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RESEARCH PAPER

Inhibition of COX-2 expression by endocannabinoid 2-arachidonoylglycerol is mediated via PPAR-γ

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BACKGROUND AND PURPOSE
Endocannabinoids have both anti-inflammatory and neuroprotective properties against harmful stimuli. We previously demonstrated that the endocannabinoid 2-arachidonoylglycerol (2-AG) protects hippocampal neurons by limiting the inflammatory response via a CB_1 receptor-dependent MAPK/NF-κB signalling pathway. The purpose of the present study was to determine whether PPARγ, an important nuclear receptor, mediates 2-AG-induced inhibition of NF-κB phosphorylation and COX-2 expression, and COX-2-enhanced miniature spontaneous excitatory postsynaptic currents (mEPSCs).

EXPERIMENTAL APPROACH
By using a whole-cell patch clamp electrophysiological recording technique and immunoblot analysis, we determined mEPSCs, expression of COX-2 and PPARγ, and phosphorylation of NF-kB in mouse hippocampal neurons in culture.

KEY RESULTS
Exogenous and endogenous 2-AG-produced suppressions of NF-κB-p65 phosphorylation, COX-2 expression and excitatory synaptic transmission in response to pro-inflammatory interleukin-1β (IL-1β) and LPS were inhibited by GW9662, a selective PPARγ antagonist, in hippocampal neurons in culture. PPARγ agonists 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and rosiglitazone mimicked the effects of 2-AG on NF-κB-p65 phosphorylation, COX-2 expression and mEPSCs, and these effects were eliminated by antagonism of PPARγ. Moreover, exogenous application of 2-AG or elevation of endogenous 2-AG by inhibiting its hydrolysis with URB602 or JZL184, selective inhibitors of monoacylglycerol lipase (MAGL), prevented the IL-1β- and LPS-induced reduction of PPARγ expression. The 2-AG restoration of the reduced PPARγ expression was blocked or attenuated by pharmacological or genetic inhibition of the CB_1 receptor.

CONCLUSIONS AND IMPLICATIONS
Our results suggest that CB_1 receptor-dependent PPARγ expression is an important and novel signalling pathway in endocannabinoid 2-AG-produced resolution of neuroinflammation in response to pro-inflammatory insults.

LINKED ARTICLES
This article is part of a themed issue on Cannabinoids in Biology and Medicine. To view the other articles in this issue visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

Abbreviations
2-AG, 2-arachidonoylglycerol; eCBs, endocannabinoids; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; RIM, rimonabant; MAGL, monoacylglycerol lipase; mEPSCs, miniature spontaneous excitatory postsynaptic currents; URB, URB602

Introduction

Endocannabinoids (eCBs) are endogenous signalling mediators involved in a variety of physiological, pharmacological and pathological processes (Freund *et al.*, 2003; Kano *et al.*, 2009; Marrs *et al.*, 2010; Pertwee *et al.*, 2010). The eCB system consists of eCBs, cannabinoid receptors (CB_1 and CB_2), enzymes synthesizing eCBs and metabolizing eCBs and cannabinoid transporters. Emerging evidence now suggests that eCBs possess anti-inflammatory and neuroprotective properties against harmful insults (Marsicano *et al.*, 2003; Walter and Stella, 2004; Panikashvili *et al.*, 2001; 2005, 2006; Eljaschewitsch *et al.*, 2006; Mackie, 2006; Centonze *et al.*, 2007; Zhang and Chen, 2008; Chen *et al.*, 2011). Neuroinflammation is the immune system's response to infection and injury in the CNS and has been implicated in the pathogeneses and neuropathology of many prevalent neurological and neurodegenerative diseases, such as multiple sclerosis, Alzheimer's and Parkinson's diseases (Chen, 2010). However, there are currently no effective medications to prevent chronic inflammation and alleviate or treat chronic inflammation-induced brain disorders. As eCBs have the ability to restrict inflammation, they may have the potential to prevent neuropathology and treat neuroinflammation-induced brain disorders. While eCB modulation of both GABAergic and glutamatergic synaptic transmission and plasticity via a CB_1 receptor-dependent mechanism has been extensively investigated (Alger, 2002; Freund *et al.*, 2003; Piomelli, 2003; Chevaleyre *et al.*, 2006; Mackie, 2006; Kano *et al.*, 2009), the mechanisms underlying the ability of eCB to limit neuroinflammation and provide neuronal protection have not been elucidated (Sarne and Mechoulam, 2005; Van der Stelt and Di Marzo, 2005; Fowler *et al.*, 2010).

PPARs are members of the nuclear receptors/transcription factors super-family. Three different PPAR genes (PPARα, PPARδ – also called PPARβ and PPARγ) have been identified. These nuclear receptors regulate expression of the genes involved in metabolism, cell differentiation and inflammation (Daynes and Jones, 2002; Kozak *et al.*, 2002; Luna-Medina *et al.*, 2005; Drew *et al.*, 2006; Bensinger and Tontonoz, 2008; Bright *et al.*, 2008; Necela *et al.*, 2008). Growing evidence suggests that PPARs are likely a target for eCBs (Lenman and Fowler, 2007; O'Sullivan, 2007; Sun *et al.*, 2007; O'Sullivan and Kendall, 2010; Pertwee *et al.*, 2010; Pistis and Melis, 2010). However, little information is available as to whether PPARs are involved in this inhibitory effect of eCBs on neuroinflammation. 2-Arachidonoyl glycerol (2-AG), the most abundant eCB and a full agonist for CB_1 and CB_2 receptors (Sugiura *et al.*, 2006; Kano *et al.*, 2009), has been shown to be an important endogenous signalling mediator protecting neurons from pro-inflammatory, excitotoxic stimuli and other harmful insults (Gopez *et al.*, 2005; Panikashvili *et al.*, 2001; 2005, 2006; Melis *et al.*, 2006; Kreutz *et al.*, 2007). Rockwell *et al.* (2006) demonstrated that 2-AG-induced suppression of IL-2 in Jurkat T cells is mediated by activation of PPARγ through a $CB_{1/2}$ receptor-independent mechanism. We demonstrated previously that direct application of 2-AG or elevation of endogenous 2-AG by inhibiting its hydrolysis protects hippocampal neurons from the effects of pro-inflammatory IL-1β and LPS, excitotoxic glutamate and kanaic acid and β-amyloid stimuli by limiting COX-2 expression via a CB_1 receptor-dependent NF-κB signalling pathway (Zhang and Chen, 2008; Chen *et al.*, 2011). In this report, we show that 2-AG-induced suppression of NF-κB phosphorylation, COX-2 expression and COX-2 elevation-enhanced excitatory synaptic transmission in hippocampal neurons in culture in response to pro-inflammatory insults is mediated by the CB_1 receptor-dependent expression of PPARγ.

Methods

Cell culture

Primary hippocampal neurons from mouse pups (P0 to P1) were cultured as described previously (Sang *et al.*, 2005; 2007, 2010; Zhang and Chen, 2008; Chen *et al.*, 2011), according to the guidelines approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center in New Orleans. Briefly, hippocampi were dissected out under a microscope and triturated in serum-free culture medium after the meninges had been removed. Tissue was incubated in oxygenated trypsin for 10 min at 37°C and then mechanically triturated. Cells were spun down and resuspended in Neurobasal/B27 medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 0.5 mM L-glutamine, penicillin/streptomycin and 25 μM glutamate. Cells (1×10^6) were loaded into poly-D-lysine-coated six-well plates for Western blot analysis and 35 mm culture dishes for electrophysiological recordings. Medium was changed every 3 days with the same medium without glutamate until use. The percentage of astroglial cells in the culture was ~5% at 10 days *in vitro* (DIV), as estimated by staining with a neuronal marker NeuN, astrocytic marker GFAP and microglial marker OX-42 in conjunction with the 4′,6-diamidino-2-phenylindole staining as described previously (Sang *et al.*, 2005; Zhang and Chen, 2008). Cultures were used between 10 and 21 DIV.

Electrophysiological recordings

Miniature spontaneous excitatory postsynaptic currents (mEPSCs) were recorded in hippocampal neurons in culture under voltage clamp using an Axopatch-200B amplifier (Molecular Devices, Palo Alto, CA) as described previously (Sang *et al.*, 2005; 2006, 2007, 2010; Zhang and Chen, 2008). Recording pipettes (4–5 MΩ) were pulled from borosilicate glass with a micropipette puller (Sutter Instrument, Novato, CA). The internal pipette solution contained (in mM) 115.0 Cs gluconate, 15.0 CsCl, 4.0 NaCl, 10.0 HEPES, 0.5 EGTA, 4.0 Mg_2ATP and 0.5 Na_2GTP (pH 7.25 with CsOH). The membrane potential was held at −70 mV. The external solution contained (in mM): 130.0 NaCl, 2.5 KCl, 1.0 MgCl2, 10.0 HEPES, 1.25 NaH_2PO4, 2.0 $CaCl_2$ and 25.0 glucose (pH 7.4 with NaOH). To isolate mEPSCs, tetrodotoxin (TTX, 0.5 to 1 μM), a voltage-gated Na^+ channel blocker and bicuculline (10 μM), a $GABA_A$ receptor blocker, were included in the external solution. All experiments were performed at room temperature (22–24°C). The frequency, amplitude and kinetics of mEPSCs were analysed using the MiniAnalysis program (Synaptosoft, Fort Lee, NJ).

