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Maintenance of the innate seizure threshold by cyclooxygenase-2 is not influenced by the translational silencer, T-cell intracellular antigen-1

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Abstract

Activity of neuronal cyclooxygenase-2 (COX-2), a primary source of PG synthesis in the normal brain, is enhanced by excitatory neurotransmission and this is thought to be involved in seizure suppression. Results herein showing that the incidence of pentylenetetrazole (PTZ)-induced convulsions is suppressed in transgenic mice overexpressing COX-2 in neurons supports this notion. T-cell intracellular antigen-1 (TIA-1) is an mRNA binding protein that is known to bind to COX-2 mRNA and repress its translation in non-neuronal cell types. An examination of the expression profile of TIA-1 protein in the normal brain indicated that it is expressed broadly by neurons, including those that express COX-2. However, whether TIA-1 regulates COX-2 protein levels in neurons is not known. The purpose of this study was to test the possibility that deletion of TIA-1 increases COX-2 expression in neurons and consequently raises the seizure threshold. Results demonstrate that neither the basal nor seizure-induced expression profiles of COX-2 were altered in mice lacking a functional Tia1 gene suggesting that TIA-1 does not contribute to regulation of COX-2 protein expression in neurons. The acute PTZ-induced seizure threshold was also unchanged in mice lacking TIA-1 protein, indicating that this promiscuous RNA binding protein does not influence the innate seizure threshold. Nevertheless, the results raise the possibility that the level of neuronal COX-2 expression may be a determinant of the innate seizure threshold and suggest that a better understanding of the regulation of COX-2 expression in the brain could provide new insight into the molecular mechanisms that suppress seizure induction.

Graphical abstract

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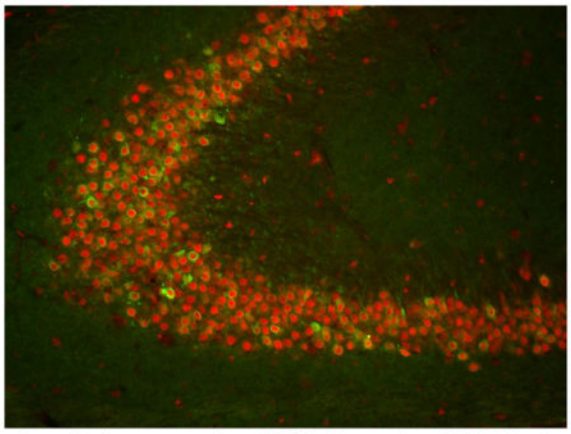

Constitutive expression of COX-2 (green) and TIA-1 (red) in CA3 pyramidal cells

Hypothesis:

The normal level of COX-2 mRNA translation in neurons is controlled by the translational repressor, TIA-1

Keywords

T-cell intracellular antigen-1; Cyclooxygenase-2; Pentylenetetrazole; Seizures; Seizure threshold; gene expression

INTRODUCTION

Cyclooxygenase-2 (COX-2) is one of two isoforms of COX enzymes that catalyze the first committed step in the metabolism of arachidonic acid (AA) to prostaglandins (PGs)(Smith et al., 2000). PGs are potent paracrine and autocrine lipid mediators that contribute to a broad spectrum of physiological and pathophysiological activities that range from gastric acid secretion and blood flow to inflammation and tumor growth. In general, whereas COX-1 is constitutively expressed throughout the body, the most prominent COX-2 expression under normal conditions outside of barrier (gastrointestinal) and immune (thymus) tissues is found in the kidneys and brain (Kirkby et al., 2016). In the normal brain, the activity of COX-2 contributes to physiological functions, including regulation of neuronal excitability and plasticity (Hewett et al., 2006). Under pathophysiological conditions, it may be detrimental or beneficial depending on the cellular source and context of the extracellular milieu. For instance, whereas it promotes neurodegeneration caused by excitotoxicity (Hewett et al., 2000; Gobbo and O'Mara, 2004; Kawaguchi et al., 2005), it reduces acute seizure severity and incidence of convulsions. In the latter case, COX-dependent PG production increases rapidly and transiently in the brain following acute convulsive seizures and pharmacological inhibitors of COX-2 activity or inactivation of the COX-2 gene (PTGS2) reduce the innate seizure threshold (Steinhauser and Hertting, 1981; Baik et al., 1999; Kunz and Oliw, 2001; Toscano et al., 2008; Claycomb et al., 2012), an effect that is thought to be due to the loss of anticonvulsant PGs (Förstermann et al., 1982; S.

Akarsu et al., 1998). These observations suggest that the COX-2 pathway of AA metabolism contributes to the maintenance of an elevated seizure threshold in the normal brain.

COX-2 is expressed constitutively in discrete subpopulations of glutamatergic neurons of the normal central nervous system (CNS), where it is localized to somata and dendritic arborizations, particularly in the primary neurons of the CA3 region of the hippocampus, cortical layers 2/3, and lateral amygdala (Yamagata et al., 1993; Kaufmann et al., 1996; Joseph et al., 2006). Neuronal expression is coupled to endogenous postsynaptic N-Methyl-D-Aspartate type glutamate receptor (NMDAR) activity and as such, can be modified by changes in excitatory neuronal activity. In this regard, antagonism of the NMDAR or deletion of its gene suppresses basal COX-2 mRNA expression by cortical neurons both in the brain and in culture (Yamagata et al., 1993; Hewett et al., 2016). Moreover, neuronal COX-2 mRNA and protein expression in the brain are upregulated strongly in response to excitatory stimulation (Yamagata et al., 1993).

Control of COX-2 expression is complex, involving transcriptional, posttranscriptional, and posttranslational mechanisms (Tanabe and Tohnai, 2002; Kang et al., 2007). While the regulation of COX-2 expression has been studied extensively in non-neuronal cell types, much less is known about its regulation in neurons. Numerous transcriptional regulatory proteins are known to bind to *cis*-acting elements in the 5′ regulatory region of the COX-2 gene and recent evidence from cultures of cortical neurons indicates that basal COX-2 expression is modulated transcriptionally by both Cyclic AMP Response Element Binding protein and Sp-1 (Hewett et al., 2016). The 3′ untranslated region (3′UTR) ofCOX-2 mRNA is a second regulatory region that has the capacity to bind numerous RNA binding proteins (RBPs), which can affect stability and/or translation of the transcript (Dixon et al., 2000). T-cell intracellular antigen-1 (TIA-1) is one such RBP. In non-neuronal cell types, inactivation of the TIA-1 gene (Tia1) was associated with an increase in COX-2 protein expression indicating that TIA-1 functions to repress COX-2 mRNA translation (Dixon et al., 2003; Phillips et al., 2004). Whether TIA-1 performs a similar function in the regulation of COX-2 expression in neurons has yet to be explored.

The goal of the present study was to test the possibility that TIA-1 contributes to COX-2 expression in neurons and by doing so influences the innate seizure threshold. Specifically, because inhibitors of COX-2 activity lower the acute seizure threshold and TIA-1 deletion enhances COX-2 expression, it is reasoned that inactivation of the TIA-1 gene will increase neuronal COX-2 expression in the brain and consequently will increase resistance to acute seizures. This hypothesis presupposes, however, that seizure threshold can be elevated by increasing expression of COX-2 in neurons and that TIA-1 regulates COX-2 expression in neurons. The former was examined herein by transgenic overexpression of COX-2 in neurons and the latter was assessed by genetic inactivation of the TIA-1 gene.

EXPERIMENTAL PROCEDURES

Animal Housing

Mice were housed on a 12h light/dark schedule in the vivarium at Syracuse University, which is accredited by the Association for Assessment and Accreditation of Laboratory

Animal Care International. Standard mouse chow and water were provided *ad libitum*. Except for breeding units, mice were housed three to five per cage. All animal procedures conformed to the National Academy of Sciences *Guide for the Care and Use of Laboratory Animals* (NRC, 2011) and were approved by the Syracuse University Institutional Animal Care and Use Committee (IACUC).

