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Δ^9-THC-Caused Synaptic and Memory Impairments Are Mediated through COX-2 Signaling

Rongqing Chen,[1,4] Jian Zhang,[1,4] Ni Fan,[1,4] Zhao-qian Teng,[1,4] Yan Wu,[1,4] Hongwei Yang,[1] Ya-ping Tang,[3] Hao Sun,[1] Yunping Song,[1] and Chu Chen[1,2,*]
[1]Neuroscience Center of Excellence
[2]Department of Otorhinolaryngology
[3]Department of Cell Biology and Anatomy
School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA
[4]These authors contributed equally to this work
*Correspondence: chen502@gmail.com
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SUMMARY

Marijuana has been used for thousands of years as a treatment for medical conditions. However, untoward side effects limit its medical value. Here, we show that synaptic and cognitive impairments following repeated exposure to Δ^9-tetrahydrocannabinol (Δ^9-THC) are associated with the induction of cyclooxygenase-2 (COX-2), an inducible enzyme that converts arachidonic acid to prostanoids in the brain. COX-2 induction by Δ^9-THC is mediated via CB1 receptor-coupled G protein $\beta\gamma$ subunits. Pharmacological or genetic inhibition of COX-2 blocks downregulation and internalization of glutamate receptor subunits and alterations of the dendritic spine density of hippocampal neurons induced by repeated Δ^9-THC exposures. Ablation of COX-2 also eliminates Δ^9-THC-impaired hippocampal long-term synaptic plasticity, spatial, and fear memories. Importantly, the beneficial effects of decreasing β-amyloid plaques and neurodegeneration by Δ^9-THC in Alzheimer's disease animals are retained in the presence of COX-2 inhibition. These results suggest that the applicability of medical marijuana would be broadened by concurrent inhibition of COX-2.

INTRODUCTION

Marijuana has been used for thousands of years to treat chronic pain, multiple sclerosis, cancer, seizure disorders, nausea, anorexia, and inflammatory and neurodegenerative diseases (Robson, 2001; Russo, 2007). However, the undesirable neuropsychological and cognitive side effects greatly limit the medical use of marijuana (Carlini, 2004). The major intoxicating effects of cannabis are the impairments in synaptic and cognitive function (Pope et al., 2001; Solowij et al., 2002; Messinis et al., 2006). These untoward effects are also the primary consequences of cannabis abuse. However, there are no currently FDA-approved effective medications for prevention and treatment of these cannabis-related disorders.

As it is clear now, Δ^9-tetrahydrocannabinol (Δ^9-THC) is the major psychoactive ingredient of marijuana (Gaoni and Mechoulam, 1964), and its effects are largely mediated through cannabinoid receptors (CB1R or CB2R), which are pertussis toxin (PTX)-sensitive G-protein-coupled receptors (Howlett, 1998; Pertwee et al., 2010). Previous studies demonstrate that deficits in long-term synaptic plasticity, learning, and memory by Δ^9-THC exposure are primarily mediated through CB1R expressed in the brain (Lichtman and Martin, 1996; Hoffman et al., 2007; Puighermanal et al., 2009; Fan et al., 2010; Han et al., 2012). However, the molecular mechanisms underlying the synaptic and cognitive deficits elicited by repeated Δ^9-THC exposure are largely unknown.

In the present study, we unexpectedly observed that Δ^9-THC increases expression and activity of cyclooxygenase-2 (COX-2), an inducible enzyme that converts arachidonic acid to prostanoids both in vitro and in vivo via a CB1R-dependent mechanism. This action is opposite to the observations where the endogenous cannabinoid 2-arachidonylglycerol (2-AG) induces a CB1R-dependent suppression of COX-2 activity and expression in response to proinflammatory and excitotoxic insults (Zhang and Chen, 2008). The differential modulation of COX-2 by the exogenous cannabinoid Δ^9-THC and endogenous cannabinoid 2-AG appears to result from intrinsic properties of the CB1R-coupled G protein. The COX-2 induction by Δ^9-THC is mediated via G$\beta\gamma$ subunits, whereas COX-2 suppression by 2-AG is mediated through the Gαi subunit. Interestingly, the impairments in hippocampal long-term synaptic plasticity, spatial, and fear memories induced by repeated Δ^9-THC exposure can be occluded or attenuated by pharmacological or genetic inhibition of COX-2. Finally, the beneficial effects of reducing Aβ and neurodegeneration by Δ^9-THC are retained in the presence of COX-2 inhibition. Our results reveal a signaling pathway that is linked to synaptic and cognitive deficits induced

