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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 were 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 suggest TSP1 in particular may control CACNA2D2 levels and could be a modifier of seizure susceptibility. © 2015 Published by Elsevier Ltd. on behalf of IBRO.

Key words: seizure, ictogenesis, epileptogenesis, gene expression, voltage-gated calcium channel, mouse model.

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 expressions (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 the development of post-traumatic epilepsy (Li et al., 2012). It is not known how TSP1 and TSP2 differ

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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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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 injections 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 TSP 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.

EXPERIMENTAL PROCEDURES

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-h 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 the next 30 min. 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, 1972; 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 a 30-min video recording mice were returned to their home cage and rested for 48 h.. Mice were injected every 48 h 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 (four 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 Reagent (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.

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