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Altered GABAergic Neurotransmission is Associated with Increased Kainate-Induced Seizure in Prostaglandin-Endoperoxide Synthase-2 Deficient Mice

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Abstract

Excitotoxicity involves over activation of brain excitatory glutamate receptors and has been implicated in neurological, neurodegenerative and neuropsychiatric diseases. Metabolism of arachidonic acid (AA) through the phospholipase A₂ (PLA₂)/ prostaglandin-endoperoxide synthase (PTGS) pathway is increased after excitotoxic stimulation. However, the individual roles of the PTGS isoforms in this process are not well established. We assessed the role of the PTGS isoforms in the process of excitotoxicity by exposing mice deficient in either PTGS-1 (PTGS-1^{-/-}) or PTGS-2 (PTGS-2^{-/-}) to the rototypic excitotoxin, kainic acid (KA). Seizure intensity and neuronal damage were significantly elevated in KA-exposed PTGS-2^{-/-}, but not in PTGS-1^{-/-}, mice. The increased susceptibility was not associated with an alteration in KA receptor binding activity or mediated through the CB1 endocannabinoid receptor. The frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) was decreased in the CA1 pyramidal neurons of PTGS-2^{-/-} mice, suggesting an alteration of GABAergic function. In wild-type mice, six weeks treatment with the PTGS-2 selective inhibitor celecoxib recapitulated the increased susceptibility to KA-induced excitotoxicity observed in PTGS-2^{-/-} mice, further supporting the role of PTGS-2 in the excitotoxic process. The increased susceptibility to KA was also associated with decreased brain levels of PGE₂, a biomarker of PTGS-2 activity. Our results suggest that PTGS-2 activity and its specific products may modulate neuronal excitability by affecting GABAergic neurotransmission. Further, inhibition of PTGS-2 but not PTGS-1, may increase the susceptibility to seizures.

Keywords

Fluoro-Jade B; Excitotoxicity; Endocannabinoid; [³H]-Kainate Autoradiography; Racine Seizure Scale; GABA

1. Introduction

The prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase (COX), isoforms, PTGS-1 and PTGS-2, are both expressed under basal conditions in the brain [25,

67]. These enzymes catalyze the first step in the transformation of arachidonic acid (AA) to prostaglandins and thromboxanes [60], bioactive metabolites of AA known to mediate physiological and pathological processes in the brain [23,24]. Release and metabolism of AA through the phospholipase A₂ (PLA₂)/ PTGS pathway is increased during excitotoxicity [12], a process that involves the over activation of brain excitatory neurotransmission. Kainic acid (KA), the prototypic excitotoxin, binds to the KA receptors in the brain and induces seizures that result in inflammation, oxidative damage and neuronal death, processes that have been implicated in neurological, neurodegenerative and psychiatric diseases [16,20,40,49,52,66, 68,71,72]. Although it has been demonstrated that PTGS plays a role in excitotoxicity, the distinct role of the two PTGS isoforms has not been fully established.

Previous attempts to study the role of PTGS isoforms in the process of excitotoxicity by using non steroidal anti-inflammatory drugs (NSAIDs), pharmacological inhibitors of PTGS, have not fully characterized specific roles of each isoform. However, a distinct dichotomy in the effect of gross PTGS inhibition on the process of excitotoxicity has been suggested, with both protective and detrimental effects of PTGS inhibition being demonstrated in studies using pharmacological inhibitors [4,34]. While these studies have examined the effects of acute inhibition of PTGS before or after excitotoxic stimuli [5,9,18,33,34,61], the consequences of long-term inhibition of PTGS activity on the process of excitotoxicity remain to be addressed. A thorough understanding of these consequences is clinically relevant since a common therapeutic approach to treating pain and inflammation involves long-term treatment with NSAIDs.

In order to clarify the specific roles of PTGS-1 and PTGS-2 in the process of KA-induced excitotoxicity and to determine the effect of long-term PTGS inhibition on this process, we employed a novel approach using mice deficient in either PTGS-1 (PTGS-1^{-/-}) or PTGS-2 (PTGS-2^{-/-}). We demonstrate for the first time that PTGS-2^{-/-} mice, but not PTGS-1^{-/-} mice, are more susceptible than wild type mice to systemically-injected, KA-induced seizures and neuronal damage. Further, we confirm our observation by demonstrating that mice pretreated for six weeks with celecoxib, a PTGS-2 specific inhibitor, also have augmented susceptibility to KA-induced excitotoxicity. Whole cell recording in hippocampal slices and measurement of brain prostaglandin levels revealed alterations in PTGS-2^{-/-} mouse neurophysiology and neurochemistry that may contribute to the increased susceptibility to KA-induced excitotoxicity. Specifically, the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) was decreased in the CA1 pyramidal neurons of PTGS-2^{-/-} mice, suggesting an alteration of GABAergic function. Augmented susceptibility to excitotoxicity was not mediated by endocannabinoid CB1 receptor signaling due to AA metabolites catabolized by PTGS-2, but not PTGS-1 [30-32,70], that are known to alter neuronal excitability [3,28].