Immunoblot

Hippocampal neurons in cultures were extracted and immediately homogenized in a one-to-one volume of modified

radioimmunoprecipitation assay lysis buffer consisting of a number of protease inhibitors. Supernatants were fractionated on 10% SDS-PAGE gels (Bio-Rad Lab, Hercules, CA, USA) and transferred onto PVDF membranes (Bio-Rad). The membrane was incubated with anti-COX-2 polyclonal antibodies (dilution of 1:1000; Cayman Chemical, Ann Arbor, MI), anti-NF-κB-p65 and phospho-NF-κB antibodies (1:1000; Cell Signaling, Danvers, MA) and anti-PPARγ (1:1000; Abcam, Cambridge,MA) at 4°C overnight. The blot was washed and incubated with a secondary antibody (goat anti-rabbit, 1:10 000; Vector Laboratories, Burlingame, CA) at room temperature for 1 h. Proteins were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, UK). The densities of specific bands were quantified by densitometry using FUJIFILM Multi Gauge software (version 3.0) (Fujifilm USA Inc., Valhalla, NY, USA). Band densities were normalized to the total amount of protein loaded in each well as determined by mouse anti-β-actin (1:4000; Sigma, St. Louis, MO) as described previously (Zhang and Chen, 2008; Sang *et al*., 2005; 2010).

Chemicals and drugs

2-AG, AEA, URB602, JZL184, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), GW9662 and rosiglitazone were purchased from Cayman Chemical. T0070907 was purchased from Tocris Bioscience (Ellisville, MO, USA). These chemicals were dissolved in DMSO to make stock solutions at concentrations of 20 to 50 mM, distributed in small vials and then diluted with the external solution or culture medium to the desired concentrations just before experiments. Rimonabant (provided by Chemical Synthesis and Drug Supply Program, the National Institute of Mental Health) was dissolved in DMSO to make up stock solutions at concentrations of 50 to 100 mM. All other drugs and chemicals were obtained from Sigma, unless stated otherwise. To rule out potentially nonspecific effects of the solvents, the same amount of ethanol or DMSO was included in the control external solution and culture medium. Nomenclature of cannabinoid receptors follows Alexander *et al*. (2009).

Data analysis

Data are presented as mean ± SEM. Unless stated otherwise, ANOVA with Fisher's PLSD test or Student–Newman–Keuls test was used for statistical comparison when appropriate. The Kolmogorov–Smirnov test was used for comparisons of mEPSCs distribution. Differences were considered significant when $P < 0.05$.

Results

Inhibition of COX-2 and NF-kB phosphorylation by 2-AG is mediated by PPARγ

To determine whether 2-AG-inudced anti-inflammatory effects involve PPARs, we targeted PPARγ since it represses the expression of inflammatory genes in response to pro-inflammatory stimuli (Bensinger and Tontonoz, 2008). We used phosphorylation of NF-κB-p65 and expression of COX-2 as biomarkers for neuroinflammation in cultured hippocampal neurons as described previously (Zhang and Chen, 2008). As shown in Figure 1A,B, phosphorylation of NF-κB-p65 and expression of COX-2 were significantly elevated in hippocampal neurons in culture treated with IL-1β (10 ng·mL^{-1}) or LPS (1 μg·mL^{-1}), commonly used pro-inflammatory stimuli. This elevation was inhibited or attenuated by exogenous application of 2-AG (3 μM), consistent with our previous observations (Zhang and Chen, 2008). However, the suppressive effect of 2-AG on NF-κB-p65 phosphorylation and COX-2 expression induced by IL-1β or LPS was blocked by GW9662 (5 μM), a selective PPARγ antagonist. This suggests that the 2-AG-produced suppression of IL-1β- and LPS-induced NF-κB-p65 phosphorylation and COX-2 expression is mediated by PPARγ. This was further confirmed by the results showing that IL-1β or LPS down-regulated the expression of PPARγ, and this down-regulation was prevented or restored by 2-AG (Figure 1C,D).

PPARγ is involved in 2-AG-induced suppression of COX-2-enhanced excitatory synaptic transmission

An elevation of COX-2 expression by pro-inflammatory IL-1β or LPS enhances mEPSCs (Sang *et al*., 2005; Zhang and Chen, 2008). We thus treated cultures with IL-1β or LPS for 16 and 24 h, respectively, and recorded mEPSCs in hippocampal neurons in the absence and presence of 2-AG. As expected, IL-1β or LPS, which elevates COX-2 expression, significantly augmented the frequency but not the amplitude of mEPSCs (Figure 2). This enhancement was suppressed in the presence of 2-AG (1 μM). However, application of GW9662 reversed this 2-AG-induced suppression, suggesting a role for PPARγ in the 2-AG-induced suppression of COX-2-enhanced mEPSCs. To further ascertain the effect of PPARγ inhibition on 2-AG-induced suppression of mEPSCs in IL-1β- or LPS-treated cultures, we used another selective PPARγ antagonist, T0070907 (T007) (Lee *et al*., 2002). Similar to GW9662, T007 (1 μM) also blocked the effect of 2-AG (Figure S1). We also measured the kinetics of mEPSCs in IL-1β- or LPS-treated neurons in the absence and presence of 2-AG or GW9662. There were no significant differences in the rise or decay time constants between the vehicle controls and the treated neurons (data not shown).

Endogenous 2-AG-induced suppression of neuroinflammation is mediated by PPARγ

To raise the levels of endogenous 2-AG, we used two selective MAGL inhibitors, URB 602 and JZL184. Since JZL184 displays higher selectivity and potency than URB602 for MAGL over FAAH (Hohmann *et al*., 2005; Long *et al*., 2009a,b), we used URB 602 at 10 μM or JZL184 at 1 μM. As seen in Figure 3A–D, administration of URB602 or JZL184 significantly reduced IL-1β- or LPS-induced phosphorylation of NF-κB-p65 and expression of COX-2. URB602- and JZL184-induced suppression of IL-1β- or LPS-induced NF-κB-p65 phosphorylation and COX-2 expression was blocked by GW9662. To determine whether an elevation of endogenous 2-AG is also capable of restoring the IL-1β- or LPS-reduced expression of PPARγ, we detected the expression of PPARγ in the absence and presence of URB602 or JZL184. As shown in Figure 3E–H, URB602 (10 μM) or JZL184 (1 μM) restored the IL-1β- or LPS-induced down-regulation of PPARγ. From these results, it appears that both exogenous and endogenous 2-AG-induced

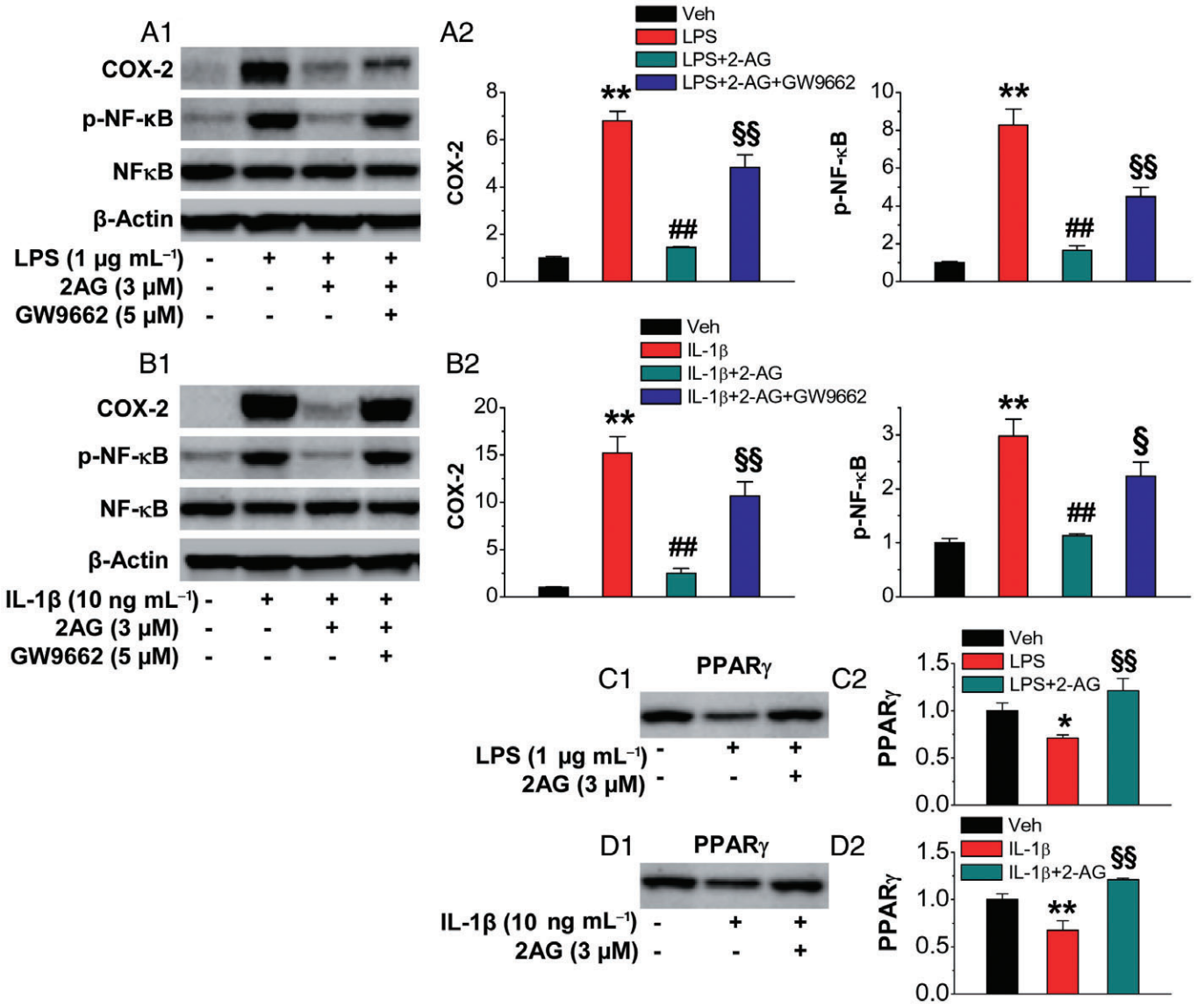

Figure 1

Exogenous application of 2-AG suppresses NF-κB phosphorylation and COX-2 expression and elevates PPARγ expression in response to pro-inflammatory IL-1β and LPS insults. Hippocampal neurons in culture were treated with IL-1β (10 ng·mL^{-1}) for 6 h or LPS (1 μg·mL^{-1}) for 16 h in the absence and presence of 2-AG or GW9662 (5 μM). The different time points used for the treatments of IL-1β and LPS were based on our previous studies where we identified that IL-1β- or LPS-induced COX-2 expression and NF-kB phosphorylation reached the peaks at these time points (Zhang and Chen, 2008). 2-AG or GW9662 was added 30 min before IL-1β or LPS application. (A1–A2) Immunoblot analysis of 2-AG suppression of LPS-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 ($n = 3$). (B1–B2) Immunoblot analysis of 2-AG suppression of IL-1β-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 ($n = 3$). (C1–C2) 2-AG restores LPS-induced down-regulation of PPARγ ($n = 3$). (D1–D2) 2-AG restores IL-1β-induced down-regulation of PPARγ ($n = 3$). **$P < 0.01$, compared with the vehicle control; ##$P < 0.01$ compared with IL-1β or LPS; §$P < 0.05$, §§$P < 0.01$ compared with IL-1β+2-AG or LPS+2-AG.

inhibition of COX-2 expression and NF-κB phosphorylation are mediated by PPARγ.