Animal breeding

The COX-2 overexpressing transgenic mouse line (L300), which employs a neuron-specific fragment of the Thy-1 gene promoter to drive expression of a human COX-2 transgene, was provided by Katrin Andreasson (Stanford University Medical Center). Brain COX-2 expression levels in this mouse line were reported to be several fold higher relative to their non-transgenic counterparts and this was accompanied by a 4 to 10-fold increase in basal levels of several PG in the brain (Andreasson et al., 2001; Vidensky et al., 2003). Hemizygous L300 mice were bred with wild-type C57BL/6J mice in the Syracuse University vivarium to obtain male littermates for studies. The Tia1 gene mutant mouse line ($Tia1^{-/-}$) was obtained from cryostorage at The Jackson Laboratory (stock # 009248) and a colony established by crossing heterozygous founder mice ($Tia1^{+/-}$) with their wild-type ($Tia1^{+/+}$) C57BL/6J counterparts (stock #000664). $Tia1^{+/+}$ and $Tia1^{-/-}$ littermates were generated for studies by crossing $Tia1^{+/-}$ breeding pairs from this colony. Genotyping of both mouse lines was performed at weaning by PCR analysis of genomic DNA isolated from tail biopsies. To ensure the integrity of the C57BL/6J genetic background, lines were backcrossed with wild-type female C57BL/6J mice from The Jackson Laboratory every 3–5 generations. A total number of 110 adult male mice were used in all studies. Specific numbers of animals for individual studies are reported in corresponding figure legends.

Dosing Paradigms

The acute PTZ-induced seizure model was used to examine changes in the innate seizure threshold. All mice were acclimated to handling for 5–7 days prior to studies. Injection solutions were prepared freshly by dissolving pentylenetetrazole (PTZ; Sigma Chemical Co., St. Louis, MO) in 0.9% saline, which was sterilized by filtration and administered in a volume of 10 ml/kg body weight, i.p. Male littermates were randomly segregated at weaning without knowledge of genotype and treated with a single dose of PTZ or saline vehicle between 8–12 weeks of age. Seizure activity was monitored for 25 minutes after PTZ administration and graded by an observer blinded to genotype using a previously established scoring system (Claycomb et al., 2011): **stage 0**, normal behavioral activities; **stage 1**, hypoactivity; **stage 2**, ≥ two isolated myoclonic jerks; **stage 3**, generalized clonic convulsions with preservation of righting; **stage 4**, generalized clonic or tonic clonic convulsions with loss of righting. Maximal seizure score and latency to seizure onset were recorded for individual mice and the incidence of convulsions (stage 3 or 4) in a given cohort was determined by the ratio of # of mice convulsed per total # treated.

Immunofluorescence staining

Mice were deeply anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine administered i.p. in saline and perfused transcardially with 0.05 M phosphate-buffered saline

(PBS) followed by 4% paraformaldehyde fixative (PFA) in PBS. After post-fixing at 4° C for 8–16 hours in PFA, brains were saturated with 20% sucrose in PBS and rapidly frozen on dry ice prior to storage (−80° C). Serial 12 μm thick coronal sections were cut within 1.34 to 2.06 mm posterior to bregma using a cryostat (Microm HM550, Thermo Scientific) and mounted on microscope slides prior to storage at −20° C. Sections were washed in PBS and incubated in PBS containing 0.25% Triton-X100 (PBT). Permeabilized sections were incubated overnight at 4° C in blocking solution containing PBT with 5% normal donkey serum (Jackson ImmunoResearch Inc.) and 1% bovine serum albumin (BSA; Sigma, A2153) then incubated overnight at 4° C with antigen-specific primary antibody diluted in PBT containing 1% normal donkey serum and 1% BSA. After washing 5 times with PBT, sections were incubated at 20° C with secondary antibody for 4 hours, washed again, then incubated for 5 minutes with PBS containing 1 μg/ml DAPI (Life Technology). Images were captured with ZEN 2 software (Version 2.0.0.0) using an Axio Imager. A2 microscope fitted with an AxioCam MRc digital camera (Zeiss United States). Images from individual studies were processed identically using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA).

Primary and secondary antibodies for immunostaining were goat anti-TIA-1 (1 μg/ml, Santa Cruz Biotechnology Cat# sc-1751, RRID:AB_2201433), rabbit anti-COX-2 (1:200, Cayman Chemical Cat# 160126-1, RRID:AB_327872), mouse anti-GFAP (1:50, Genetex, GeneTex Cat# GTX73615, RRID:AB_378828), Alexa Fluor 594 donkey anti-Goat IgG (7.5 μg/ml, Jackson ImmunoResearch Labs Cat# 705-585-147, RRID:AB_2340433), Alexa Fluor 488 AffiniPure Donkey anti-rabbit IgG (7.5 μg/ml, Jackson ImmunoResearch Labs Cat# 711-545-152, RRID:AB_2313584), and Alexa Fluor 488 AffiniPure Donkey anti-mouse IgG (7.5 μg/ml, Jackson ImmunoResearch Labs Cat# 715-545-150, RRID:AB_2340846). Specificity of the anti-TIA-1 antibody was confirmed using comparable sections from $Tia1^{+/+}$ and $Tia1^{-/-}$ mice processed in parallel. For GFAP/TIA-1 double staining, sections were processed for antigen-retrieval as described (Abcam, http://www.abcam.com/protocols/ihc-antigen-retrieval-protocol).

Quantification of fluorescent intensity

Images were converted to 8 bit grayscale and fluorescent intensity quantified using NIH Image J, version 1.50i (Schneider et al., 2012). For each brain, two images were taken from coronal sections at −1.7 ± 0.12 relative to bregma. Maximal threshold was set to 255 and minimal threshold was set to 55 (TIA-1) or 40 (COX-2), using the default auto thresholding method. The mean integrated density in 8 non-overlapping regions of interest (ROIs) with fixed size (100 x 100 $pixel^2$) was calculated for CA1 and CA3 pyramidal and DG granule cell layers of the hippocampal formation. Results were averaged across duplicate sections from each brain.

Neuron and astrocyte culturing

Pure cultures of cortical neurons and astrocytes were prepared from gestational day 15 C57BL/6J fetuses or postnatal day 1–3 pups, respectively, as detailed previously (Hamby et al., 2006; Hewett et al., 2016). For cortical neuron cultures, single cell suspensions were plated at a density of ~5.5 hemispheres/plate on 24 well polyethyleneimine-coated tissue

culture dishes (400 μL/well). Cultures were exposed to cytosine arabinoside (Sigma, St. Louis, MO, 1μM) over *days in vitro* (DIV) 2–4 to inhibit growth of glial cells and used for experimentation on DIV 7. For cortical astrocyte cultures, single cell suspensions were plated at a density of two hemispheres/plate on Falcon Primaria 24 well culture plates (BD Biosciences, Lincoln Park, NJ). Upon reaching confluence, cultures were treated with cytosine arabinoside (8 μM) for 4 to 6 days then with L-leucine methyl ester (50–75 mM, 30–90 minutes) one day before experimentation on DIV 30 (Hamby et al., 2006).

Measurement of TIA-1 mRNA expression

TIA-1 mRNA was measured in lung, spleen, liver, kidney, heart, and brain from adult male C57BL/6J mice (8 weeks old), as well as from cultured cortical neurons (DIV 7) and cortical astrocytes (DIV 30). Total RNA was extracted from homogenized tissue or cell samples using TRIZOL reagent (Invitrogen, Carlsbad, CA) and first-strand cDNA synthesis was performed as described previously (Hewett et al., 1999). TIA-1 mRNA levels were measured using real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) as follows: cDNA was subjected to qRT-PCR in a 20 μL singleplex reaction containing either mouse TIA-1 (Mm00441742_m1) or mouse β-actin (Mm01205647_m1) Assay-On-Demand probes along with TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. The TIA-1 assay detects mRNA splice variants 1a and 1b that contain or lack exon 5, respectively. Reactions were performed using Mastercycler ep RealPlex2 Real-Time PCR System (Eppendorf North America) and expression assessed using the comparative cycle threshold method (ΔΔCT), where CT values of the transcript of interest were normalized to β-actin CT values from the same sample to derive ΔCT. These values were compared to a calibrator ΔCT value (lung or astrocyte) to determine the relative fold increase in mRNA.

Statistical analysis

All statistical analyses were performed using GraphPad Prism, Version 4.03 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). For qRT-PCR, statistics were performed on the logarithmic transformation of $2^{-\Delta\Delta CT}$ values. TIA-1 mRNA tissue expression was analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. TIA-1 mRNA expression between astrocytes and neurons was analyzed by unpaired t-test. Quantitative protein expression of TIA-1 or COX-2 immunoreactivity was analyzed by two-way ANOVA on the logarithmic transformation of gray values. Latency to convulsion after acute PTZ injection between genotypes was assessed using two-tailed Mann Whitney test. Datasets representing proportions (percentage mortality or incidence of convulsions) were analyzed using a two-tailed Fisher's exact test. Significance was set at $p < 0.05$. In figure legends, p values ≥ 0.0001 were reported as their exact values, whereas p values that are less than 0.0001 were reported as "$p < 0.0001$". For fisher's exact test and Mann Whitney test, only p values were reported. For one-way ANOVA, two-way ANOVA, Brown-Forsythe test and F-test for equal variance, in addition to p values, F values were reported as F(dfn, dfd) where dfn is the numerator of df (degree of freedom) and dfd is the denominator of df. For unpaired t-test, t values and df were reported in addition to p values.