2. Methods and Materials

2.1 Animal Housing

All procedures were performed under an animal protocol approved by the NICHD ACUC in accordance with NIH guidelines on the care and use of laboratory animals. Male COX-1^{-/-}, COX-1^{+/+}, COX-2^{-/-}, COX-2^{+/-}, and COX-2^{+/+} mice maintained on a C57Bl/6-129/Ola background for over 35 generations [35,41] were received at our animal facility at 6 weeks of age from a private NIEHS colony maintained by Taconic Farms (Germantown, NY) separately from their commercially available colony. In order to prevent the inclusion of strain or genetic background confounders between COX deficient and wild type mice, all of the mice used in this study were derived from a maintenance colony of COX-1^{+/+} or COX-2^{+/+} males and females, as previously described [35,41,64]. Specifically, all mice used in this study were progeny derived from heterozygous by heterozygous matings and therefore all contained the same strain and genetic background. This is an important point to recognize since genetic

background and mouse strain has been demonstrated to alter the susceptibility to KA-induced excitotoxicity [38,39,54,55]. For celecoxib pretreatment, PTGS-2^{+/+} mice were given free access for six weeks to a diet containing 0, 3000 or 6000 ppm celecoxib. Celecoxib is a COX-2 specific inhibitor with a selectivity ratio of >375 [13]. CelebrexTM capsules (400 mg; Pfizer Inc, New York, NY) were obtained from the NIH Division of Veterinary Medicine and were incorporated into feed by Research Diets, Inc. (New Brunswick, NJ) [73].

2.2 Kainate Injections and Seizure Observation

Mice (males, 12-14 week-old) were injected intraperitoneally (i.p., injection volume 100-300 μ l) with 10 mg/kg KA (Biomol International, Plymouth Meeting, PA; 2 mg/ml in 0.9% saline) or vehicle and then videotaped for 2 h after injection. A dose-response pilot study (data not shown) ranging from 10-25 mg/kg determined that 10 mg/kg KA dissolved in 0.9 % saline caused seizures but did not result in fatalities in wild type mice. Seizure intensity was rated using a modified Racine seizure scale [48,55]: 0 = no behavioral alteration; 1 = immobility, mouth and facial movements, facial clonus; 2= head nodding, forelimb and/or tail extension, rigid posture; 3= forelimb clonus, repetitive movements; 4= rearing, forelimb clonus with rearing, rearing and falling; 5= continuous rearing and falling, jumping; 6= severe tonic-clonic seizures. Mice were assigned a Racine seizure score (RSS) for each 5 min interval over the course of the 2 h session, after which a median RSS was calculated for the entire 2 h session for each mouse.

2.3 Pretreatment with the CB1 Receptor Antagonist AM-251

Thirty minutes before KA injection, mice were injected i.p. with 30 mg/kg of the CB1 antagonist, AM-251 (Tocris Bioscience, Ellisville, MO), dissolved in DMSO, Tween-80, ethanol and 0.9% saline or vehicle as previously described [2,59]. AM-251 peak brain concentrations have previously been observed at 30 minutes post injection and 30 mg/kg is a dosage at which a plateau in brain to plasma ratio is reached [17,59].

2.4 Histochemistry and Autoradiography

Sagittal brain sections from mice euthanized 24 h after KA injection were cut on a cryostat (Bright Instrument Company, LTD; Huntingdon, England). Cresyl violet staining was performed as described [46]. FJB (Histo-Chem, Inc., Jefferson, AR) staining was performed as described previously using postfixed tissue samples [56]. FJB and cresyl violet staining were quantified using an ordinal pathology index [6]; 0=no pathology; 1=mild pathology; 2=moderate pathology; 3=severe pathology.

[³H] KA receptor autoradiography was performed as previously described [22,43]. Slides were apposed to Biomax MR Film (Kodak, Rochester, NY) for 6 to 8 weeks and images were analyzed using NIH Image. Optical density for specific brain regions was transformed to tissue equivalent by using autoradiographic microscans (GE Healthcare Life Sciences, Piscataway, NJ).

2.5 Measurement of Plasma Celecoxib Levels by HPLC

Plasma was collected by cardiac puncture (Vacutainer PST gel and lithium heparin tube; Becton-Dickenson, Franklin Lakes, NJ) from the cage mates of mice used in KA experiments. Celecoxib concentration was determined by HPLC with UV detection as previously described [74].

2.6 Determination of Brain Prostaglandin Concentration by ELISA

KA-naïve mice pretreated with celecoxib were euthanized by head-focused microwave fixation (90% power, 0.9 sec; Cober Electronics, Stamford, CT). Brains were prepared and analyzed for prostaglandin measurement as previously described [7].

2.7 Whole Cell Recordings of CA1 Pyramidal Neurons

Slice preparation and whole cell recording were performed as previously described [44,65]. Mice (3-4 weeks of age) brains were placed in an ice-cold slicing solution containing: 85 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 4 mM MgCl₂, 1 mM NaH₂PO₄, 25 mM NaHCO₃, 25 mM glucose, 75 mM sucrose, pH 7.4; 95% O₂ and 5% CO₂. Coronal slices (250-300 µm) prepared using a Vibratome 3000 Plus Sectioning System (Vibratome, St Louis, MO) were incubated in the slicing solution at 32 °C for 30 min. Slices recovered for 30 min at 32 °C in carbogen-bubbled artificial cerebrospinal fluid (ACSF): 120 mM NaCl, 3.1 mM KCl, 1.25 mM Na₂HPO₄, 26 mM NaHCO₃, 5 mM dextrose, 1 mM MgCl₂, 2 mM CaCl₂ and 0.01 mM D-Serine, 305 mOsm, pH 7.4. CA1 pyramidal neurons were viewed with an upright microscope (Axioscope, Zeiss, Germany) equipped with Nomarski optics. Drugs were added to the ACSF and delivered locally with a Y-tube device [42]. Stock solutions of bicuculine metabromide (BMR; to confirm GABA postsynaptic currents (IPSCs) via inhibition of GABA receptors) and tetrodotoxin (TTX; to observe miniature (m)IPSCs) were dissolved in water.

