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Increased sensitivity to kindling in mice lacking TSP1

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Abstract

The development of a hyperexcitable neuronal network is thought to be a critical event in epilepsy. Thrombospondins (TSPs) regulate synaptogenesis by binding the neuronal α2δ subunit of the voltage-gated calcium channel. TSPs regulate synapse formation during development and in the mature brain following injury. It is unclear if TSPs are involved in hyperexcitability that contributes to the development of epilepsy. Here we explore the development of epilepsy using a pentylenetetrazole (PTZ) kindling model in mice lacking TSP1 and TSP2.

Unexpectedly, we found increased sensitivity to PTZ kindling in mice lacking TSP1, while mice lacking TSP2 kindled similar to wild-type. We found that the increased seizure susceptibility in the TSP1 knockout (KO) mice was not due to a compensatory increase in TSP2 mRNA as TSP1/2 KO mice were sensitive to PTZ, similar to the TSP1 KO mice. Furthermore, there were similar levels of TGF-B signal activation during kindling in the TSP1 KO mice compared to wild-type. We observed decreased expression of voltage-dependent calcium channel subunit CACNA2D1 mRNA in TSP1, TSP2, and TSP1/2 KO mice. Decreased CACNA2D2 mRNA was only detected in mice that lacked TSP1 and α2δ−1/2 protein levels in the cortex was lower in the TSP 1/2 KO mice. CACNA2D2 knockout mice have spontaneous seizures and increased PTZ seizure susceptibility. Here we report similar findings, TSP1, and TSP1/2 KO mice have low levels of CACNA2D2 mRNA expression and α2δ−1/2 receptor level in the cortex, and are more susceptible to seizures. CACNA2D2 mutations in mice and humans can cause epilepsy. Our data suggests TSP1 in particular may control CACNA2D2 levels and could be a modifier of seizure susceptibility.

Keywords

seizure; ictogenesis; epileptogenesis; gene expression; voltage-gated calcium channel; mouse model

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Introduction

Epilepsy is a common disorder occurring in ~ 3% of the population and a common sequela of brain injury (Hesdorffer and Begley, 2013). Kindling is the process of neuronal networks developing recurrent spontaneous seizures. Epilepsy is thought to be an imbalance between excitatory and inhibitory neurotransmission. A large number of changes in gene expression and a variety of pathophysiological processes involving abnormal synaptic connectivity are likely to contribute to seizure sensitivity (Goldberg and Coulter, 2013).

Thrombospondins (TSPs) are a family of five genes, divided into two groups based on structural similarities; TSP1 and TSP2 are in group A and TSP3, TSP4, and TSP5 are in group B. TSP1-TSP4 are expressed throughout the central nervous system (CNS), though with different temporal and spatial expression (O'Shea et al., 1990, Iruela-Arispe et al., 1993, Lawler et al., 1993, Christopherson et al., 2005). Astrocyte-derived TSPs regulate excitatory synapse formation in the cerebellar cortex (Christopherson et al., 2007) and peripheral nervous systems (Kim et al., 2012; Hennekinne et al., 2013; Mendus et al., 2014) during development. TSP1 modulates presynaptic plasticity in hippocampal neurons (Crawford et al., 2012). Moreover, after stroke and injury to the mature cortex, levels of TSP1 and TSP2 are upregulated and appear to contribute to synaptic growth and remodeling (Lin et al., 2003; Liauw et al., 2008, Li et al., 2012).

All TSPs share epidermal growth factor (EGF)-like repeat domains shown to mediate synaptogenesis (Eroglu et al. 2009). TSP1-TSP5 are thought to bind to the α2σ subunit of the voltage-dependent calcium channel and regulate synapse formation through the extracellular Von Willebrand Factor A (VWF-A) repeat domains of this auxiliary calcium channel subunit (Eroglu et al., 2009). There is wide spread brain expression of three homologous α2δ subunits of the voltage dependent calcium channel (α2δ−1, α2δ−2, and α2δ−3), though little is understood about TSP1-TSP5 specificity for each homolog (Cole et al., 2005). TSP1, TSP2 and TSP1/2 KO mice have multiple systemic abnormalities but no one has reported an abnormal neurologic phenotype in uninjured mice (Adams and Lawler, 2011). It has been shown in chronic models of neocortical injury that TSPs increase, there is upregulation in mRNA of the TSP neuronal receptor CACNA2D1 and aberrant synaptogenesis, potentially contributing to development of post-traumatic epilepsy (Li et al., 2012). It is not known how TSP1 and TSP2 differ in promoting synaptic rearrangement and if they contribute to epilepsy following brain insult.

