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Letter

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Detection of cyclooxygenase-2-derived oxygenation products of the endogenous cannabinoid 2-arachidonoylglycerol in mouse brain

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Abstract

Cyclooxygenase-2 (COX-2) catalyzes the formation of prostaglandins, which are involved in immune regulation, vascular function, and synaptic signaling. COX-2 also inactivates the endogenous cannabinoid (eCB) 2-arachidonoylglycerol (2-AG) via oxygenation of its arachidonic acid backbone to form a variety of prostaglandin glyceryl esters (PG-Gs). Although this oxygenation reaction is readily observed *in vitro* and in intact cells, detection of COX-2-derived 2-AG oxygenation products has not been previously reported in neuronal tissue. Here we show that 2-AG is metabolized in the brain of transgenic COX-2-overexpressing mice and mice treated with the lipopolysaccharide to form multiple species of PG-Gs that are detectable only when monoacylglycerol lipase is concomitantly blocked. Formation of these PG-Gs is prevented by acute pharmacological inhibition of COX-2. These data provide evidence that neuronal COX-2 is capable of oxygenating 2-AG to form a variety PG-Gs *in vivo* and support further investigation of the physiological functions of PG-Gs.

Keywords

Endocannabinoid, prostaglandin glyceryl esters, cannabinoid, monoacylglycerol lipase, Lumiracoxib, lipopolysaccharide, prostamide

Introduction

Endogenous cannabinoids (eCBs) are a class of bioactive lipid signaling molecules implicated in a variety of physiological processes including appetite regulation, energy homeostasis, cardiovascular function, motivation, anxiety, and sleep[1-5]. Within neurons, the eCBs 2-AG and anandamide (AEA) are generated in an activity-dependent manner and serve primarily as retrograde signaling molecules that reduce presynaptic neurotransmitter release via activation of type-1 cannabinoid receptors[6]. eCBs may also have direct postsynaptic effects on membrane potential and neuronal excitability[7, 8]. Despite these well-established roles of eCBs, how these lipids are metabolized via non-canonical routes to generate potentially distinct signaling molecules is an active area of investigation.

The exact biosynthesis of AEA is poorly understood, but AEA is primarily metabolized by neuronal fatty-acid amide hydrolase (FAAH) to arachidonic acid (AA)[9]. However, COX-2 can also metabolize AEA to form prostaglandin ethanolamides (PG-EAs; prostamides)[10]. The prostamides include PGE_2-EA, PGD_2-EA, and $PGF_{2\alpha}$-EA and have been previously detected *in vivo*, supporting the notion that COX-2 serves as a non-canonical eCB metabolic enzyme. For example, $PGF_{2\alpha}$-EA is detected upon COX-2 upregulation in both rodent dorsal spinal neurons after carrageenan-induced knee inflammation[11] and in preadipocyte cells where $PGF_{2\alpha}$-EA acts to prevent adiopogenesis[12]. It has been suggested that the ability to detect prostamides *in vivo* may be facilitated by their relative metabolic stability and lack of metabolism by FAAH[13].

2-AG is a highly abundant eCB that mediates retrograde inhibition at central glutamatergic and GABAergic synapses[14]. Synaptic 2-AG is generated via the sequential actions of phospholipase C and diacylglycerol lipase-α, in response to a variety of activity-dependent mechanisms. In addition to its signaling functions, 2-AG is thought to be the most common

source of AA for PG production in the CNS[15]. The synaptic actions of 2-AG are terminated predominantly via monoacylglycerol lipase (MAGL), but also by α/β-hydrolase domain 6 and 12, as well as COX-2[16]. Indeed, 2-AG is efficiently oxygenated by COX-2 *in vitro* to form prostaglandin glyceryl esters (PG-Gs), and 2-AG-mediated retrograde synaptic inhibition can be enhanced by pharmacological inhibition of COX-2[10, 17-20]. Despite being detected in macrophages following lipopolysaccharide (LPS) administration[21-23] and in inflamed rat hindpaw[24], COX-2-derived oxidative metabolites of 2-AG in brain have remained elusive. It is possible that PG-Gs are hydrolytically unstable *in vivo* or rapidly further metabolized, and thus have eluded detection. Given that PG-Gs have been recently identified as endogenous agonists of P2Y6 receptors[25] and implicated as neuronal signaling molecules[16] and pain modulators in the periphery[24], a clearer understanding of the conditions under which PG-Gs are formed is needed.

Here we use an LPS model of neuroinflammation, a transgenic COX-2 overexpression model, and pharmacological approaches combined with stable isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) to detect and quantify PG-G formation in mouse brain for the first time. These data provide evidence that neuronal COX-2 can produce PG-Gs in the brain when 2-AG levels are elevated via MAGL inhibition and provide direct support for the contention that neuronal COX-2 metabolizes 2-AG *in vivo*.