Whole-cell voltage-clamp recordings of spontaneous (s)IPSCs and mIPSCs were made with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA). Currents were filtered at 2 kHz, digitized at 5-10 kHz and analyzed using Clampfit 9 (Molecular Devices) or Mini Analysis (Synaptosoft Inc., Synaptosoft, Decatur, GA). Series resistance was typically less than 15 MΩ and was checked throughout the experiments. Electrodes, pulled from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA), were filled with 135 mM CsCl, 10 mM HEPES, 5 mM ATP.Mg, 0.2 mM GTP.Na, and 10 mM BAPTA, adjusted to pH 7.2 with KOH. We did not use AMPA receptor antagonists as they block presynaptic receptors on GABAergic terminals [11] and have non specific actions on GABA release [8]. However, AMPA-mediated mEPSCs were easily identified by the very rapid decay (<2 ms) and excluded from the analysis using a template specific for mIPSCs. Recordings with unstable baseline (>10% difference from the mean baseline current) were not included in the analyses.

2.8 Statistics

Non-parametric Kruskal-Wallis ANOVA and Mann-Whitney U-test were used for statistical analysis of seizure intensity and pathology index since these are ordinal measures. Chi-Square was used for analysis of mortality rates in AM-251 pretreatment experiments. All other data are expressed as mean ± SD, or SEM where stated, and were analyzed with one-way ANOVA and Bonferroni post-hoc test. p values <0.05 were considered statistically significant.

3. Results

3.1 KA-Induced Seizure Intensity is Increased in PTGS-2^{-/-}, but not PTGS-1^{-/-} or PTGS-2^{+/-}, Mice

Median seizure intensity was increased in PTGS-2^{-/-} mice when compared to PTGS-2^{+/-} and PTGS-2^{+/+} mice after KA injection (Figure 1A). Median seizure intensity in PTGS-2^{-/-} mice resulted in a Racine seizure score (RSS) of 3, a value significantly greater than in PTGS-2^{+/+} (Median =1, range=0-2) or in PTGS-2^{+/-} mice (Median=1, range=0-1). There was no significant difference in seizure intensity between PTGS-2^{+/+} and PTGS-2^{+/-} mice or between PTGS-1^{+/+} and PTGS-1^{-/-} mice (Figure 1B). Maximal seizure level attained was also significantly higher in PTGS-2^{-/-} mice (RSS=5, range= 3-6) when compared to PTGS-2^{+/+}

(RSS=4, range=1-5) and PTGS-2^{+/-} (RSS=1, Range= 1-5). No difference in latency to onset of seizure activity existed (PTGS-2^{+/+} = 6.1 ± 2.7 min vs. PTGS-2^{-/-} = 6.6 ± 2.4 min; p = 0.52).

KA-induced mortality was only observed in the PTGS-2^{-/-} mice. Of the 18 PTGS-2^{-/-} mice observed in this study, 5 (28%) reached a maximal seizure intensity that included tonic-clonic seizures. Of these 5 mice, 3 died within the 2 h observation period. No tonic-clonic seizure activity was observed in any PTGS-2^{+/+}, PTGS-2^{+/-}, PTGS-1^{+/+}, or PTGS-1^{-/-} mice injected with 10 mg/kg KA.

3.2 Fluoro Jade B Positive Neurons are only Observed in the Brains of PTGS-2^{-/-} Mice Exposed to Kainate

FJB positive neurons were observed in the brains of PTGS-2^{-/-} mice at 24 h after injection with KA (Figure 1C). All 15 PTGS-2^{-/-} mice observed for seizures that survived for 24 h expressed FJB positive neurons in the CA1/CA2 and CA3 hippocampal fields, as well as in the amygdala (anterior amygdaloid area) and thalamus (laterodorsal thalamic nuclei and the lateral posterior thalamic nuclei [45]). FJB staining was only detected in PTGS-2^{-/-} mice and not in KA-exposed PTGS-2^{+/-} or PTGS-2^{+/+} mice. No FJB staining was observed in PTGS-1^{+/+} or ^{-/-} mice at 24 h after 10, 15 or 20 mg/kg KA, in any brain region examined. However, FJB positive neurons were observed in both PTGS-1^{+/+} and PTGS-1^{-/-} mice exposed to 25 mg/kg KA but pathology index scores and incidence of staining did not differ significantly between genotype at this dosage of KA (data not shown). A trend for a decrease in cresyl violet hypochromaticity pathology index (p=0.09) was observed in the CA3 hippocampal field of PTGS-2^{-/-} mice 24 h after KA injection (Figure 1C).

Overall, there was a correlation between the median seizure intensity observed and the extent of FJB staining (Spearman Rank Correlation= 0.727; p=0.00001, n=54). This suggests that the major effect of PTGS-2 deletion is an alteration in neuronal excitability that causes an increase in seizure intensity which secondarily results in more FJB staining in PTGS-2^{-/-} mice exposed to KA.