We tried to determine if TSP1 and/or TSP2 are necessary for the development of epilepsy in vivo by using a kindling model of epilepsy. Repeated injection of initially subconvulsive doses of pentylenetetrazole (PTZ) lead to induction of brief, focal seizures with increasing intensity of focal and tonic-clonic seizures. Once fully kindled, animals remain sensitive to further chemoconvulsants for their life span (Barton et al., 2001). The anti-seizure drugs gabapentin and pregabalin specifically bind to α2δ−1 and α2δ−2 subunits of the voltage-gated calcium channel and block their intracellular signaling; both drugs are potent inhibitors of PTZ kindling (Gee et al., 1996; Watanabe et al., 2010).

We hypothesized that mice lacking TSP1 and TSP2 would have delayed kindling possibly via reduced strengthening of excitatory synapses during kindling (Christopherson et al., 2005). To test our hypothesis we used mice lacking TSP1, TSP2 or both TSP1 and 2 (TSP1/2) to compare the rate of kindling to wild-type (WT) littermates. We found no delay in PTZ kindling, suggesting that lack of TSP1 and TSP2 does not slow the rate of kindling. Unexpectedly, mice lacking TSP1 had an increased sensitivity to PTZ and rapidly kindled in comparison to WT and TSP2 KO mice. We further explored how lack of TSP1 and TSP2 altered the expression of the neuronal thrombospondin receptor CACNA2D1 and CACNA2D2. Lack of TSP1, TSP2, or TSP1/2 caused a dramatic decrease of CACNA2D1 mRNA and mice specifically lacking TSP1 (TSP1 KO and TSP1/2 KO mice) had lower CACNA2D2 mRNA and α2δ−1/2 protein. This suggests that TSP1, with possible contributions from TSP2, regulates RNA and protein expression of their putative receptor targets α2δ−1/2. Loss of TSP1 had unexpected pro-ictogenesis consequences to PTZ.

Materials and methods

Experimental Animals

All animal care and experiments were carried out in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA) and protocols approved by the Stanford University Administrative Panel on Laboratory Animal Care. Animals were exposed to a 12 hour day/night cycle under controlled temperature and humidity with free access to food and water. All efforts were made to minimize the number of animals used and their suffering. Young adult male mice (7–8 weeks old) lacking TSP1 (Lawler et al., 1998), TSP2 (Kyriakides et al., 1998) or both TSP1/2 (Agah et al., 2002) and their WT littermates were used for all experiments. TSP1, TSP2 and TSP1/2 KO mice did not show any overt neurological phenotypes. Reported phenotypes of TSP KO mice have been described previously (Adams and Lawler, 2011).