RESULTS

Effect of neuronal COX-2 overexpression on acute seizure threshold

Constitutive overexpression of COX-2 in neurons of L300 transgenic mice brains was confirmed herein (Fig. 1). Compared to non-transgenic mice, COX-2 immunoreactivity was elevated in neuronal populations that endogenously express mouse COX-2 constitutively, including cortical layer 2/3 pyramidal neurons and pyramidal neurons of the CA3 region of the hippocampus. In addition, no COX-2 expression was observed in granule neurons of the dentate gyrus (DG), which do not express endogenous COX-2 in the normal C57BL/6J mouse brain. An examination of the seizure threshold in these mice demonstrated that the incidence of PTZ-induced convulsive seizures was markedly reduced compared to non-transgenic littermates (Fig. 2A). Thus, only 2 of 17 L300 mice (12%) with high levels of neuronal COX-2 had convulsive seizures after PTZ administration compared to 14 of 19 non-transgenic mice (74%) with normal levels of neuronal COX-2. The convulsions elicited in the two L300 mice were indistinguishable from their non-transgenic littermates. In this regard, the latencies to convulsions between the two genotypes were similar (Fig. 2B) and all convulsions regardless of genotype resolved spontaneously within one minute from onset, after which the animals entered a postictal state of hypomobility. These results suggest that the level of COX-2 expression by neurons is inversely related to seizure threshold.

TIA-1 mRNA expression profile in the brain

Prior to assessing the role of TIA-1 in the regulation of neuronal COX-2 expression and seizure threshold maintenance, a comprehensive analysis of its expression profile in the brain was performed. High levels TIA-1 mRNA were found in the normal brain. Indeed, a quantitative analysis of its tissue distribution demonstrated that the relative expression in various tissues was brain > heart > kidney > liver ≈ spleen ≈ lung (Fig. 3A). Within the brain, high levels of TIA-1 mRNA were detected in the cerebral cortex and hippocampus. A comparison between cultures of cortical neurons and astrocytes showed that neurons expressed 4-fold higher TIA-1 than astrocytes (Fig. 3B), suggesting that neurons are a primary contributor to TIA-1 mRNA expression in this brain region.

TIA-1 protein expression profile in the brain

To examine in detail the distribution of constitutive TIA-1 expression in the brain, a comprehensive analysis of protein expression was performed. Prior to performing this analysis, the specificity of the TIA-1 antibody was assessed in the mouse brain (Fig. 4). Strong TIA-1 protein expression was detected in the dentate gyrus (DG) of wild-type mice possessing two functional Tia1 gene alleles ($Tia1^{+/+}$). This was reduced in brains from mice containing only one functional allele of the Tia1 gene ($Tia1^{+/-}$). No staining was detected in brains from mice in which both of the Tia1 gene alleles were inactivated ($Tia1^{-/-}$). This gene dosage effect confirms the specificity of the TIA-1 antibody and verifies the genotype of Tia1 mutant mice at the protein level.

TIA-1 protein was expressed broadly in the brain under basal conditions (Fig. 5 and 6). Widespread neuronal expression was observed throughout the cerebral cortex, thalamus, hypothalamus, amygdala, and caudoputamen (Fig. 5A). It is also noteworthy that whereas

many cells of the medial habenula express TIA-1, many fewer cells express the protein in the lateral habenula. Particularly high constitutive levels were detected in the hippocampal formation, where the primary excitatory neurons of the CA3 and CA1, as well as the DG, strongly express TIA-1 protein (Fig. 5B–D). Although the protein appeared to be concentrated in the nucleus of these cells, low levels were also detected in the cytoplasm of neurons in the less densely populated CA3 layer (Fig. 5D). A small number of cells outside of the primary cell layers expressed TIA-1 protein as well, including within the hilus of DG (Fig. 5C). A subset of TIA-1-positive cells appeared to co-express GFAP (Fig. 6), consistent with mRNA expression in astrocyte cultures (Fig. 3B). TIA-1 expression was detected in layers 2–6 of the somatosensory cerebral cortex (Fig. 7A) and particularly prominent expression was observed in layer 2 of the retrosplenial and piriform cortical regions (Fig. 7B and C, respectively). Scattered TIA-1-positive cells, possibly oligodendrocytes and/or white matter astrocytes, were detected in the myelinated fiber tracts of the fimbria and corpus callosum (Fig. 7E and F, respectively). Expression was also detected in numerous other brain regions, including choroid epithelium, caudoputamen, hypothalamus, and amygdala (Fig. 7G–J, respectively). These observations are in agreement with the TIA-1 mRNA profile in the normal mouse brain (Allen Brain Atlas (Tia1 - RP_041122_01_B01 sagittal, © 2004, Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: http://mouse.brain-map.org/). Finally, the level of TIA-1 protein expression was not altered by a convulsive stimulus, suggesting that its expression is not linked to excitatory neuronal activity (Figs. 8 and 10A).

Effect of Tia1 gene deletion on neuronal COX-2 protein expression in the brain

Next, the expression profile of COX-2 protein in the hippocampus of $Tia1^{+/+}$ mice was compared to $Tia1^{-/-}$ littermates. In $Tia1^{+/+}$ mice under basal conditions, constitutive expression of COX-2 protein was detected in only a few granule neurons of the DG, whereas it was broadly expressed by pyramidal neurons of the CA3 region (Fig. 9A and E, respectively). This expression pattern was modified following a PTZ-induced convulsion. Specifically, expression was induced strongly in neurons of the DG and enhanced in CA3 neurons (Fig. 9C and G, respectively). Importantly, this expression profile was not altered in mutant mice lacking TIA-1 as hypothesized. Thus, the COX-2 expression patterns in the DG and CA3 regions of $Tia1^{+/+}$ and $Tia1^{-/-}$ mice were comparable under both basal conditions and after the PTZ-induced convulsion (Fig. 9B/F and 9D/H, respectively). Note that COX-2 protein expression was undetectable in the pyramidal neurons of the CA1 in both genotypes and this was not changed following an acute PTZ-induced convulsion (data not shown). Together, these results indicate that TIA-1 does not control COX-2 expression in neurons under normal conditions or in response to a marked increase in excitation induced by inhibition of endogenous GABA neurotransmission by PTZ. Quantitative analysis of the expression changes further supports this contention (Fig. 10B).

Effect of Tia1 gene inactivation on the innate seizure threshold

To test the possibility that TIA-1 influences the innate seizure threshold independent of regulating COX-2 expression, the PTZ-induced seizure threshold was assessed in mice lacking TIA-1 protein (Fig. 11). The results indicate that seizure severity and incidence of or latency to convulsions was not altered in $Tia1^{-/-}$ mice relative to $Tia1^{+/+}$. These results were

the same regardless of whether mice were treated with 42 or 54 mg/kg PTZ (Fig. 11A–C and 11D–F, respectively). Within this dose range, convulsion-induced mortality was low regardless of genotype. Thus, TIA-1 does not appear to influence the sensitivity of mice to acute PTZ-induced seizures.