3.3 Lack of Differential Effect of Kainate on Seizure Intensity in PTGS-1^{-/-} Mice at Higher Doses

No difference in median seizure intensity and maximal seizure intensity existed between PTGS-1^{+/+} and PTGS-1^{-/-} mice at 15, 20 or 25 mg/kg KA (Table 1), suggesting that the lack of effect observed at 10 mg/kg was not due to a sub-threshold dosage of KA being used in our experiments.

3.4 [³H] KA Binding is not Altered in PTGS-2^{-/-} Mice

No significant difference in KA binding was observed between PTGS-2^{+/+}, PTGS-2^{+/-}, and PTGS-2^{-/-} mice or between PTGS-1^{+/+} and PTGS-1^{-/-} mice in any of the regions examined (Table 2), suggesting that the ability of KA to bind the KA receptor is not altered by PTGS deletion.

3.5 Pretreatment with the CB1 Antagonist, AM-251, Increases Seizure Intensity and Neuronal Damage in Kainate-Exposed PTGS-2^{+/+} and PTGS-2^{-/-} Mice

Since PTGS-2, but not PTGS-1, can catabolize endocannabinoids [31], one possibility is that the increased susceptibility of PTGS-2^{-/-} mice to KA-induced seizures was due to an excess of endocannabinoid CB1 receptor-mediated signaling, a process known to increase the excitability of hippocampal pyramidal cells by inhibition of the GABAergic system [3,28]. To explore this possibility, we pretreated mice 30 min before KA injection with the CB1 receptor antagonist AM-251. If an endocannabinoid excess was involved, this pretreatment would be

expected to reverse the susceptibility of PTGS-2^{-/-} mice to KA. However, AM-251 pretreatment augmented KA-induced seizure intensity in both PTGS-2^{+/+} and PTGS-2^{-/-} mice (Figure 2A & 2B). Median seizure intensity after KA injection was increased in PTGS-2^{+/+} from a Racine seizure score of 1 (range=1-2) to 3 (range=1-4) in AM-251 pretreated mice (Figure 2A). In PTGS-2^{-/-} mice, median seizure intensity after KA injection increased from a Racine seizure score of 3 (range= 2-6) to 4 (range 3-6) in AM-251 pretreated mice (Figure 2B).

Pretreatment of PTGS-2^{-/-} mice with AM-251 before KA injection significantly increased the mortality rate after KA injection (AM-251 pretreated= 73% vs. vehicle=10%; Chi-Square (1 degree of freedom) = 9.64, $p=0.001$). A trend for increased mortality in AM-251 pretreated PTGS-2^{+/+} mice did exist (AM-251 pretreated = 29% vs. vehicle =0%; Chi-Square (1 degree of freedom) = 3.43, $p=0.064$). In addition to increasing seizure intensity in KA-injected PTGS-2^{+/+} mice, AM-251 pretreatment resulted in the presence of FJB positive neurons in PTGS-2^{+/+} mice exposed to 10 mg/kg KA (Figure 2C).

3.6 Whole Cell Recordings from CA1 Pyramidal Neurons from PTGS-2^{-/-} Mice Reveal Defects in GABAergic Neurotransmission

Whole-cell recordings of sIPSCs from neurons voltage-clamped at -60 mV were observed as inward currents (Fig 3A). The average resting potential, capacitance and input resistance of CA1 pyramidal neurons from both genotypes were similar (data not shown). The frequency of sIPSC occurrence was decreased by 61% in PTGS-2^{-/-} mice (6.4 ± 0.6 Hz vs. 2.5 ± 0.45 Hz; Figure 3C and 3D). There was no significant effect of genotype on sIPSC amplitude, rise time or decay time (Figure 3A and 3B). No effect of genotype was observed on mIPSC amplitude, rise time or decay time although a trend for a decrease in frequency was observed (Figure 3). Further, no effect of genotype was observed on the extent of tonic GABAergic activation [57] measured in the presence of the GABA_A receptor antagonist BMR (18.4 ± 14.0 vs. 15.8 ± 10.9 pA, $p>0.05$). Additionally, there was no effect of genotype on the tonic response to KA (3 μ M) in the presence of TTX (PTGS-2^{+/+} = 35.2 ± 14.1 pA vs. PTGS-2^{-/-} = 33.8 ± 15.0 pA, $p>0.05$), suggesting the electrophysiological characteristics of the KA receptor are not altered by PTGS deletion.

3.7 Long-term Pretreatment of Mice with Celecoxib Increases Kainate Induced Seizure Intensity and Neuronal Damage

Mice pretreated with 6000 ppm celecoxib showed KA-induced seizure intensity similar to PTGS-2^{-/-} mice (median RSS= 3, range= 1-4; Figure 4A). Pretreatment of mice with 6000 ppm celecoxib caused a significantly increased seizure intensity (median RSS=2, range= 2-4) when compared to 0 ppm (median RSS=1, range= 0-2) or 3000 ppm (median RSS=1, range= 1-2) celecoxib. Thirty five percent of the mice exposed to 6000 ppm celecoxib also demonstrated KA-induced FJB neuronal staining exclusively in the CA3 hippocampal field at 24 h post injection (Figure 4B). The increased seizure intensity and FJB staining was associated with plasma celecoxib concentrations of 13.4 ± 2.6 μ g/ml and 18.2 ± 5.8 μ g/ml in mice receiving 3000 or 6000 ppm celecoxib in the diet, respectively. These plasma concentrations are within the same order of magnitude of steady state concentrations (2-3 μ g/ml) observed in humans exposed chronically to 400-800 mg of celecoxib, doses normally used for the treatment of conditions such as rheumatoid arthritis and familial adenomatous polyposis [13]. Since only the unbound concentration (approximately 98%) of celecoxib is free to distribute across the blood brain barrier [14], this would place the range of brain concentrations of celecoxib in the human population and our study at approximately 160-940 nM, well above the IC₅₀ (39nM) of celecoxib for COX-2 [19].

