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# WORTMANNIN ATTENUATES SEIZURE-INDUCED HYPERACTIVE PI3K-Akt-mTOR SIGNALING, IMPAIRED MEMORY, and SPINE DYSMORPHOLOGY iN RATS

Wortmannin attenuates seizure-induced aberrations

**Angela N. Carter[1,4], Heather A. Born[3,4], Amber T. Levine[1,4], An T. Dao[3,4], Amanda J. Zhao[3,4], Wai Ling Lee[3,4] and Anne E. Anderson[1,2,3,4,5]**

[1]*The Departments of Neuroscience, Baylor College of Medicine, Houston, Texas*
[2]*Neurology, Baylor College of Medicine, Houston, Texas*
[3]*Pediatrics at Baylor College of Medicine, Houston, Texas*
[4]*The Gordon and Mary Cain Foundation Laboratories, Texas Children's Hospital, Houston, Texas USA*
[5]*Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, Texas USA*

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**Correspondence should be addressed to** Dr. Anne Anderson, 1250 Moursund Street, Jan and Dan Duncan Neurological Research Institute Suite 1225.14, Texas Children's Hospital and Baylor College of Medicine, Houston, Texas 77030. Telephone: 832-824-3976, Email: annea@bcm.edu

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**Running title:** Wortmannin attenuates seizure-induced aberrations

**Authors:** Angela N. Carter[1,4], Heather A. Born[3,4], Amber T. Levine[1,4], An T. Dao[3,4], Amanda J. Zhao[3,4], Wai Ling Lee [3,4], and Anne E. Anderson[1-5]

The Departments of [1]Neuroscience, [2]Neurology, and [3]Pediatrics at Baylor College of Medicine, Houston Texas. [4]The Gordon and Mary Cain Foundation Laboratories and [5]Jan and Dan Duncan Neurological Research Institute at Texas Children's Hospital, Houston, Texas, USA

**Author Contributions:** ANC designed and conducted experiments, made figures, analyzed data, and wrote paper. HAB, ATL, and ATD performed electrode implantations for video encephalography. AJZ helped analyze spine morphology. WLL helped conduct experiments and data analysis, and edited paper drafts. AEA oversaw the design and analyses of all experiments, generation of figures, and the manuscript preparation as well as provided the final approval of the version to be published.

Correspondence should be addressed to Dr. Anne Anderson, 1250 Moursund Street, Jan and Dan Duncan Neurological Research Institute Suite 1225.14, Texas Children's Hospital and Baylor College of Medicine, Houston, Texas 77030. **Telephone**: 832-824-3976, **Email**: annea@bcm.edu

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**Keywords:** learning and memory, seizures, phosphosinositide-3 kinase (PI3K), mechanistic target of rapamycin (mTOR), mTOR inhibitor, wortmannin, spine morphology

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**ABSTRACT**

Numerous studies have shown epilepsy-associated cognitive deficits, but less is known about the effects of one single generalized seizure. Recent studies demonstrate that a single, self-limited seizure can result in memory deficits and induces hyperactive phosphoinositide 3-kinase/Akt (protein kinase B)/mechanistic target of rapamycin (PI3K/Akt/mTOR) signaling. However, the effect of a single seizure on subcellular structures such as dendritic spines and the role of aberrant PI3K/Akt/mTOR signaling in these seizure-induced changes are unclear. Using the pentylenetetrazole (PTZ) model, we induced a single generalized seizure in rats and: 1) further characterized short and long-term hippocampal and amygdala-dependent memory deficits, 2) evaluated whether there are changes in dendritic spines, and 3) determined whether inhibiting hyperactive PI3K/Akt/mTOR signaling rescued these alterations. Using the PI3K inhibitor wortmannin, we partially rescued short and long-term memory deficits, and altered spine morphology. These studies provide evidence that pathological PI3K/Akt/mTOR signaling plays a role in seizure-induced memory deficits as well as aberrant spine morphology.

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**Significance Statement**

Epilepsy-associated memory deficits were originally thought to only arise in chronic epilepsy. However, the current studies demonstrate that a single generalized seizure can result not only short, but also long-term memory deficits. Furthermore, the mechanisms of how a single generalized seizure impairs memory are not well known. We find that the seizure-induced memory impairments are transient and are linked, in part, to dysregulated signaling of a memory related cascade (PI3K/Akt/mTOR) and possibly also disruptions in spine morphology, both of which are crucial for memory formation. Our studies are clinically relevant as we demonstrate that a single generalized seizure can profoundly impair memory, particularly long-term memory, despite the transient nature of the molecular and structural perturbations.

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## INTRODUCTION

Seizures are characterized by transient hypersynchronous neuronal activity that is often associated with altered neuronal function (Banerjee et al., 2009; Berg et al., 2010; Fisher et al., 2014; Thom, 2014). The presence of 2 or more unprovoked seizures defines epilepsy, one of the most common neurological disorders (Berg et al., 2010; Fisher et al., 2014). Along with seizures, epilepsy is also characterized by a number of comorbidities with cognitive disorders being the most prevalent (Helmstaedter et al., 2003; Nolan et al., 2004; van Rijckevorsel, 2006; Lodhi and Agrawal, 2012; Kanner, 2016). While many studies have explored how epilepsy-associated comorbidities arise, it is not clear how one self-limited generalized seizure affects cognition, in particular learning and memory. Recent studies have shown that a single generalized seizure can result in deficits in short and long-term hippocampal dependent memory, as well as impaired long-term amygdala-dependent memory (Mao et al., 2009; Holley and Lugo, 2016).

In addition to memory deficits, a single generalized seizure has been shown to induce hyperactivation of the phosphoinositide 3-kinase/Akt (protein kinase B)/mechanistic target of rapamycin (PI3K/Akt/mTOR) signaling cascade (Zhang and Wong, 2012). PI3K and mTOR are serine/threonine kinases that, under physiological conditions, are necessary for learning and memory (Hoeffer and Klann, 2010). Signaling of the PI3K/Akt/mTOR pathway results in protein synthesis and cell growth (Kim et al., 2002; Sarbassov et al., 2005). In particular, PI3K/Akt/mTOR signaling has been shown to promote dendritic spine morphogenesis and remodeling (Richter and Klann, 2009; Hoeffer and Klann, 2010; Huang et al., 2013). To facilitate memory formation, dendritic spines modulate in morphology such that the spine shape converts from a long, thin immature protrusion to that of a wider and shorter mature morphology, a process that requires PI3K/Akt/mTOR signaling (Kumar et al., 2005). As such, this pathway plays a critical role in learning and memory (Kumar et al., 2005; Ehninger et al., 2008; Trinh and Klann, 2013). The fact that PI3K/Akt/mTOR signaling is dysregulated following a single generalized seizure suggests that

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pathological activation of this cascade may underlie seizure-induced memory deficits. In addition, whether a single generalized seizure results in alterations in dendritic spines, and the role of PI3K/Akt/mTOR dysregulation in this process remains unclear.

In the present studies, we corroborate and extend previous findings by demonstrating that a single pentylenetetrazole (PTZ)-induced generalized seizure is associated with both short and long-term hippocampal as well as amygdala-dependent memory deficits. Using the PI3K/Akt/mTOR inhibitor wortmannin (Wort) (Brunn et al., 1996), we expand previous studies by examining the role of seizure-induced pathological PI3K/Akt/mTOR signaling on memory deficits and dendritic spine morphology. We show, for the first time, that a single generalized seizure is associated with hippocampal dendritic spine loss and immaturity. In our pharmacological studies using Wort, we demonstrate partial rescue of the behavioral and dendritic spine changes that result from a single generalized seizure.

## MATERIALS AND METHODS

### Animals

All procedures complied with and were approved by the Institutional Animal Care and Use Committee of [Author Institution] and conformed to National Institutes of Health guidelines for the Care and Use of Laboratory animals. Sprague-Dawley rats of either sex (Envigo, USA) were used for biochemistry and behavioral studies. All animals were housed at the [Author Institution] housing facilities (USA). Animals were provided food *ad libiditum* and kept on a 14 hour light, 10 hours dark cycle at 22°C in the Center for Comparative Medicine housing facilities (USA). Prior to any experimental manipulation, all animals were handled for approximately 2 minutes in both the induction and behavioral suites.

### Generalized Seizure Induction

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A single generalized seizure was induced on postnatal (P) day 39-42 animals through intraperitoneal (i.p.) administration of the chemoconvulsant pentylenetetrazole (PTZ; 75mg/kg). Control animals received an equal volume of the vehicle saline (Sham; Sigma-Aldrich, St. Louis, MO, USA). The age and dose were chosen based upon work from previous studies as well as to improve survivability following induction (Brewster et al., 2013; Zhang and Wong, 2012) and dose response studies (data not shown). We found that PTZ at 75mg/kg was sufficient to result in 75% of animals exhibiting a generalized seizure with a 71% survival rate. In our hands, lower doses of PTZ resulted in less than 50% of animals having generalized seizures. Higher dose of PTZ were not studies due to survivability. For the molecular biology and behavioral studies, we observed the animals and recorded the seizure stages using a modified Racine scale (Racine, 1972; Luttjohann et al., 2009). The modified Racine scale ranged from 1 to 6 and was as follows: 1) rigid posture or immobility; 2) tail clonus; 3) partial clonus with fore- or hind-limb clonus and head bobbing; 4) rearing with whole body clonus; 5) rearing and falling; and 6) tonic-clonic with loss of posture or jumping. A seizure was considered generalized when a rat exhibited stage 4 behavior corresponding to generalized seizure activity on video-electroencephalography (see below). Any animal that did not exhibit a single, self-limited generalized convulsive seizure was not included in the behavioral testing or biochemistry studies.