Discussion

Results from several studies employing various animal models showed that inhibitors of COX-2 activity enhanced the susceptibility for convulsions (Steinhauser and Hertting, 1981; Förstermann et al., 1982, 1984; Baik et al., 1999; Sanchez-Hernandez et al., 1999; Kunz and Oliw, 2001; Claycomb et al., 2012; Arnao et al., 2017). This is consistent with observations in human cases as well (Sanchez-Hernandez et al., 1999; Arnao et al., 2017). Together, these results provide compelling support for the notion that this pathway of PG synthesis functions as a homeostatic control mechanism to maintain the innate seizure threshold in the normal brain. Results herein showing that seizure threshold is elevated in mice overexpressing COX-2 in neurons complement the results from these pharmacological studies. On the other hand, results from other pharmacological studies argue against this conclusion and even suggest a proconvulsant action of the COX-2 pathway (Okada et al., 2001; Dhir et al., 2006; Akula et al., 2008; Oliveira et al., 2008; Claycomb et al., 2011; Serrano et al., 2011; Citraro et al., 2015; Temp et al., 2017). The observations that some COX inhibitors potentiated the action of certain antiepileptic drugs is consistent with this possibility (Kaminski et al., 1998; Dhir et al., 2008). The reason for these disparate results remain to be resolved. However, several factors may influence the outcome with COX inhibitors (Radu et al., 2017). For example, unique properties of individual inhibitors or differences in their treatment paradigms may have contributed to the inconsistencies in some cases. In this regard, only a subset of COX inhibitors affected seizure threshold in some studies (Wallenstein, 1985; Temp et al., 2017), perhaps owing to pharmacokinetic differences or off-target effects (Lehmann et al., 1997; Süleyman et al., 2007; Fowler, 2012; Karlsson and Fowler, 2014). Additionally, whereas the COX-2 selective inhibitor, rofecoxib, lowered the seizure threshold of PTZ when administered orally in a bolus dose of 30 mg/kg 3 hours prior to PTZ (Claycomb et al., 2012), subacute delivery of 30 mg/kg/day rofecoxib in diet for 5 days had no effect (Claycomb et al., 2011). It is possible that persistent inhibition in the latter study elicited compensatory gene expression changes that masked the role of COX-2 (Lehmann et al., 1997; Claycomb et al., 2011). It is more difficult to reconcile results from another study showing that acute rofecoxib treatment raised PTZ seizure threshold (Akula et al., 2008). However, the PTZ treatment paradigms (bolus i.p. vs. i.v. infusion, respectively) and mouse strains used (C57BL/6 vs. “Laka”, respectively) were key differences between this study and the other (Claycomb et al., 2012).

Studies using gene deletion approaches to assess the role of COX-2 in seizure threshold similarly yielded conflicting results. Thus, whereas global deletion of the COX-2 gene (PTGS2) in all cell populations of the mouse brain lowered the seizure threshold relative to wild-type littermates (Toscano et al., 2008), no effect was observed using a conditional cre-lox approach to target PTGS2 deletion in forebrain neurons exclusively (Serrano et al., 2011). This difference might suggest that neurons other than those in the forebrain account for the function of COX-2 in maintenance of seizure threshold. However, both studies

assessed the role of COX-2 in models of temporal lobe seizures, and as such, both genetic approaches deleted COX-2 expression in key populations of glutamatergic neurons relevant to this model (e.g., hippocampus, cerebral cortex, and thalamus). Alternatively, the results from the global deletion approach might be related to loss of COX-2 function in non-neuronal cell types, which would not be targeted in the conditional approach. Pertinently, COX-2 is expressed constitutively under normal conditions in endothelial cells of the brain microvascular endothelium, where it may modulate blood flow to the brain in response to neuronal activity (Parfenova et al., 1997; Niwa et al., 2000). Alternatively, the different outcomes could be related to genetic background differences between the global and forebrain-specific PTGS2 gene deficient mouse lines (C57Bl/6–129/Ola and C57Bl/6, respectively) or to differential alterations in early development. In the latter case, whereas the PTGS2 gene was deleted throughout all phases of development in the globally deficient mice, it was deleted postnatally in the conditional knockout mice due to the characteristics of the promoter used to target cre expression. It is relevant to note, however, that the native PTGS2 gene reportedly becomes transcriptionally active in neurons in the second week postnatally (Yamagata et al., 1993; Kaufmann et al., 1996), suggesting that similar developmental concerns might apply to both genetic approaches.

Because of the concerns with approaches that block COX-2 activity or expression, the present study employed a transgenic overexpression approach to investigate the role of COX-2 in the innate seizure threshold. If it is true that COX-2 contributes to maintenance of the innate seizure threshold as posited herein and by others, then it is reasonable to expect that enhanced neuronal COX-2 activity would increase the seizure threshold. The results from the L300 COX-2 overexpressing mouse line herein showing a marked reduction in the incidence of convulsive seizures suggest that this is indeed the case and further strengthen the contention that COX-2 functions to maintain the innate seizure threshold. The explanation for this phenotype is not clear, although it is presumed that it would be manifest at the cellular level by a corresponding dampening of the electrical response elicited by PTZ. Whether this is true warrants further investigation. Results from the L300 mice suggest that knowledge of the determinants of constitutive COX-2 expression in neurons may lead to a better understanding of the molecular mechanisms that maintain the innate seizure threshold.

It should be noted that kainic acid-induced seizures were more severe in a different transgenic mouse line similarly modified to expression a human COX-2 transgene broadly in neurons (Kelley et al., 1999). This line, however, employed the neuron-specific enolase (NSE) promoter to drive neuronal overexpression of the transgene, which could explain the contrasting outcomes in several ways. First, transcription from the Thy-1 promoter of the L300 mouse line used herein is initiated around postnatal day 8 (Vidal et al., 1990; Aigner et al., 1995; Caroni, 1997; Andreasson et al., 2001), which closely parallels the upregulation of endogenous COX-2 mRNA and protein expression during the second week of postnatal development (Yamagata et al., 1993; Kaufmann et al., 1996). In contrast, the NSE promoter reportedly becomes transcriptionally active early during differentiation of embryonic stem cells in culture and during late gestation *in vivo* (Forss-Petter et al., 1990; Alouani et al., 1993), raising the probability for developmental effects of ectopic COX-2 overexpression. Second, the neuronal distribution of COX-2 overexpression in adult offspring differed between the two lines to some degree. For example, unlike the Thy-1 promoter, strong

expression of the COX-2 transgene was reported in the DG of the NSE promoter mice. This could conceivably lead to differences in the PG profile or response due to cell-type specific expression of PG synthases and/or receptors, respectively. Third, neuronal activity is induced rapidly and widely by PTZ via antagonism of GABAergic transmission (Morgan et al., 1987; Brevard et al., 2006), whereas kainic acid directly increases excitation in a progressive and focal manner, beginning in the hippocampal formation specifically in the DG (Popovici et al., 1990; Airaksinen et al., 2012). Fourth, differences in the level of COX-2 overexpression in specific populations of neurons could potentially influence outcome.

It is intuitive to assume that the actions of COX-2 in the brain are due to PGs (Seregi et al., 1984). In the L300 mouse brain, the levels of PGE_2 and PGI_2 and $PGF_{2\alpha}$ were elevated constitutively ~10- and 4-fold over basal levels, respectively (Vidensky et al., 2003). However, which of the PGs mediates the contribution of COX-2 to seizure threshold regulation remains to be determined unequivocally. Although PGD_2 would seem to be a reasonable candidate (S. Akarsu et al., 1998; Kaushik et al., 2014), PGD_2 was increase <2-fold (Vidensky et al., 2003). Anticonvulsant properties have been ascribed to $F_{2\alpha}$ as well (Kim et al., 2008), leaving open the possibility that the PG may be involved. On the other hand, because COX-2 can contribute to the biometabolism endocannabinoids to prostamide products (Woodward et al., 2013; Karlsson and Fowler, 2014), the possibility that results from L300 mice are due to depletion of endocannabinoids and not increased PG cannot be negated. However, endocannabinoids are known to possess antiepileptic properties and their depletion in brains reduced seizure threshold (Wallace et al., 2002; Monory et al., 2006; Naydenov et al., 2014; Shubina et al., 2015; Sugaya et al., 2016). Conversely, it is possible that the antiepileptic actions of certain COX-2 inhibitors may be due, at least in part, to their ability to enhance endocannabinoid levels (Fowler, 2012; Karlsson and Fowler, 2014). Finally, the metabolites generated from endocannabinoid metabolism by COX-2 possess novel biological properties that might contribute to the effects of COX-2 overexpression in L300 mice (Yang et al., 2008; Iannotti et al., 2016). Whether or not these products are antiepileptic, however, remains to be determined.