3.8 Long-term Pretreatment of Mice with Celecoxib Decreases Brain PGE₂ Concentration

In PTGS-2^{+/+} mice chronically pretreated with celecoxib at concentrations that increased KA-induced seizure activity (Figure 4A) and PTGS-2^{-/-} mice resulted in decreased PGE₂ concentration in mouse brain when compared to untreated or wild-type mice (Table 3). No effect under these conditions was observed on mouse brain concentrations of PGF_{2α}, 6-keto PGF_{1α}, or TXB₂ (Table 3).

Discussion

In this study, we demonstrate the differential susceptibility of PTGS-1^{-/-} and PTGS-2^{-/-} mice to KA-induced excitotoxicity. Using a systemic dose of KA that caused minimal seizures in wild-type mice, we have shown that PTGS-2^{-/-}, but not PTGS-1^{-/-}, mice have elevated seizure intensity and an associated increase in neuronal damage. We also show that pretreatment of mice for 6 weeks with celecoxib increases the susceptibility to KA. Additionally, we demonstrate that deficits in GABAergic neurotransmission and PGE₂ brain concentration are associated with the increased susceptibility to KA in PTGS-2^{-/-} mice. Overall our findings suggest a novel role for PTGS-2, but not PTGS-1, in gating KA-induced seizure intensity.

The results of previous studies employing acute pharmacological inhibitors of PTGS to study the process of excitotoxicity have uncovered a distinct dichotomy in the central nervous system response to the sequence of PTGS inhibition in relation to excitotoxin exposure. This dichotomy represents two distinct roles for PTGS in regulating the physiological processes of neuronal excitation and the progression of the inflammatory reaction in response to neuronal cell damage. Acute pretreatment with PTGS inhibitors such as celecoxib, nimesulide and flurbiprofen has been shown to increase seizure activity in response to excitotoxic doses of KA or pentylenetetrazole [4,61], supporting the suggested role for PTGS in regulation of neuronal excitability. Conversely, PTGS inhibition by genetic or pharmacological means after neuronal damage has been initiated results in neuroprotection [9,15,18,21,26,63]. While this dichotomy is well defined for the studies mentioned above, it has also been demonstrated that mice over expressing a human form of PTGS-2 solely in neurons, had increased KA induced mortality [27]. These results described by Kelley et al are difficult to reconcile with the observation described in our study since the only endpoint measured in their study was mortality as opposed to ours which measured seizure intensity and neuronal damage. In addition, the distribution of PTGS-2 in these mice was restricted to neurons thereby making their model difficult to compare to ours which has a universal lack of PTGS-2. Furthermore, the assertion of Takemiya et al [63] that PTGS-2 deficient mice exhibit less neuronal loss after icv KA injections when compared to wild type mice suffers from the lack of both a stereology-based neuronal count [36] and of a demonstrated similarity in the baseline number of neurons in saline exposed PTGS-2 deficient and wild type mice. While we do not dispute the possible involvement of PTGS-2 in determining the extent of neuronal death, our study overwhelmingly supports the observations of those who have demonstrated that acute pharmacological abrogation of PTGS activity before KA injection increases seizure intensity. Furthermore, ours is the first study to directly compare the roles of both PTGS isoforms in regulating susceptibility of mice to systemically-administered, KA-induced seizures and to demonstrate altered GABAergic transmission in PTGS-2^{-/-} mice. The altered susceptibility observed in our study is also supported by previous studies suggesting a differential and dynamic contribution of the two PTGS isoforms to excitotoxin-induced prostaglandin synthesis in the rat hippocampus [47, 69]. Therefore, even though the basic function of PTGS is to metabolize AA to prostaglandins, it seems that PTGS-1 and PTGS-2 activity, and possibly their specific end products, are important determinants of susceptibility to excitotoxin-induced seizures.

Since it has been suggested that exogenous treatment with prostaglandins may alter neuronal excitability [1,50,51], disruption of the normal brain prostaglandin profile by genetic