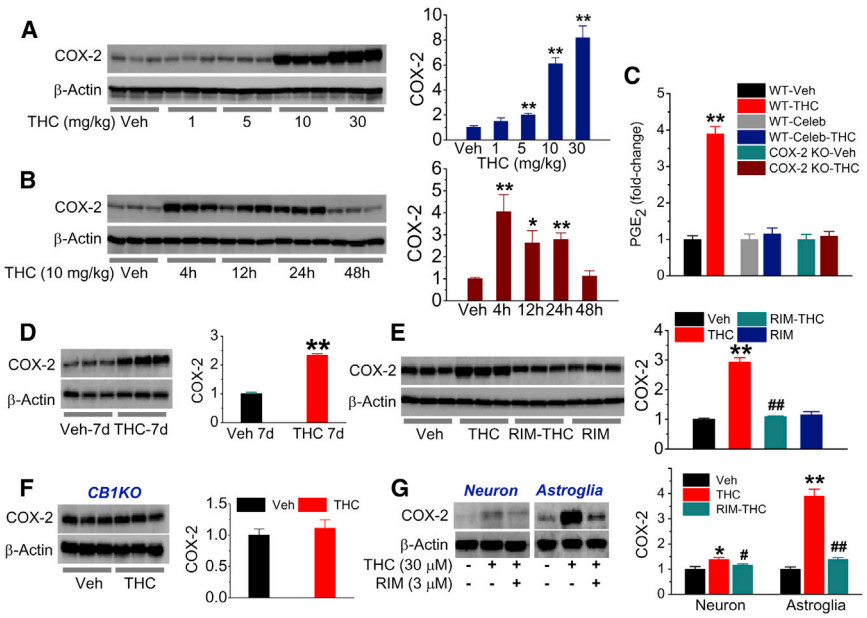

Figure 1. Δ^9-THC In Vivo Exposure Induces CB1R-Dependent Activation and Elevation of COX-2 Expression in the Hippocampus

(A and B) Δ^9-THC induces a dose- and time-dependent increase in hippocampal COX-2 expression (n = 5).

(C) Δ^9-THC increases synthesis of PGE_2, and the increase is blocked by Celebrex (Celeb) or genetic inhibition of COX-2 (COX-2 knockout). PGE_2 was detected 4 hr after Δ^9-THC injection (10 mg/kg). Celebrex (10 mg/kg) was injected 30 min prior to Δ^9-THC injection (n = 10/group).

(D) COX-2 is persistently elevated in animals that received repeated injections of Δ^9-THC (10 mg/kg, i.p.) once a day for 7 consecutive days. COX-2 was analyzed 24 hr after secession of the last injection (n = 3).

(E) COX-2 induction by Δ^9-THC (10 mg/kg) is blocked by Rimonabant (RIM, 5 mg/kg). Hippocampal COX-2 was detected 4 hr after Δ^9-THC injection (n = 3). RIM was injected 30 min prior to Δ^9-THC injection.

(F) Δ^9-THC fails to increase COX-2 in CB1R knockout mice (n = 3).

(G) Δ^9-THC increases COX-2 both in neurons and astroglial cells in culture, and the increase is blocked by RIM. COX-2 was assayed 12 hr after treatments (n = 6). All the data are presented as mean ± SEM; *p < 0.05, **p < 0.01 compared with the vehicle controls, #p < 0.05, and ##p < 0.01 compared with Δ^9-THC (one-way ANOVA, Fisher's PLSD). See also Figures S1 and S7.

by Δ^9-THC exposure, suggesting that Δ^9-THC would display its beneficial properties with fewer undesirable side effects when its COX-2 induction effect is inhibited, which may form a therapeutic intervention for medical treatments.