Results and Discussion

We first optimized our analytical detection of PG-Gs by LC/MS/MS using deuterated PGE_2-G and PGD_2-G as authentic standards. PGE_2-G, PGD_2-G and $PGF_{2\alpha}$-G were identified and quantified by LC-MS/MS analysis. These three analytes and the internal standards (PGE_2-G-d5 and $PGF_{2\alpha}$-G-d5) were ionized by complexation with the ammonium cation, resulting in an

$[M+NH_4]^+$ complex. Multiple product ions were observed when this complex was subjected to collision induced dissociation (CID). For example, the CID spectrum of the $[PGE_2\text{-}G+NH_4]^+$ complex (*m/z* = 444.3) is shown in Fig. 1a, where the product ions at *m/z* 391.2, 317.3, and 299.0 are observed. Fragmentation of the $[PGD_2\text{-}G+NH_4]^+$ complex gives a similar spectrum (not shown). For both PGE_2-G and PGD_2-G, the selected reaction monitoring (SRM) transition *m/z* 444.2 – 391.1 was used for quantitative analysis as this transition gave the greatest signal:noise ratio. Table S1 gives the SRM transitions for all analytes reported here. The chromatographic system we employed here was able to resolve the *sn*-1 and *sn*-2 isomers of PGE_2-G, PGD_2-G and $PGF_{2\alpha}$-G (Fig. S1). PG-Gs are monoacylglycerols and thus will undergo isomerization from the presumed original *sn*-2 conformation to the thermodynamically favored *sn*-1 conformation. Importantly, LC-MS/MS analysis using alternative SRM transitions (*m/z* 444.2 – 317.1 & 444.2 – 299.0) generated nearly identical chromatograms to that generated by *m/z* 444.2 – 391.1 transition, providing compelling support for our accurate and reliable detection of authentic PG-Gs using our analytical approach (Fig. S2 a-b).