abrogation of either PTGS-1 or PTGS-2 may result in an altered profile that modulates susceptibility to excitotoxicity. In this regard, certain prostaglandins, such as PGE₂, may protect against induction of seizures. Specifically, electroconvulsive shock and pentylenetetrazole induced seizures can be blocked by pretreatment with PGE₂ intracerebroventricular (icv) injections [50,51]. Further, convulsion-prone gerbils have 50% lower brain levels of PGE₂ [58] than normal gerbils, an observation consistent with the decrease in brain levels of PGE₂ reported here (Table 4) and in previous studies [4,7,61]. In contrast, PGE₂ levels are increased in PTGS-1^{-/-} mice [10] suggesting the possibility that PGE₂ may regulate neuronal inhibition. While further experiments are needed, it is possible that a difference in brain prostaglandin profiles between PTGS-1^{-/-} and PTGS-2^{-/-} mice may alter the neurophysiology of mice resulting in their differential susceptibility to KA-induced seizure. Supporting this hypothesis, differential expression of GABA-A receptor beta 1 (GABRB1) and GABA transporter 3 (GAT3) in the brains of PTGS-2^{-/-}, but not PTGS-1^{-/-}, mice has been demonstrated [64] suggesting an effect of decreased PTGS-2 activity on the GABAergic system. We further characterize this concept in the present study by demonstrating an electrophysiological defect in GABAergic neurotransmission in PTGS-2^{-/-} mice. In fact, the magnitude of decrease observed in our study for sIPSC frequency has also been observed in the hippocampus of seizure prone rats [29], suggesting that decreased sIPSC frequency is associated with decreased GABA-mediated inhibition and therefore increased susceptibility to excitotoxicity. While it is possible that the decrease in sIPSCs observed in our study is related to the decreased PTGS-2 activity and PGE₂ brain concentrations, the mechanism of this relationship is still not fully understood and further experiments involving the effect of pharmacological and genetic inhibition of PTGS-2 on the GABAergic system are required. However, our study is the first to describe alterations in GABA neurotransmission in a model of decreased brain prostaglandin production, thereby suggesting that genetic ablation of PTGS-2 is associated with an increased susceptibility to excitotoxicity, possibly resulting from decreased GABAergic inhibition.

We also demonstrate no involvement of CB1 receptor activation in the increased susceptibility of PTGS-2^{-/-} mice to KA-induced excitotoxicity. Previous studies have suggested that activation of the CB1 receptors on GABA interneurons by retrograde endocannabinoid signaling induced by depolarization results in an increase in synaptic excitation due to a depolarization-induced disinhibition [3,28]. Since it is known that PTGS-2, but not PTGS-1, can catabolize endocannabinoids [30-32], we hypothesized that PTGS-2^{-/-} mice experience an excess of endocannabinoid production which would be expected to increase synaptic excitation [3,28] and that inhibition of the CB1 endocannabinoid receptor by AM-251 pretreatment before KA-injection would abrogate the enhanced sensitivity of PTGS-2^{-/-} mice to KA by blocking the CB1-mediated disinhibition. Contrary to our hypothesis, antagonism of the CB1 receptor prior to KA administration augmented, rather than diminished, the KA-induced seizure activity and neuronal damage, suggesting that the fraction of endocannabinoid that undergoes PTGS-2 metabolism does not significantly contribute to this process of seizure susceptibility. Our observation is consistent with a previous suggestion that activation of glutamatergic neuron CB1 receptors acts as an “on-demand” system for protecting against excitotoxicity [37]. While our study eliminates the possibility that activation of the CB1 receptor through endocannabinoid excess mediates the observed susceptibility to KA in PTGS-2^{-/-} mice, it does not rule out the involvement of endocannabinoids in this process. It has recently been demonstrated that novel PTGS-2-derived oxygenated metabolites of 2-arachidonylethanolamide (2-AG), such as PGE₂-glycerol, can modulate the frequency of inhibitory postsynaptic currents in neuronal cultures in a CB1 independent fashion [53]. Therefore, it is possible that the lack of PTGS-2 in PTGS-2^{-/-} mice results in a decreased production of these metabolites of 2-AG and in the subsequent alteration in the process of excitotoxicity.

While abnormalities in the macula densa and juxtaglomerular apparatus have been previously described in adult PTGS-2 deficient mice [21], Iadecola and colleagues [21] have also reported that PTGS-2 deficient mice show creatinine levels in the normal range, suggesting normal renal function in adult PTGS-2 deficient mice. Further, since the dicarboxylate excitatory amino acid analogs are mainly eliminated by glomerular filtration [62] and no alterations in the glomeruli and renal tubules have been characterized [21], the renal clearance of KA is unlikely to be altered in PTGS-2 deficient mice. Additionally, we found that the time to onset of seizure activity was not different between PTGS-2 deficient and wild type mice, suggesting no alteration in either the absorption of KA from the peritoneum nor in its distribution to the brain. To further support the fact that a reduction of PTGS-2 activity in the brain is the major determinant of seizure susceptibility in our model, we show that mice pretreated chronically with celecoxib also have an increased susceptibility to KA-induced seizures and neuronal damage. This suggests that it is the decreased activity of PTGS-2 in the brain that is mediating the susceptibility to KA-induced seizure and the involvement of compensatory changes or of renal lesions, while important to consider, do not contribute significantly to the observations made in the PTGS deficient mice.

Beyond the fact that a strong correlation existed between the amount of FJB staining and seizure intensity, it is difficult to provide a mechanistic reason for the differences in FJB staining between wild type and knockout mice since we did not investigate the manner and mechanism of cell death in this study. As far as the difference in location and propensity for FJB staining between PTGS-2 deficient mice and celecoxib-treated mice is concerned, a possible explanation may be attributed to the fact that PTGS-2 deficient mice have a complete lack of PTGS-2 whereas it is unlikely that 100% of PTGS-2 activity was inhibited by the conditions of celecoxib treatment used in this study. This difference in PTGS-2 activity, while not important for the generation of differences in seizure intensity, may contribute to the anatomical distribution and quantity of FJB staining observed in our study.