While the posttranscriptional regulation of COX-2 expression has been studied extensively in other cell types, the extent to which these mechanisms control expression of COX-2 in glutamatergic neurons is not known. TIA-1 has been shown to suppress basal expression of COX-2 in embryonic fibroblasts (Dixon et al., 2003) as well as induction of COX-2 expression by proinflammatory mediators in macrophages (Phillips et al., 2004) and this is likely by virtue of its ability to bind to the COX-2 mRNA 3′UTR and repress translation. It does not appear, however, to perform a similar function in neurons. Despite the fact that COX-2 and TIA-1 proteins are constitutively expressed CA3 pyramidal neurons, basal COX-2 protein expression was not altered in brains of mutant mice lacking TIA-1. Furthermore, in granule cells of the DG, which are largely devoid of COX-2 expression under basal conditions, induction of COX-2 expression by an acute convulsive seizure was not affected by deletion of TIA-1. Thus, TIA-1 regulates neither constitutive nor activity-dependent neuronal COX-2 expression. In addition to its broad mRNA binding specificity (Lopez de Silanes et al., 2005), TIA-1 protein interacts extensively with other proteins in the brain (Vanderweyde et al., 2016). However, the lack of an effect of Tia1 gene deletion on PTZ-induced acute seizures suggests that TIA-1 does not function in the maintenance of the

innate seizure threshold of the brain either by affecting COX-2 expression or by mechanisms other than via COX-2 regulation. Nevertheless, the prominent and widespread expression of TIA-1 in neurons, particularly in excitatory neurons of the cerebral cortex and hippocampal formation, suggests that this protein may modulate physiological activities, either through its role as a pre-RNA splice regulator or silencer of mRNA translation, or via its ability to interact with other neuronal proteins. Although the TIA-1 deficient mice appear to behave normally, a more careful analysis of the role of this protein in normal brain function warrants further investigation. The significance of the comparative difference in mRNA expression between neurons and astrocytes reported herein is not clear, particularly since this cell-type difference was not observed by others (Zhang et al., 2014).

It could be argued that compensatory mechanisms in the TIA-1 mutant mice may have masked its role in the regulation of COX-2 expression in neurons. For example, the COX-2 mRNA ARE also serves as a docking site for TIAR, an RBP that has high homology with TIA-1 (Dember et al., 1996; Cok et al., 2004). Thus, the possibility that TIAR may compensate for the absence of TIA-1 in neurons cannot be excluded. Indeed, TIAR is constitutively expressed in the brain and its mRNA expression profile is remarkably similar to TIA-1 (Beck et al., 1996). However, the observation that COX-2 expression was enhanced in embryonic fibroblasts derived from the same TIA-1 mutant mouse line employed herein argues against this possibility (Dixon et al., 2003). On the other hand, it is possible that TIA-1 indeed has no role and that another RBP regulates COX-2 expression post-transcriptionally in neurons. In this case, TIA-1 would not be expected to bind to the ARE of the COX-2 mRNA in neurons, a possibility that requires further investigation.

TIA-1 is best known for its role under conditions of cellular stress, during which it facilitates formation of stress granules (SGs). SGs are large complexes of proteins and translationally silent mRNAs that reprogram translation under cellular stress conditions (Gilks et al., 2004). SGs are thought to be an adaptive response to pathological conditions where cells may be subjected to oxidative or endoplasmic reticulum stress (Arimoto-Matsuzaki et al., 2016). In the CNS, TIA-1 mediates the stress responses associated with brain ischemia, tauopathies, and other neurological disease models where SG formation progresses with the severity of the disease and contributes to neurodegeneration (Kayali et al., 2005; DeGracia et al., 2007; Ratovitski et al., 2012; Vanderweyde et al., 2012, 2016). It is possible, however, that TIA-1 and SGs contribute to the changes in neuroplasticity leading to a reduction in seizure threshold in models of temporal lobe epileptogenesis, such as kindling or *status epilepticus*. It is interesting to note in this regard that the pathophysiological effects of tau have been linked to its interaction with TIA-1 and evidence suggesting that depletion of tau ameliorates the epileptic phenotype in a model of Dravet Syndrome. Together, these observations suggest a possible relationship between the two proteins in epileptogenesis (Gheyara et al., 2014; Vanderweyde et al., 2016). The role of TIA-1 in epileptogenesis is currently under investigation in the PTZ-induced kindling model.

Although the results herein suggest that TIA-1 is not involved in neuronal COX-2 expression or acute seizure responses, recent results from cultures of cortical neurons have demonstrated that COX-2 is indeed regulated at the posttranscriptional level in an NMDAR-dependent manner and that the ARE region of the 3′UTR is necessary (YG and JAH,

unpublished results). AREs have been recognized for some time as key determinants of gene expression (Chen and Shyu, 1995). The ARE in the COX-2 mRNA 3′UTR is located within 80 bases of the stop codon in the coding sequence. This sequence is 89% AU-rich and contains seven AUUUA pentanucleotide motifs, which can be divided into two separate AU domains: 1) a 5′ domain consisting of three overlapping AUUUA pentameric nucleotide motifs, and 2) an adjacent 3′ domain containing four sequential AUUUA pentamers. The nanomeric consensus sequence, UUAUUUA(A/U)(A/U), which has been suggested to be the key determinant of mRNA expression (Lagnado et al., 1994), is embedded within each of these domains. Lastly, a sequence of eight U nucleotides is positioned just 3′ of the second AUUUA domain. In addition to TIA-1 and TIAR, a number of additional mRNA stability factors and translational regulators bind to this region of the COX-2 mRNA, including CELF2 (CUGBP2), HNRNPD (AUF1), hnRNPK and the ELAVL proteins, HuR and HuD (Mukhopadhyay et al., 2003; Cok et al., 2004; Shanmugam et al., 2008). Like TIA-1, each of these RBPs are broadly expressed throughout the brain (Allen Brain Atlas). Whether these or other novel RBPs bind to and regulate COX-2 expression in neurons is currently under investigation. It is hoped that a better understanding of the molecular mechanisms that link NMDAR activity to changes in COX-2 expression could identify approaches for the development of novel antiepileptic therapies that target enhancement of the level of COX-2 expression in neurons.

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. The authors have no conflict of interest to disclose.

ABBREVIATIONS

COX-2	Cyclooxygenase-2
L300	COX-2 overexpressing mouse line
PG	Prostaglandin
PTZ	pentylenetetrazole
RBP	RNA Binding Protein
TIA-1	T-cell intracellular antigen-1
Tia1	Tia1 gene
−/−	homozygous Tia1 mutant
+/−	heterozygous Tia1 mutant

+/+ wild-type Tia1

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Highlights

- Neuronal over-expression of COX-2 suppressed convulsive seizure induction
- Constitutive TIA-1 expression was detected in neurons throughout the normal brain
- The TIA-1 expression profile was not altered following an acute convulsive seizure
- Neither basal nor convulsion-induced neuronal COX-2 expression were altered in mice lacking TIA-1
- TIA-1 deletion did not alter the acute seizure threshold