PTZ administration and behavioral testing

Pentylenetetrazole (PTZ) was prepared daily and dissolved in saline before intra-peritoneal (IP) injection (Sigma-Aldrich, Corp., St. Louis, MO, USA). The PTZ dose was first tested on WT animals and a dose of 25 mg/kg was settled on as a subconvulsive dose. Animals were placed in transparent plastic chambers (18 cm wide, 30 cm long, 15 cm high) and their behavior was observed before PTZ treatment. After animals displayed resting posture they were injected with a single dose of 25 mg/kg PTZ IP. Animals were then observed and video recorded for next 30 minutes. Mice behavior was classified using the video by an observer blinded to genotype and scored base on the criteria that were previously used in other reports (Racine, 1971, Dhir, 2012): 0 – normal, 1 – immobilization and facial movements; 2 – head nodding; 3- short myoclonic jerks and bilateral forelimb movement; 4 – rearing, generalized clonic seizure; 5 – rearing and falling with loss of posture. After PTZ injection and 30 minute video recording mice were returned to their home cage and rested for 48 hours.. Mice were injected every 48 hours with PTZ till they were fully kindled. Mice were considered fully kindled at the time of the fifth stage V seizure. All mice were sacrificed three hours after their fifth stage V seizure.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Snap-frozen tissues of whole hippocampus or cortex were taken for RNA extraction with an RNAqueous®−4PCR Total RNA Isolation Kit (Ambion, Grand Island, NY, USA). Procedure was performed according to the manufacturer's protocol. RNA was measured using NanoDrop* Lite Spectrophotometer (Thermo Scientific, Wilmington, USA) and concentration adjusted to 100 ng/ml. Random samples (4 out of 10) were also analyzed with Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) for RNA integrity and purity. All tested samples had RNA integrity number nine and above. Extracted RNA was converted to cDNA with a TaqMan® Reverse Transcription Reagents (Applied Biosystems, Grand Island, NY, USA) using manufacturer protocol. The cDNA was measured with NanoDrop Lite Spectrophotometer (Thermo Scientific) prior to the polymerase chain reaction. Quantitative real-time PCR was carried out in triplicate using TaqMan® Gene Expression Assays (Applied Biosystems, Grand Island, NY, USA). The proprietary probes and primers for TSP1 (assay ID Mm01335418_m1), TSP2 (assay ID Mm01279240_m1), CACNA2D1 (Mm00486607_m1), and CACNA2D2 (Mm00457825_m1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Mm03302249_g1) was used as the internal standard. All Taqman qPCR assays were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using a MicroAmp® Fast Optical 96-Well Reaction Plate (Applied Biosystems). The accompanying StepOne v2.3 software and Microsoft Office Excel (Microsoft Corporation, Redmond, WA, USA) were used for data analysis. Data are shown as normalized expression of the gene of interest after comparative CT (ΔΔ CTq) analysis. Relative expression ratios of genes of interest in KOs compared to those in WT.

Western blot

Protein was isolated from snap-frozen whole hippocampus or cortex. Tissues were kept on ice and sonicated (25 mg of tissue was lysed in 250 μl of lysis buffer with protease and phosphatase inhibitor cocktails). The samples were incubated on ice for 30 minutes and then spun for 10 minutes at 14 rpm at +4°C. Protein concentration was measured using Pierce™BCA Protein Assay Kit (Thermo Scientific). Samples were diluted with lysis buffer to a final protein concentration of 4 mg/ml. Novex® NuPAGE® SDS-PAGE Gel System was used. After mixing with NuPAGE loading buffer and DTT samples were loaded into a NuPAGE® Bis-Tris Pre-Cast gel with final concentration of 40 mg per well. Proteins were separated for 90 minutes at 100V. Transfer was done using iBlot ®Dry Blotting System (Invitrogen, Grand Island, NY, USA) with a nitrocellulose membrane according to the manufacturer's instructions. Primary antibodies were: rabbit monoclonal anti-AKT1, RAC-alpha serine/threonine-protein kinase (phospho S473) antibody (#ab81283, Abcam, UK); rabbit polyclonal anti-AKT antibody (#9272, Cell Signaling Technology, Danvers, MA, USA); mouse monoclonal anti-a-tubulin antibody (#T5168, Sigma-Aldrich, Corp., St. Louis, MO, USA). Following the primary antibody, incubation blots were washed five times in 0.2% TWEEN 20 (Fisher Scientific Company, ON, Canada) in TBS buffer with vigorous shaking and incubated with secondary antibodies for 1 hour at room temperature. Secondary antibodies were: goat polyclonal anti-Mouse IgG (H+L) Secondary Antibody, HRP conjugate (#31430, Pierce Antibodies Thermo Scientific), donkey polyclonal anti-Rabbit IgG (H+L) Secondary Antibody, Peroxidase AffiniPure (#711035152, Jackson

ImmunoResearch Laboratories, West Grove, PA, USA). Western blots were visualized by enhanced chemiluminescence using either SuperSignal®West Pico Chemiluminescent Substrate Kit (Thermo Scientific) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Membranes were exposed on X-ray CL-Xposure films (Thermo Scientific).

Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA), StepOne v2.3 (Applied Biosystems), and Microsoft Office Excel (Microsoft Corporation) were used for data analysis. Image J software (NIH, USA) was used to measure the protein levels in Western blots. One way ANOVA with unpaired two-tailed *t* tests were used for comparisons between different groups. Error bars represent standard errors of the mean (SEMs). Significant *P* values in the text correspond to <0.05 (*) and <0.01 (**).

Results

Seizure sensitivity to PTZ is increased in TSP1 KO and TSP1/2 KO mice

To determine if lack of TSP1 or TSP2 leads to changes in response to PTZ kindling, we treated young adult male mice with PTZ every 48 hours and scored responses to repeated doses from mice lacking TSP1, TSP2, TSP1/2, or WT mice. We did not see differences in seizure activity between TSP2 KO and WT mice; it took on average eight injection of PTZ to develop generalized convulsive seizures in WT and TSP2 KO mice (Figure 1A). In contrast, TSP1 KO mice were highly sensitive to PTZ and developed stage V tonic-clonic seizures as early as the 2nd injection (Figure 1A). We decided to test TSP1/2 KO mice to see whether these mice have PTZ sensitivity similar to TSP1 KO. We hypothesize that TSP2 overexpression in the TSP1 KO mice might compensate for lack of TSP1, creating increased PTZ sensitivity. TSP1/2 KO mice had increased sensitivity to PTZ kindling similar to the TSP1 KO.

Deletion of TSP1 or TSP2 did not result in a compensatory upregulation of the remaining group A TSP family member

Previous experiments with TSP1 KO and TSP2 KO mice have shown that these genes might compensate for each other's loss (Christopherson et al., 2005). To see whether loss of TSPs compensate within group A of the TSP family, we measured expression of TSP1 and TSP2 in TSP2 KO and TSP1 KO mice, respectively, and compared this expression to WT mice (Figure 2). We saw similar levels of TSP1 and TSP2 gene expression in TSP2 KO and TSP1 KO mice, respectively as compared to WT mice. This was true for both hippocampus (data not shown) and cortex. Expression of GAPDH was used as the internal standard to confirm sample integrity (data not shown).

TGF B signaling is not altered in TSP1 and TSP1/2 KO mice

TSP1 but not TSP2 is responsible for the majority of transforming growth factor β1 (TGFβ −1) activation in vivo (Schultz-Cherry et al., 1994; Schultz-Cherry et al., 1995; Crawford et al., 1998; Ribeiro et al., 1999). Moreover, mutants lacking TGF-β1 activation had similar overall phenotypes to TSP1 KO mice (Crawford et al., 1998). TGF-β1 can be protective

against the degeneration of neurons in the hippocampus after seizures (Li et al., 2013). As TGF-β1 activation leads to increase in nuclear phosphorylation of AKT1, we measured phospho-AKT1 over total AKT1 ratio in brain tissues of mice lacking TSPs and compared the result to same ratio in WT mice (Figure 3). We did not observe a difference in the level of AKT1 phosphorylation between TSP1 KO, TSP2 KO, TSP1/2 KO, and WT mice cortex (Figure 3). Overall we could not find evidence for a difference in TGF-β signaling between the TSP1 KO and TSP2 KO mice.