RESULTS

Δ^9-THC Induces Dose- and Time-Dependent Increase in COX-2 Expression

Identification of CBRs led to discovery of several endogenous cannabinoids, including anandamide (AEA) and 2-arachidonylglycerol (2-AG), which are the most-studied endocannabinoids involved in a variety of physiological, pharmacological, and pathological processes (Kano et al., 2009; Pertwee et al., 2010). 2-AG, the most abundant endocannabinoid, plays significant roles in synaptic modification, resolution of neuroinflammation, and neuronal survival (Alger, 2009; Chevaleyre et al., 2006; Lovinger, 2008; Panikashvili et al., 2001; Zhang and Chen, 2008). In particular, its anti-inflammatory and neuroprotective effects in response to proinflammatory and neurotoxic insults appear to be through limiting COX-2 signaling (Chen et al., 2011; Du et al., 2011; Zhang and Chen, 2008). Because acute inhibition of COX-2 by selective COX-2 inhibitors has been shown to decrease hippocampal long-term potentiation (LTP) and impairs memory consolidation (Chen et al., 2002; Teather et al., 2002; Cowley et al., 2008), we thus wondered whether impairments of synaptic plasticity and memory by marijuana result from a COX-2 suppressive effect. To assess this, we first analyzed hippocampal expression and activity of COX-2 in mice that received Δ^9-THC. Unexpectedly, in vivo exposure to Δ^9-THC produced a dose- and time-dependent induction of COX-2 in the brain, rather than suppression (Figures 1A and 1B), whereas expression of COX-1 was unaffected by Δ^9-THC (Figure S1A available online). The increase in COX-2 expression induced by Δ^9-THC was accompanied by elevated production of prostaglandin E_2 (PGE_2), which could be inhibited by the selective COX-2 inhibitor Celebrex or genetic inhibition of COX-2 (Figures 1C and S1B). To confirm the ability of exogenous cannabinoids to induce COX-2, we assessed COX-2 expression and PGE_2 production in animals injected with the synthetic cannabinoid CP55,940 (CP). As expected, CP produced more pronounced effects on COX-2 expression and PGE_2 synthesis (Figures S1C–S1E). The increase in PGE_2 could be blocked by NS398, another selective COX-2 inhibitor. In addition, we observed that COX-2 expression was steadily elevated in animals injected with Δ^9-THC once daily for 7 consecutive days, although the magnitude of increase in COX-2 was not as intensified as that of a single injection (Figure 1D). This indicates that expression of COX-2 is persistently elevated upon repeated exposure to Δ^9-THC (Figure S7).

COX-2 Induction by Δ^9-THC Is CB1R Dependent

Because undesirable side effects elicited by cannabinoids are primarily mediated by CB1R (Lichtman and Martin, 1996; Hoffman et al., 2007; Han et al., 2012), we wondered whether COX-2 induction by Δ^9-THC is mediated via CB1R. As shown in Figures 1E and1F, Δ^9-THC-induced increase in COX-2 in the hippocampus was blocked either by Rimonabant (RIM), a selective CB1R antagonist, or by genetic deletion of CB1R. To determine whether the increase in COX-2 by Δ^9-THC occurs in neurons or astroglial cells, we made different conditions in cultures as described previously (Zhang and Chen, 2008). We found that, although Δ^9-THC induced a CB1R-dependent increase in COX-2 expression both in neuronal and astroglial cell-enriched

cultures, the increase was more pronounced in astroglial cell-enriched cultures than in neuronal culture (Figure 1G). Our data provide convincing evidence that COX-2 induction by Δ^9-THC both in vivo and in vitro is mediated via CB1R.

COX-2 Induction by Δ^9-THC Is via CB1R-Coupled G Protein $\beta\gamma$ Subunits

Because the suppression of COX-2 by 2-AG in response to proinflammatory stimuli occurs via a CB1R-dependent mechanism (Zhang and Chen, 2008), we questioned why the exogenous cannabinoid Δ^9-THC increases COX-2 and why the endogenous cannabinoid 2-AG suppresses COX-2 acting through the same CB1R-dependent mechanism, and we speculated that CB1R may not be the key molecule responsible for differential regulation of COX-2 expression upon exposure to cannabinoids. CB1R is coupled to a PTX-sensitive Gi/o protein, and activation of CB1R releases $G\beta\gamma$ subunits from the GTP-bound $G\alpha i$ subunit (Howlett, 1998; Pertwee et al., 2010). Earlier studies show that activation of CB1R is capable of inducing $G\beta\gamma$-mediated response (Guo and Ikeda, 2004; Wilson et al., 2001; Yao et al., 2003). We hypothesized that $G\beta\gamma$ and $G\alpha i$ may differentially mediate COX-2 induction or suppression by exogenous Δ^9-THC or endogenous 2-AG. To test this prediction, we first overexpressed $G\beta\gamma$ subunits by transfection with plasmids carrying $\beta1$ and $\gamma2$ subunits in NG108-15 cells, which express native CB1R (Figures S2A and S2B). Whereas Δ^9-THC still increased expression of COX-2 mRNA in culture transfected with the control vector, it did not increase COX-2 in culture overexpressing $\beta1$ and $\gamma2$ subunits (Figure 2A). In subsequent experiments, $\beta1$ and $\gamma2$ subunits were silenced by small hairpin RNA (shRNA). Knockdown of $\beta1\gamma2$ by shRNA suppressing endogenous $\beta1\gamma2$ also blocked COX-2 induction by Δ^9-THC in NG108-15 cells, and the blockade was rescued by concurrently expressing shRNA-resistant $\beta1\gamma2$ (Figures 2A and S2E). This indicates that COX-2 induction by Δ^9-THC is likely mediated through $G\beta\gamma$. To further confirm that $G\beta\gamma$ mediates COX-2 induction by Δ^9-THC, we treated mixed culture of hippocampal neurons and astroglial cells (~5%–10%) with a membrane-permeable $G\beta\gamma$-binding peptide mSIRK to disrupt the function of $G\beta\gamma$ (Delaney et al., 2007; Goubaeva et al., 2003). As a negative control, we used a variant mSIRK with a point mutation of Leu^9 to Ala (L^9A-mSIRK). As shown in Figure 2B, disruption of $G\beta\gamma$ activity by mSIRK also blocked COX-2 induction by Δ^9-THC, whereas it failed to block the suppression of COX-2 by 2-AG in response to LPS, a commonly used COX-2 inducer (Zhang and Chen, 2008). PTX treatment also blocked Δ^9-THC-induced increase in COX-2. Interestingly, application of 2-AG failed to suppress Δ^9-THC-induced increase in COX-2 (Figures 2B and S2I). To test the prediction that $G\alpha i$ mediates COX-2 suppressive effect by 2-AG, we silenced $G\alpha i$ using a lentiviral vector in mixed culture of neurons and astroglial cells (Figure S2C). As illustrated in Figures 2C and S2D, silencing $G\alpha i1$, but not $G\alpha i2$ or $G\alpha i3$, blocked the suppression of COX-2 by 2-AG in response to the LPS stimulus, and this blocking effect was rescued by concurrently expressing shRNA-resistant $G\alpha i1$ (Figures 2C and S2E). Knockdown of $G\alpha i1$, $G\alpha i2$, or $G\alpha i3$ did not block COX-2 induction by Δ^9-THC (Figures 2C and S2D). These results indicate that COX-2 induction by Δ^9-THC is likely mediated via $G\beta\gamma$, whereas COX-2 suppression by 2-AG is likely mediated through $G\alpha i1$ (Figure S7).