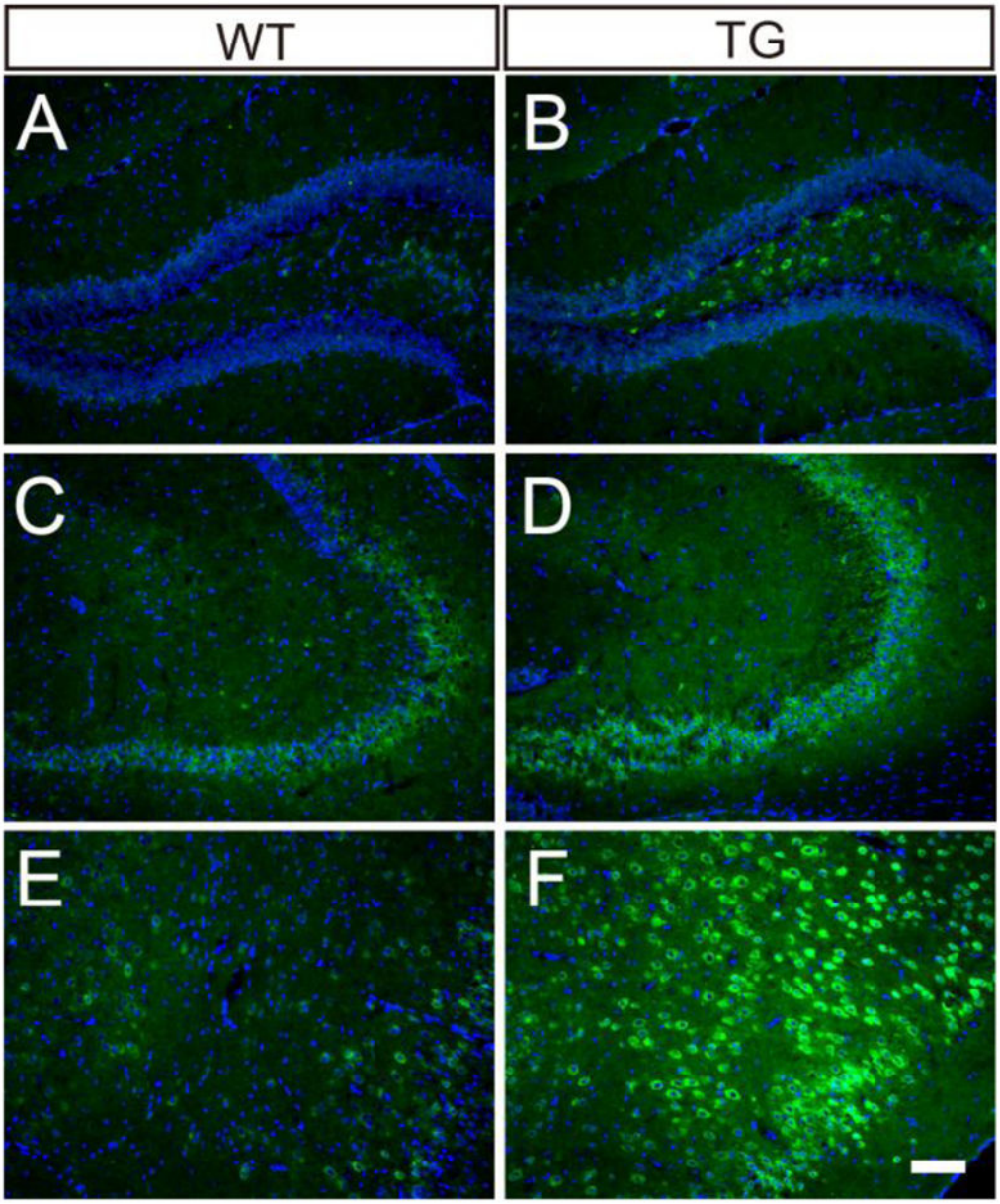

Fig. 1.

L300 transgenic (TG) mice constitutively overexpress COX-2 in populations of neurons that constitutively express endogenous COX-2. COX-2 protein (green) and cell nuclei (DAPI, blue) were visualized by epifluorescence microscopy in 12μm thick coronal sections obtained at bregma −1.70 ± 0.12 mm. (A, B), dentate gyrus (DG); (C, D), CA3; (E, F), Pyriform cortex. Images represent N = 2 mice per genotype. White scale bar represents 100 μm.

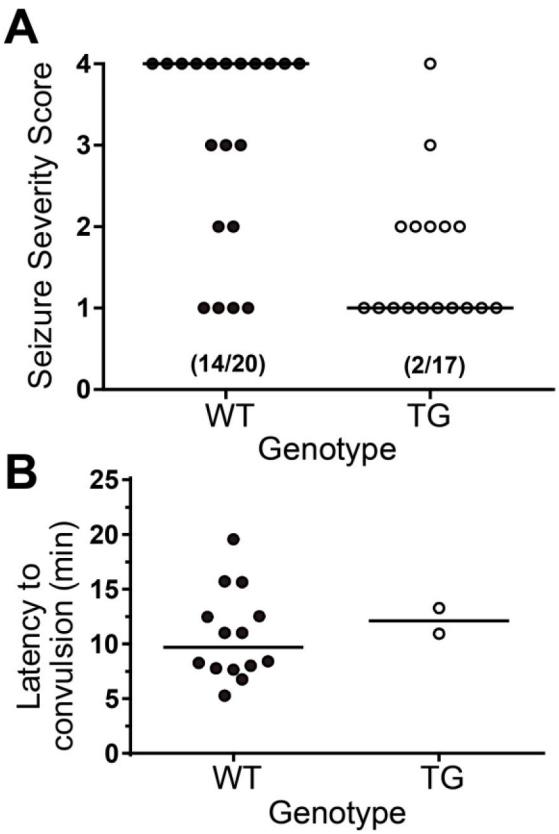

Fig. 2.

The innate seizure threshold is elevated in transgenic mice overexpressing COX-2 in neurons. L300 transgenic mice (TG, ●, N = 17) and their wild-type littermates (WT, ○, N = 20) were treated with32 mg/kg PTZ. (A), Seizure severity. Each point represents the maximum seizure score for individual mice. Horizontal lines indicate median seizure score. Ratio in parentheses indicates incidence of convulsions, which was significantly different between genotypes ($p = 0.0007$, two-tailed Fisher's exact test). (B), Latency to convulsive seizures. Horizontal lines indicate mean values.

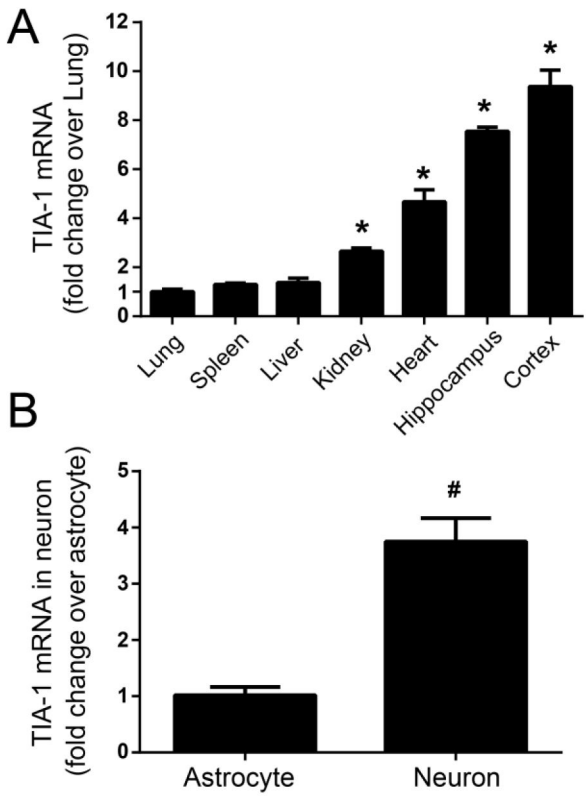

Fig. 3.

TIA-1 mRNA is expressed constitutively at high levels in normal mouse brain tissue and cells. (A), Tissue expression. Relative TIA-1 mRNA in mouse tissues was assessed by qRT-PCR analysis. Results are expressed as Mean ± Standard Error of the Mean relative to lung (N = 3 mice). Equal variance was validated by Brown-Forsythe test ($p = 0.792$, F(6, 14) = 0.5087). *, significantly higher than lungs ($p < 0.0001$, Dunnett's multiple comparisons test following one-way ANOVA on log-transformed $2^{-\Delta\Delta}$ values, F(6, 14) = 117.4). (B), Neural cell expression. TIA-1 mRNA expression was assessed in primary cultures of astrocytes and neurons by qRT-PCR. Results are expressed as Mean ± Standard Error of the Mean relative to astrocytes (N = 3 independent cultures). Equal variance was validated by F test ($p = 0.8207$, F(2, 2) = 1.437). #, significantly higher than astrocytes ($p = 0.002$, unpaired t-test on log-transformed $2^{-\Delta\Delta}$ values, t = 7.168, df = 4).

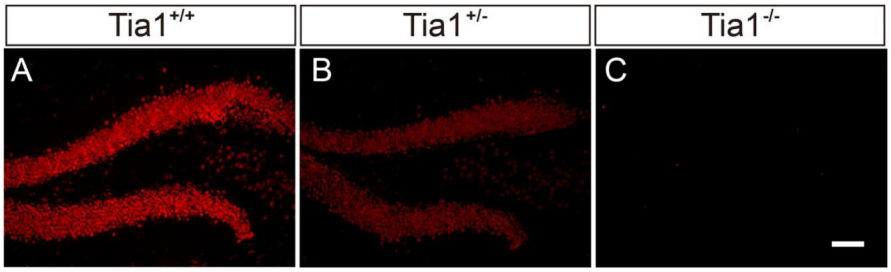

Fig. 4.

Verification of TIA-1 protein deletion and validation of anti-TIA-1 antibody. Brains of adult $Tia1^{+/+}$ (A), $Tia1^{+/-}$ (B), and $Tia1^{-/-}$ (C) animals were fixed, sectioned (12 μm) and stained with anti-TIA-1 antibody (red). White scale bar represents 100 μm. Images are representative of brains from two mice per genotype.