Decreased CACNA2D1/D2 in cortex of mice lacking TSP1 and TSP2

Previously, mice lacking the CACNA2D2 gene, a putative TSP receptor, were found to be extremely sensitive to PTZ. Absence of CACNA2D2 causes epilepsy in mice and a severe form of epilepsy in children (Ivanov et al., 2004; Edvardson et al., 2013; Pippucci et al., 2013). We performed qPCR test to measure expression levels of CACNA2D1 and CACNA2D2 in the brains of TSP1, TSP2 and TSP1/2 KO and WT mice (Figure 4). We saw a decrease in CACNA2D1 in the brains of all the knockout mice but a reduction in CACNA2D2 in only the TSP1 and TSP1/2 KO mice (Figure 4A and B). This indicates that lack of TSP2 suppresses mRNA expression of CACNA2D1 and lack of TSP1 suppresses both CACNA2D1 and CACNA2D2 mRNA. We looked at the protein level of these receptors (Figure 5) and saw significantly reduced α2δ−1/2 receptor level in the cortex of TSP1/2 mice, while TSP2 mice had α2δ−1/2 receptor level similar to WT mice. Unfortunately, we were not able to look at individual levels of α2δ−1 and α2δ−2 because of antibody cross reactivity (Dolphin, 2013).

Discussion

Here we report increased PTZ seizure sensitivity in mice lacking TSP1, while TSP2 KO animals had PTZ induced seizures similar to WT mice. There was no compensatory up regulation in TSP1 and TSP2 mRNA in the cortex of the TSP2 and TSP1 KO mice respectively. TSP1, TSP2, and TSP1/2 KO mice had down regulation of the CACNA2D1 mRNA compared to wild-type mice. TSP1 and TSP1/2 KO mice had a down regulation of CACNA2D2 mRNA and decreased expression of the α2δ−1/2 protein in the cortex compared to WT and TSP2 KO mice.

PTZ has multiple potential mechanisms for seizure induction, including $GABA_A$ inhibition, increased glutamate receptor surface expression, and activation of voltage gated Ca^{2+} channels (Hassan et al., 1999; Omrani et al. 2003; Hansen et al., 2004). The anti-epileptic drug gabapentin binds to the TSP receptor α2δ−1 and α2δ−2, blocking α2δ subunit activity of the voltage gated calcium channel. Gabapentin is a potent suppressor of PTZ induced seizures in rodents (Gee et al., 1996; Field et al., 2006; Maneuf, Luo and Lee, 2006). The α2δ subunits help direct and stabilize the voltage gated calcium channels to the presynaptic region and drive synaptic vesicle release (Hoppa et al., 2012). CACNA2D1 is expressed ubiquitously through different tissues and has robust expression in neuronal tissues and excitatory cells; CACNA2D2 is highly expressed in GABAergic interneurons (Ellis et al., 1988; Gong et al., 2001; Cole at al., 2005).Transgenic deletion of CACNA2D2 causes seizures and increases PTZ seizure sensitivity (Barclay et al., 2001; Brodbeck et al., 2002;

Brill et al., 2004; Ivanov et al., 2004). TSP1 deletion in our study caused a decrease in CACNA2D2 mRNA, α2δ−1/2 protein, and increased seizure sensitivity to PTZ though no direct causal relationship could be proven.

An additional mechanism for TSP1 loss to contribute to hyperexcitability is a study showing TSP1 can cause muting, a mechanism of presynaptic silencing of glutamate synapses on hippocampal neurons *in vitro* (Crawford et al., 2012). However, the study only looked at TSP1 and did not study whether TSP2 has a similar ability to cause muting. Overall TSP1 and TSP2 may play distinct and overlapping roles in regulating synapse formation and function, but only loss of TSP1 caused increased sensitivity to PTZ seizures.

Unexpectedly TSP1 deletion contributes to down regulation of CACNA2D1 and CACNA2D2 mRNA and TSP2 deletion caused down regulation of CACNA2D1 mRNA. Why loss of a ligand causes decreased receptor is unclear. We looked for but found no compensatory up-regulation TSP1 and TSP2 mRNA in TSP2 and TSP1 KO mice, respectively, and a similar decrease of CACNA2D1 and CACNA2D2 mRNA expression in the TSP1/2 KO animals. *In vitro*, TSPs cause synapse induction and synaptic maturation, which might include regulation of the genes involved in induction of the presynaptic voltage gated calcium channels (Eroglu et al., 2009). Four α2δ subunits of the voltage gated calcium channels have been cloned, CACNA2D1-CACNA2D4, with first 1,2 and 3 genes having been shown to be highly express in neuronal tissues (Cole et al., 2005). Bioinformatic analysis of $\alpha_2\delta$ sequences shows that all $\alpha_2\delta$ subunits contain certain domains, including a Von Willebrand Factor A (VWF-A or VWA) domain (Whittaker et al., 2002) that all TSPs may bind. The specificity and affinity of TSPs for specific $\alpha_2\delta$ isoforms needs to be more carefully studied and could have important therapeutic implications.