Akt, ERK, p38MAPK, and NF-κB Are Downstream Signaling of $G\beta\gamma$

To determine downstream signaling pathways of $G\beta\gamma$, we detected phosphorylation of Akt, ERK, and p38MAPK by overexpression or knockdown of $G\beta\gamma$ in the presence and absence of Δ^9-THC. As shown in Figures 2D and S2F, Δ^9-THC induced phosphorylation of these signaling molecules, and the phosphorylation was inhibited by knockdown or overexpression of $G\beta1\gamma2$. Inhibition of phosphorylation of these mediators by shRNA was rescued by concurrently expressing shRNA-resistant $G\beta1\gamma2$ (Figure 2D). These data indicate that COX-2 induction by Δ^9-THC is likely through signaling of these downstream molecules of $G\beta\gamma$. To further characterize this signaling pathway that regulates COX-2 expression by Δ^9-THC, we targeted NF-κB, which is a transcription factor regulating expression of genes, including the COX-2 gene (*ptgs2*). We observed that Δ^9-THC induced NF-κB phosphorylation in NG-108-15 cells, and this phosphorylation was inhibited by overexpression or knockdown of $G\beta\gamma$ and was rescued by concurrently expressing shRNA-resistant G $\beta1\gamma2$ (Figures 2E and S2G). To determine regulation of COX-2 transcription by NF-κB, we performed a chromatin immunoprecipitation (ChIP) analysis in mixed culture of neurons and astroglial cells. As shown in Figure 2E, a binding activity of NF-κB p65 was detected in the promoter positions (−419 to −428 bp) of *ptgs2*, and this interaction was enhanced by Δ^9-THC and inhibited by SC-514, a specific IKKβ inhibitor that inhibits p65-associated transcriptional activation of the NF-κB pathway. To further confirm the involvement of NF-κB in Δ^9-THC-induced increase in COX-2, COX-2 expression and NF-κB phosphorylation by Δ^9-THC were determined in the absence and presence of SC-514. Inhibition of IKKβ blocked Δ^9-THC-induced COX-2 and NF-κB phosphorylation (Figure 2E). Phosphorylation of Akt, ERK, p38MAPK, and NF-κB was confirmed in the hippocampus of animals that received Δ^9-THC (Figure S2H).

Inhibition of COX-2 Eliminates Impairments in Hippocampal Long-Term Synaptic Plasticity

If sustained elevation of COX-2 expression and activity following repeated Δ^9-THC exposure contribute to impairments in long-term synaptic plasticity and cognitive function, then inhibition of COX-2 should be able to eliminate or attenuate the impairments. To test this hypothesis, we recorded hippocampal LTP in mice receiving daily injections of Δ^9-THC (10 mg/kg, the dosage used by other studies such as Fan et al. [2010], Hoffman et al. [2007], Puighermanal et al. [2009], and Tonini et al. [2006]), NS398, Δ^9-THC+NS398, or vehicle for 7 consecutive days. We found that COX inhibition by NS398 rescued decreased hippocampal LTP induced by repeated in vivo exposure to Δ^9-THC for 7 days both at CA3-CA1 synapses (Figure 3A) and perforant path synapses in the dentate gyrus (Figure S3A). Similarly, genetic inhibition of COX-2 also prevented LTP deterioration induced by Δ^9-THC at both CA3-CA1 synapses (Figure 3B) and the perforant path (Figure S3B). To verify whether persistent overexpression of COX-2 impairs LTP, we recorded LTP in