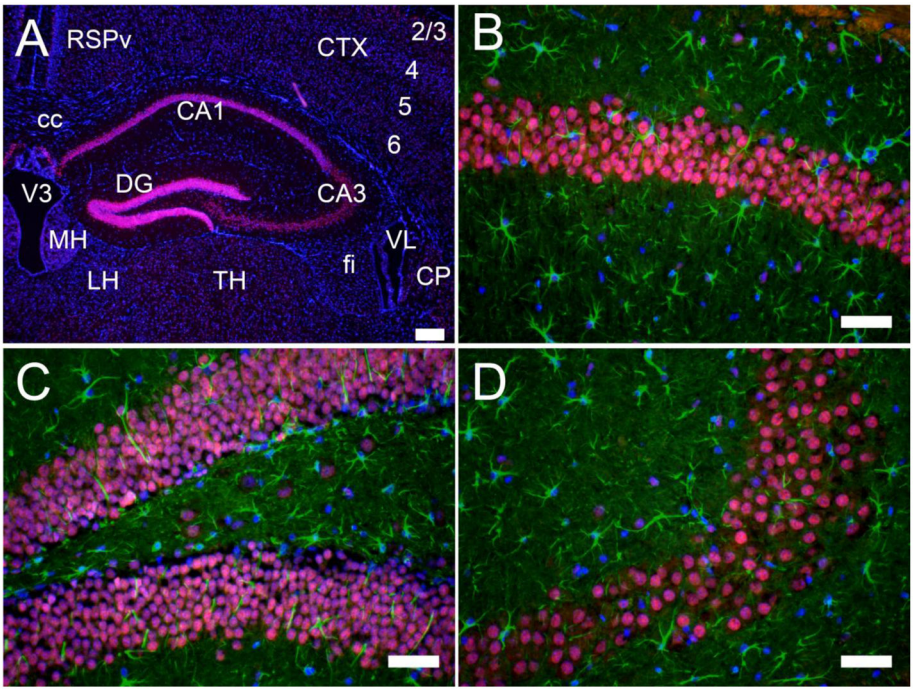

Fig. 5.

TIA-1 protein is expressed in the primary neuronal layers of the hippocampal formation. Coronal sections (12μm, bregma −1.70 ± 0.12 mm) were obtained from normal C57BL/6J mouse brains and stained for TIA-1 (red), GFAP (green) and DAPI (blue). Magenta indicates co-localization of TIA-1 and DAPI. (A), Brain hemisphere along the dorsal-ventral axis between the cerebral cortex (CTX) and thalamus (TH) and laterally between the third (V3) and lateral ventricles (VL); RSPv, ventral retrosplenial cortex; DG, dentate gyrus; CA1/CA3, hippocampal layers; CP, caudoputamen; MH/LH, medial/lateral habenula; cc, corpus callosum; fi, fimbria. White scale bar represents 200 μm. (B), CA1; (C), DG and hilus; and (D), CA3. White bar represents 50 μm. Image panels B–D were taken from a different section than panel A. Images are representative of brains from two $Tia1^{+/+}$ mice.

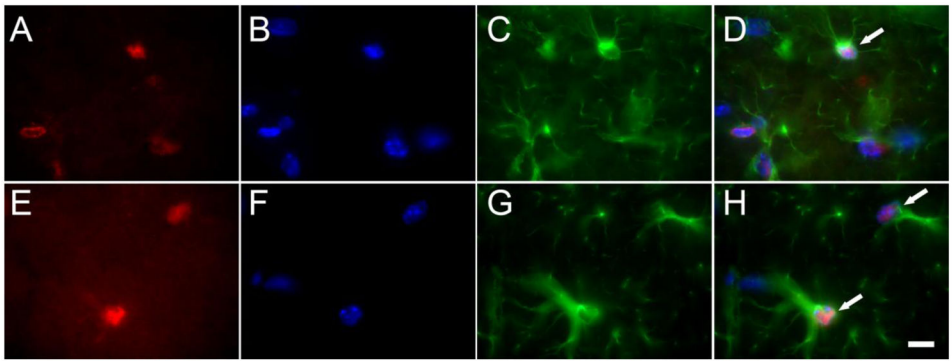

Fig. 6.

Hippocampal astrocytes express TIA-1 protein. Images were acquired from the molecular layer of dentate gyrus of adult Tia1$^{+/+}$ mouse brains (12μm). (A, E), TIA-1 (red); (C, G), GFAP (green); (B, F), nuclei (blue). (D, H), overlay of A–C and E–G, respectively. Arrows, astrocytes expressing TIA-1 protein (magenta/green). White scale bar, 10 μm. A–D and E–H are representative images from two mice.

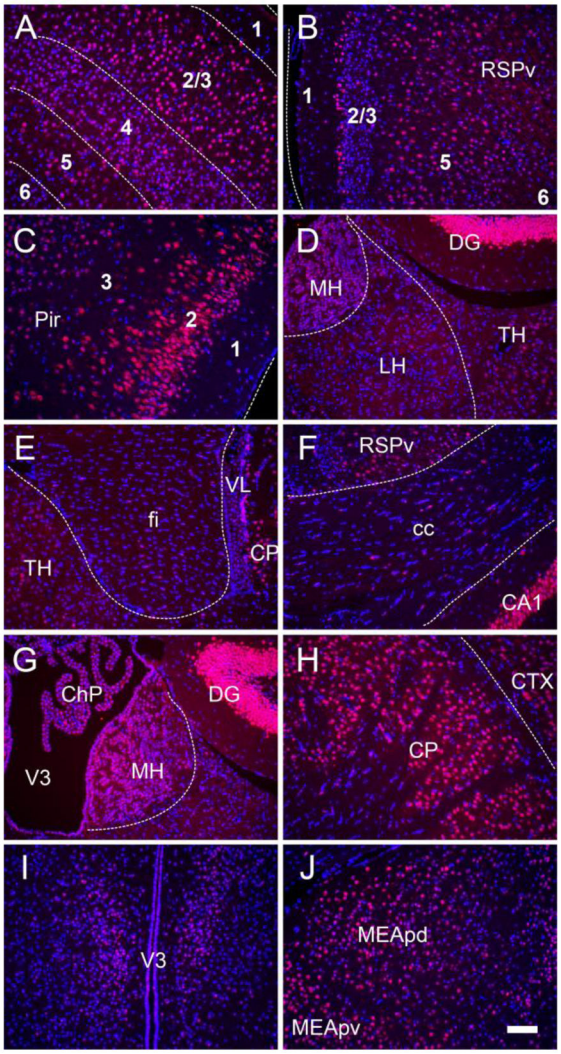

Fig. 7.

TIA-1 protein expression profile in the brain of wild-type (Tia1$^{+/+}$) mice. TIA-1 protein (red), GFAP protein (green), and cell nuclei (DAPI, blue) were visualized by epifluorescence microscopy in 12μm thick coronal sections obtained at bregma −1.70 ± 0.12 mm. Magenta indicates co-localization of TIA-1 and DAPI. TIA-1 expression in: (A), somatosensory cortical layers 1–6; (B), ventral retrosplenial cortical layers 1–6 (RSPv); (C), piriform cortical layers 1–3 (Pir); (D), medial (MH) and lateral habenula (LH); (E), thalamus (TH) and fimbria (fi); (F), corpus callosum (cc); (G) choroid plexus (ChP), lateral ventricle (LV); (H), caudoputamen (CP); (I), hypothalamus, 3rd ventricle (V3); (J), medial amygdalar nuclei, anterodorsal (MEApd) and anteroventral (MEApv) parts. CTX, cerebral cortex; DG, dentate gyrus; CA1, *cornu ammonis* area 1. White scale bar, 100 μm. Images are representative of two mouse brains.

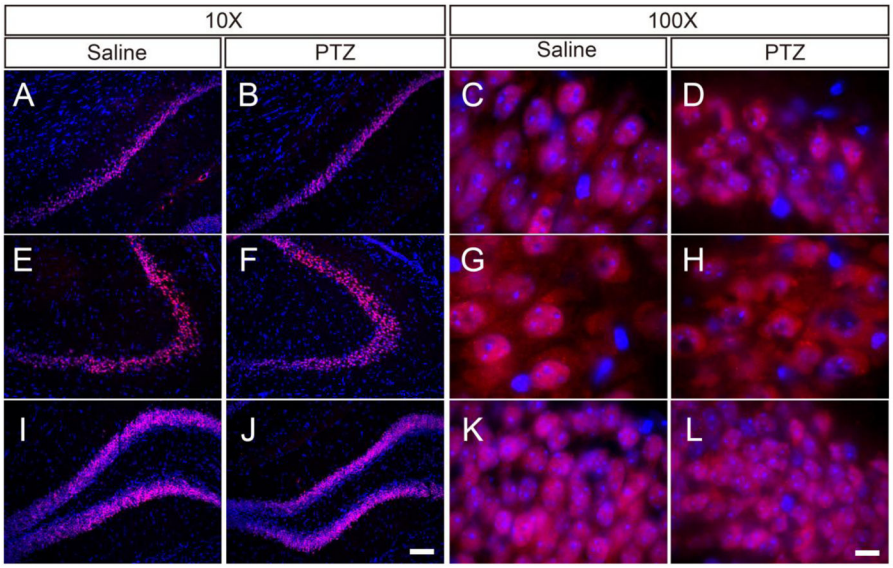

Fig. 8.