PPARγ is involved in endogenous 2-AG-induced suppression of COX-2-enhanced excitatory synaptic transmission

If the exogenous application of 2-AG is capable of suppressing the COX-2-enhanced mEPSCs, then elevating the level of endogenous 2-AG by inhibiting its hydrolysis, as described above, should also be able to inhibit the enhanced frequency of mEPSCs in IL-1β- or LPS-treated cultures. Moreover, if PPARγ mediates this endogenous 2-AG-induced suppression of NF-κB phosphorylation and COX-2 expression, then blockade of PPARγ should be able to reverse the endogenous 2-AG-induced suppression of COX-2-enhanced mEPSCs. As seen in Figures 4 and 5, treating the culture with URB602 or JZL184 significantly reduced IL-1β- or LPS-induced

enhancement of mEPSCs frequency, suggesting that an elevation of endogenous 2-AG also is capable of preventing the increase in excitatory synaptic transmission induced by COX-2. The URB602- or JZL184-produced suppression was blocked by GW9662. This suggests that, similar to the effects of exogenously applied 2-AG, the elevation of endogenous 2-AG produced by inhibiting MAGL is sufficient to reduce the release of excitatory neurotransmitter glutamates induced by COX-2 and PPARγ mediates this inhibitory effect of endogenous 2-AG on excitatory synaptic transmission.

PPARγ agonists suppress phosphorylation of NF-κB, expression of COX-2 and enhancement of mEPSCs induced by IL-1β and LPS

To determine whether PPARγ agonists or activators mimic the actions of 2-AG in resolving IL-1β- or LPS-induced NF-κB-p65 phosphorylation, COX-2 expression and enhanced mEPSCs, we used 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and rosiglitazone (Ros), both PPARγ agonists (Park *et al.*, 2003). As seen in Figure 6, administration of 15d-PGJ_2 (2 μM) or Ros (1 μM) significantly reduced IL-1β- or LPS-induced phosphorylation of NF-κB-p65 and expression of COX-2; these effects of 15d-PGJ_2 and Ros were blocked by GW9662. Similarly, 15d-PGJ_2 and Ros also suppressed IL-1β- or LPS-induced enhancement of mEPSCs, and again this suppression was blocked by antagonism of PPARγ (Figures 7 and 8).

CB_1 receptor mediates 2-AG-produced restoration of reduced PPARγ expression by LPS

To determine whether 2-AG-induced increase in PPAR expression is dependent on CB_1 receptors, we conducted another set of experiments where the culture was treated with rimonabant (RIM), a selective CB_1 receptor antagonist, in the presence of LPS and 2-AG, URB602 or JZL184. As shown in Figure 9A–C, LPS significantly decreased the expression of PPARγ, and this decrease was restored by 2-AG (3 μM), URB602 (10 μM) or JZL184 (1 μM). However, the action of 2-AG, URB602 or JZJ184 on PPARγ expression was blocked by RIM (1 μM), suggesting a CB_1 receptor-mediated effect. To further confirm that restoration of the LPS-suppressed PPARγ expression by direct application of 2-AG or enhancement of endogenous 2-AG is mediated via a CB1 receptor, we treated cultured hippocampal neurons from mice deficient in the CB_1 receptor with LPS in the presence of 2-AG, URB602 or JZl184. As shown in Figure 9D, 2-AG, URB602 or JZl184 failed to restore the LPS-induced suppression of PPARγ, confirming that the action of 2-AG on PPARγ expression is CB_1 receptor-dependent. To determine whether endogenous 2-AG-produced suppression of LPS- or IL-1β-induced enhancement of mEPSCs is also mediated via a CB_1 receptor, we recorded mEPSCs in culture treated with RIM in the presence of LPS or IL-1β and URB602. As illustrated in Figure S2, URB602-induced suppression of the increase in mEPSCs induced by LPS- or IL-1β was blocked by RIM (1 μM). These results suggest that 2-AG-induced increase in PPARγ expression is mediated primarily via the CB_1 receptor.

We need to mention here that 2-AG or URB602 alone did not alter the basal expression of COX-2 or the basal activity of mEPSCs. This is consistent with previously described observations (Zhang and Chen, 2008). In addition, we demonstrated that treating the culture with JZL184, rosiglitazone, 15d-PGJ_2 or GW alone did not alter the basal activity of mEPSCs (data not shown). In particular, we provide evidence that JZL184, Ros, 15d-PGJ_2 or GW9662 alone did not significantly alter the basal expression of COX-2 (Figure S3). This suggests that 2-AG probably functions as an important signalling mediator maintaining the homeostasis of brain function.

Discussion and conclusion

In the present study, we provide evidence that exogenous application of 2-AG or the elevation of endogenous 2-AG, produced by inhibiting its hydrolysis with selective MAGL inhibitors URB602 and JZl184, is capable of suppressing NF-kB-p65 phosphorylation and COX-2 expression. This expands upon our previous work where we discovered that 2-AG protects neurons against harmful insults by limiting the inflammatory response (Zhang and Chen, 2008; Chen *et al.*, 2011). In particular, we demonstrate here that the 2-AG-produced suppression NF-κB-p65 phosphorylation, COX-2 expression and mEPSC enhancement by pro-inflammatory IL-1β- or LPS is mediated via PPARγ. This suggests PPARγ is a target for 2-AG in protecting neurons against pro-inflammatory insults. Since the anti-neuroinflammatory effects and restoration of the LPS-reduced PPARγ expression by exogenous and endogenous 2-AG are largely blocked by pharmacological or genetic inhibition of the CB_1 receptor, the actions of 2-AG were not through direct interaction with nuclear PPARγ. More likely, they are mediated primarily by CB_1 receptor-dependent changes in PPARγ expression.

Arachidonoyl ethanolamide (AEA or anandamide) and 2-AG are the two most studied eCBs. However, despite their similar chemical structure, 2-AG and AEA display distinct profiles in their synthesis, metabolism, cannabinoid receptor binding affinity and synaptic modulation (Freund *et al.*, 2003; Sugiura *et al.*, 2006; Kano *et al.*, 2009). In particular, AEA and 2-AG exhibit differences in their ability to limit neuroinflammation and protect neurons from harmful insults. For instance, 2-AG has been shown to protect neurons from brain ischaemia, traumatic brain injury and pro-inflammatory stimuli (Panikashvili *et al.*, 2001; 2005, 2006; Melis *et al.*, 2006; Kreutz *et al.*, 2007; Zhang and Chen, 2008). However, AEA shows a paradoxical phenomenon in its neuroprotective effects against inflammatory and excitotoxic stimuli and even induces neurotoxicity *per se* (Cernak *et al.*, 2004; Movsesyan *et al.*, 2004; Sarne and Mechoulam, 2005; Van der Stelt and Di Marzo, 2005; Fowler *et al.*, 2010). This implies that 2-AG is probably an endogenously intrinsic signalling mediator protecting neurons against harmful insults and maintaining tissue homeostasis (Yang and Chen, 2008). Since the exogenous application or endogenous elevation of 2-AG suppressed the pro-inflammatory IL-1β- or LPS-induced phosphorylation of NF-κB-p65 and expression of COX-2 (two key markers in inflammation), strengthening the 2-AG signalling pathway by inhibition of MAGL will be beneficial in resolving neuroinflammation, which is the root of many neurological disorders and neurodegenerative diseases (Chen, 2010). MAGL is the enzyme that hydrolyzes 85% of 2-AG in the brain