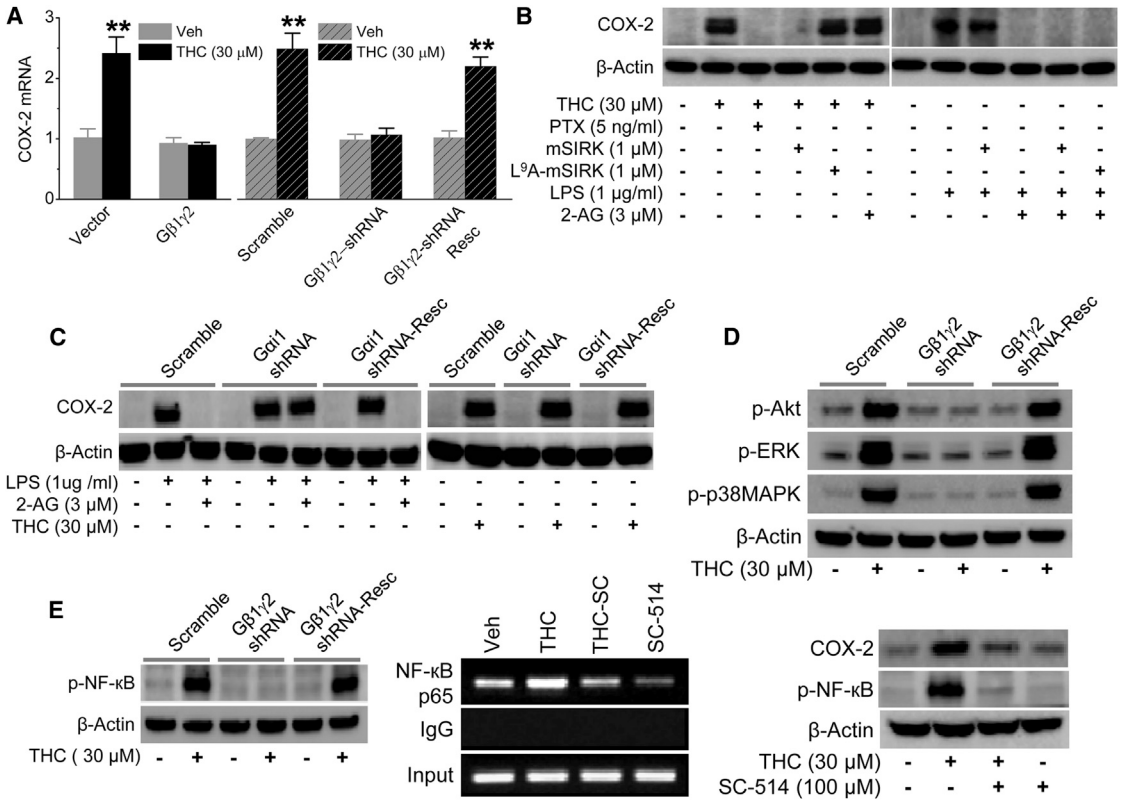

Figure 2. Gβγ Subunits Mediate Δ^9-THC-Elevated COX-2 Expression

(A) Overexpression or knockdown of β1 and γ2 subunits eliminates Δ^9-THC-increased COX-2 mRNA detected by qPCR in NG108-15 cells. Error bars represent ± SEM; **$p < 0.01$ compared with the vehicle control (ANOVA, Fisher's PLSD, $n = 6$). NG108-15 cells were transfected with pcDNA3.1 plasmids encoding $G\beta_1$ and $G\gamma_2$ subunits, the pLL3.7 vector expressing Gβ1 and Gγ2 shRNA, or the vector expressing shRNA-resistant Gβ1γ2 in the absence and presence of Δ^9-THC.

(B) Disruption of Gβγ subunits blocks Δ^9-THC-elevated COX-2 but does not prevent suppression of COX-2 by 2-AG in response to LPS stimulus in mixed culture of hippocampal neurons and astroglial cells (~10%). The culture was treated with a membrane-permeable Gβγ-binding peptide mSIRK or a single point mutated (Leu 9 to Ala) Gβγ-binding peptide mSIRK (L^{9A}-mSIRK) in the absence and presence of Δ^9-THC, LPS, PTX, 2-AG.

(C) Silencing the Gαi1 subunit blocks 2-AG-suppressed COX-2 but does not affect the elevation of COX-2 by Δ^9-THC in mixed culture of neurons and astroglial cells treated with the lentiviral vector expressing Gαi1 shRNA or shRNA-resistant Gαi1.