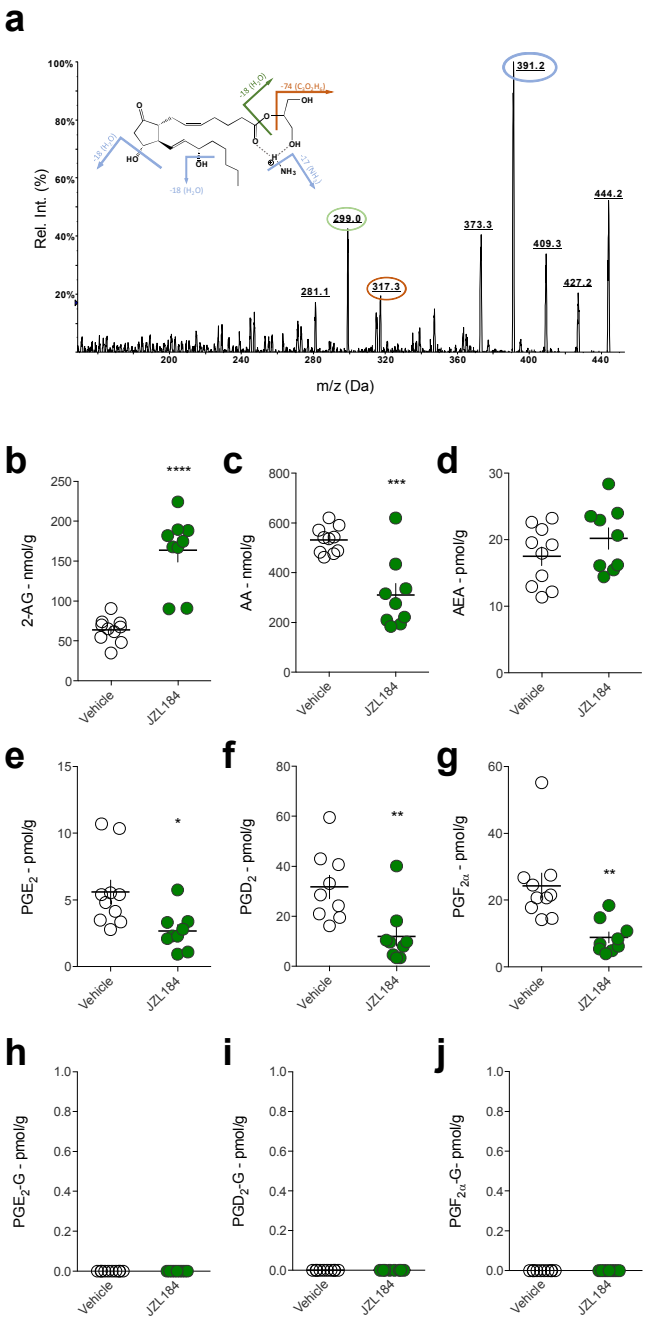

Figure. 1. PG-Gs are not detected in C57/Bl6J mouse brain after MAGL inhibition. (a) CID spectra of PGE_2-G. Upon CID, the ammonium complex of PGE_2-G (*m/z* 444) undergoes sequential loss of NH_3 and two molecules of H_2O to generate the product ions at *m/z* 427, 409 and 391, respectively. Subsequently, *m/z* 391 species will undergo a loss of H_2O and C_3H_6 O_2 (74 Da) – in either order – to generate the product ions at *m/z* 373, 317 and 299. Circled peak 391 was used for quantitative analysis while 299 and 317 were used for confirmatory analysis (see Fig. S1). (b-d) Effects of JZL184 on 2-AG, AA, and AEA levels in mouse brain. (e-g) Effects of JZL184 on prostaglandin levels in mouse brain. (h-j) Effects of JZL184 on PG-G levels in mouse brain, relative to vehicle-treated mice. Each point (circle) represents a biological replicate (one mouse). Veh n=10, JZL184 n=9. Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by t-test.

To determine whether PG-Gs are present in mouse brain, we extracted lipids from one hemisphere of C57/Bl6J mouse brains and analyzed them for PG-G levels. Under these conditions, we were unable to detect any native PG-G species, consistent with our previous attempts and previously published studies[20, 24] (Fig. 1h-j, vehicle-treated mice). We hypothesized that COX-2-mediated generation of PG-Gs might be substrate-limited, and thus the lack of detectable PG-Gs may be due to insufficient levels of 2-AG available for oxygenation by COX-2. To test this hypothesis, we administered the MAGL inhibitor JZL184 (40 mg/kg) to elevate 2-AG levels. As expected, 2-AG levels were significantly elevated after JZL184 treatment, whereas free AA levels were reduced, and AEA levels were unchanged (Fig. 1b-d). Also, consistent with previous studies, levels of PGs were reduced in JZL184-treated mice relative to vehicle-treated mice[15] (Fig. 1e-g). Despite elevated 2-AG levels present in the brains of JZL184-treated mice, we were still unable to detect PG-Gs under these conditions (Fig. 1h-j). It has been suggested that PG-Gs are metabolized by MAGL[26], therefore the lack of detectable PG-Gs after JZL184 treatment was quite surprising and suggests that elevation of 2-AG combined with PG-G degradation inhibition may not be sufficient for generation of detectable levels of PG-Gs. Alternatively, the levels of 2-AG may not be sufficiently high after JZL184 treatment to allow for PG-G formation.

We next considered the possibility that COX-2 levels are too low to generate detectable levels of PG-Gs under basal conditions. To test this hypothesis, we utilized COX-2-Tg mice that overexpress human COX-2 (hCOX-2) in neurons. COX-2-Tg mice showed high levels of hCOX-2 in the cerebellum and prefrontal cortex (PFC) consistent with previous studies[27] (Fig. 2a). Levels of PGs, but not free AA, were highly elevated in COX-2-Tg mice (Fig. 2b-c), however we were unable to detect PG-Gs in vehicle-treated COX-2-Tg mice (Fig 2d-f). In

contrast, when COX-2-Tg mice were treated with JZL184, a variety of PG-Gs were easily detected (Fig. 2d-f).