Gabapentin and pregabalin are used as antinociceptive and antiseizure drugs and bind to the $\alpha_2\delta$−1 and $\alpha_2\delta$−2 receptors. Mutation of the gabapentin or pregabalin interaction site of the $\alpha_2\delta$−1 receptor causes decreased efficacy for treating pain and preventing seizures (Field et al. 2006). Due to a down regulation of CACNA2D1 mRNA in the TSP1, TSP2, and TSP1/2 KO mice they should be less sensitive to gabapentin blockade of PTZ induced seizures, though those experiments need to be carried out in the future to test this hypothesis. In contrast, mutating the gabapentin $\alpha_2\delta$−2 receptor binding site had minimal impact on pain or seizure therapy, suggesting that suppressing $\alpha_2\delta$−2 is not the therapeutic mechanism of gabapentin. Several mouse models with loss of CACNA2D2 result in epilepsy phenotypes and several families have been reported with CACNA2D2 mutations and severe epilepsy (Barclay et al., 2001; Brodbeck et al., 2002; Brill et al., 2004; Ivanov et al., 2004; Edvardson et al., 2013; Pippucci et al., 2013). Mechanistically inhibiting $\alpha_2\delta$−1 and increasing $\alpha_2\delta$−2 activity might be a better mechanism for treating epilepsy.

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Abbreviations

CNS	central nervous system
EGF	epidermal growth factor
IP	intraperitoneal injection
KO	knock out
PTZ	pentylenetetrazole
PNS	peripheral nervous system
qPCR	quantitative polymerase chain reaction
TSP	thrombospondin
TGF-β	transforming growth factor β
VWF-A	Von Willebrand Factor
WT	wild-type
AKT-1	RAC-alpha serine/threonine-protein kinase

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1. TSP1 KO mice had increased PTZ kindling sensitivity
2. TSP2 KO and wild-type mice had similar rates of PTZ kindling
3. TSP1 KO mice had decreased brain expression of α2δ
4. Thrombospondins appear to regulate expression of their neuronal receptors in vivo

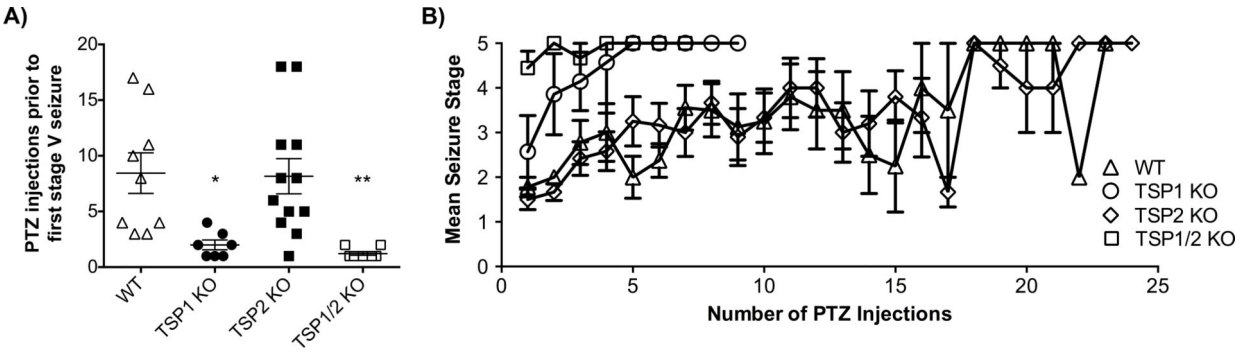

Figure 1. Pentylenetetrazole kindling of mice lacking TSP1, TSP2, TSP1/2 and WT controls.