**Surgery, electrode implantation, and video-electroencephalography (vEEG)**

At P32-35, cortical and hippocampal depth electrodes were implanted using methods described in (Sunnen et al., 2011; Brewster et al., 2013). Rats were anesthesized with isoflurane and positioned in a stereotaxic frame. Following a 1-2 cm midline sagittal incision, 3 subdural electrodes and 1 hippocampal-depth electrode were implanted (Plastics One, Roanake, VA, USA). The coordinates (determined relative to Bregma) were as follows: 2 subdural electrodes over somatosensory cortex (1 mm posterior, 3 mm lateral) and the third electrode 4 mm posterior, -3 mm lateral to Bregma; the hippocampal-depth electrode was positioned in area CA1 (4 mm

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127 posterior, 2.8 mm lateral) at the depth of 2.8 mm; and the ground electrode was sutured in the
128 cervical paraspinous region. The electrodes were held in place with Metabond (Parkell Inc.,
129 Edwood NY, USA) and dental cement (Co-Oral-Ite Dental Mfg. Co., Diamond Springs, CA, USA).
130 Following a 1 week recovery, animals were habituated in a recording suite and using the
131 NicoletOne acquisition system (Natus, San Carlos, CA, USA) baseline EEG activity was recorded
132 for 1 hour. Afterwards, animals received PTZ or saline as described above. The recordings were
133 maintained continuously for 24 hours thereafter (a total of 25 hours). These animals were not used
134 for any additional experimental or behavioral paradigms due to the electrode implantation.

135 **Pharmacological inhibition of** PI3K/Akt/mTOR **signaling**

136 Wortmannin (Wort; Selleck Chemicals, USA) was first dissolved in dimethyl sulphoxide (DMSO;
137 Sigma Aldrich, St. Louis, USA) and then added to a vehicle solution of 1% Tween 80, 30% PEG
138 400. Ten minutes post seizure or saline injection animals received either vehicle (Veh) or Wort
139 2.4mg/kg i.p. We chose the Wort dose based upon pilot dose response studies (data not shown)
140 and previously reported data (Zhang and Wong, 2012). To verify inhibition of PI3K/Akt/mTOR
141 signaling, tissue was collected and processed for western blotting.

142 **Western blotting**

143 Separate animal cohorts were induced and whole hippocampi were collected at different time
144 points following a single generalized seizure. Briefly, animals were deeply anesthetized using
145 Isoflurane (Piramal HealthCare, Bethlehem, PA, USA) and rapidly decapitated. Hippocampi were
146 rinsed in 1x PBS solution, frozen, and stored at -80°C. Tissue preparation and western blotting on
147 whole hippocampal tissue was performed using the methods described in (Brewster et al., 2013;
148 Nguyen et al., 2015). Optical densities of immunoreactive bands were obtained using
149 ImageStudioLite (LI-COR Biosciences, Lincoln, NE, USA) and normalized to GAPDH (EMD
150 Millipore, Billerica, MA, USA) as a loading control. Subsequently, the GAPDH-normalized bands of
151 phosphorylated protein were normalized to GAPDH-normalized bands of total protein. Antibodies

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against phospho (P)-Akt at T308, P-Akt at S473, and Akt were used as markers of PI3K activation, while antibodies against P-S6 S240/244 and S6 were used as readouts of mTOR activation (Cell Signaling Technologies, Danver, MA, USA).

**Fear Conditioning Protocol**

For all behavioral tests, the experimenters were blind to the treatment groups. One hour post generalized seizure or sham injection, animals were subjected to fear conditioning (FC). FC was performed in sound attenuated chambers with a metal grid pan for flooring (Med Associates, St. Albans, VT, USA). Briefly, animals were placed in a novel arena and allowed to explore for 2 minutes. Afterwards, they were subjected to two pairings of an unconditioned stimulus (US) of a 0.75 mA shock and a conditioned stimulus (CS; 72 dB white noise) with a 2 minute delay between each pairing. Freezing behavior in a single cohort for the training paradigm was evaluated prior to further testing. Animals were then tested for short-term memory (3 hours post seizure) or long-term memory (24 hours post seizure). To test for contextual memory deficits, animals were tested (without the US) in the same arena in which they were trained and the time spent freezing out of a total of five minutes was scored. To test for cued memory, the animals were placed in a novel arena in a sound attenuated chamber different from the one in which they were originally trained, one hour after contextual memory testing. After a two minute exploration period (Pre CS), the white noise was presented for the remaining three minutes (CS). Time spent freezing was collected for the Pre CS and CS conditions. Freezing, defined as the absence of any movement, was scored manually by the same experimenters for each cohort. Retention for contextual and cued memory was quantified using percent freezing. To determine if the seizure-induced memory deficits were long lasting, we performed FC training at 16 hours post seizure and tested animals 24 hours following training (i.e. 40 hours post seizure) to screen for any long-term contextual and cued memory deficits.

**Golgi-Staining and spine analyses**

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Golgi-Cox staining was performed according to manufacturer instructions (FD Rapid GolgiStain Kit, FD Neurotechnologies, Columbia, MD, USA). A separate cohort of animals were induced as before and 3 hours post generalized seizure brains were extracted, washed with 1X phosphate-buffered saline (PBS), and processed as per manufacturer's instructions. Using a vibratome, hippocampal tissue sections of 150 micron (μm) thickness were collected (Leica VT 1000S, Buffalo Grove, IL, USA). The sections were then mounted on microscope slides, and dried for up to 1 week. Finally, the slides were dehydrated with increasing volumes of ethanol (50 to 100 percent) and coverslipped with Cytoseal 60 solution (ThermoScientific, Waltham, MA, USA). Images of hippocampal area CA1 were obtained using a Zeiss Axio Imager M2 microscope and AxioVision software. At least 100 optical sections of 0.5 μm thickness were imaged and collated into an uncompressed z-stack image. Secondary and tertiary dendrites with lengths of at least 30 μm were selected and their spine widths and lengths traced using the protocols outlined previously (Risher et al., 2014). For each animal, 4-6 neurons were analyzed and 5-10 dendrites per neuron were analyzed yielding a total of 27,090 spines. Finally, statistical analyses were performed. The experimenters were blind to treatment group throughout the process of image collection, tracing, and spine analyses.

**Statistics**

Prism 5 software was used for statistical analyses (GraphPad, La Jolla, CA, USA). For two group comparisons, an unpaired two-tailed Student's t-test with Welch's correction to control for unequal standard deviations was used. For experiments with more than 2 groups, a parametric one-way analysis of variance (ANOVA) was performed. To correct for multiple comparisons, the Holm-Sidak post hoc test was used. When applicable, parametric two-way Repeated Measures ANOVA using the Holm-Sidak post hoc test was performed. For all tests, significance was set at $p \leq 0.05$. A table detailing data distribution (i.e., parametric versus non parametric distribution) has been included

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(Table 1). In addition, the statistical analyses, sample sizes, group effects, and p-values for each experiment are included (Table 2).

## RESULTS

### PTZ induction results in a single, self-limited generalized seizure

Previous studies have demonstrated that a single generalized seizure results in impaired short and long-term hippocampal as well as amygdala-dependent memory (Mao et al., 2009; Holley and Lugo, 2016). Furthermore, hyperactive signaling of the PI3K/Akt/mTOR cascade has been shown following a single generalized seizure (Zhang and Wong, 2012). For our studies, we sought to determine whether pharmacological inhibition of the PI3K/Akt/mTOR pathway would restore seizure-induced memory deficits. First, using continuous video-electroencephalographic (vEEG) recordings, we confirmed that a single injection of PTZ (75mg/kg) resulted in one self-limited generalized seizure. While recordings were obtained from both the cortex and hippocampus, Figure 1 depicts representative traces from the hippocampal-depth electrode, as the PTZ injection induced electrographic seizure activity involving both the cortex and hippocampus synchronously and there was no observable difference in EEG activity between the regions. The mean latency to a generalized seizure was 8 minutes and 25 seconds with a mean duration of 56 seconds. There was no significant effect of animal sex on the latency to a generalized seizure.

We found that at baseline (i.e., prior to induction), there was no observable difference in EEG activity between PTZ-induced and Sham (PTZ vehicle injection) animals (Figure 1A). Furthermore, following saline administration (black arrow), Sham animals did not exhibit any changes in behavior or EEG activity (Figure 1B, top trace). However, PTZ administration led to behavioral manifestations of a generalized seizure (e.g., fore- and hind-limb clonus with rearing, Racine stage 4 and greater). vEEG recordings verified the behavioral seizure manifestations associated with high-amplitude (compared to EEG baseline activity) spike activity in the hippocampus (seizure onset and offset denoted by red bars Figure 1B, bottom trace) and in the

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cortex (subdural recordings, data not shown). Using continuous vEEG recordings, we confirmed that no additional seizures or epileptiform activity occurred, including the time points used for behavioral testing and training (1, 3, 16 and 24 hours post induction, Figure 1C). Thus, our data show that a single, self-limited, generalized seizure was induced by PTZ (75mg/kg).

**Inhibition of aberrant** PI3K/Akt/mTOR **signaling partially rescues seizure-induced memory deficits**

Based upon previous studies demonstrating learning and memory deficits as well as hyperactive PI3K/Akt/mTOR signaling following a single generalized seizure (Mao et al., 2009; Zhang and Wong, 2012; Holley and Lugo, 2016), we hypothesized that aberrant activation of this pathway contributes to seizure-induced memory deficits. To test our hypothesis, we determined whether pharmacological inhibition of PI3K/Akt/mTOR signaling could rescue hippocampal and amygdala-dependent memory deficits that result following a single PTZ-induced seizure. Hereafter, we refer to hippocampal and amygdala-dependent memory as contextual and cued memory, respectively.

Prior to any inhibitor studies, we first verified that a single generalized seizure would not impair acquisition of the fear conditioning (FC) task. Animals were trained 1 hour post seizure and assessed for freezing activity prior to and following the presentation of the conditioned stimulus (Pre CS-US and Post CS-US). In animals trained for short-term memory, we found that Pre CS-US, both PTZ and Sham animals exhibited basal levels of freezing with no significant differences between the two groups (3.77 ± 3.89 vs. 0.67 ± 0.87%, Figure 2B, left panel). Following the presentation of the conditioned stimulus (Post CS-US), there was a significant increase in the freezing levels for both PTZ (36.02 ± 30.90 vs. 3.77 ± 3.89%, $p<0.01$) and Sham (58.55 ± 18.57 vs. 0.67 ± 0.87%, $p<0.001$) animals relative to the Pre CS-US condition. However, there was no significant difference between the PTZ and Sham rats indicating that the animals can acquire the task (Figure 2B, left panel). Likewise, when training the animals for long-term memory, we found no significant difference in the freezing levels of the PTZ relative to the Sham rats (5.64 ± 4.30 vs.