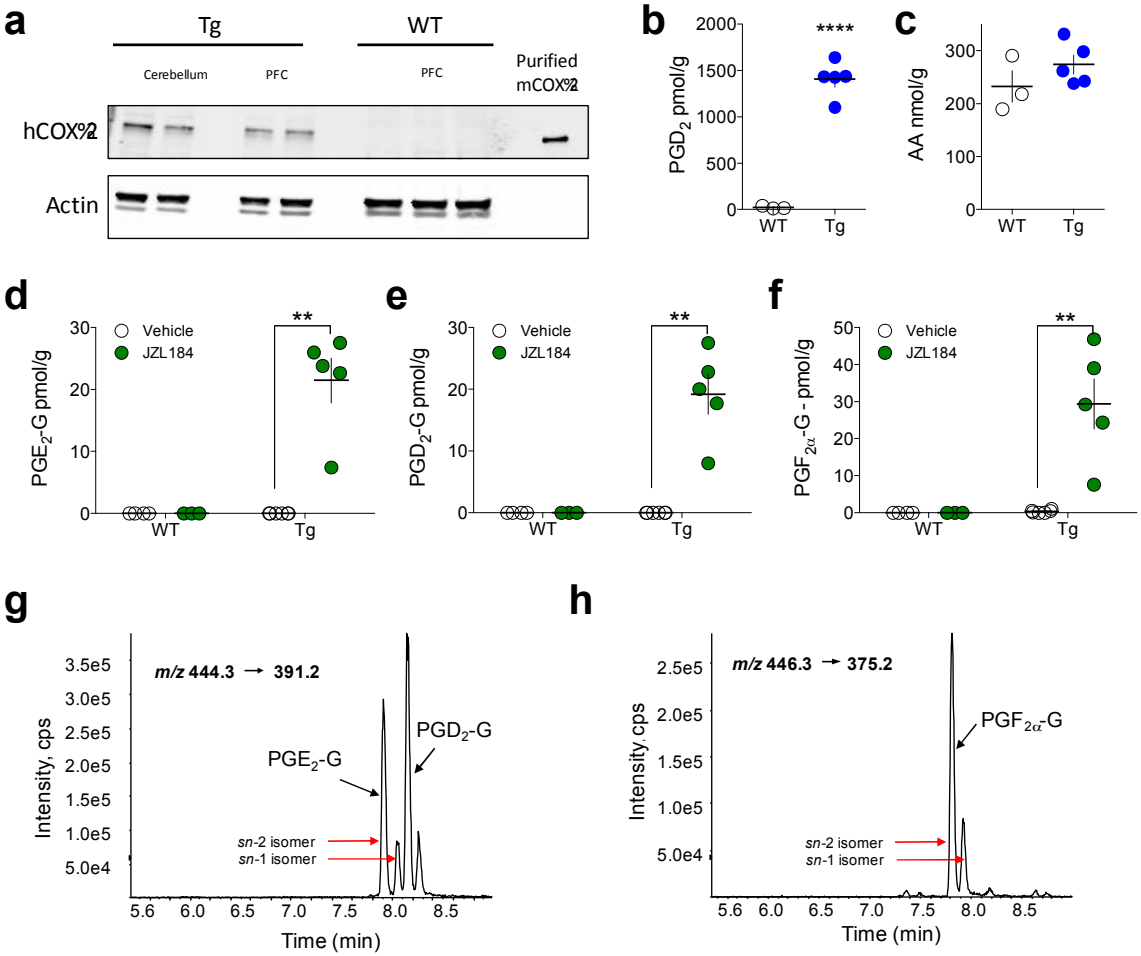

Figure 2. Detection of PG-Gs in COX-2-Tg mice after MAGL inhibition. (a) Validation of COX-2 overexpression in COX-2-Tg mice by western blot. (b-c) COX-2-Tg mice (Tg; n=5, WT; n=3) exhibit high levels of PGD_2 but not AA. (d-f) Effects of JZL184 on PG-G levels in WT and COX-2-Tg mice (WT Veh n=4, WT JZL n=3, Tg Veh n=6, Tg JZL n=5). (g-h) Representative chromatograms of endogenous PGE_2-G, PGD_2-G, and $PGF_{2\alpha}$-G in COX-2-Tg mouse brain after JZL184 treatment. ** $p<0.01$, **** $p<0.0001$ by t-test (b) or Holm-Sidak test after two-way ANOVA (d-f). Each point (circle) represents a biological replicate (one mouse). Error bars represent S.E.M. Also see Supplementary Fig. 2 (c-d) for representative chromatogram of native PG-Gs using alternate SRM transitions.

Specifically, we detected both *sn*-1 and *sn*-2 isomers of PGE_2-G, PGD_2-G and $PGF_{2\alpha}$-G in the brains of COX-2-Tg mice treated with JZL184 (40 mg/kg) (Fig. 2d-f and g-h). The *sn*-2 isomer

predominated in all cases (Fig. 2g-h and Fig. S2c-d for confirmatory analysis using alternate SRM transitions). Detection of PG-Gs in COX-2-Tg mice was not due to higher levels of 2-AG after JZL184 treatment relative to WT mice, as 2-AG levels were comparable in WT and COX-2-Tg mice after JZL184 treatment (Fig. S3).

In order to confirm the increase in PG-Gs observed in COX-2-Tg mice after JZL184 treatment was indeed mediated via enzymatic activity of COX-2, we pretreated COX-2-Tg mice with the highly selective COX-2 inhibitor lumiracoxib (LMX; 5 mg/kg). LMX treatment resulted in high drug levels in brain tissue (Fig. 3a) and prevented formation of PG-Gs in COX-2-Tg mice treated with JZL184 (Fig 3b-d). LMX also reduced PG levels in COX-2-Tg mice treated with JZL184 (Fig. 3e-h).