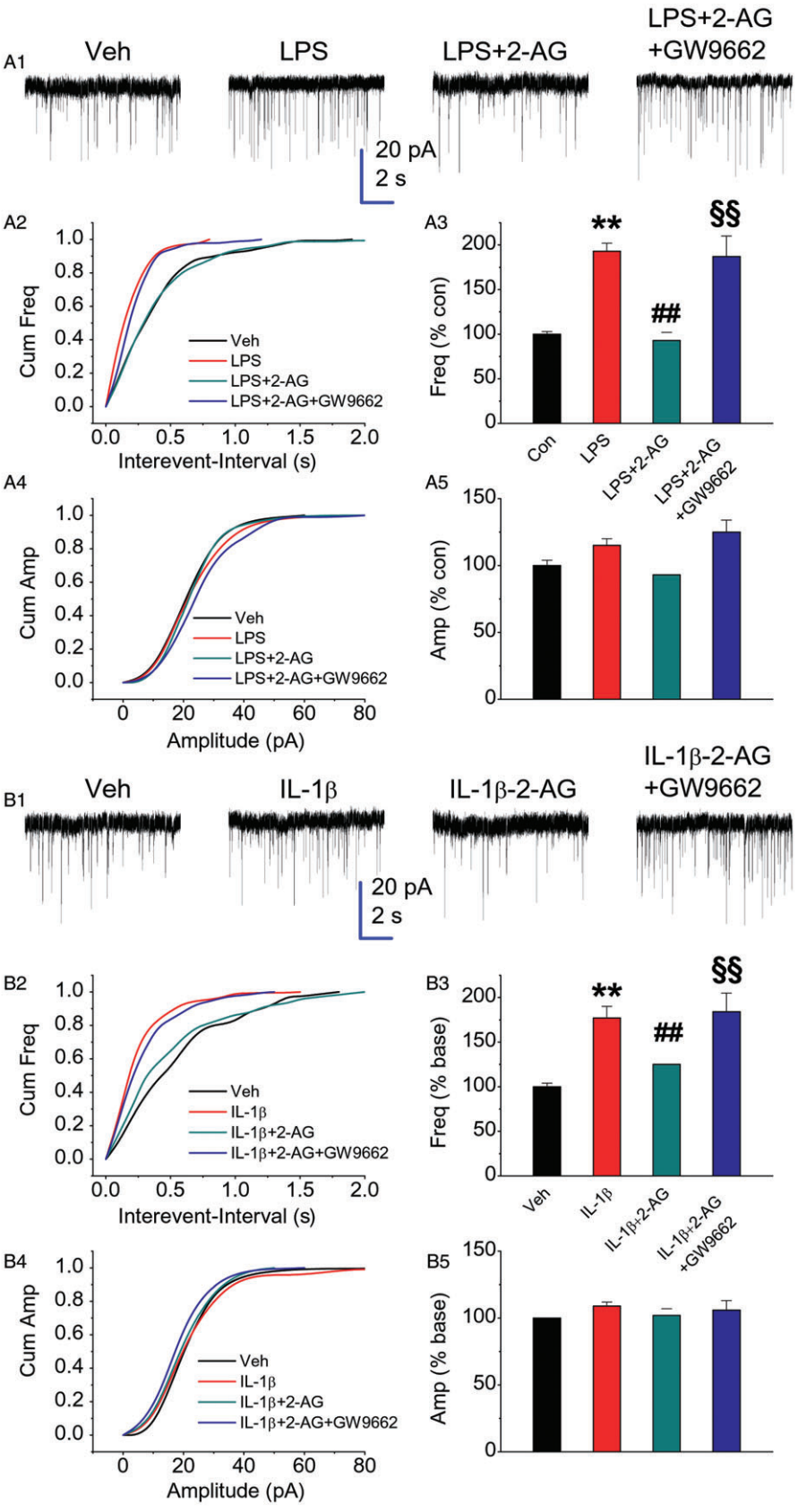
A1
Veh
LPS
LPS+2-AG
LPS+2-AG
+GW9662
20 pA
2 s
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Figure 2

2-AG inhibits COX-2 elevation-induced enhancement of mEPSCs. Hippocampal neurons in culture were treated with IL-1β (10 ng·mL^{-1}) for 16 h or LPS (2 μg·mL^{-1}) for 24 h in the absence and presence of 2-AG (1 μM) or GW9662 (5 μM). The different time points used for the treatment of IL-1β and LPS were based on our previous studies where we identified that IL-1β or LPS significantly enhanced synaptic activity at these time points (Sang *et al.*, 2005; Zhang and Chen, 2008). (A1) Representative sweeps of mEPSCs recorded in vehicle control-, LPS-, LPS + 2-AG- and LPS + 2-AG+GW9662-treated neurons. Scale bar: 20 pA/2 s. (A2) Cumulative probability of mEPSCs frequency recorded in neurons with different treatments. (A3) Mean percentage changes in the frequency of mEPSCs in neurons with different treatments. (A4) Cumulative probability of mEPSCs amplitude. (A5) Mean percentage changes in the amplitude of mEPSCs. (B1) Representative sweeps of mEPSCs recorded in vehicle control-, IL-1β-, IL-1β+2-AG (1 μM)- and IL-1β+2-AG+GW9662 (5 μM)-treated neurons. (B2) Cumulative probability of mEPSCs frequency recorded in neurons with different treatments. (B3) Mean percentage changes in the frequency of mEPSCs. (B4) Cumulative probability of mEPSCs amplitude. (B5) Mean percentage changes in the amplitude of mEPSCs. $^{**}P < 0.01$ compared with the vehicle control; $\#\#P < 0.01$ compared with IL-1β or LPS; §§$P < 0.01$ compared with IL-1β+2-AG or LPS+2-AG (n = 24–32).

(Blankman *et al.*, 2007). Thus, inhibiting MAGL with selective MAGL inhibitors will raise the levels of endogenous 2-AG (Hohmann *et al.*, 2005; Comelli *et al.*, 2007; King *et al.*, 2007; Long *et al.*, 2009a; 2009b; Pan *et al.*, 2009). As such, this may lead to potential interventions for preventing, alleviating and treating brain disorders associated with neuroinflammation.

Although PPARγ was originally shown to regulate lipid metabolism and adipocyte differentiation, there is accumulating evidence indicating that PPARγ possesses anti-inflammatory and neuroprotective properties, induced by regulating the transcription of genes involved in inflammation (Jiang *et al.*, 1998; Ricote *et al.*, 1998; Daynes and Jones, 2002; Luna-Medina *et al.*, 2005; Drew *et al.*, 2006; Bensinger and Tontonoz, 2008; Bright *et al.*, 2008; Necela *et al.*, 2008; Racke and Drew, 2008). PPARγ regulates gene transcription by binding to conserved DNA sequences termed peroxisome proliferator response elements (PPRE) as heterodimers with retinoic X receptor (Bensinger and Tontonoz, 2008). Increasing evidence suggests that eCBs are probably PPARγ activators (O'Sullivan, 2007; Sun *et al.*, 2007; O'Sullivan and Kendall, 2010; Pertwee *et al.*, 2010; Pistis and Melis, 2010). It has been shown that 2-AG suppression of the expression of IL-2, an autocrine/paracrine T-cell growth factor, is mediated via a $CB_{1/2}$ receptor-independent activation of PPARγ (Rockwell *et al.*, 2006), suggesting that 2-AG may be able to directly activate nuclear PPARγ by crossing both the plasma and nuclear membranes (O'Sullivan, 2007; Sun *et al.*, 2007; O'Sullivan and Kendall, 2010; Pistis and Melis, 2010). However, 2-AG may also be able to activate PPARγ and restore neuroinflammation-induced down-regulation of PPARγ expression through a CB_1 receptor-dependent pathway. In the present study, we observed that exogenous and endogenous 2-AG-produced suppression of NF-κB-p65 phosphorylation and COX-2 expression in response to pro-inflammatory IL-1β or LPS are blocked by antagonism of PPARγ with a selective PPARγ inhibitor. Moreover, 2-AG prevented the IL-1β- or LPS-induced down-regulation of PPARγ. It has been shown previously that COX-2 participates in synaptic transmission and plasticity via PGE_2, which facilitates the synaptic release of the excitatory neurotransmitter glutamate (Chen *et al.*, 2002; Chen and Bazan, 2005; Sang *et al.*, 2005; Slanina and Schweitzer, 2005; Akaneya and Tsumoto, 2006; Yang *et al.*, 2008). We demonstrated that the frequency of the mEPSCs in cultured hippocampal neurons is significantly elevated by an increase in COX-2 expression, and this enhancement is suppressed by 2-AG (Zhang and Chen, 2008). However, 2-AG-produced suppression of COX-2-enhance mEPSCs was blocked or attenuated by antagonism of PPARγ. Our results provide convincing evidence that PPARγ probably mediates 2-AG-produced inhibition of COX-2 expression and NF-kB phosphorylation.

Based on our previous and present results, PPARγ may not be directly targeted by 2-AG because inhibition of the CB_1 receptor eliminates or decreases the effects 2-AG on neuroinflammation and neuroprotection. For instance, we demonstrated previously that 2-AG-produced neuroprotection and suppression of COX-2 expression in response to pro-inflammatory and other harmful insults are mediated via CB_1 receptor-dependent inhibition of MAPK/NF-κB phosphorylation (Zhang and Chen, 2008; Chen *et al.*, 2011). In addition, inhibition of the CB_1 receptor blocked URB602-produced suppression of IL-1β- or LPS-enhanced mEPSCs (Figure S2). In particular, pharmacological and genetic inhibition of the CB_1 receptor significantly attenuated restorative effect of 2-AG on IL-1β- or LPS-induced down-regulation of PPARγ (Figure 9). If the actions of 2-AG on NF-κB phosphorylation and COX-2 expression are through crossing both the plasma and nuclear membranes to directly activate PPARγ, then inhibition of the CB_1 receptor, which is expressed on the surface plasma membrane, should fail to inhibit 2-AG-induced expression of PPARγ that regulates the expression of genes involved inflammation. In addition, antagonism of the CB_1 receptor should fail to block 2-AG suppression of COX-2-induced increase in mEPSCs. Apparently, this is not the case. This indicates that the signalling mechanisms mediating 2-AG-induced activation/expression of PPARγ in our study are different from those implicated by Rockwell *et al.*, (2006). The exact mechanism for this discrepancy between the two studies is still not clear, but it is probably due to the different preparations used.