(D) Δ^9-THC induces phosphorylation of Akt, ERK, and p38MAPK. The phosphorylation is inhibited by knockdown of Gβγ2, and the inhibition is rescued by expressing shRNA-resistant Gβ1γ2.

(E) Left: Δ^9-THC induces phosphorylation of NF-κB, and the effect is blocked by Gβ1γ2 shRNA in NG108-15 cells. Middle: binding of NF-κB p65 in the promoter region of the COX-2 gene (*ptgs2*) by ChIP analysis. Right: Δ^9-THC-induced NF-κB phosphorylation and COX-2 expression are blocked by IKKβ inhibition in mixed culture of neurons and astroglial cells.

See also Figures S2 and S7.

animals repeatedly treated with LPS, which increases COX-2. As we expected, repeated injection of LPS significantly reduced LTP, and this decrease was prevented by inhibition of COX-2 (Figure S3C). These data suggest that persistent elevation of COX-2 in the brain will be detrimental to integrity of synaptic structure and plasticity. Because a single dose of Δ^9-THC produced an increase in COX-2 expression, we wondered whether this increase alters synaptic function. To this end, we recorded long-term depression (LTD) induced by low-frequency stimulation (LFS) at hippocampal CA3-CA1 synapses and found that LTD is impaired by a single Δ^9-THC exposure. However, LTD is normal in COX-2 knockout animals that received a single injection of Δ^9-THC (Figure S4). This information suggests that a single Δ^9-THC exposure induces a COX-2-associated impairment in LTD (Mato et al., 2004, 2005).

Impairments in Spatial and Fear Memories by Δ^9-THC Are Occluded by COX-2 Inhibition

Administration of marijuana or Δ^9-THC impairs learning and memory. If this impairment is associated with COX-2 induction, then inhibition of COX-2 would prevent or attenuate the deficits. To test this prediction, we determined the effect of COX-2 inhibition on spatial learning and memory using the Morris water maze test in mice that received repeated Δ^9-THC exposure in wild-type (WT) and COX-2 KO mice. As shown in Figures 4B and 4C, pharmacological or genetic inhibition of COX-2 prevented Δ^9-THC-impaired spatial learning and memory. To further determine the role of COX-2 in Δ^9-THC-impaired memory, hippocampus-dependent contextual memory was determined using the fear conditioning protocol (Chen et al., 2006a). As seen in Figure 4A, repeated Δ^9-THC exposure impaired fear

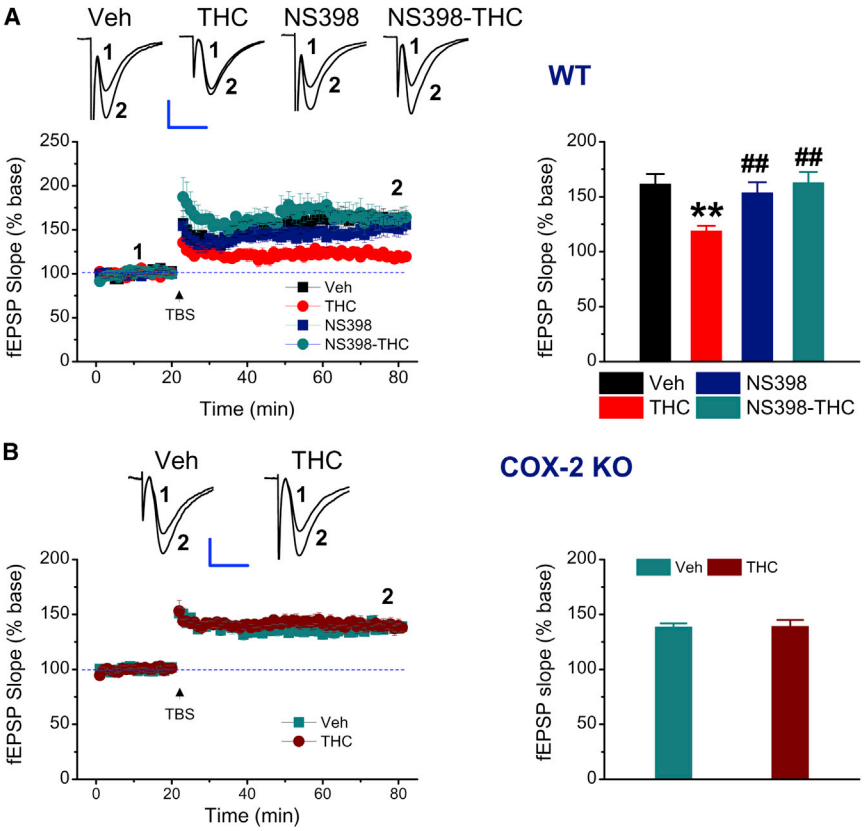

Figure 3. Inhibition of COX-2 Eliminates Deficits in LTP by Repeated Δ^9-THC Exposure

(A) Top: representative field excitatory post-synaptic potentials (fEPSPs) recorded at hippocampal CA3-CA1 synapses from WT animals repeatedly injected with vehicle, Δ^9-THC (10 mg/kg), NS398 (10 mg/kg), or Δ^9-THC+NS398 once daily for 7 consecutive days. LTP was measured 24 hr after cessation of the last injection. Left: time courses of changes in fEPSP slope under different treatment. Right: mean values of the potentiation of fEPSPs averaged from 56 to 60 min following TBS (n = 6– 8 slices/5–6 animals).