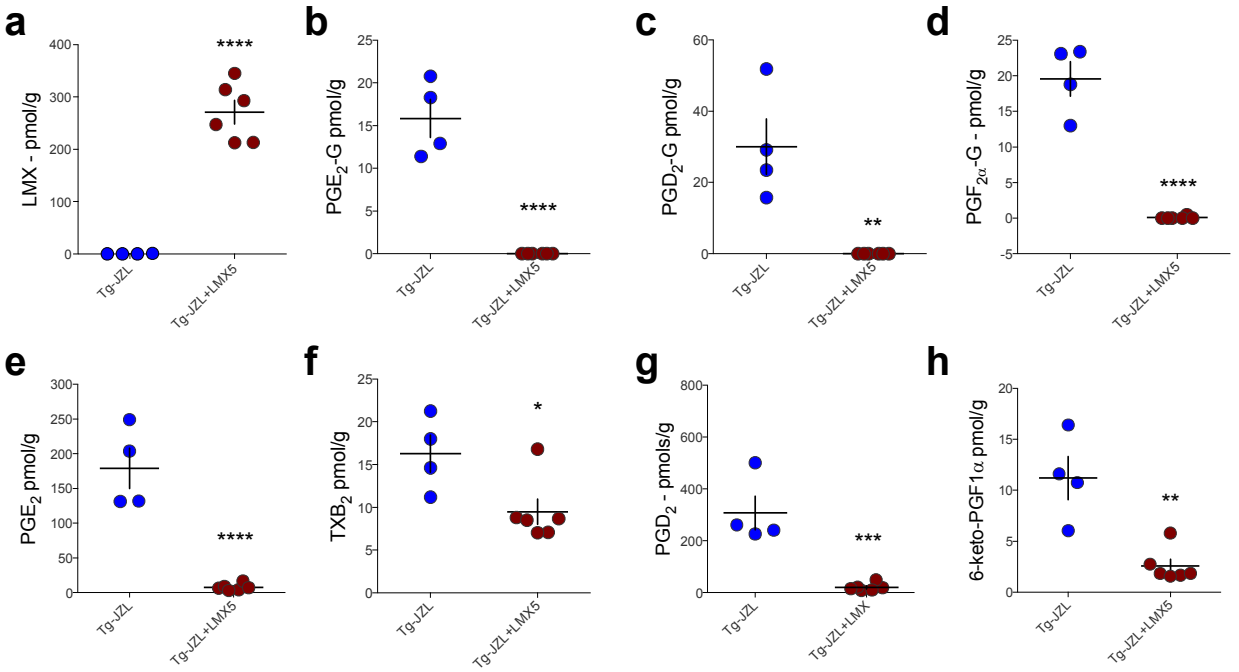

Figure 3. Lumiracoxib (LMX) reduces PG-G levels in COX-2-Tg mice treated with JZL184. (a) LMX is detected at high levels in brain after i.p. administration (5 mg/kg). (b-d) LMX treatment reduced PG-G levels in JZL184-treated COX-2-Tg mice. (e-h) LMX decreased PG levels in JZL184-treated COX-2-Tg mice (Tg-JZL n=4, Tg-JZL+LMX n=6). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by unpaired t-test. Each point (circle) represents a biological replicate (one mouse). Error bars represent S.E.M.

We also investigated the effects of two putative substrate-selective COX-2 inhibitors, *R*-flurbiprofen[28] (9 mg/kg) and LM-4131[29] (10 mg/kg), in combination with JZL184 (40 mg/kg) in COX-2-Tg mice. Both *R*-flurbiprofen and LM-4131 prevented the formation of PG-Gs, and also reduced levels of all PG species, in COX-2-Tg mice treated with JZL184 (Fig. S4). These data suggest that under the conditions used here, neither compound demonstrated clear substrate-selective profile *in vivo*. However, we found that ~10% of LM-4131 converted to indomethacin, and also detected *S*-flurbiprofen in *R*-flurbiprofen-treated mice (Fig. S5), suggesting pharmacokinetic issues could complicate interpretation of these findings since neither indomethacin nor *S*-flurbiprofen are substrate-selective.

Lastly, the detection of PG-Gs have been reported in other *in vivo* models of increased COX-2 expression, namely under inflammatory conditions[30]. To assess the potential COX-2-mediated generation of PG-Gs using an inflammatory model of increased COX-2 expression, we induced neuroinflammation in mice using lipopolysaccharide (LPS) treatment. We pretreated WT mice with LPS (3 mg/kg, once daily for two days), and JZL184 2h prior to sacrifice. LPS alone and LPS combined with JZL184 increased PGE_2 and 6-keto-$PGF_{1\alpha}$ (Fig. 4 a-d) while only LPS combined with JZL184 significantly increased PG-G levels (Fig. 4 e-f), albeit to levels approximately 100-fold lower than seen in COX-2-Tg mice treated with JZL184.