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1.54 ± 1.56%, Figure 2B, right panel) in the Pre CS-US condition. Post CS-US both PTZ (53.48 ± 34.51 vs. 5.64 ± 4.30%, $p<0.001$) and Sham rats (66.28 ± 13.21 vs. 1.54 ± 1.56%, $p<0.001$) and displayed significantly elevated freezing levels relative to the Pre CS-US condition. However, there was no significant difference in freezing levels between the PTZ and Sham animals Post CS-US (Figure 2B, right panel). Therefore, our data show that a single, PTZ-induced seizure did not impair the responsiveness to an aversive cue as the PTZ animals display freezing behavior that is not statistically significant from Shams.

Next, we determined whether pharmacological inhibition of the PI3K/Akt/mTOR cascade would rescue memory deficits following a single generalized seizure. Ten minutes after a PTZ-induced generalized seizure, Sham and PTZ animals received either vehicle (Veh) or the PI3K inhibitor wortmannin (Wort). One cohort of animals was tested for short-term contextual and cued memory three hours post induction (Cohort 1, Figure 3A). A second cohort of animals were induced and trained as above, but tested at 24 hours post seizure for long-term contextual and cued memory (Cohort 2, Figure 3A).

We found that, following a single generalized seizure, the animals displayed impairments in short-term contextual memory. Freezing time was significantly reduced in PTZ + Veh relative to Sham + Veh animals (21.40 ± 11.16 vs. 49.28 ± 15.47%, respectively, $p<0.001$, Figure 3B). Wort was sufficient to rescue the seizure-induced reduction in freezing levels in PTZ + Wort compared to PTZ + Veh animals (36.94 ± 15.32 vs. 21.40 ± 11.16%, respectively, $p<0.05$). There were no significant differences in the freezing levels in Sham + Wort animals relative to Sham + Veh controls (41.96 ± 17.05% vs. 49.28 ± 15.47%, respectively, Figure 3B).

We assessed whether blocking seizure-induced hyperactive PI3K/Akt/mTOR signaling with Wort treatment rescued cued memory deficits. When placed in a novel context, rats from all of the treatment groups exhibited basal freezing levels. In the Pre CS condition, there was no significant difference in the percent freezing in PTZ + Veh relative to Sham + Veh animals (4.54 ± 5.60 vs.

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11.36 ± 9.99%, respectively, Figure 3C, left panel). However, there was a significant reduction in freezing levels in PTZ + Veh relative to PTZ + Wort rats (4.54 ± 5.60 vs. 21.67 ± 9.77%, respectively, $p<0.001$, Figure 3C, left panel) prior to cue presentation. There was no significant difference in the percent freezing between Sham + Veh (11.36 ± 9.99%), Sham + Wort (19.57 ± 13.85%) and PTZ + Wort animals (Figure 3C). Once the cue (CS) was presented, all experimental groups displayed elevated freezing levels relative to the Pre CS condition. However, there was a significant reduction in the percentage of time freezing in PTZ + Veh relative to Sham + Veh animals (31.18 ± 17.24 vs. 74.11 ± 24.86%, respectively, $p<0.001$, Figure 3C). Wort treatment was not sufficient to restore the significantly reduced freezing levels in PTZ + Wort relative to the Sham + Veh animals (50.54 ± 21.72 vs. 74.11 ± 24.86%, respectively, $p<0.05$, Figure 3C). In contrast, there was a significant increase in the percentage of time freezing in PTZ + Wort animals relative to the PTZ + Veh group (50.54 ± 21.72 vs. 31.18 ± 17.24%, respectively, $p<0.05$, Figure 3C) consistent with a partial rescue. Finally, there were no significant differences in freezing levels in Sham + Wort relative to Sham + Veh rats (81.15 ± 10.89 vs. 74.11 ± 24.86%, respectively, Figure 3C, right panel). These data suggest that by blocking the seizure-induced elevation in PI3K/Akt/mTOR signaling, we were able to partially rescue deficits in short-term contextual and cued memory.

We also evaluated whether blocking elevated PI3K/Akt/mTOR signaling could rescue seizure-induced deficits in long-term contextual memory. Similar to that observed in the short-term memory test, there were significantly decreased freezing levels in PTZ + Veh relative to Sham + Veh controls (2.61 ± 4.36 vs. 42.40 ± 22.21%, respectively, $p<0.001$, Figure 3D) in the long-term contextual memory test. However, unlike in the short-term contextual memory test, there was a significant reduction in freezing levels in PTZ + Wort compared to Sham + Veh animals (2.53 ± 5.23 vs. 42.40 ± 22.21%, respectively, $p<0.001$, Figure 3D). There was no significant difference

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between the Sham + Veh and Sham + Wort groups (42.40 ± 22.21 vs. 28.44 ± 26.22%, respectively, Figure 3D).

As we observed in the short-term cued memory test, there was a partial rescue with Wort treatment following a single generalized seizure in the long-term cued memory test. We first evaluated the percentage of time freezing for animals placed in a novel environment prior to cue presentation. All Pre CS treatment groups exhibited basal levels of freezing, with the PTZ + Veh (0.75 ± 1.63%), PTZ + Wort (1.32 ± 1.67%), and Sham + Wort (2.22 ± 5.27%) having significantly reduced freezing levels relative to Sham + Veh animals (11.40 ± 16.64%, $p<0.05$, Figure 3E). As we observed in the short-term cued memory test, the Pre CS freezing levels for all treatment groups were lower than the CS condition. Upon CS presentation, there were significantly lower freezing levels in the PTZ + Veh compared to Sham + Veh rats (7.82 ± 9.10 vs. 63.38 ± 18.43%, respectively, $p<0.001$, Figure 3E, right panel). Comparable to what we observed in the short-term cued memory test, PTZ + Wort animals displayed significantly lower freezing levels compared to Sham + Veh ($p<0.001$), but freezing the levels were significantly higher in PTZ + Wort compared to PTZ + Veh rats (27.72 ± 22.89 vs. 7.82 ± 9.10%, respectively, $p<0.05$, Figure 3E), suggesting a partial rescue. Additionally, there was no significant differences in the freezing levels between the Sham + Veh and the Sham + Wort groups (63.38 ± 18.43 vs. 60.47 ± 24.76%, respectively, Figure 3E) in the CS condition of the long-term cued memory test. These results suggest that by blocking PI3K/Akt/mTOR signaling following a single generalized seizure, we were able to partially rescue long-term contextual and cued memory deficits.

To verify that Wort effectively blocked the seizure-induced dysregulation of PI3K/Akt/mTOR signaling, we performed western blotting of downstream effectors of the PI3K/Akt/mTOR pathway in hippocampal homogenates 1 hour following seizure induction corresponding to the time at which the animals were trained (Figures 3F and 3G). For these studies, we used antibodies that recognize phosphorylated S6 (P-S6) at S240/244 and S6 as well as phosphorylated Akt (P-Akt) at

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T308 and Akt. The levels of P-S6 and P-Akt were used as readouts of PI3K/Akt/mTOR activation. Our analyses revealed a significant increase in the P-S6 levels in PTZ + Veh as compared to Sham + Veh rats (240.50 ± 70.65 vs. 100 ± 58.44%, respectively, $p<0.01$, Figure 3F) 1 hour post seizure. There was a significant reduction of P-S6 levels in PTZ + Wort compared to PTZ + Veh animals (65.80 ± 41.12 vs. 240.50 ± 70.65, respectively, $p<0.01$, Figure 3F). There was no significant difference in the P-S6 levels in Sham + Veh compared to Sham + Wort animals (100 ± 58.44 vs. 76.67 ± 29.26%, respectively, Figure 3F).

Similar results were found with P-Akt. Following a single generalized seizure, there was a significant increase of P-Akt levels in PTZ + Veh animals relative to Sham + Veh controls (229.50 ± 63.32 vs. 100 ± 50.44%, respectively, $p<0.05$, Figure 3G). P-Akt levels were significantly reduced in PTZ + Wort relative to PTZ + Veh rats (86.75 ± 82.84 vs. 229.50 ± 63.32%, respectively, $p<0.05$, Figure 3G). There was no significant difference in the P-Akt levels in Sham + Veh compared to Sham + Wort animals (100 ± 50.44 vs. 68.33 ± 46.52%, respectively, Figure 3G). Taken together, these data indicate that a single injection of Wort was sufficient to block seizure-induced increases in PI3K/Akt/mTOR signaling. Furthermore, by inhibiting activation of this cascade, we were able to partially rescue the seizure-induced short and long-term memory deficits.

**Inhibition of aberrant PI3K/Akt/mTOR signaling rescues seizure-induced aberrant spine morphology but not spine loss in hippocampal CA1**

Previous studies have reported altered dendritic structure and decreases in spine number following prolonged seizure activity (or status epilepticus) (Ouyang et al., 2007; Zeng et al., 2007). However, it is unclear how dendritic spine morphology is affected following a single brief and self-limited generalized seizure. Given the important role of spine morphology in learning and memory, and the known role of the PI3K/Akt/mTOR pathway in regulating spine structure, we evaluated whether there were seizure-induced spine changes and whether seizure-induced hyperactive PI3K/Akt/mTOR signaling is associated with these changes.

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We determined whether a single generalized seizure was sufficient to alter the number of dendritic spines. In the current studies, we focused our analyses on the secondary and tertiary dendrites in stratum radiatum (SR) of hippocampal area CA1. There was a significant reduction the number of dendritic spine protrusions in PTZ + Veh relative to Sham + Veh animals (0.70 ± 0.44 vs. 0.89 ± 0.50 spines/μm, respectively, $p<0.001$, Figure 4B). A further decrease in the number of dendritic spines was observed in the PTZ + Wort relative to PTZ + Veh rats (0.58 ± 0.34 vs. 0.70 ± 0.44 spines/μm, respectively, $p<0.05$, Figure 4B). There was also a significant reduction in the number of dendritic spine protrusions in Sham + Wort relative to the Sham + Veh animals (0.71 ± 0.5 vs. 0.89 ± 0.50 spines/μm, respectively, $p<0.01$, Figure 4B), suggesting that inhibition of PI3K/Akt/mTOR signaling leads to a decrease in spine number in Sham animals. Furthermore, a single self-limited generalized seizure also resulted in a decreased spine number, which could not be rescued by blocking hyperactive PI3K/Akt/mTOR signaling.