(B) Top: representative fEPSPs recorded from COX-2 knockout (KO) mice injected with vehicle or Δ^9-THC (10 mg/kg) once daily for 7 consecutive days. Left: time courses of changes in fEPSP slope induced by Δ^9-THC. Right: mean values of the potentiation of fEPSPs averaged from 56 to 60 min following TBS (n = 8–12 slices/6–8 animals).

Error bars represent ± SEM; **p < 0.01 compared with vehicle controls; ##p < 0.01 compared with Δ^9-THC (ANOVA with Bonferronni post hoc test). Scale bars in (A1) and (B1), 0.3 mV/10 ms. See also Figures S3 and S4.

memory, and this impairment was attenuated by COX-2 inhibition. These results suggest that COX-2 plays a critical role in synaptic and cognitive function deterioration consequent to repeated in vivo Δ^9-THC exposure (Figure S7).

Cataleptic effect and hypomotility are behavioral responses upon administering Δ^9-THC (Burstein et al., 1989; Long et al., 2009). We observed that the cataleptic and locomotor depressive effects of Δ^9-THC were attenuated or prevented by pharmacological or genetic inhibition of COX-2 (Figure S5). This means that cannabis-elicited catalepsy and locomotor depression are associated with the COX-2 induction.

Functional Synaptic Integrity in Δ^9-THC-Treated Animals Is Maintained by COX-2 Inhibition

Impaired long-term synaptic plasticity and memory induced by Δ^9-THC are largely associated with altered expression and function of glutamate receptors (Fan et al., 2010; Han et al., 2012). Recent evidence shows that adolescent chronic treatment with Δ^9-THC results in reduced density of dendritic spines and lowered length and number of dendrites in the hippocampus (Rubino et al., 2009). We used Thy1-GFP-expressing transgenic mice to detect morphology of dendritic spines (Chen et al., 2012). As seen in Figure 5A, repeated Δ^9-THC exposure significantly reduced density of dendritic spines of CA1 pyramidal neurons, especially mushroom spines in which AMPA and NMDA receptors are expressed. We found that the reduction in spines was prevented by pharmacological or genetic inhibition of COX-2. (We should mention here that the comparatively low number of mushroom-type spines in Figure 5A may be due to the scoring criteria.) Meanwhile, Δ^9-THC-reduced expression of PSD-95, an important post-synaptic marker, was rescued by COX-2 inhibition (Figure 5B). However, Δ^9-THC did not alter expression of synaptophysin (Syn), a presynaptic marker. This information indicates that increased COX-2 by repeated Δ^9-THC exposure decreases dendritic spines and postsynaptic density. We show previously that repeated Δ^9-THC exposure for 7 days induces CB1R-dependent decreases in functional and surface expression of AMPA and NMDA receptor subunits (Fan et al., 2010). We speculated that reduced expression of glutamate receptor subunits in the hippocampus of animals that received repeated in vivo Δ^9-THC exposure is likely regulated by a homeostatic mechanism. Δ^9-THC increased synthesis of COX-2 and its reaction product PGE_2, which stimulates glutamate released from presynaptic nerve terminals and astroglial cells, resulting in an extracellular accumulation of glutamate (Figure S6A). The increased extracellular glutamate may also result from the reduced uptake of glutamate by glutamate transporters because expression of these transporters was downregulated by repeated exposure to Δ^9-THC (Figure S6B). To this end, we used immunostaining to determine expressions of synaptic and extrasynaptic GluA1, GluN2A, and GluN2B in the hippocampal CA1 area. As shown in Figures 5C and 5D, hippocampal expressions of both synaptic and extrasynaptic GluA1, GluN2A, and GluN2B were significantly reduced by repeated Δ^9-THC exposure, and the reduction was attenuated or prevented by COX-2 inhibition. This was consistent with the observations where total and surface expressions of GluA1, GluN2A, and GluN2B detected by immunoblot in WT mice were significantly decreased following