There was increased sensitivity to PTZ in TSP1 KO and TSP1/2 KO mice compared to WT and TSP2 KO mice. (A) Number of PTZ injection to reach first stage V seizure in mice with different genotype. Average number of injections is shown for WT (n=10), TSP1 KO (n=7), TSP2 KO (n=12), TSP1/2 KO (n=9). (B) Mean seizure stage in mice with different genotype: WT is shown as open triangle, TSP1 KO open circle, TSP2 KO open square and TSP1/2 KO open square. The X axis represents number of PTZ injection. Number of mice at each time point decreased as the animals became fully kindled (5 stage V seizures). Results are expressed as mean +/− SEM. Significant difference: ANOVA *P < 0.05, **P < 0.01.

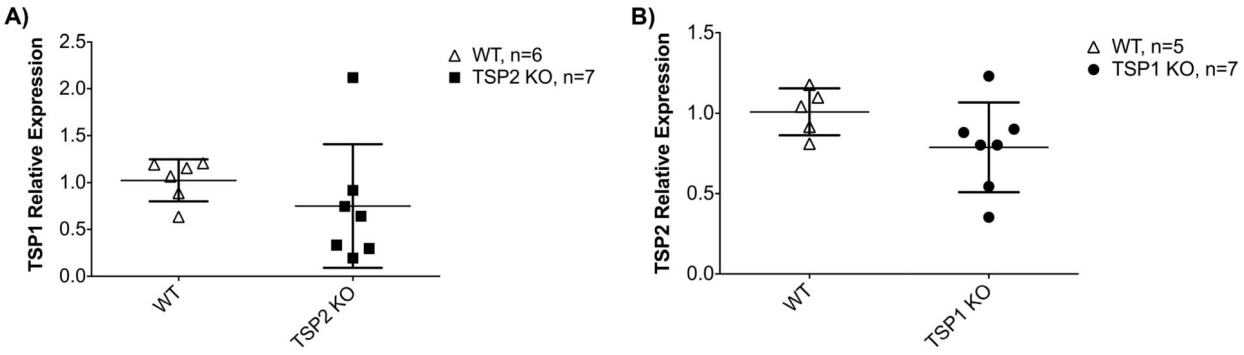

Figure 2. mRNA expression of TSP1 and TSP2 in PTZ kindled mice.

Levels of TSP1 (A) and TSP2 (B) mRNA in cortex of either WT, TSP1 KO or TSP2 KO mice after they were fully kindled. Results are expressed as mean +/− SEM.

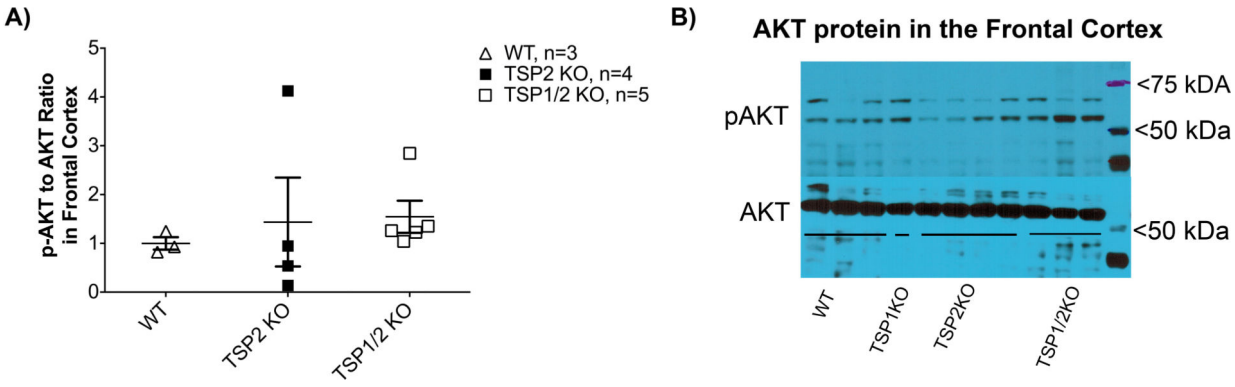

Figure 3. AKT signaling in cortex of PTZ kindled mice.