To ascertain how a single generalized seizure affects spine morphology, we traced the length and width of dendritic spines to obtain spine length-to-width ratios (LWR) for animals in each experimental group. A single generalized seizure resulted in a significant increase in the LWR in the dendritic spines of PTZ + Veh relative to Sham + Veh animals (3.60 ± 0.83 vs. 3.20 ± 0.82%, respectively, $p<0.001$, Figure 4C). Blocking the seizure-induced increase in PI3K/Akt/mTOR signaling using Wort resulted in a significant reduction in the LWR in PTZ + Wort relative to PTZ + Veh animals (3.10 ± 0.62 vs. 3.60 ± 0.83%, respectively, $p<0.001$), but had no significant effect on Sham + Wort relative to Sham + Veh controls (3.10 ± 0.70 vs. 3.20 ± 0.82%, respectively, Figure 4C). Because we observed a significant increase in the LWR following a single generalized seizure, we hypothesized that there would be an increase in long, immature spine types. As such, we categorized and quantified dendritic spines of differing morphologies and determined whether inhibiting hyperactive PI3K/Akt/mTOR signaling was sufficient to block these changes. The analyses demonstrated a significant increase in the number of immature spines (red

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arrows) in PTZ + Veh compared to Sham + Veh animals (0.38 ± 0.21 vs. 0.33 ± 0.17 immature spines/μm, respectively, $p<0.05$, Figures 4A and 4D). There was a significant reduction in the number of immature spines in PTZ + Wort compared to PTZ + Veh animals (0.31 ± 0.20 vs. 0.38 ± 0.21 immature spines/μm, respectively, $p<0.01$). There was no significant difference in the number of immature spines in Sham + Wort relative to Sham + Veh animals (0.31 ± 0.21 vs. 0.33 ± 0.17 immature spines/μm respectively, Figures 4A and 4D). Thus, a single generalized seizure was associated with an increase in immature, or immature dendritic spines, and by inhibiting PI3K/Akt/mTOR hyperactivity we were able to block this seizure-induced increase.

Next, we examined whether the number of mature dendritic spines (red arrowheads), were altered following a single generalized seizure and effect of PI3K/Akt/mTOR blockade on this spine type. Indeed, we found a significant reduction in the number of mature spines in PTZ + Veh animals relative to the Sham + Veh group (0.22 ± 0.19 vs. 0.27 ± 0.19 mature spines/μm, respectively, $p<0.05$, Figures 4A and 4E). The number of mature spines remained reduced in PTZ + Wort compared to the Sham + Veh animals (0.20 ± 0.13 vs. 0.27 ± 0.19 mature spines/μm, respectively, $p<0.05$). Finally, our analyses revealed no difference in the number of mature spines in Sham + Wort relative to the Sham + Veh rats (0.26 ± 0.20 vs. 0.27 ± 0.19 mature spines/μm, respectively, Figures 4A and 4E). Thus, in parallel with an increase in immature spines, there was a decrease in mature spines following a single generalized seizure. In addition, while PI3K/Akt/mTOR inhibition was sufficient to block the increase in immature spine protrusions, the concurrent decrease in mature spines was not rescued.

**Seizure-induced memory deficits and altered PI3K/Akt/mTOR signaling are transient**

We evaluated whether long-term memory deficits following a single generalized seizure are transient. Seizures were induced in the rats as described before (Cohort 3) but the animals were trained 16 hours post seizure and tested 24 hours post training for long-term contextual and cued memory (40 hours post seizure, Figure 5A). We found that in contextual long-term memory, there

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was no significant difference in the percentage of time freezing between Sham and PTZ groups (48.88 ± 27.95 vs. 45.72 ± 20.59%, respectively, Figure 5B, left panel). Similarly, when animals were tested for cued long-term memory, our analyses showed no significant differences in the percent freezing during either the Pre CS condition in Sham compared to PTZ rats (10.79 ± 10.30 vs. 10.98 ± 13.86%, respectively) or in the CS condition (63.66 ± 8.27 vs. 70.92 ± 6.81% respectively, Figure 5B) in the Post CS condition. Thus, by this time point there was recovery of the long-term memory deficits induced by a single generalized seizure, suggesting that these alterations were transient.

We assessed PI3K/Akt/mTOR signaling at 16 hours following a single generalized seizure (training time point for Cohort 3) and found that it had returned to basal levels (Figure 5C). At 16 hours post seizure, there was no significant difference in P-S6 levels between Sham and PTZ animals (100 ± 40.72 vs. 76.13 ± 22.64%, respectively), nor was there a significant difference in the P-AKT levels in Sham compared to PTZ rats (100 ± 8.45 vs. 79.26 ± 8.65%, respectively, Figure 5C, right panel). These data suggest that both the memory deficits and hyperactive signaling of PI3K/Akt/mTOR following a single generalized seizure are transient and resolve within 16 hours of the seizure.

## DISCUSSION

In the present study, we demonstrated that a single generalized seizure resulted in contextual and cued memory deficits, dysregulated PI3K/Akt/mTOR signaling, and altered dendritic spine morphology. Furthermore, we showed that by inhibiting seizure-induced hyperactivation of PI3K/Akt/mTOR signaling, we were able to rescue short-term contextual memory, partially rescue short and long-term cued memory, and block the increase in hippocampal immature spines induced by a single generalized seizure. Inhibition of PI3K/Akt/mTOR signaling did not restore the seizure-induced long-term contextual memory deficits or the decrease in mature spines. We found that these changes were transient as rats that underwent a single generalized seizure had no

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significant differences in long-term cued or contextual memory compared to Shams when trained 16 hours following a single generalized seizure, at which point PI3K/Akt/mTOR signaling was no longer hyperactivated. These findings suggest that dysregulation of the PI3K/Akt/mTOR pathway and aberrant spine morphology may contribute to seizure-induced memory deficits. Furthermore, there is a window of time during which memory is disrupted following a single generalized seizure, with later recovery, which has potential clinical relevance.

Our findings using rats are consistent with and extend previous work. Compared with previous studies using the single generalized seizure model, Mao et al. reported impaired short and long-term contextual fear memory in rats (Mao et al., 2009). However, they did not test contextual learning. Holley and Lugo reported that a single generalized seizure in mice resulted in deficits in long-term cued memory, had no effect on locomotion, and did not impair cued fear acquisition (Holley and Lugo, 2016).

In epilepsy models, where animals have multiple spontaneous seizures, a significant decrease in the number of spine protrusions in hippocampal dendrites has been recorded (Jiang et al., 1998; Zha et al., 2005; Zeng et al., 2007; Guo et al., 2012; Brewster et al., 2013; Nie et al., 2015). In the current studies, we demonstrate that a single, self-limited generalized seizure is sufficient to reduce spine number. To our knowledge, this has not previously been shown. Moreover, we found that of the spines that remained, there was a significant increase in immature and a concurrent decrease in mature spine types. A significant increase in the number of immature spines has been evident in chronic epilepsy where cognitive impairments are described and in genetic models of cognitive disorders such as Fragile X syndrome, autism, and tuberous sclerosis (Penzes et al., 2011; Pathania et al., 2014; Kim et al., 2016).

Other studies using epilepsy models report altered structure of the dendritic shaft and decreases in the number of dendritic branches (Jiang et al., 1998; Ouyang et al., 2007; Zeng et al., 2007; Guo et al., 2012; Brewster et al., 2013). Using the single seizure model, we did not observe

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any overt morphological changes in the dendrites themselves based upon qualitative visual inspection (data not shown). The observed memory deficits following a single generalized seizure may be, at least in part, due to shifts in spine morphology (maturity) and number. If this is the case, we expect these changes to be transient.

Hyperactive PI3K/Akt/mTOR signaling has been well characterized in epilepsy-associated memory deficits (Ehninger et al., 2008; Brewster et al., 2013; Cambiaghi et al., 2013; Trinh and Klann, 2013; Lugo et al., 2014). Recently, Zhang and Wong reported that a single generalized seizure was sufficient to cause dysregulated signaling of PI3K/Akt/mTOR pathway (Zhang and Wong, 2012). In the current studies, we show that by blocking seizure-induced hyperactive PI3K/Akt/mTOR signaling at the time of training, we are able to rescue deficits in hippocampal-dependent and partially rescue amygdala-dependent memory. We were able to block hyperactive PI3K/Akt/mTOR signaling following a single generalized seizure. A previous study demonstrated that pathway inhibition prior to seizure induction also suppressed PI3K/Akt/mTOR pathway activation (Zhang and Wong, 2012). In the current studies, dysregulated PI3K/Akt/mTOR activity appears to partially contribute to seizure-induced deficits in hippocampal and amygdala-dependent memory. When we blocked hyperactive PI3K/Akt/mTOR signaling following a single generalized seizure, we partially rescued both cued short and long-term memory. Thus, our results suggest that amygdala-dependent memory deficits may only partially be attributed to dysregulation of the PI3K/Akt/mTOR pathway. In contrast, PI3K/Akt/mTOR inhibition following a single generalized seizure rescued only short-term contextual memory suggesting that with hippocampal-dependent memory, there are additional mechanisms that contribute to the long-term memory deficits that are possibly related to the conversion from short to long-term memory.

Pathological PI3K/Akt/mTOR pathway activation seems to correlate with the seizure-induced memory deficits because 16 hours post seizure, when the PI3K/Akt/mTOR pathway is no longer activated, the deficits in contextual and cued memory were not observed. Additional future

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studies are needed to further evaluate the role of PI3K/Akt/mTOR dysregulation and other molecular mechanisms underlying seizure-induced memory deficits and dendritic changes.