Effect of acute convulsive seizures on TIA-1 protein expression in the hippocampal formation. Cohorts of $Tia1^{+/+}$ mice were treated with saline or 54 mg/kg PTZ (N=4). Brains from PTZ-treated animals were harvested 3 hours following convulsions, fixed, sectioned (12 μm) and stained with TIA-1 antibody (red) and DAPI (blue). Brains from saline-treated mice were processed in parallel. (A, E, I, C, G, K) Representative images from a saline-treated mouse. (B, F, J, D, H, L) Representative images from PTZ-treated mouse. (A–D) CA1; (E–H) CA3; (I–L) dentate gyrus. High/low magnification images showing the same brain structure were taken from different sections. White scale bar, 100 μm (J) and 10 μm (L). Images are representative of four mice from each treatment group.

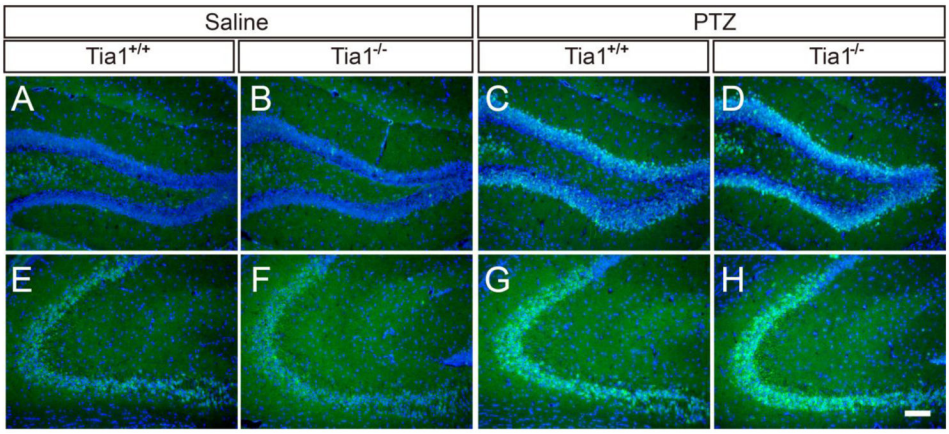

Fig. 9.

Basal and convulsion-induced COX-2 expression in the hippocampal formation. $Tia1^{+/+}$ and $Tia1^{-/-}$ littermate mice were treated with saline or 54 mg/kg PTZ. Brains from saline- and PTZ-treated animals that had convulsive seizures were harvested 3 hours later, fixed, sectioned (12 μm) and stained with COX-2 antibody (green) and DAPI (blue). (A, B, E, F) COX-2 expression in a saline-treated mouse brain. (C, D, G, H) COX-2 expression in a PTZ-treated mouse brain. (A–D) Dentate gyrus; (E–H) CA3. White scale bar, 100 μm. Images are representative of three mice per genotype.

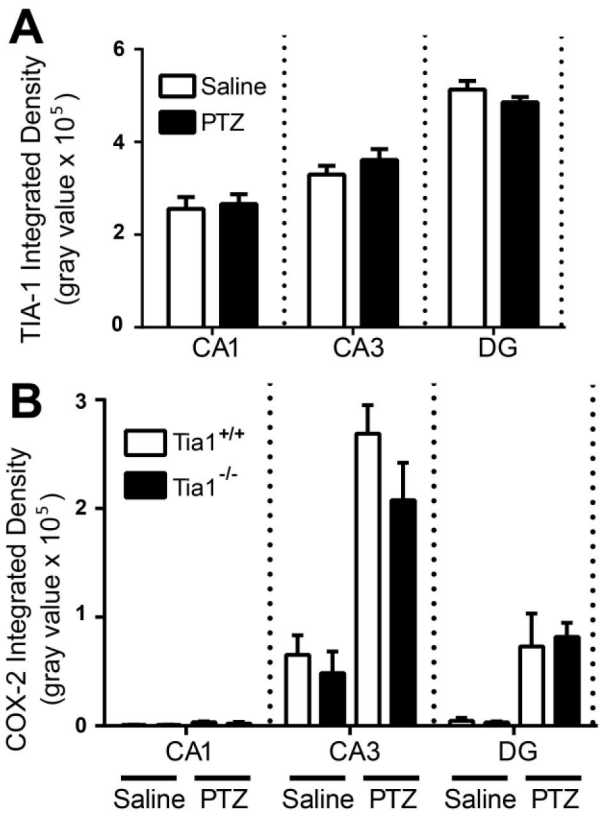

Fig. 10.

Quantification of basal and convulsion-induced TIA-1 and COX-2 expression in the hippocampal formation. Fluorescent intensity in brain images was quantified using NIH Image J (version 1.50i). (A) TIA-1 protein expression in CA1, CA3 and DG. Results were analyzed by two-way ANOVA on log-transformed gray values. Results are the average of duplicate sections from four mice. Equal variance was validated by Brown-Forsythe test (p = 0.2014, F(5, 18) = 1.636). A significant difference was detected between brain regions (p < 0.0001, F(2, 18) = 51.24) but not between treatment (p = 0.6181, F(1, 18) = 0.2574). (B) COX-2 protein expression in CA1, CA3 and DG. Results are the average of duplicate sections from three mice. CA3, equal variance was validated by Brown-Forsythe test (p = 0.57, F(3, 8) = 0.7153). Significant difference between treatments (p = 0.0017, F(1, 8) = 21.46)) but not genotypes (p = 0.3185, F(1, 8) = 1.132)). DG, equal variance was validated by Brown-Forsythe test (p = 0.6669, F(3, 8) = 0.5421). Significant difference between treatments (p = 0.0001, F(1, 8) = 45.69)) but not genotypes (p = 0.8467, F(1, 8) = 0.03988)). Minimal COX-2 protein expression was detected in CA1.

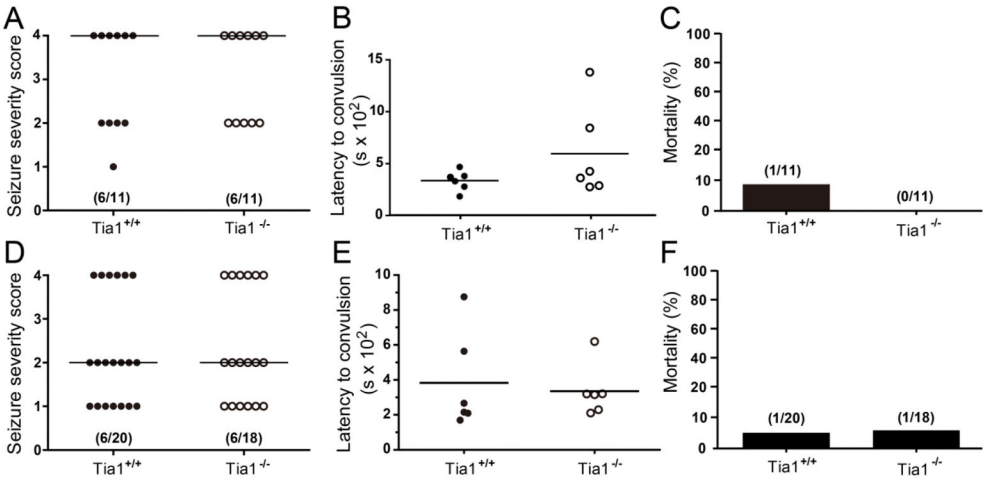

Fig. 11.

TIA-1 does not influence the innate seizure threshold. Seizure severity is shown for $Tia1^{+/+}$ (●) and $Tia1^{-/-}$ (○) littermates that were treated with PTZ i.p. (A–C) 54 mg/kg; N = 11 per genotype). (D–F) 42 mg/kg; N = 20 and 18 for $Tia1^{+/+}$and $^{-/-}$, respectively. Symbols indicate maximum seizure scores for individual mice. Horizontal lines indicate median seizure score. Ratio in parentheses indicates incidence of convulsions. Genotype responses are not significantly different ($p = 1.0$, two tailed Fisher's exact test). (B, E) Latency to convulsive seizures. Symbols indicate latencies for mice in A and D. Horizontal lines indicate mean values. No significant difference was detected between genotypes ($p = 0.5714$ and $p = 0.474$, for B and E respectively, two-tailed Mann Whitney test). (C, F) Mortality. No significant difference was detected between genotypes ($p = 1.0$, two-tailed Fisher's exact test).