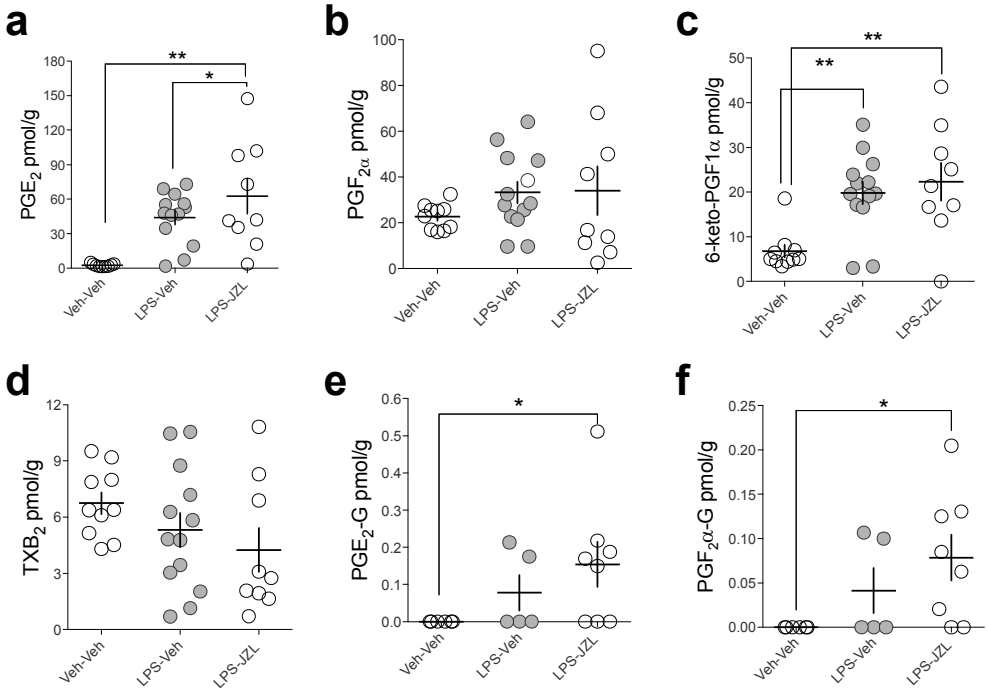

Figure 4. LPS combined with JZL184 pretreatment increases PG and PG-G levels in brain. (a-d) LPS (3 mg/kg, twice over 48 hours) and LPS+JZL increased PGE_2 and 6-keto-PGF1α levels in WT mice (Veh-Veh n=10, LPS-Veh n=13, LPS-JZL n=9). (e-f) LPS+Veh and LPS+JZL did not significantly increase PG-Gs in WT mice (Veh-Veh n=6, LPS-Veh n=5, LPS-JZL n=8). * $p<0.05$, ** $p<0.01$ by Fisher's LSD test after one-way ANOVA. Each point represents biological replicate (one mouse). Error bars represent S.E.M.

Here we show that PG-Gs are generated in mouse brain under conditions of increased neuronal COX-2 expression combined with elevated 2-AG levels. Given that our approach to elevating 2-AG utilized inhibition of MAGL, which has also been suggested to hydrolyze PG-Gs[26], it is likely that PG-G hydrolysis inhibition facilitated *in vivo* detection of PG-Gs under these conditions. This is in contrast to prostamides, which are not hydrolyzed by FAAH[13], which may account for their previous *in vivo* detection[11, 12]. Our data also indicate a requirement for COX-2 enzymatic activity in the generation of PG-Gs, since formation was prevented by the highly selective COX-2 inhibitor LMX. In addition to LMX, we utilized two other compounds that have demonstrated substrate-selective activity (i.e. preferentially inhibiting 2-AG

oxygenation by COX-2 over AA) in various assays, *R*-flurbiprofen[28] and LM-4131[29]. Both compounds prevented the formation of most PGs and PG-Gs to a similar degree indicating a lack of substrate-selectivity *in vivo*. Our data indicate that *in vitro* substrate-selectivity of these compounds does not translate to *in vivo* substrate-selectivity, possibly due to *in vivo* conversion to non-substrate-selective metabolites. Development of metabolically stable substrate-selective probes is needed to determine whether substrate-selectivity measured in recombinant enzymatic and cellular assays can be measured *in vivo*.