The demonstration that genetic or chronic pharmacological abrogation of PTGS-2, but not PTGS-1, activity increases susceptibility to KA-induced excitotoxicity raises an important issue regarding the possible consequence of long term inhibition of brain PTGS-2 activity in humans. Chronic pharmacological inhibition of PTGS with NSAIDs in humans is a therapeutic strategy commonly used to treat chronic pain and inflammation. Due to the gastrointestinal side-effects of PTGS-1 inhibition, selective PTGS-2 inhibitors were developed for the treatment of chronic inflammation. While we have demonstrated that PTGS-2^{-/-} mice and mice chronically exposed to a PTGS-2 inhibitor are more susceptible to seizures and the resulting neuronal damage, it is unclear if chronic clinical usage of PTGS-2 selective inhibitors in humans decreases the level of brain PTGS activity to that observed in PTGS-2^{-/-} mice [14]. However, our demonstration that selective reduction in PTGS-2 activity increases susceptibility to KA-induced excitotoxicity suggests that chronic PTGS-2 inhibition may be a risk factor for increased seizure susceptibility. We suggest that this previously unrecognized relationship between chronic inhibition of PTGS-2 and an increased risk of seizure intensity should be examined in the human population using epidemiological methods.

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Figure 1. PTGS-2^{-/-}, but not PTGS-1^{-/-} or PTGS-2^{+/-}, have elevated KA-induced seizure intensity
 a) Frequency histogram of Racine Seizure Score (RSS) demonstrates increased median seizure intensity in PTGS-2^{-/-} mice (open bars; Kruskal-Wallis ANOVA $H = 40.09$, $p = 0.00001$) exposed to 10 mg/kg KA when compared to PTGS-2^{+/-} (hatched bars; Mann-Whitney U-test $z = 4.88$, $p < 0.00001$) and PTGS-2^{+/+} (filled bars; Mann-Whitney U-test $z = 4.75$, $p < 0.00001$) mice. There was no significant difference in seizure intensity between PTGS-2^{+/+} and PTGS-2^{+/-} mice (Mann-Whitney U-test $z = 0.57$, $p > 0.05$). RSS represents median seizure intensity over a 2 h observation period.

b) Median seizure scores for PTGS-1^{-/-} mice (open bars) and PTGS-1^{+/-} mice (filled bars) demonstrate no significant difference (Mann-Whitney U-test $z = 0.00001$, $p = 0.999$) in seizure intensity after injection with 10 mg/kg KA.

c) Representative photomicrographs of Fluoro Jade B (FJB) and cresyl violet-stained brain slices are depicted for PTGS-2^{-/-} mice exposed to KA. Magnification is at 40× objective and reference bar represents 50 μm. FJB positive neurons and cresyl violet hypochromaticity were only observed in the brains of PTGS-2^{-/-} mice exposed to 10mg/kg kainate. PTGS-2^{-/-} mice that died during the 2 h observation session (n=3) did not have FJB positive neurons, despite exhibiting severe tonic-clonic seizures (data not shown). No FJB positive neurons were observed in saline injected mice of any genotype (data not shown).

Figure 2. AM251 Pretreatment Augments Seizure Activity in PTGS-2^{-/-} and PTGS-2^{+/+} Mice Exposed to Kainate

Frequency histogram of Racine Seizure Score (RSS) demonstrates increased median seizure intensity in a) PTGS-2^{+/+} (Mann Whitney U test: $z = 3.72$, $p = 0.0001$) and b) PTGS-2^{-/-} mice (Mann Whitney U test: $z = 1.94$, $p = 0.03$) pretreated with 30 mg/kg AM-251 thirty min before exposure to 10 mg/kg KA (open bars), when compared to mice pretreated with vehicle (filled bars). RSS represents median seizure intensity over a 2 h observation period. c) Representative photomicrographs of Fluoro Jade B (FJB) and cresyl violet-stained brain slices are depicted for PTGS-2^{+/+} and PTGS-2^{-/-} mice exposed to KA thirty minutes after pretreatment with 30 mg/kg AM-251 or vehicle. All mice shown above received KA after the pretreatment. 24 h

after KA injection, a significant increase in FJB staining was observed in the CA1/CA2 (Mann Whitney U test, $z=2.26$, $p=0.01$) and CA3 (Mann Whitney U test, $z=1.89$, $p=0.03$) hippocampal fields of PTGS-2^{+/+} mice only. Magnification is at 40× and reference bar represents 50 μm. AM-251 pretreatment did not significantly worsen or ameliorate the extent of FJB staining in the CA1/CA2, CA3 hippocampal fields, thalamus or amygdala of PTGS-2^{-/-} mouse brain after KA exposure. Irrespective of genotype, no FJB stained neurons were observed in AM-251 or vehicle- pretreated mice that received saline instead of KA.

Figure 3. Whole Cell Recordings from CA1 Pyramidal Cells Reveals Defect in GABAergic Neurotransmission in PTGS-2^{-/-} Mice

a) Representative whole cell recordings of CA1 pyramidal cell GABA sIPSCs and mIPSCs from PTGS^{+/+} and PTGS^{-/-} mice. b) sIPSC and mIPSC rise time and decay time constant (Tau) do not differ between PTGS^{+/+} and PTGS^{-/-} mice. c) Representative sweeps of mIPSC and sIPSC whole cell recordings. d) Frequency of occurrence (t-statistic= 5.17; n=21 cells from 4 mice; p=0.00001) but not amplitude (t-statistic= 0.51; p=0.61) of sIPSCs is decreased in PTGS^{-/-} mice mIPSC frequency (t-statistic= 1.67; n=18 cells from 4 mice; p=0.10) and amplitude (t-statistic= 0.12; p=0.90) are not affected by genotype.