A recent study shows that the suppression of IL-2 in T cells produced by AEA or 2-AG is COX-2-dependent (Rockwell *et al.*, 2008). 2-AG and AEA are substrates for COX-2 and can be oxidatively metabolized by COX-2 to prostaglandin glycerol esters or ethanolamides (Kozak *et al.*, 2004; Sang and Chen, 2006; Yang and Chen, 2008). However, it is not clear which COX-2 metabolites mediate the 2-AG- or AEA-induced IL-2 suppression. We demonstrated previously that the effects of COX-2 metabolites of 2-AG on synaptic transmission and neurodegeneration are opposite to that of their precursor 2-AG (Sang *et al.*, 2006, 2007; Yang *et al.*, 2008; Zhang and Chen, 2008). For instance, acute application of PGE_2-G, a major COX-2 metabolite of 2-AG, enhances excitatory synaptic transmission, while 2-AG inhibits it in cultured hippocampal neurons. Moreover, treatment of neurons in culture

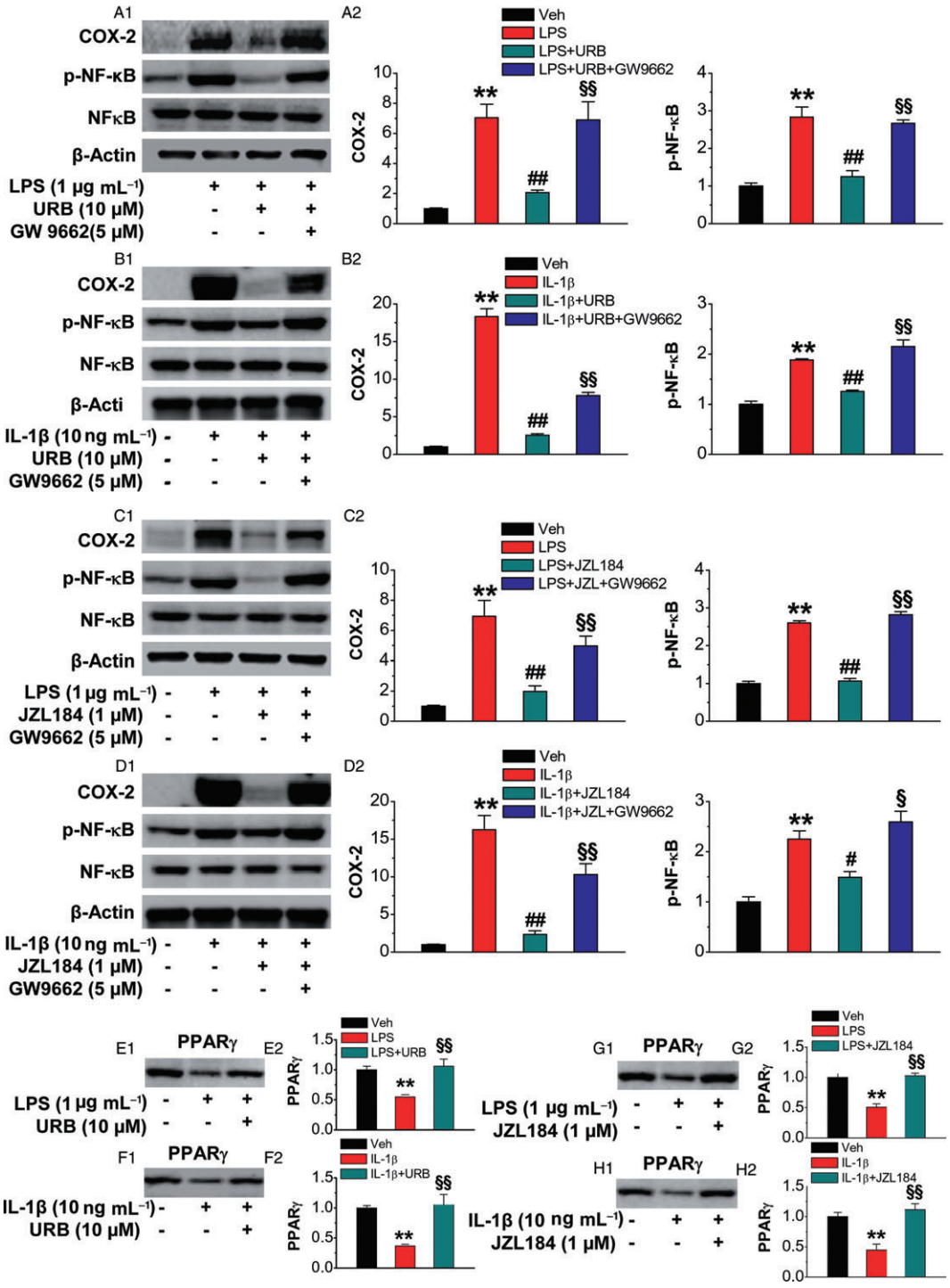

Figure 3

Endogenous 2-AG suppresses NF-κB phosphorylation and COX-2 expression and prevents down-regulation of PPARγ expression induced by IL-1β and LPS. Hippocampal neurons in culture treated with IL-1β (10 ng·mL^{-1}) or LPS (1 μg·mL^{-1}) were the same as described in Figure 1. Selective MAGL inhibitors URB602 (URB, 10 μM) and JZL184 (1 μM) were added to the culture 30 min before IL-1β or LPS application in order to elevate endogenous 2-AG. (A1–A2) Immunoblot analysis of URB suppression of LPS-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 ($n = 3$). (B1–B2) Immunoblot analysis of URB suppression of IL-1β-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 ($n = 3$). (C1–C2) Immunoblot analysis of JZL184 suppression of LPS-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 ($n = 3$). (D1–D2) Immunoblot analysis of JZL184 suppression of IL-1β-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 ($n = 3$). (E1–H2) Elevation of endogenous 2-AG by inhibiting MAGL with URB602 or JZL184 restores LPS- or IL-1β-induced down-regulation of PPARγ ($n = 3$ per group). $^{**}P < 0.01$, compared with the vehicle control; $^{\#\#}P < 0.01$ compared with IL-1β or LPS; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ compared with IL-1β + URB or JZL184, LPS+URB or JZL184.

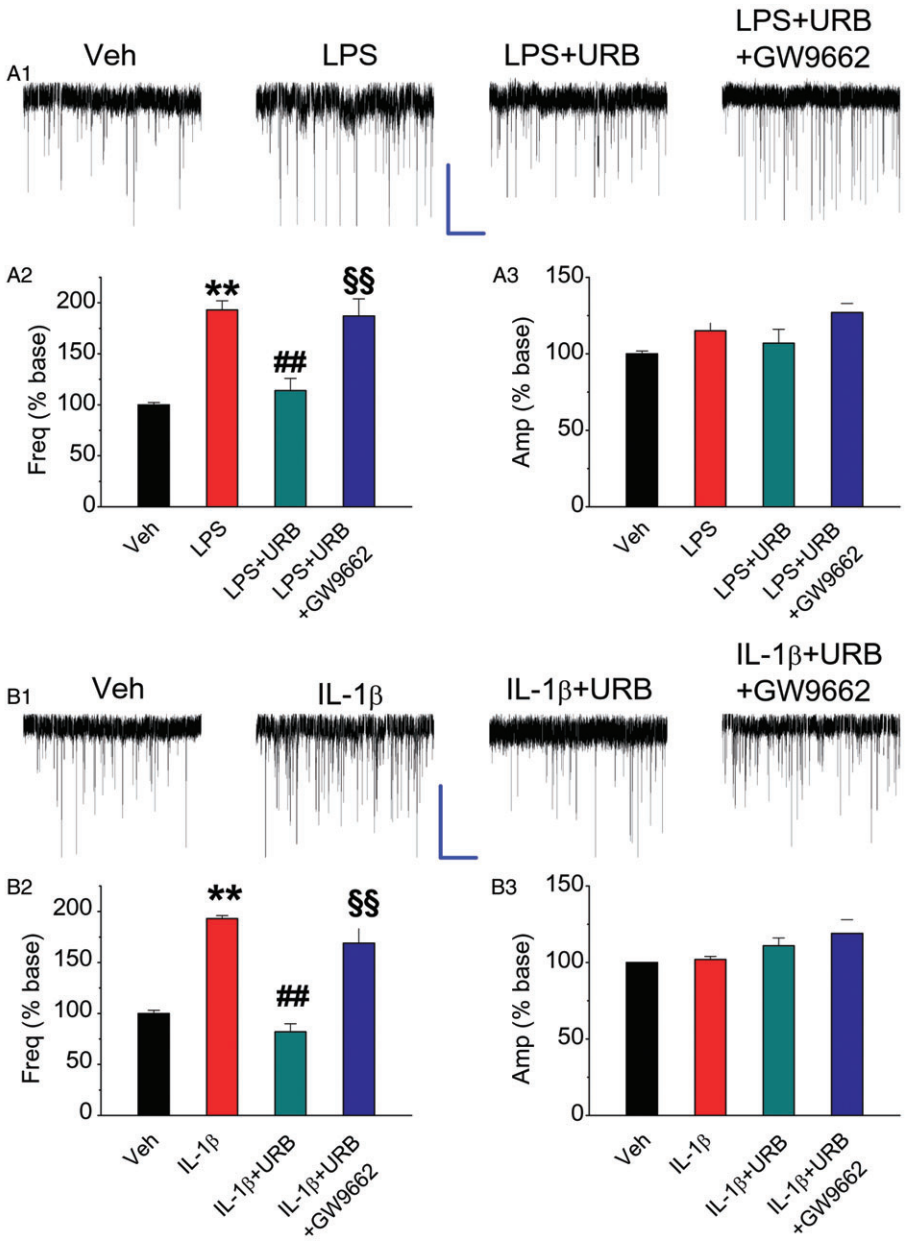

Figure 4

MAGL inhibitor URB602 attenuates COX-2 elevation-induced enhancement of mEPSCs. Hippocampal neurons in culture treated with IL-1β or LPS were the same as described in Figure 2. (A1) Representative sweeps of mEPSCs recorded in vehicle control-, LPS-, LPS+URB (10 μM)- and LPS+URB+GW9662 (5 μM)-treated neurons. Scale bar: 20 pA/2 s. (A2) Mean percentage changes in the frequency of mEPSCs in neurons with different treatments. (A3). Mean percentage changes in the amplitude of mEPSCs. (B1) Representative sweeps of mEPSCs recorded in vehicle control-, IL-1β-, IL-1β + URB (10 μM)- and IL-1β + URB+GW9662 (5 μM)-treated neurons. (B2)Mean percentage changes in the frequency of mEPSCs. (B3) Mean percentage changes in the amplitude of mEPSCs. $^{**}P < 0.01$ compared with the vehicle control; $^{\#\#}P < 0.01$ compared with IL-1β or LPS; §§$P < 0.01$ compared with IL-1β + URB or LPS+URB (n = 20–34).

with PGE_2-G induces neurodegeneration and apoptosis and increases NF-κB phosphorylation, while 2-AG protects neurons against neurodegeneration and apoptosis and inhibits NF-κB phosphorylation. Therefore, in our study it is unlikely that 2-AG-produced suppression of COX-2 expression and NF-kB-p65 phosphorylation and increase of PPARγ expression are mediated through COX-2 metabolites of 2-AG.