Since results from our lab and others demonstrate that a single generalized seizure results in aberrant PI3K/Akt/mTOR signaling, it is conceivable that excessive protein translation may underlie the observed memory deficits. However, there are recent reports that activation of mTOR downstream targets may not lead to increased translation (Biever et al., 2015). AKT signaling has been reported to regulate actin polymerization, spine number, and subsequently, synaptic potentiation and memory (Huang et al., 2013). Moreover, Kumar et al. found that constitutive activation of Akt signaling results in increased expression of filopodic spines in cultured hippocampal neurons (Kumar et al., 2005). Based upon these studies and our findings showing that inhibiting the seizure-induced elevation in PI3K/Akt/mTOR signaling blocked the seizure-induced increase in immature spine types, it is possible that these changes in spine morphology are, at least in part, the result of aberrant PI3K-AKT signaling.

In summary, our data show that a single generalized seizure and the associated memory impairments are linked, in part, to dysregulated signaling of PI3K/Akt/mTOR cascade and altered spine morphology and that these changes are transient. Based on these findings and those from other labs, there is a window of time following a single generalized seizure in rodents during which there are significant short- and long-term memory deficits with associated disruption of PI3K/Akt/mTOR signaling and dendritic spines, both of which are important determinants of memory. These studies have translational relevance in clinical epilepsy as these findings indicate that a single generalized seizure significantly impacts memory for up to 24 hours.

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## REFERENCES


Banerjee PN, Filippi D, Allen Hauser W (2009) The descriptive epidemiology of epilepsy-A review. Epilepsy Res 85:31–45.

Berg AT, Berkovic SF, Brodie MJ, Buchhalter J, Cross JH, Van Emde Boas W, Engel J, French J, Glauser TA, Mathern GW, Moshé SL, Nordli D, Plouin P, Scheffer IE (2010) Revised terminology and concepts for organization of seizures and epilepsies: Report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia 51:676–685.

Biever A, Puighermanal E, Nishi A, David A, Panciatici C, Longueville S, Xirodimas D, Gangarossa G, Meyuhas O, Herve D, Girault JA, Valjent E (2015) PKA-dependent phosphorylation of ribosomal protein S6 does not correlate with translation efficiency in striatonigral and striatopallidal medium-sized spiny neurons. J Neurosci 35:4113–4130 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25762659.

Brewster AL, Lugo JN, Patil V V., Lee WL, Qian Y, Vanegas F, Anderson AE (2013) Rapamycin Reverses Status Epilepticus-Induced Memory Deficits and Dendritic Damage. PLoS One 8.

Brunn GJ, Williams J, Sabers C, Wiederrecht G, Lawrence JC, Abraham RT (1996) Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J 15:5256–5267.

Cambiaghi M, Cursi M, Magri L, Castoldi V, Comi G, Minicucci F, Galli R, Leocani L (2013) Behavioural and EEG effects of chronic rapamycin treatment in a mouse model of Tuberous Sclerosis Complex. Neuropharmacology 67:1–7.

Ehninger D, Han S, Shilyansky C, Zhou Y, Li W, Kwiatkowski DJ, Ramesh V, Silva a J (2008) Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. Nat Med 14:843–848 Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18568033.

eNeuro Accepted Manuscript

521 Fisher RS, Acevedo C, Arzimanoglou A, Bogacz A, Cross JH, Elger CE, Engel J, Forsgren L,
522 French JA, Glynn M, Hesdorffer DC, Lee BI, Mathern GW, Moshé SL, Perucca E, Scheffer IE,
523 Tomson T, Watanabe M, Wiebe S (2014) ILAE Official Report: A practical clinical definition of
524 epilepsy. Epilepsia 55:475–482.
525 Guo D, Arnspiger S, Rensing NR, Wong M (2012) Brief seizures cause dendritic injury. Neurobiol
526 Dis 45:348–355.
527 Helmstaedter C, Kurthen M, Lux S, Reuber M, Elger CE (2003) Chronic epilepsy and cognition: a
528 longitudinal study in temporal lobe epilepsy. Ann Neurol 54:425–432 Available at:
529 http://www.ncbi.nlm.nih.gov/pubmed/14520652.
530 Hoeffer CA, Klann E (2010) mTOR signaling: At the crossroads of plasticity, memory and disease.
531 Trends Neurosci 33:67–75.
532 Holley AJ, Lugo JN (2016) Effects of an acute seizure on associative learning and memory.
533 Epilepsy Behav:51–57.
534 Huang W, Zhu PJ, Zhang S, Zhou H, Stoica L, Galiano M, Krnjević K, Roman G, Costa-mattioli M
535 (2013) mTORC2 controls actin polymerization required for consolidation of long-term memory.
536 Nat Neurosci 16:441–448 Available at:
537 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3615448&tool=pmcentrez&renderty
538 pe=abstract%5Cnhttp://dx.doi.org/10.1038/nn.3351.
539 Jiang M, Lee CL, Smith KL, Swann JW (1998) Spine loss and other persistent alterations of
540 hippocampal pyramidal cell dendrites in a model of early-onset epilepsy. J Neurosci 18:8356–
541 8368 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9763479.
542 Kanner AM (2016) Management of psychiatric and neurological comorbidities in epilepsy. Nat Rev
543 Neurol 12:106–116 Available at:
544 http://www.nature.com/doifinder/10.1038/nrneurol.2015.243%5Cnhttp://www.ncbi.nlm.nih.gov/
545 pubmed/26782334.

eNeuro Accepted Manuscript

546 Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini
547 DM (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the
548 cell growth machinery. Cell 110:163–175.
549 Kim H-J, Cho M-H, Shim WH, Kim JK, Jeon E-Y, Kim D-H, Yoon S-Y (2016) Deficient autophagy in
550 microglia impairs synaptic pruning and causes social behavioral defects. Mol Psychiatry:1–9
551 Available at: http://www.nature.com/doifinder/10.1038/mp.2016.103.
552 Kumar V, Zhang M-X, Swank MW, Kunz J, Wu G-Y (2005) Regulation of dendritic morphogenesis
553 by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. J Neurosci 25:11288–11299.
554 Lodhi S, Agrawal N (2012) Neurocognitive problems in epilepsy. Adv Psychiatr Treat 18:232–240.
555 Lugo JN, Smith GD, Arbuckle EP, White J, Holley AJ, Floruta CM, Ahmed N, Gomez MC,
556 Okonkwo O (2014) Deletion of PTEN produces autism-like behavioral deficits and alterations
557 in synaptic proteins. Front Mol Neurosci 7:27 Available at:
558 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3997048&tool=pmcentrez&renderty
559 pe=abstract.
560 Luttjohann A, Fabene PF, van Luijtelaar G (2009) A revised Racine's scale for PTZ-induced
561 seizures in rats. Physiol Behav 98:579–586.
562 Mao RR, Tian M, Yang YX, Zhou QX, Xu L, Cao J (2009) Effects of pentylenetetrazol-induced brief
563 convulsive seizures on spatial memory and fear memory. Epilepsy Behav 15:441–444.
564 Nguyen LH, Brewster AL, Clark ME, Regnier-Golanov A, Sunnen CN, Patil V V., D'Arcangelo G,
565 Anderson AE (2015) MTOR inhibition suppresses established epilepsy in a mouse model of
566 cortical dysplasia. Epilepsia 56:636–646.
567 Nie D, Chen Z, Ebrahimi-Fakhari D, Di Nardo A, Julich K, Robson VK, Cheng Y-C, Woolf CJ,
568 Heiman M, Sahin M (2015) The Stress-Induced Atf3-Gelsolin Cascade Underlies Dendritic
569 Spine Deficits in Neuronal Models of Tuberous Sclerosis Complex. J Neurosci 35:10762–
570 10772 Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.4796-

eNeuro Accepted Manuscript

14.2015%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/26224859%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4518051.

Nolan MA, Redoblado MA, Lah S, Sabaz M, Lawson JA, Cunningham AM, Bleasel AF, Bye AME (2004) Memory function in childhood epilepsy syndromes. J Paediatr Child Health 40:20–27.

Ouyang Y, Yang XF, Hu XY, Erbayat-Altay E, Zeng LH, Lee JM, Wong M (2007) Hippocampal seizures cause depolymerization of filamentous actin in neurons independent of acute morphological changes. Brain Res 1143:238–246.

Pathania M, Davenport EC, Muir J, Sheehan DF, López-Doménech G, Kittler JT (2014) The autism and schizophrenia associated gene CYFIP1 is critical for the maintenance of dendritic complexity and the stabilization of mature spines. Transl Psychiatry 4:e374 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3966042&tool=pmcentrez&rendertype=abstract.

Penzes P, Cahill ME, Jones K a, VanLeeuwen J-E, Woolfrey KM (2011) Dendritic spine pathology in neuropsychiatric disorders. Nat Neurosci 14:285–293 Available at: http://dx.doi.org/10.1038/nn.2741.

Racine RJ (1972) Modification of seizure activity by electrical stimulation: II. Motor seizure. Electroencephalogr Clin Neurophysiol 32:281–294.

Richter JD, Klann E (2009) Making synaptic plasticity and memory last: Mechanisms of translational regulation. Genes Dev 23:1–11.

Risher WC, Ustunkaya T, Alvarado JS, Eroglu C (2014) Rapid golgi analysis method for efficient and unbiased classification of dendritic spines. PLoS One 9.

Sarbassov DD, Guertin D a, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098–1101.

Sunnen CN, Brewster AL, Lugo JN, Vanegas F, Turcios E, Mukhi S, Parghi D, D'Arcangelo G, Anderson AE (2011) Inhibition of the mammalian target of rapamycin blocks epilepsy

eNeuro Accepted Manuscript

progression in NS-Pten conditional knockout mice. Epilepsia 52:2065–2075.

Thom M (2014) Review: Hippocampal sclerosis in epilepsy: A neuropathology review. Neuropathol Appl Neurobiol 40:520–543.

Trinh MA, Klann E (2013) Translational control by eIF2?? kinases in long-lasting synaptic plasticity and long-term memory. Neurobiol Learn Mem 105:93–99.

van Rijckevorsel K (2006) Cognitive problems related to epilepsy syndromes, especially malignant epilepsies. Seizure 15:227–234.

Zeng L-H, Xu L, Rensing NR, Sinatra PM, Rothman SM, Wong M (2007) Kainate seizures cause acute dendritic injury and actin depolymerization in vivo. J Neurosci 27:11604–11613 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3934634&tool=pmcentrez&rendertype=abstract.