The function of PG-G signaling is not well understood. At the synaptic level, exogenous PG-Gs can increase inhibitory neurotransmission, possibly via release of intracellular calcium[16]; these effects are opposite those seen with 2-AG which *decreases* inhibitory neurotransmission via CB1 receptor activation[6, 16]. Therefore, it is possible that elevated neuronal COX-2 expression represents a mechanism by which traditional eCB signaling can be "switched" from inhibition to facilitation of presynaptic neurotransmitter release through generation of PG-Gs acting through an as of yet unidentified receptor target. Interestingly, the recent report that PG-Gs activate P2Y6 receptors[25], which are known to trigger calcium release in astrocytes[31], suggests that PG-Gs may indirectly regulate neurotransmission via astrocyte-neuronal crosstalk. Based on our findings, the conditions under which PG-G signaling occurs would most likely require elevated COX-2 expression and increased levels of 2-AG production, such as those observed after brain injury or inflammation for example[32-36]. That LPS injection combined with JZL184 treatment led to the detection of PG-Gs (albeit at very low levels) further supports this notion. Future studies should be aimed at determining the precise physiological or pathophysiological conditions under which PG-Gs are generated and the functional significance of these signaling molecules.

Methods

Animals: Male and female COX-2-Tg mice (Jackson Laboratories, Bar Harbor, ME; JAX stock #010703) that overexpress COX-2 under the neuron specific Thy-1 promoter or wild-type littermates derived from heterozygous breeding pairs were used as subjects. Mice were 60-90 days of age at time of experimentation. COX-2-Tg and wild-type (WT) progeny were genotyped using PCR analysis of tail genomic DNA. Briefly, tail tissue from tail snips were placed on 95^{o}C heat block in lysis solution for one hour with neutralizing solution applied immediately after. Samples were centrifuged at 4000 rpm for 3 min at 4^{o}C. 2µl of each sample was loaded, with REDTaq DNA polymerase (Sigma-Aldrich, St. Louis, MO), autoclaved H_2O, and hCOX-2 DNA primers. Following PCR, the results were visualized with gel electrophoresis, using an agarose gel run at 105 V, 3.00 A, for 45 min. Transgenic bands were defined with a top band at 500 kb, and WT mice had top band as well as bottom band (700 kb)[27]. All WT mice referenced in these experiments are native WT littermates of the COX-2-Tg colony. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Vanderbilt University Office of Animal Welfare.

Drugs: The MAGL inhibitor JZL184 (40 mg/kg) and *R*-flurbiprofen (9 mg/kg) were purchased from Cayman Chemicals, Ann Arbor, MI; Lumiracoxib (5 mg/kg) from Selleck Chemicals, TX, USA; and LM-4131 (10 mg/kg), was synthesized as previously described[29]; and were administered via intraperitoneal injection (1 ml/kg DMSO) 3 h before sacrifice. LPS (Sigma-Aldrich, St. Louis, MO), was administered via intraperitoneal injection (10 ml/kg; pyrogen-free saline), with two injections of LPS given, 24 h apart. Four hours after the second injection, mice

were treated with JZL184 (40 mg/kg). Mice were sacrificed 3 hours after JZL184 or DMSO injection.

Western Blot: Western blot analysis for COX-2 was performed as previously described using a rabbit-anti hCOX-2 antibody[28] (Cayman Chemicals, Ann Arbor, MI).

LC-MS/MS: Whole mouse brains were frozen on dry ice and stored at -80°C prior to processing and analysis. Brain tissue was prepared for LC-MS/MS analysis as previously described. All LC-MS/MS analysis was performed on a Shimadzu Nexera system in-line with a SCIEX 6500 QTrap as previously described[37]. The QTrap was equipped with a TurboV Ionspray source and operated in both positive and negative ion mode. SCIEX Analyst software (ver 1.6.2) was used to control the instruments and acquire and process the data. The deuterated internal standards AEA-d4, 2-AG-d5, AA-d8, PGE_2-d4, PGD_2-d4, $PGF_{2\alpha}$-d4, 6-keto-$PGF_{2\alpha}$-d4, TXB_2-d4, PGE_2-G-d5 were purchased from Cayman Chemicals (Ann Arbor, MI). Authentic unlabeled standards of these analytes also were purchased from Cayman.

For PG, eCB, and AA analysis, the analytes were chromatographed on an Acquity UPLC BEH C18 reversedphase- column (5.0 x 0.21 cm; 1.7 μm) which was held at 40°C. A gradient elution profile was applied to each sample; %B was increased from 15% (initial conditions) to 99% over 4.0 min and held at 99% for another minute. Then the column was returned to initial conditions for 1.5 min prior to the next injection. The flow rate was 330 μL/min and component A was water with 0.1% formic acid while component B was 2:1 acetonitrile:methanol (v/v) with 0.1% formic acid.