It has been proposed that there is a reciprocally negative feedback loop between NF-κB and PPARγ, suggesting that there is cross-talk between these two transcription factors (Bensinger and Tontonoz, 2008; Necela *et al.*, 2008). We speculate that under physiological conditions, inflammatory genes are tonically repressed by co-repressor complexes. However, exposure to pro-inflammatory stimuli such as IL-1β

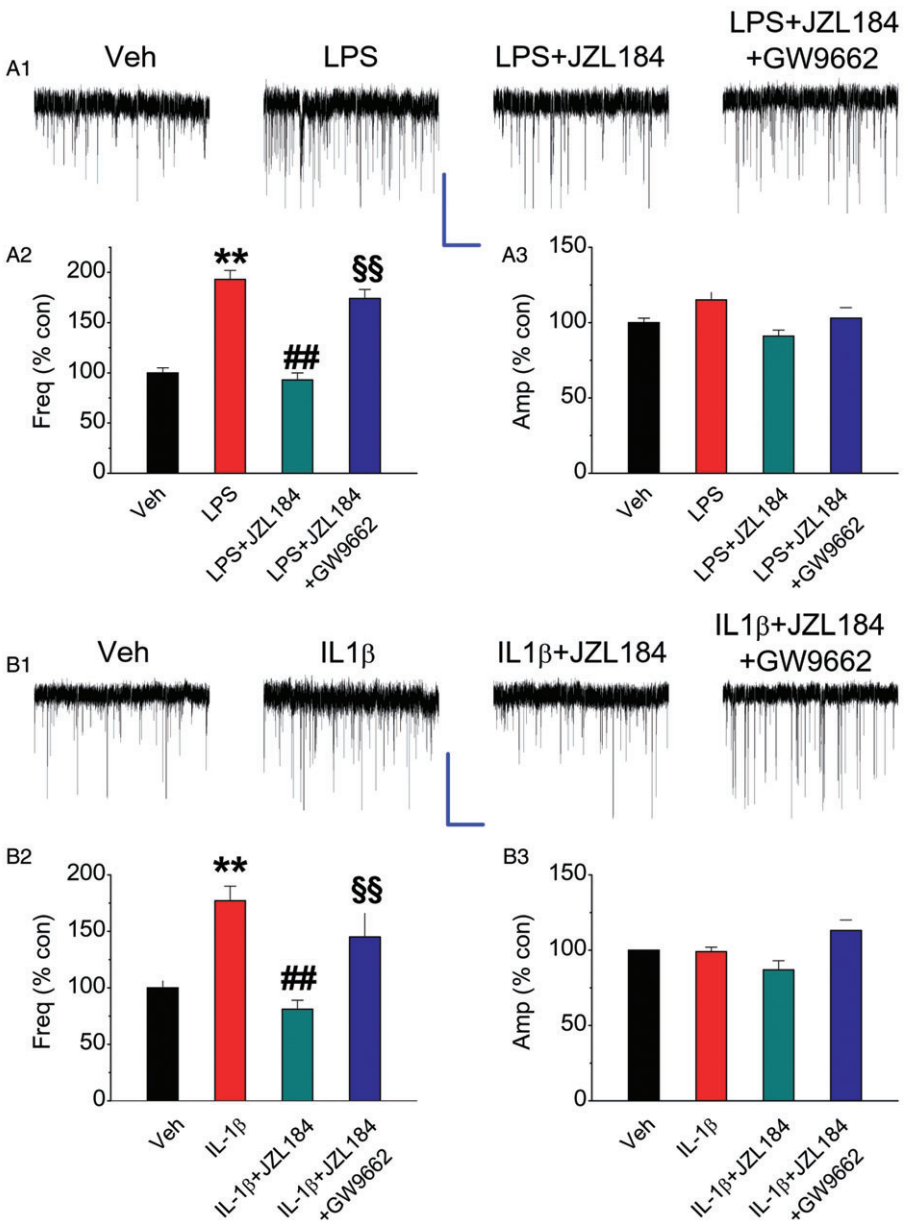

Figure 5

MAGL inhibitor JZL184 attenuates COX-2 elevation-induced enhancement of mEPSCs. Hippocampal neurons in culture treated with IL-1β or LPS were the same as described in Figure 2. (A1) Representative sweeps of mEPSCs recorded in vehicle control-, LPS-, LPS+JZL184 (1 μM)- and LPS+JZL184+GW9662 (5 μM)-treated neurons. Scale bar: 20 pA/2 s. (A2) Mean percentage changes in the frequency of mEPSCs in neurons with different treatments. (A3). Mean percentage changes in the amplitude of mEPSCs. (B1) Representative sweeps of mEPSCs recorded in vehicle control-, IL-1β-, IL-1β + JZL184 (1 μM)- and IL-1β + JZL184+GW9662 (5 μM)-treated neurons. (B2) Mean percentage changes in the frequency of mEPSCs. (B3) Mean percentage changes in the amplitude of mEPSCs. $^{**}P < 0.01$ compared with the vehicle control; $^{\#\#}P < 0.01$ compared with IL-1β or LPS; §§$P < 0.01$ compared with IL-1β + JZL184 or LPS+JZL184 (n = 23–30).

or LPS activates astroglial toll-like receptor 4 (TLR4) or IL-1 receptors, which elevate phosphorylation of MAPK and NF-κB. Increased expression and activity of NF-κB not only suppresses PPARγ expression and activity but also directly triggers inflammatory gene transcription. The CB_1 receptor is expressed both in hippocampal neurons and astroglial cells (Sinha *et al.*, 1998; Waksman *et al.*, 1999; Stella, 2004; Eljaschewitsch *et al.*, 2006; Zhang and Chen, 2008). Thus, 2-AG binds to Gi-coupled CB_1 receptors and/or CB_2 receptors. Activation of $CB_{1/2}$ receptors suppresses phosphorylation of NF-κB through ERK/p38MAPK (Zhang and Chen, 2008) and increases the expression of PPARγ, which represses inflammatory gene transcription (Bensinger and Tontonoz, 2008; Necela *et al.*, 2008). Nevertheless, the possibility that 2-AG could cross the plasma and nuclear membranes to directly induce PPARγ activation and expression, leading to repression