Zha XM, Green SH, Dailey ME (2005) Regulation of hippocampal synapse remodeling by epileptiform activity. Mol Cell Neurosci 29:494–506.

Zhang B, Wong M (2012) Pentylenetetrazole-induced seizures cause acute, but not chronic, mTOR pathway activation in rat. Epilepsia 53:506–511.

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## FIGURE LEGENDS

**Figure 1.** PTZ induces a single, self-limited, generalized seizure. Continuous video-electroencephalographic (vEEG) recordings were performed in saline injected (Sham) and PTZ-induced animals for 24 hours post induction. Depicted are representative hippocampal EEG traces from implanted rats prior to PTZ seizure induction (baseline), at the time of seizure (induction), and at time points used for behavioral training and testing. (A) Baseline: No overt difference in baseline EEG activity was observed. (B) Induction: Following saline administration (black arrow), no difference in EEG activity was observed in Sham animals (top traces). However, PTZ-seizure induction (black arrow) resulted in abnormal electrographic spikes followed immediately by a single, self-limited discharge of high amplitude spikes observed in both the cortex (not shown) and hippocampus. Red bars indicate seizure onset and termination in the hippocampus with similar findings in the cortex (not shown). At the onset of the spike discharges (electrographic seizure onset), a behavioral Racine stage 4 generalized seizure was observed. (C) Post Induction: Representative EEG traces for Sham (left) and PTZ-induced animals (right) at 1, 3, 16 and 24 hours post induction. There were no other seizure events in PTZ-induced animals when compared to Sham controls at any time point during the 24 hours post induction (n=4-5/group).

**Figure 2.** A single generalized seizure does not significantly impair fear conditioning (FC) task acquisition. (A) Timeline of seizure induction and the fear conditioning training protocol for short as well as long-term memory. (B) Training for Short-term Memory (left): When placed in a novel arena prior to the presentation of the conditioned stimulus-unconditioned stimulus (Pre CS-US), both Sham and PTZ animals displayed a low basal level of freezing which was not significantly different between the two groups. Following the presentation of the CS (Post CS-US), both Sham and PTZ animals had a significant increase in freezing levels (n=6-8/group, **p<0.01, ***p<0.001). Training for Long-term Memory (right): Again, prior to CS-US presentation, both Sham and PTZ animals

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displayed a low basal level of freezing, which was not significantly different between the two groups. Both Sham and PTZ animals had a significant increase in freezing levels Post CS-US (n=9/group, ***p<0.001). There was no significant difference between Sham and PTZ animals Post CS-US. Data are presented as mean ± SEM.

**Figure 3.** Wortmannin (Wort) partially rescues memory deficits that result from a single PTZ-induced generalized seizure. (A) Timeline detailing seizure induction, Wort administration, and the fear conditioning protocols. Two separate cohorts of animals were tested for short-term (Cohort 1) and for long-term (Cohort 2) memory. (B) Contextual Short-term Memory: PTZ + Veh animals displayed significantly reduced freezing levels relative to Sham + Veh controls (n=11-12/group). Wort treatment resulted in significantly increased freezing levels in PTZ + Wort animals relative to the PTZ + Veh condition (n=11-12/group). There was no significant difference between Sham + Veh and PTZ + Wort animals (n=11-12/group) and between the Sham + Wort group as compared to Sham + Veh animals (n=11-12/group). (C) Cued Short-term Memory: Prior to the CS presentation (left), there was no significant difference between the PTZ + Veh and Sham + Veh groups (n=12-13/group). The PTZ + Wort animals displayed significantly elevated freezing levels when compared to the PTZ + Veh group (n=12-13/group). There were no significant differences in freezing levels between the Sham + Veh and Sham + Wort conditions. During the CS presentation (right panel), the PTZ + Veh animals displayed significantly reduced freezing levels when compared to Sham + Veh controls (n=11-12/group). While there remained a significant reduction in freezing levels in the PTZ + Wort animals compared to Sham + Veh controls (n=11-12/group), PTZ + Wort animals displayed significantly increased freezing levels compared to PTZ + Veh animals (n=12-13/group). There was no difference in freezing levels between Sham + Veh and Sham + Wort animals. (D) Contextual Long-term Memory: As we observed for short-term memory, there was a significant reduction in the freezing levels in PTZ + Veh animals relative to the Sham + Veh

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control group (n=11-15/group). There was no significant difference between PTZ + Veh and PTZ + Wort animals (n=11-15/group). Wort did not significantly affect freezing levels in the Sham + Wort group relative to Sham + Veh controls (n=11-15/group). (E) Cued Long-term Memory: As compared to Sham + Veh controls, all other treatment groups displayed significantly reduced freezing levels in the Pre CS (left) condition (n=11-15/group). No other significant differences were observed. During the presentation of the CS (right panel), there were significantly reduced freezing levels in PTZ + Veh animals when compared to Sham + Veh controls (n=11-15/group). The PTZ + Wort group exhibited significantly reduced freezing levels as compared to the Sham + Veh group (n=11-15/group). However, significantly elevated freezing levels were present in PTZ + Wort animals relative to PTZ + Veh group (n=11-15/group). Wort treatment did not affect the freezing levels in Sham animals. Representative western blots (left panels) and quantifications (right panels) depicting P-S6 (F) and P-Akt (G) immunoreactivity from whole hippocampal homogenates of animals from all treatment groups 1 hour following induction are shown. (F). There were significantly elevated P-S6 levels in the PTZ + Veh animals relative to Sham + Veh controls (n=3-6/group). The PTZ + Wort group displayed significantly reduced P-S6 levels as compared to PTZ + Veh animals (n=3-6/group). P-S6 levels were not significantly changed in Sham + Wort compared to Sham + Veh animals (n=3-6/group). (G). Similarly, in PTZ + Veh animals, P-Akt levels were significantly elevated relative to Sham + Veh animals (n=3-6/group). The P-Akt levels were reduced in PTZ + Wort animals the relative to PTZ + Veh group (n=3-6/group). There was no significant difference in P-Akt levels between Sham + Wort and Sham + Veh animals (n=3-6/group). All data are represented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).

**Figure 4.** A single PTZ-induced seizure leads to dendritic spine alterations in hippocampal dendrites with partial rescue using wortmannin (Wort). Brains were collected 3 hours following a single generalized seizure and images of hippocampal area CA1 secondary and tertiary dendrites

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were obtained. (A) High magnification representative photomicrographs of dendrites in Sham and PTZ animals treated either with Veh or Wort. Mature spines are observed in Sham + Veh and Sham + Wort animals (red arrowheads), while dendritic spines from animals in the PTZ + Veh and PTZ + Wort conditions display immature shaped spines (red arrows). Wort treatment reduced the number of visible spines in both Sham and PTZ animals. Less immature spines are observed in PTZ + Wort animals. (B) Analyses revealed that a single generalized seizure resulted in a significant reduction of the number of spine protrusions per micron (μm) relative to Sham + Veh animals (n=6 animals/group, 4-6 neurons/animal, 94-113 dendrites/group). In the PTZ + Wort condition, the number of protrusions per μm was significantly reduced relative to Sham + Veh controls and further reduced relative to PTZ + Veh animals (n=3-6 animals/group, 4-6 neurons/animal, 113-135 dendrites/group). There was also a significant reduction in Sham + Wort relative to Sham + Veh (n=3 animals/group, 4-6 neurons/animal, 94 dendrites/group). (C) In PTZ + Veh animals, there was a significant increase in the length-to-width ratio (LWR) relative to Sham + Veh controls of those spines that remained (n=6 animals/group, 4-6 neurons/animal, 94-113 dendrites/group). The LWR was significantly increased in PTZ + Wort animals as compared to the PTZ + Veh condition (n=3-6 animals/group, 4-6 neurons/animal, 113-135 dendrites/group). No significant differences in LWR were found between Sham + Wort and Sham + Veh animals (n=3-6 animals/group, 4-6 neurons/animal, 113-135 dendrites/group). (D) Further analyses of spine morphology revealed that in the PTZ + Veh experimental group, there was a significant increase in the number of immature spines per μm relative to Sham + Veh animals (n=6 animals/group, 4-6 neurons/animal). Wort treatment blocked the seizure-induced increase in immature spines per μm in PTZ + Wort when compared to PTZ + Veh condition (n=3-6 animals/group, 4-6 neurons/animal, 113-135 dendrites/group). In Sham + Wort animals, there was no significant difference in the number of immature spines per μm relative to Sham + Veh animals (n=6 animals/group, 4-6 neurons/animal). (E) Finally, in PTZ + Veh animals there was a significant reduction in the number

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of mature spines per μm when compared to Sham + Veh controls (n=6 animals/group, 4-6 neurons/animal, 94-113 dendrites/group). There was no significant difference in the number of mature spines per μm in PTZ + Wort as compared to PTZ + Veh animals indicating Wort did not block the seizure-induced decrease in mature spines (n=3-6 animals/group, 4-6 neurons/animal, 94-135 dendrites/group). There was no effect of Wort on the number of mature spines per μm in Sham + Wort compared to Sham + Veh animals (n=3-6 animals/group, 4-6 neurons/animal, 94-135 dendrites/group). Data represented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).

**Figure 5.** Seizure-induced long-term contextual and cued memory deficits and PI3K/Akt/mTOR hyperactivation are transient. (A) Timeline of seizure induction and FC protocol is shown. A third group of animals (Cohort 3) was induced as before but trained 16 hours post seizure in fear conditioning. Twenty-four hours after the training (40 hours post seizure), the animals were tested for long-term contextual and cued memory. (B) Contextual Long-term Memory (left panel): When tested at 40 hours post seizure, both Sham and PTZ animals displayed elevated freezing levels with no significant difference between groups (n=11-12/group). Cued Long-term Memory (right panel): There was no significant difference between the Sham and PTZ animals in the Pre CS condition (n=11-12/group). During the CS presentation, both Sham and PTZ animals displayed significantly elevated freezing levels relative to the Pre CS condition and there was no significant difference between the two treatment groups (n=11-12/group). (C) Representative western blot from whole hippocampi and quantifications of P-S6 and P-Akt (left and right blot panels, respectively) at 16 hours following a single seizure are shown. The bar graphs from the analysis reveal that at 16 hours following a single generalized seizure there was no significant difference in the P-S6 and P-Akt levels in PTZ animals relative to Sham controls (n=5/group). Data represented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).