Figure 4. Long-term Celecoxib Pretreatment Increases Kainate Induced Seizure Intensity and Neuronal Damage

a) Frequency histogram of Racine Seizure Score (RSS) demonstrates increased median kainate-induced seizure intensity in mice pretreated for six weeks with a diet containing 6000 ppm celecoxib. Data points with different letters in the parenthesis in the legend are significantly different from each other at $p < 0.05$. b) Representative FJB stained sections demonstrate FJB positive neurons in the CA3 hippocampal field of mice pretreated with 6000 ppm celecoxib or PTGS-2^{-/-} mice. No FJB positive neurons are observed in 0 or 3000 ppm celecoxib pretreated mice. Magnification is at 40 $\times$ and reference bar represents 50 μ m. None of the mice pretreated with 0 or 3000 ppm Celecoxib expressed FJB positive neurons 24 h after KA injection.

Table 1

Median and Maximal Seizure Intensity are not Significantly Changed in PTGS-1^{-/-} Mice in Response to Kainate

Genotype	Kainate (mg/kg)	Number	Median Seizure Score (range)	Maximal Seizure Score (range)
PTGS-1 ^{+/+}	15	8	2 (1-2)	5 (4-6)
PTGS-1 ^{-/-}	15	9	2 (1-4)	5 (2-6)
PTGS-1 ^{+/+}	20	8	2 (2-3)	4 (3-5)
PTGS-1 ^{-/-}	20	9	2 (1-3)	4 (2-5)
PTGS-1 ^{+/+}	25	8	3 (1-6)	5 (4-6)
PTGS-1 ^{-/-}	25	7	4 (1-4)	6 (4-6)

No difference in median Racine seizure score (15 mg/kg: p=0.25; 20 mg/kg: p=0.50; 25 mg/kg: p= 0.30) and maximal (15 mg/kg: p=0.50; 20 mg/kg:

p=0.22; 25 mg/kg: p=0.30) Racine seizure score existed between the PTGS-1^{+/+} and PTGS-1^{-/-} mice. Data are expressed as median with range provided in parenthesis.

Table 2

Kainate binding is not altered by PTGS genotype

Genotype	Cornu Ammonis 3 (nCi/mg)	Cerebral Cortex (nCi/mg)	Striatum (nCi/mg)
PTGS-1 ^{+/+}	7.57 ± 1.06	6.12 ± 0.62	6.80 ± 0.60
PTGS-1 ^{-/-}	8.08 ± 1.22	6.51 ± 0.31	7.02 ± 0.46
PTGS-2 ^{+/+}	8.03 ± 0.81	5.75 ± 1.35	6.49 ± 1.11
PTGS-2 ^{+/-}	7.84 ± 1.88	5.52 ± 0.27	6.84 ± 0.87
PTGS-2 ^{-/-}	8.84 ± 1.38	6.00 ± 0.81	6.80 ± 1.07

Using [³H] KA autoradiography, no significant difference in [³H]-KA binding was observed between PTGS-1 ^{+/+} and PTGS-1 ^{-/-} (n=6 and 7, respectively) mice in the CA3 hippocampal field (p=0.44), cerebral cortex (p=0.17) and striatum (p=0.47). No significant difference in KA binding was observed between PTGS-2 ^{+/+}, PTGS-2 ^{+/-}, and PTGS-2 ^{-/-} mice (n=8 /group) in the CA3 hippocampal field (p=0.35), cerebral cortex (p=0.59), and striatum (p=0.76). Data are expressed as mean ± SD.

Table 3Chronic PTGS-2 Inhibition by Genetic or Pharmacological Means Decreases Brain Levels of PGE₂

Celecoxib Dose (ppm)	PGE ₂ (ng/g)	PGF _{2α} (ng/g)	6-keto PGF _{1α} (ng/g)	TXB ₂ (ng/g)
0	9.8 ± 2.3 ^A	0.21 ± 0.12	2.0 ± 1.2	0.06 ± 0.03
3000	5.5 ± 2.5 ^B	0.22 ± 0.05	2.5 ± 1.4	0.05 ± 0.02
6000	5.1 ± 1.6 ^B	0.14 ± 0.09	2.3 ± 1.4	0.05 ± 0.02
PTGS-2 KO	4.6 ± 1.3 ^C	0.18 ± 0.11	2.0 ± 1.0	0.06 ± 0.03

A decrease in PGE₂ was observed in PTGS-2^{-/-} mice and PTGS-2^{+/+} mice fed a diet for six weeks containing celecoxib (ANOVA F (3,31)=8.46; p=0.001). No effect was observed on brain concentrations of PGF_{2α} (ANOVA F(3,33)= 1.11; p=0.36), 6-ketoPGF_{1α} (ANOVA F (3,37)=0.38 p=0.77) or TXB₂ (ANOVA F (3,31)=1.43; p=0.24). Data are mean ± SD. Data points with different letters are statistically different from each other at p<0.05