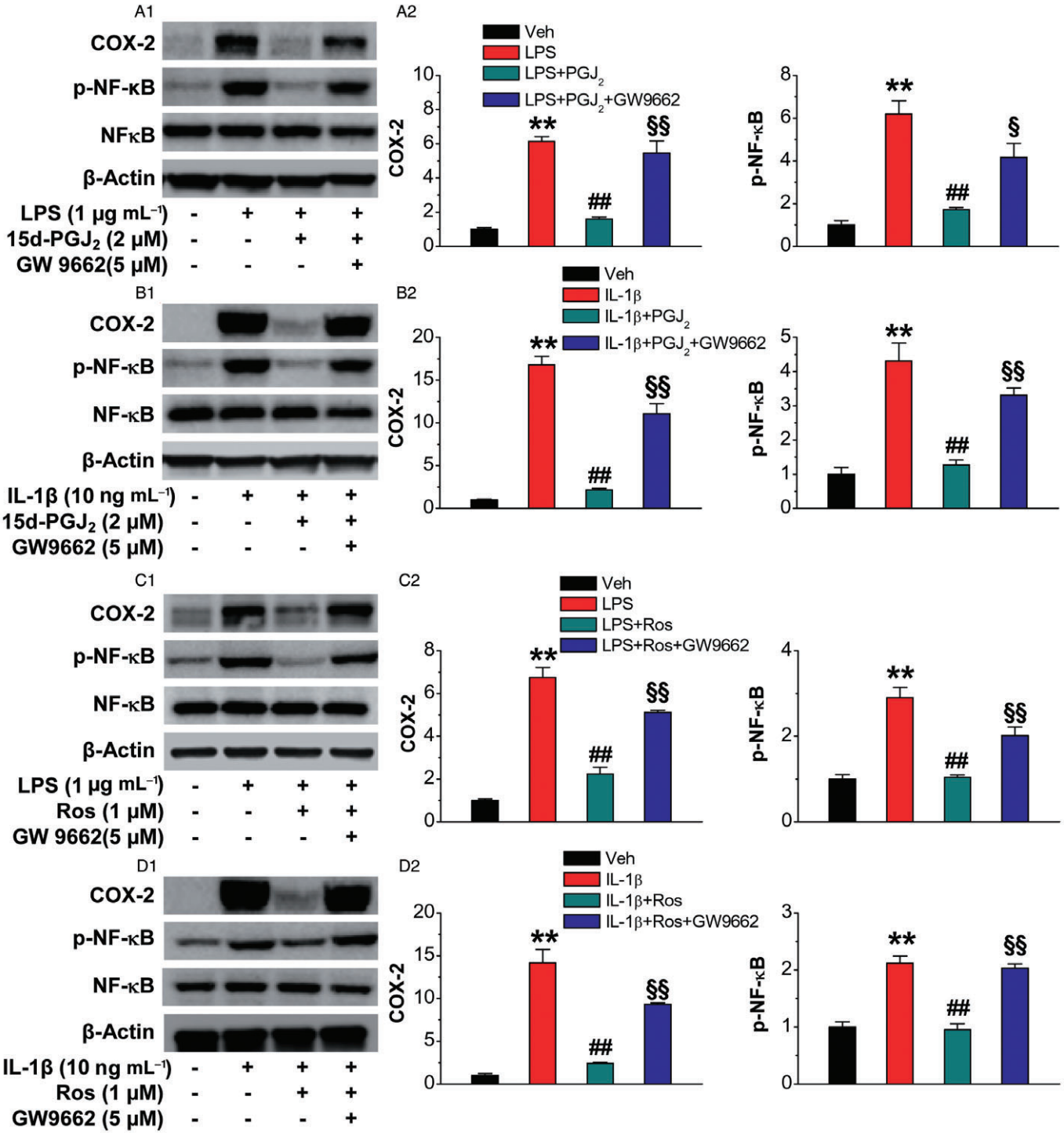

Figure 6

PPARγ agonists inhibit phosphorylation of NF-κB and expression of COX-2 induced by IL-1β-and LPS. Hippocampal neurons in culture-treated with IL-1β (10 ng·mL^{-1}) or LPS (1 μg·mL^{-1}) were the same as described in Figure 1. PPARγ agonists 15d-PGJ$_2$ (2 μM) or rosiglitazone (Ros, 1 μM) were added to the culture 30 min before IL-1β or LPS. (A1–A2) Immunoblot analysis of 15d-PGJ$_2$ suppression of LPS-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 (n = 3). (B1-–B) Immunoblot analysis of 15d-PGJ$_2$ inhibition of IL-1β-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 (n = 3). (C1–C2) Immunoblot analysis of Ros suppression of LPS-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW9662 (n = 3). (D1–D2) Immunoblot analysis of Ros suppression of IL-1β-induced NF-κB-p65 phosphorylation and COX-2 expression in the absence and presence of GW (n = 3). **P < 0.01, compared with the vehicle control; ##P < 0.01 compared with IL-1β or LPS; §P < 0.05, §§P < 0.01 compared with IL-1β+15d-PGJ$_2$ or Ros, LPS+15d-PGJ$_2$ or Ros.

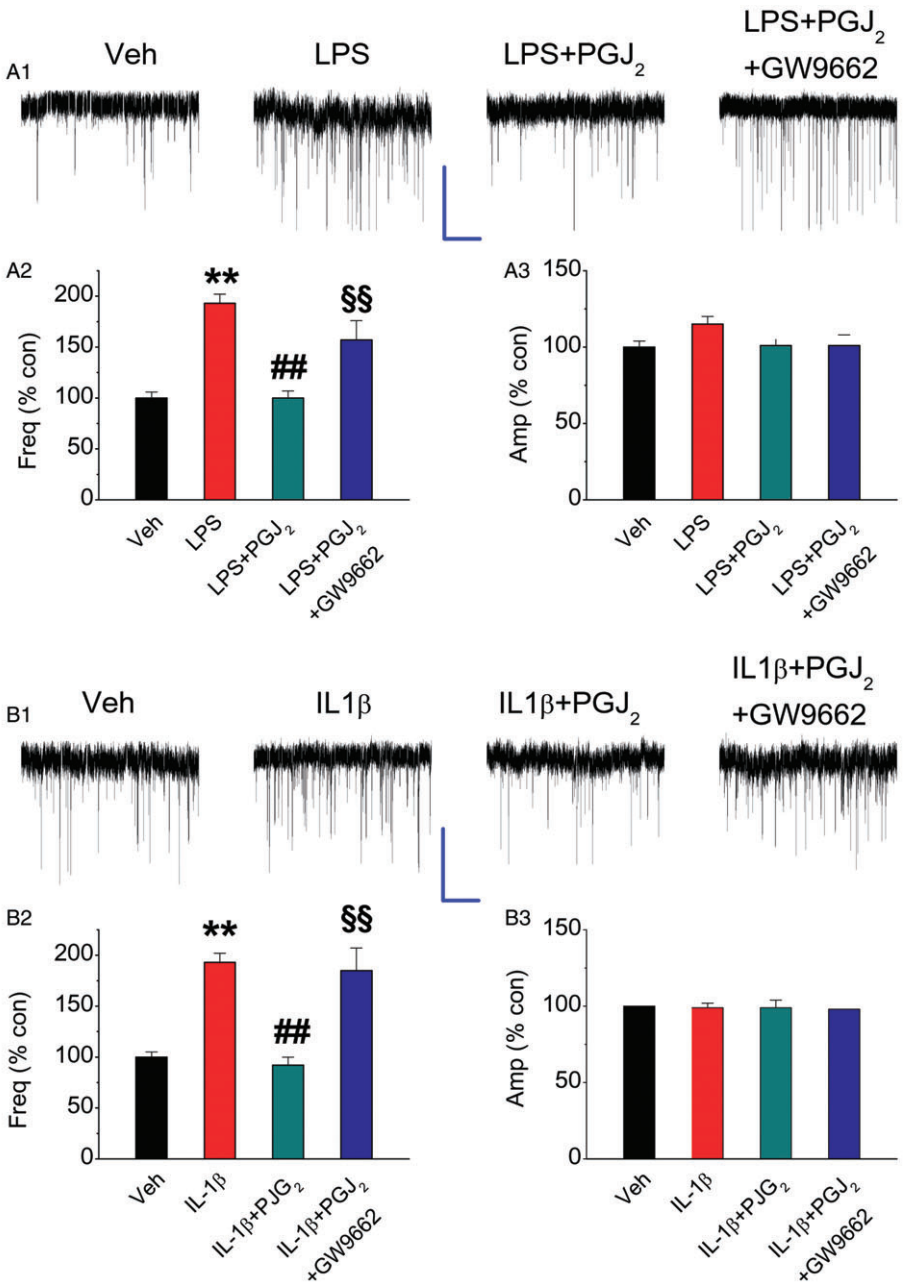

Figure 7

PPARγ agonist 15d-PGJ2 reduces COX-2 elevation-induced enhancement of mEPSCs. Hippocampal neurons in culture-treated with IL-1β or LPS were the same as described in Figure 2. (A1) Representative sweeps of mEPSCs recorded in vehicle control-, LPS-, LPS+15d-PGJ_2 (2 μM)- and LPS+15d-PGJ_2+GW9662 (5 μM)-treated neurons. Scale bar: 20 pA/2 s. (A2) Mean percentage changes in the frequency of mEPSCs in neurons with different treatments. (A3). Mean percentage changes in the amplitude of mEPSCs. (B1) Representative sweeps of mEPSCs recorded in vehicle control-, IL-1β-, IL-1β+15d-PGJ_2 (2 μM)- and IL-1β+15d-PGJ_2+GW9662 (5 μM)-treated neurons. (B2) Mean percentage changes in the frequency of mEPSCs. (B3) Mean percentage changes in the amplitude of mEPSCs. $^{**}P < 0.01$ compared with the vehicle control; $^{\#\#}P < 0.01$ compared with IL-1β or LPS; §§$P < 0.01$ compared with IL-1β+15d-PGJ_2 or LPS+15d-PGJ_2 (n = 23–30).

of NF-κB and inflammatory gene transcription, cannot be excluded. It is likely that 2-AG-initiated signalling events prevent or inhibit inflammatory gene transcription, resulting in resolution of inflammation and neuroprotection.

In this study, we provide evidence for the first time that PPARγ mediates exogenous and endogenous 2-AG-produced suppression of pro-inflammatory IL-1β- or LPS-induced NF-κB-p65 phosphorylation and COX-2 expression, the two key inflammatory markers. We also provide evidence that PPARγ mediates exogenous and endogenous 2-AG-produced inhibition of enhanced mEPSCs resulting from COX-2 elevation in hippocampal neurons in culture. 2-AG-induced PPARγ