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738 Table 1. Probability analyses performed to determine data normality for each experiment

| Figure/ Panel | Assay | Graphed Residuals (Y/N) | Normality Analysis | Probability of the Goodness of Fit (%) | Data Distribution |
|---|---|---|---|---|---|
| 2A | Training for Short-term Memory | No (graphed and analyzed individual scatterplot) | Nonlinear Regression with Akaike's Information Criterion | 98.75 | Parametric |
| 2B | Training for Long-term Memory | No (graphed and analyzed individual scatterplot) | Nonlinear Regression with Akaike's Information Criterion | 99.94 | Parametric |
| 3B | Contextual Short-term Memory | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.96 | Parametric |
| 3C/left | Cued Short-term Memory Pre CS | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.98 | Parametric |
| 3C/right | Cued Short-term Memory CS | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.97 | Parametric |
| 3D | Contextual Long-term Memory | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.91 | Parametric |
| 3E/left | Cued Long-term Memory Pre CS | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.86 | Parametric |
| 3E/right | Cued Long-term Memory CS | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.96 | Parametric |
| 3F | Western blot of phosphorylated S6 | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.97 | Parametric |
| 3G | Western blot of phosphorylated Akt | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.98 | Parametric |
| 4B | Protrusions per µM | Yes | Nonlinear Regression with Akaike's Information Criterion | 98.58 | Parametric |
| 4C | Length-to-width Ratio | Yes | Nonlinear Regression with Akaike's Information Criterion | 99.85 | Parametric |
| 4D | Immature Spines per µM | Yes | Nonlinear Regression with Akaike's Information Criterion | 98.91 | Parametric |
| 4E | Mature spines per µM | Yes | Nonlinear Regression with Akaike's Information Criterion | 86.15* | Parametric |
| 5B/left | Contextual Long-term Memory 16 hour training | Yes | Nonlinear Regression with Akaike's Information Criterion | 96.03 | Parametric |
| 5B/right | Cued Long-term Memory | No (graphed and analyzed individual scatterplot) | Nonlinear Regression with Akaike's Information Criterion | 99.94 | Parametric |
| 5C/right | Western blot of phosphorylated S6 and Akt | Yes | Nonlinear Regression with Akaike's Information Criterion (for S6 only) | 95.53 | Parametric |
| | | Yes | Nonlinear Regression with Akaike's Information Criterion (for AKT only) | 98.17 | |

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740 *Because the probability was lower than 90%, an extra sum-of-squares test was subsequently run

741 which reported that it is not valid to reject the null hypothesis (i.e. the data fit the model).

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742 Table 2. Statistical analyses performed for each experiment

| Figure/ Panel | Assay | Statistical Test | F(df, error value),p-value | Post hoc analysis/Correction |
|---|---|---|---|---|
| 2A | Training for Short-term Memory | Two-way Repeated Measures ANOVA | Interaction: F(1,12)=3.446, p=0.0881<br>Time: F(1,12)=42.49, p<0.0001<br>Experimental group: F(1,12)=1.686, p=0.2185 | Holm-Sidak's multiple comparisons test |
| 2B | Training for Long-term Memory | Two-way Repeated Measures ANOVA | Interaction: F(1,16)=1.986, p=0.1779<br>Time: F(1,16)=88.27, p<0.0001<br>Experimental group: F(1,16)=0.4603, p=0.5072 | Holm-Sidak's multiple comparisons test |
| 3C/left | Cued Short-term Memory Pre CS | Ordinary one-way ANOVA | F(3,46)=16.69, p=0.0100 | Holm-Sidak's multiple comparisons test |
| 3C/right | Cued Short-term Memory CS | Ordinary one-way ANOVA | F(3,43)=16.69, p<0.0001 | Holm-Sidak's multiple comparisons test |
| 3D | Contextual Long-term Memory | Ordinary one-way ANOVA | F(3,46)=16.72, p<0.0001 | Holm-Sidak's multiple comparisons test |
| 3E/left | Cued Long-term Memory Pre CS | Ordinary one-way ANOVA | F(3,46)=16.69, p=0.0100 | Holm-Sidak's multiple comparisons test |
| 3E/right | Cued Long-term Memory CS | Ordinary one-way ANOVA | F(3,45)=20.39, p<0.0001 | Holm-Sidak's multiple comparisons test |
| 3F | Western blot of phosphorylated S6 | Ordinary one-way ANOVA | F(3,13)=9.391, p=0.0014 | Holm-Sidak's multiple comparisons test |
| 3G | Western blot of phosphorylated Akt | Ordinary one-way ANOVA | F(3,11)=5.058, p=0.0192 | Holm-Sidak's multiple comparisons test |
| 4B | Protrusions per μM | Ordinary one-way ANOVA | F(3,613)=13.55, p<0.0001 | Holm-Sidak's multiple comparisons test |
| 4C | Length-to-width Ratio | Ordinary one-way ANOVA | F(3,645)=14.45, p=0.0009 | Holm-Sidak's multiple comparisons test |
| 4D | Immature Spines per μM | Ordinary one-way ANOVA | F(3,609)=4.505, p=0.0039 | Holm-Sidak's multiple comparisons test |
| 4E | Mature spines per μM | Ordinary one-way ANOVA | F(3,613)=4.845,p=0.0023 | Holm-Sidak's multiple comparisons test |
| 5B/left | Contextual Long-term Memory 16 hour training | Unpaired Student's t-test | t(18.31)=0.3066, p=0.7626 | Welch's correction |
| 5B/right | Cued Long-term Memory | Two-way Repeated Measures ANOVA | Interaction: F(1,21)=0.5235, p=0.4773<br>Time: F(1,21)=133.2, p<0.0001<br>Experimental group: F(1,21)=0.3028, p=0.5879 | Holm-Sidak's multiple comparisons test |
| 5C/right | Western blot of phosphorylated S6 and Akt | Unpaired Student's t-test (for S6 only)<br>Unpaired Student's t-test (for AKT only) | t(6.257)=0.5123, p=0.6260<br>t(7.997)=1.713, p=0.1250 | Welch's correction<br>Welch's correction |

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Figure 1. PTZ induces a single, self-limited, generalized seizure.

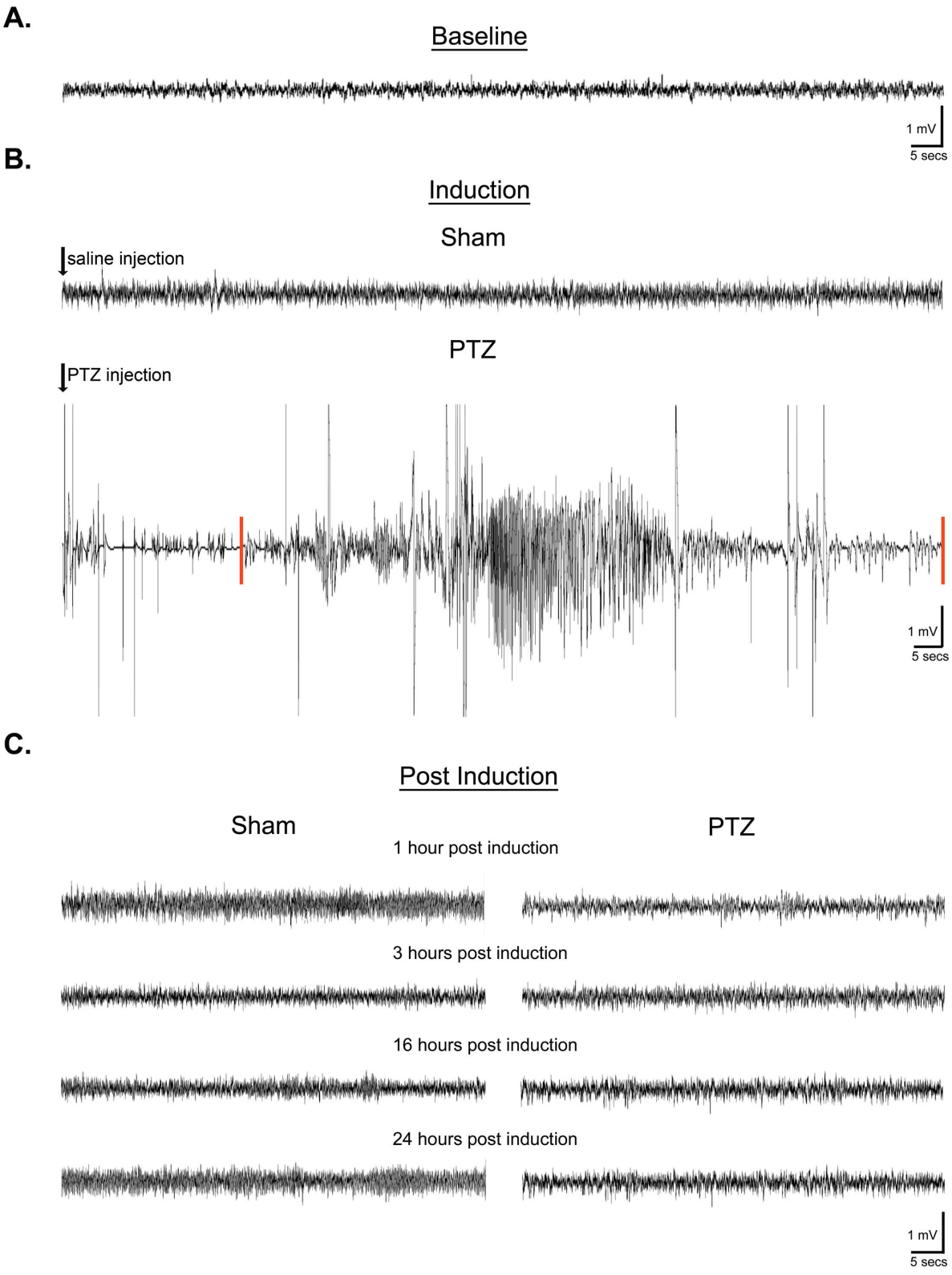

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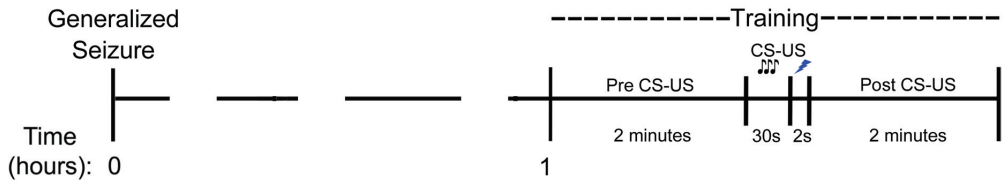
Figure 2. A single generalized seizure does not significantly impair fear conditioning (FC) task acquisition.