(A) Phospho-AKT to AKT ratio in cortex of WT and TSP1, TSP2 and TSP1/2 KO kindled mice. (B) Representative blots for 10 μg of protein from cortex of different genotype. Results are expressed as mean +/− SEM.

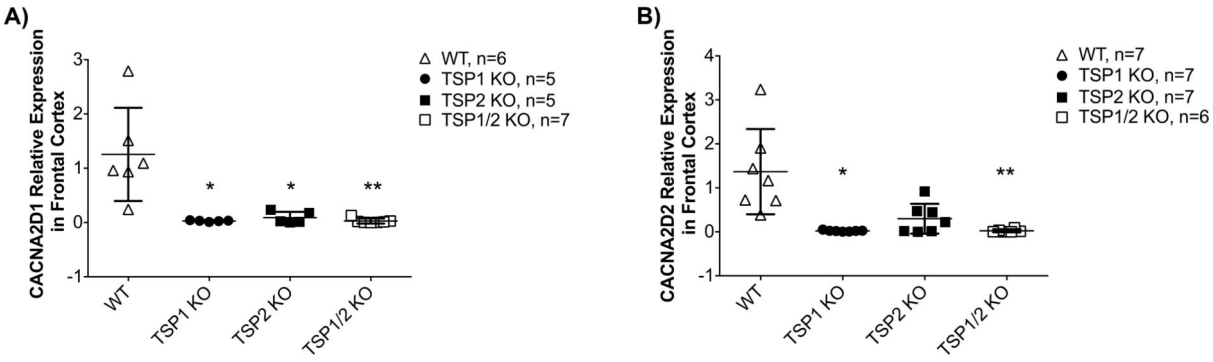

Figure 4. Expression of CACNA2D1 and CACNA2D2 mRNA in cortex of PTZ kindled mice.

(A) CACNA2D1, (B) CACNA2D2. Results are expressed as mean +/− SEM. Significant difference: ANOVA *P < 0.05, **P < 0.01.

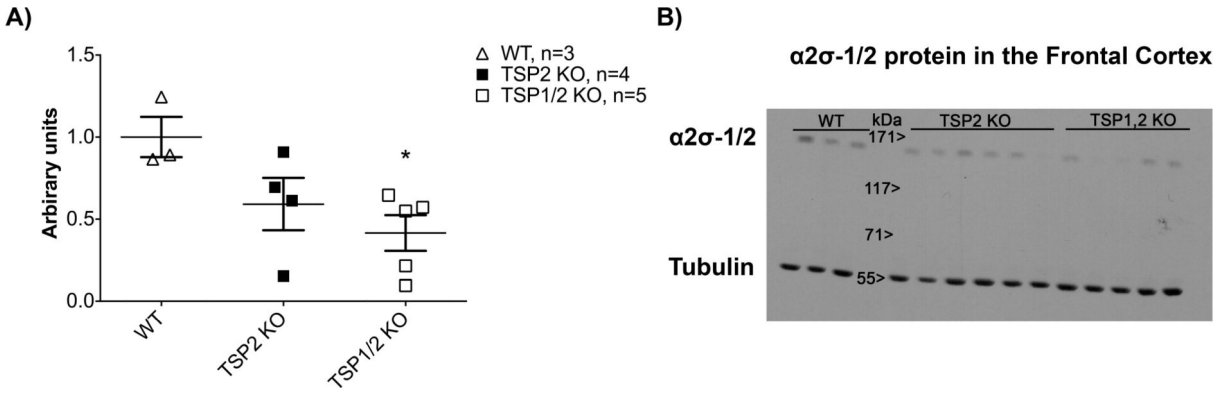

Figure 5. α2δ−1/2 receptor protein measurement in WT and TSP2 and TSP1/2 KO mice.

(A) Relative expression of α2δ−1/2 protein in cortex of PTZ –kindled mice. (B) Representative cortex protein immunoblots. Results are expressed as mean +/− SEM. Significant difference: ANOVA *P < 0.05.