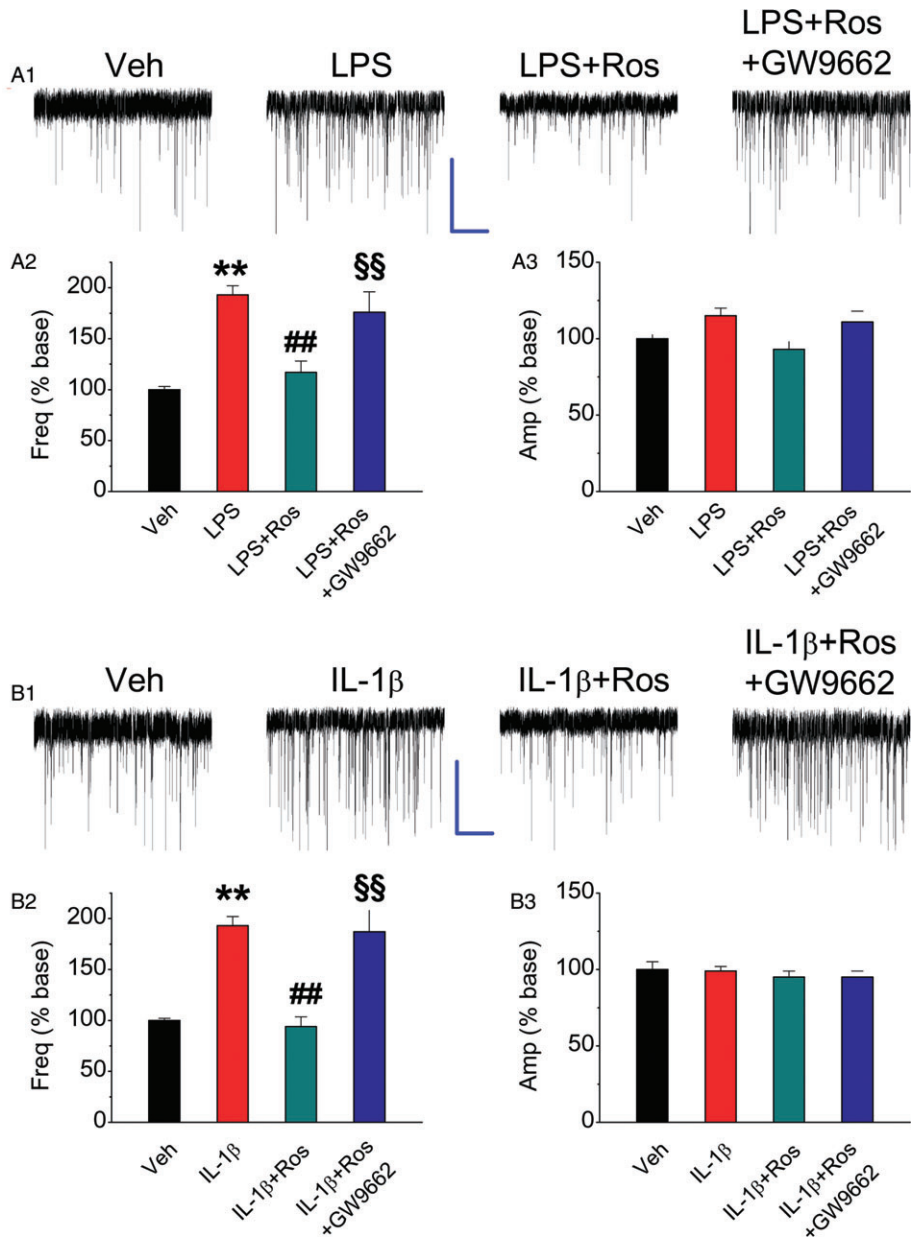

Figure 8

PPARγ activator rosiglitazone reduces COX-2 elevation-induced enhancement of mEPSCs. Hippocampal neurons in culture treated with IL-1β or LPS were the same as described in Figure 2. (A1) Representative sweeps of mEPSCs recorded in vehicle control-, LPS-, LPS + Ros (1 μM)- and LPS + Ros + GW9662 (5 μM)-treated neurons. Scale bar: 20 pA/2 s. (A2) Mean percentage changes in the frequency of mEPSCs in neurons with different treatments. (A3) Mean percentage changes in the amplitude of mEPSCs. (B1) Representative sweeps of mEPSCs recorded in vehicle control-, IL-1β-, IL-1β + Ros (1 μM)- and IL-1β + Ros+GW9662 (5 μM)-treated neurons. (B2) Mean percentage changes in the frequency of mEPSCs. (B3) Mean percentage changes in the amplitude of mEPSCs. $^{**}P < 0.01$ compared with the vehicle control; $^{\#\#}P < 0.01$ compared with IL-1β or LPS; §§$P < 0.01$ compared with IL-1β + Ros or LPS + Ros (n = 23–30).

expression appears to be dependent on the CB_1 receptor and interplay between NF-κB-p65 and PPARγ, indicating that this would be a previously unrevealed signalling pathway mediating 2-AG-produced anti-inflammatory and neuroprotective effects. However, it is still not clear how the interaction or cross-talk between NF-κB and PPARγ occurs when 2-AG activates the CB_1 receptor. More work is needed to elucidate this intriguing signalling pathway. Resolution of neuroinflammation is believed to be an efficacious therapeutic approach for prevention and treatment of neurodegenerative diseases such as Alzheimer's disease (Walker and Lue, 2007; Chen, 2010; Glass *et al.*, 2010). Therefore, the results obtained in the present study suggest that endogenous 2-AG plays an important role in regulation of innate and adaptive immune systems in maintaining tissue homeostasis, and that approaches strengthening endogenous 2-AG signalling by

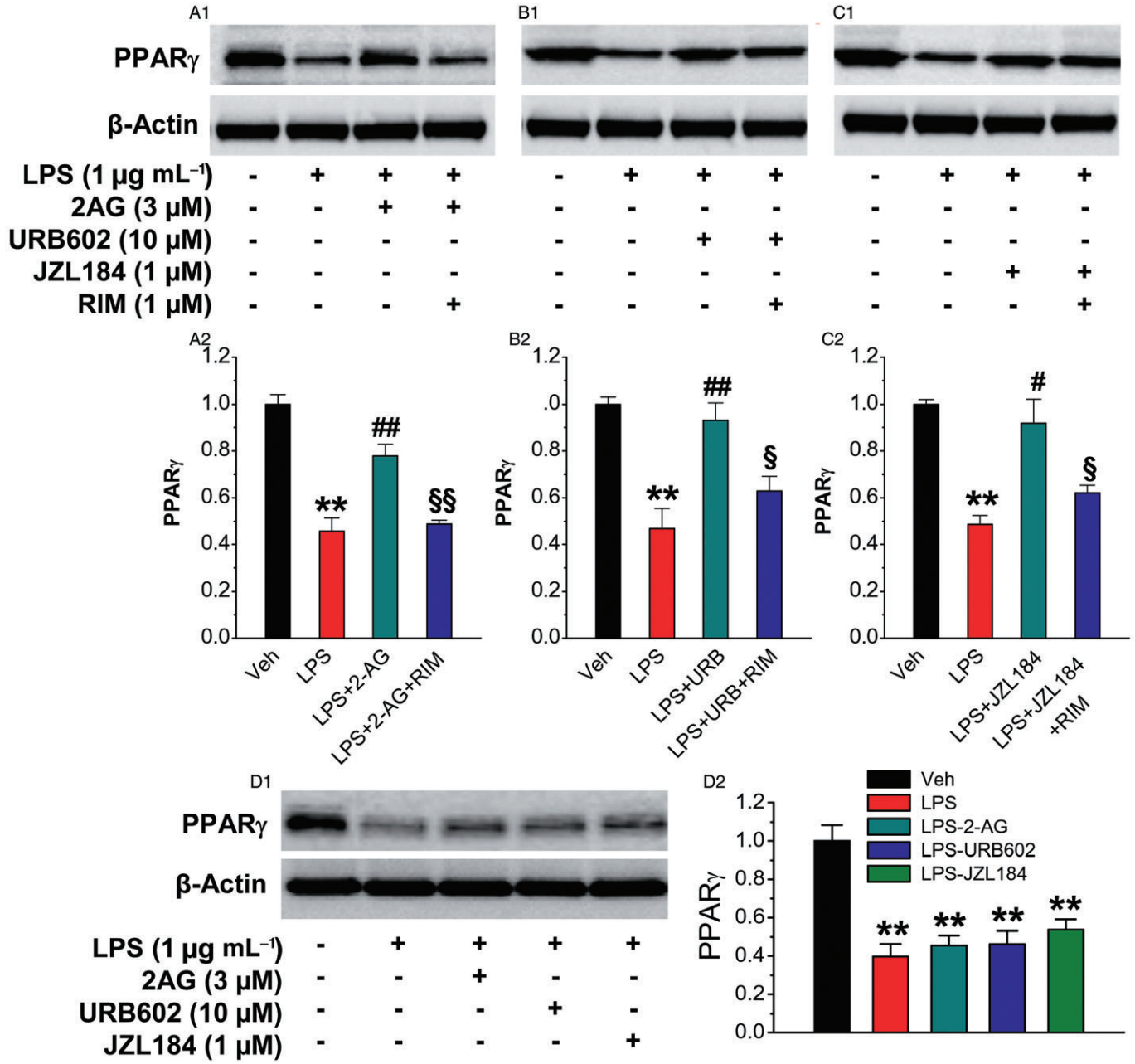

Figure 9

Exogenous and endogenous 2-AG prevents IL-1β-and LPS-induced decrease in PPARγ expression via a CB_1 receptor-dependent mechanism. Hippocampal neurons in culture were treated with LPS (1 μg·mL^{-1}) for 6 h. 2-AG (A1–A2), URB602 (B1–B2), JZL184 (C1–C2) or rimonabant (RIM, 1 μM) were administered 30 min before LPS. Expression of PPARγ was detected using the immunoblot analysis. $^{**}P < 0.01$ compared with the vehicle control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LPS; §$P < 0.05$ compared with LPS+2-AG, + URB or +JZL184 ($n = 3$ per group). Hippocampal neurons in culture from mice deficient in the CB_1 receptor were treated with LPS (1 μg·mL^{-1}) for 6 h in the absence and presence of 2-AG, URB602 or JZL184 (D1–D2). $^{**}P < 0.01$ compared with the vehicle control. There are no statistically significant differences between LPS and LPS+2-AG, LPS+URB602 or LPS+JZl184 ($n = 3$ per group).

inhibiting its hydrolysis or facilitating its synthesis will be potentially efficacious therapeutic interventions for preventing, relieving and treating chronic neuroinflammation-induced brain disorders.

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Conflicts of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Inhibition of PPARγ blocks 2-AG-produced suppression of COX-2 elevation-induced enhancement of mEPSCs. T007: T0070907 (1 μM).
Figure S2 Inhibition of the CB1 receptor eliminates URB602 suppression of COX-2 elevation-induced enhancement of mEPSCs. RIM: rimonabant (1 μM).
Figure S3 JZL184, rosiglitazone (Ros), 15d-PGJ2, or GW9662 alone do not significantly alter basal expression of COX-2 in cultured hippocampal neurons. The treatment of JZL184, Ros, 15d-PGJ2 and GW9662 was the same as that described in other figures. COX-2 expression was detected using Western blot analysis.