+	4/16/13	04/18/13	5/6/13
JS213	3/2/2013	Tsc1 fl/+	JS164	Gbx;TdT;Tsc1 fl/+	4/30/13	5/02/13	5/20/13
LN2220	10/2/2012	Tsc1 fl/+	JS164	Gbx;TdT;Tsc1 fl/+	4/30/13	5/04/13	Died (05/22/13)

Treatment Info:

Summary:

Mouse #	Age @ Sac	Dose	Drug	Sire	# of Embryos	# Reabsorbed	
JS233	~2 Months	9mg/kg	Rapa - OG	JS164	0	0	*Embryos and Reabsorbed were estimated based on appearance; mother was found dead
JS231	~2 Months	9mg/kg	Vehicle - IP	JS45	6	0	
JS232	~2 Months	9mg/kg	Rapa - IP	JS45	0	0	
JS227	~2 Months	1mg/kg	Rapa - IP	JS164	3	5	
JS229	~2 Months	1mg/kg	Vehicle - IP	JS164	9	0	
JS230	~2 Months	1mg/kg	Rapa - OG	JS41	8	0	
JS228	~2 Months	5mg/kg	Rapa - IP	JS45	0	6	
JS213	~2.5 Months	5mg/kg	Rapa - OG	JS164	9	1	
LN2220	~7.5 Months	1mg/kg	Rapa - IP	JS164	6*	7*	

Bodyweights of mice each day

BW (g)	PreTx	Tx1	Tx2	Tx3	Tx4	Tx5	Tx6	Tx7	
JS233	30.43	32.48	31.66	31.65	27.08	25.95	25.74	26	9mg Rapa OG
JS231	35.05	35.77	37.51	38.28	38.7	40.28	44.1	45.9	9mg Veh IP
JS232	33.02	33.82	34.68	34.92	29.7	28.13	27.63	27.74	9mg Rapa IP
JS227	30.59	30.85	28.85	29.7	31.07	32.57	33.47	34.77	1mg Rapa IP
JS229	36.34	38.31	39.59	40.45	43.5	46.85	49.53	53.81	1mg Veh IP
JS230	32.1	33.37	34.24	34.89	36.08	38.14	41.79	45.12	1mg Rapa OG
JS228	34.59	35.8	35.83	35.62	35.51	33.81	29.91	28.74	5mg Rapa IP
JS213	34.69	35.98	36.5	37.91	38.69	40.49	43	47.43	5mg Rapa OG
LN2220	49.35	50.25	51.7	55.23	57.2	57.87	57.16	n/a	1mg Rapa IP
	e12.0	e12.5	e13.5	e14.5	e15.5	e16.5	e17.5	e18.5	

Change in BW compared to e12.0 (pretreatment):

Change (g)	PreTx	Tx1	Tx2	Tx3	Tx4	Tx5	Tx6	Tx7
9mg Rapa OG	0	2.05	1.23	1.22	-3.35	-4.48	-4.69	-4.43
9mg Veh IP	0	0.72	2.46	3.23	3.65	5.23	9.05	10.85
9mg Rapa IP	0	0.8	1.66	1.9	-3.32	-4.89	-5.39	-5.28
1mg Rapa IP	0	0.26	-1.74	-0.89	0.48	1.98	2.88	4.18
1mg Veh IP	0	1.97	3.25	4.11	7.16	10.51	13.19	17.47
1mg Rapa OG	0	1.27	2.14	2.79	3.98	6.04	9.69	13.02
5mg Rapa IP	0	1.21	1.24	1.03	0.92	-0.78	-4.68	-5.85
5mg Rapa OG	0	1.29	1.81	3.22	4	5.8	8.31	12.74
1mg Rapa IP	0	0.9	2.35	5.88	7.85	8.52	7.81	
	e12.0	e12.5	e13.5	e14.5	e15.5	e16.5	e17.5	e18.5