**A.**




**B.**

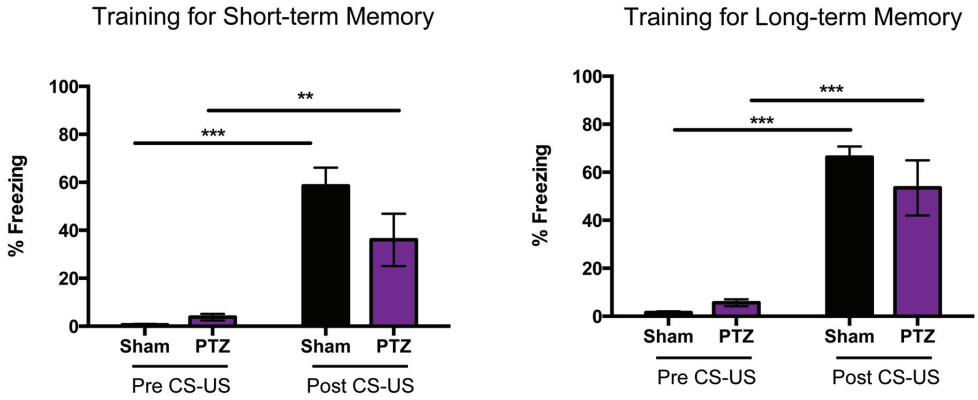


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Figure 3. Wortmannin (Wort) partially rescues memory deficits that result from a single PTZ-induced seizure.

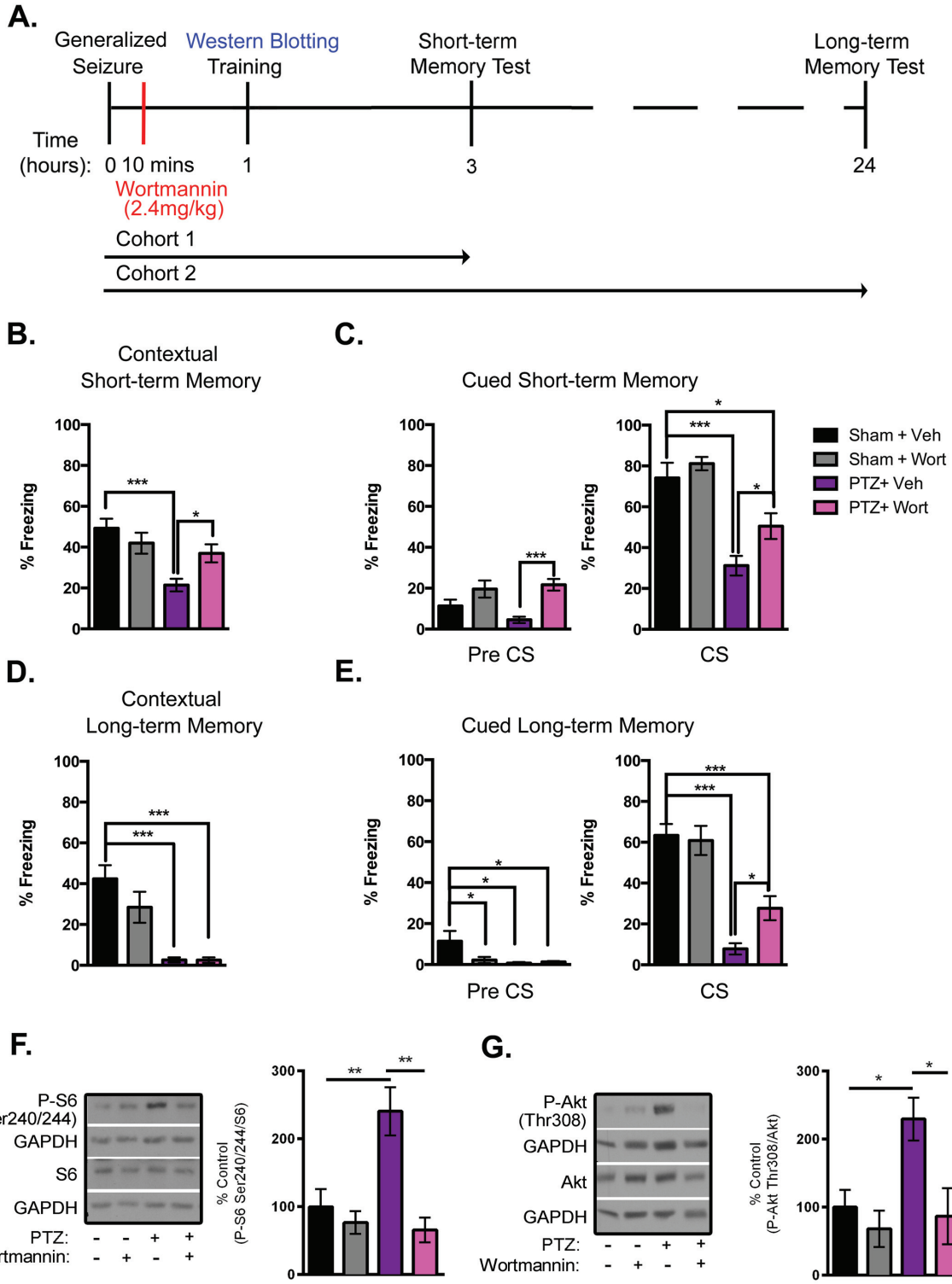

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Figure 4. A single PTZ-induced seizure leads to dendritic spine alterations in hippocampal dendrites with partial rescue using wortmannin (Wort).

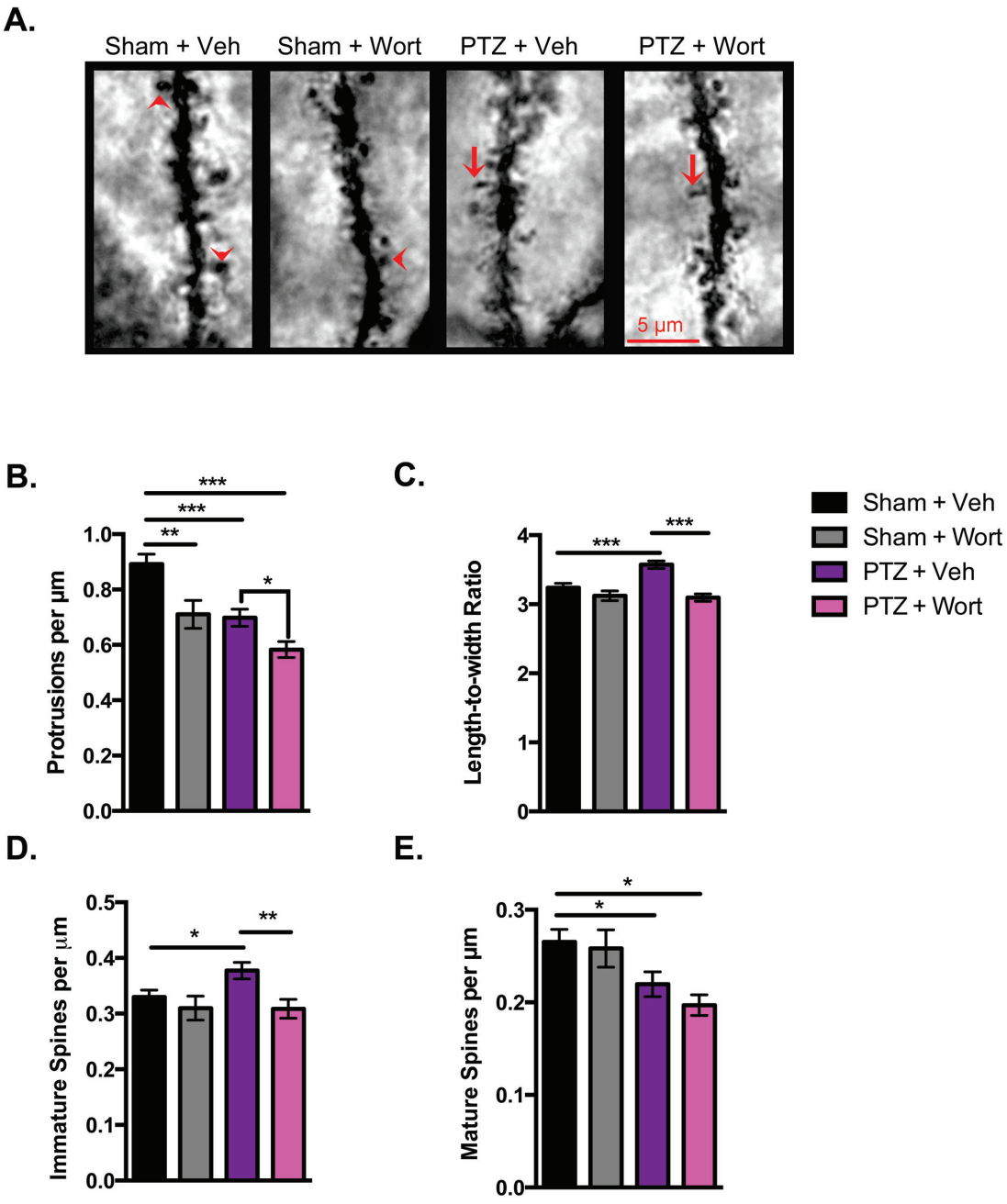

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Figure 5. Seizure-induced long-term contextual and auditory memory deficits and PI3K-mTOR hyperactivation are transient.