For PG-G analysis, the analytes were chromatographed on a Acquity BEH C18 column (10.0 x 0.21 cm; 1.7 μm) which was held at 42°C. A gradient elution profile was applied to each sample; %B was increased from 6% (initial conditions) to 50% over 8.5 min, then increased to 99% over 0.5 min and held at 99% for another 2.2 min. Finally, the column was returned to initial conditions for 2.5 min prior to the next injection. The flow rate was 300 μL/min and component A was 10 mM ammonium acetate (pH adjusted to approximately ≈ 3.3 with glacial acetic acid) while component B was acetonitrile with 5% component A.

All analytes were detected using a SCIEX 6500 QTrap via selected reaction monitoring. Table S1 gives the Q1 and Q3 *m/z* values, collision energy (CE), declustering potential (DP) and ionization mode for all analytes and their respective internal standards. Analytes were quantitated by stable isotope dilution against their deuterated internal standard. Data was normalized to tissue mass and are presented as either "pmol/g tissue" or "nmol/g tissue".

Abbreviations

Collision energy (CE), collision-induced dissociation (CID), cyclooxygenase-2 (COX-2), declustering potential (DP), endogenous cannabinoid (eCB), liquid chromatography- tandem mass spectrometry (LC-MS/MS), mass to charge ratio (*m/z*), monoacylglycerol lipase (MAGL), prefrontal cortex (PFC), prostaglandin glyceryl esters (PG-G), prostaglandin ethanolamide (PG-EA), selected reaction monitoring (SRM), transgenic (Tg), 2-arachidonoylglycerol (2-AG), anandamide (AEA), fatty-acid amide hydrolase (FAAH), arachidonic acid (AA), lipopolysaccharide (LPS), lumiracoxib (LMX).

Author Information

Author contributions: A.M. generated and genotyped mice, performed *in vivo* experiments, and prepared tissue for analysis; P.J.K. performed LC-MS/MS analytical analysis; and M.M.M. performed western blot experiments under supervision of S.P. and L.J.M. M.A., T.P., and A.G., contributed to mouse model generation, tissue preparation, and genotyping under supervision of S.P. A.M. and S.P. wrote the manuscript with input from all authors.

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Conflicts of Interest: Vanderbilt University owns patent #14/415,977 entitled "Compositions and Methods for Substrate-Selective Inhibition of Endocannabinoid Oxygenation." S.P. is a scientific consultant for Psy Therapeutics and has an active research contract with H. Lundbeck A/S.

Supporting Information

Table S1. Mass Spectral Parameters for Lipid Analysis

Figure S1. Isomerization of PG-Gs.

Figure S2. PGE_2-G and PGD_2-G Chromatography.

Figure S3. 2-AG levels in WT and COX-2-Tg mice.

Figure S4. *R*-flurbiprofen and LM-4131 reduce PG-G levels in COX-2-Tg mice treated with JZL184.

Figure S5. *In vivo* metabolism of LM-4131 and *R*-flurbiprofen.

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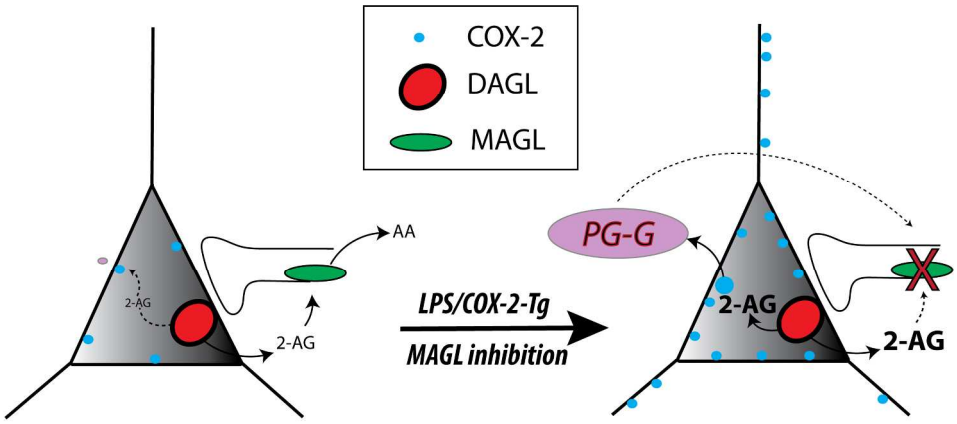

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