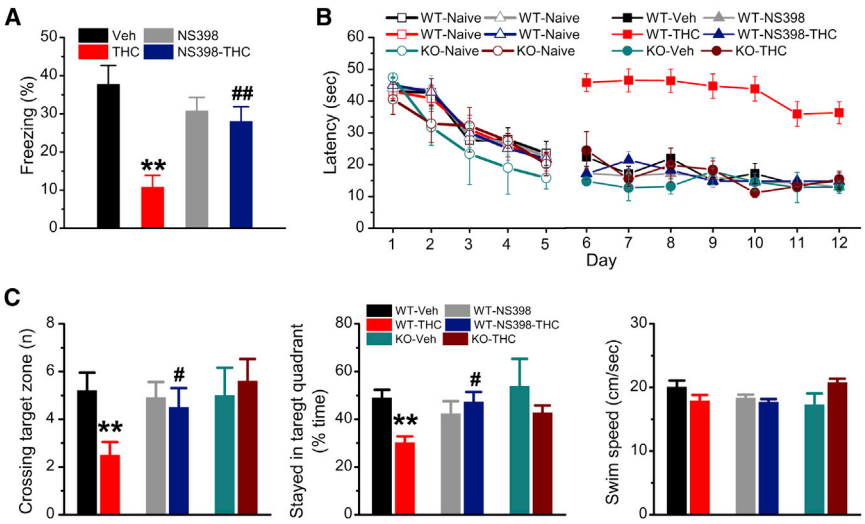

Figure 4. Impaired Spatial and Fear Memories by Repeated Δ^9-THC Exposure Are Occluded by COX-2 Inhibition

(A) Impaired fear memory is attenuated by COX-2 inhibition. 24 hr after a footshock conditioning, animals were administered with Δ^9-THC (10 mg/kg) or NS398 (10 mg/kg) once a day for 7 days. Freezing behavior was recorded 24 hr after the cessation of the last injections.

(B) COX-2 KO and WT mice received training in the Morris water maze for 5 days without any treatments (naive). Starting at day 6, WT animals received vehicle, Δ^9-THC (10 mg/kg), NS398 (10 mg/kg), Δ^9-THC+NS398, once a day for 7 days. COX-2 KO mice received vehicle or Δ^9-THC (10 mg/kg) for 7 days. Tests were performed 30 min following the injections.

(C) Probe trial test, which was conducted 24 hr after the cessation of the last Δ^9-THC injection. Left: the number of times crossed the target zone. Middle: the amount of time stayed in the target quadrant. Right: swim speed in different treatments in probe trial tests.

Error bars represent ± SEM; **$p < 0.01$ compared with the vehicle control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with Δ^9-THC (n = 9–12 animals/group, two-way ANOVA, Bonferronni post hoc test). See also Figures S5 and S7.

exposure to Δ^9-THC for 7 days, but the decreases were not seen in COX-2 knockout mice (Figure 6). These results indicate that reduced expression of glutamate receptor subunits and density of dendritic spines are associated with the COX-2 induction effect of Δ^9-THC (Figure S7).

The Beneficial Effects of Decreasing Aβ and Neurodegeneration by Δ^9-THC Are Preserved in the Presence of COX-2 Inhibition

A critical issue is whether COX-2 inhibition would eliminate the beneficial effects of marijuana. To answer this question, we used 5XFAD APP transgenic mice, an animal model of Alzheimer's disease (AD) as described previously (Chen et al., 2012), to determine whether Δ^9-THC is capable of reducing Aβ and neurodegeneration and whether these effects are retained when COX-2 is inhibited. As shown Figures 7A and 7B, treatment of Δ^9-THC once daily for 4 weeks significantly reduced the numbers of Aβ plaques and degenerated neurons in the absence and presence of Celebrex in AD animals. This information indicates that the beneficial effects of Δ^9-THC are preserved while COX-2 is inhibited. Meanwhile, we revealed that the reduction of Aβ by Δ^9-THC is not through inhibiting expression of β-site amyloid precursor protein cleaving enzyme 1 (BACE1), an enzyme responsible for synthesis of Aβ, but is likely through elevating neprilysin, an important endopeptidase that degrades Aβ (Figure 7C).

DISCUSSION

The results presented here demonstrate that impaired synaptic and cognitive function induced by repeated Δ^9-THC exposure is associated with a previously unrevealed CB1R-Gβγ-Akt-ERK/MAPK-NF-κB-COX-2 signaling pathway. It has been long known that use of marijuana induces neuropsychiatric and cognitive deficits, which greatly limit medical use of marijuana. Synaptic and memory impairments are also the consequence of cannabis abuse. However, the molecular mechanisms underlying undesirable effects by cannabis are largely unknown. We discovered in this study that pharmacological or genetic inhibition of COX-2 eliminates or attenuates synaptic and memory impairments elicited by repeated Δ^9-THC exposure, suggesting that these major adverse effects of cannabis on synaptic and cognitive function can be eliminated by COX-2 inhibition, which would broaden the use of medical marijuana.

CB1R is the primary target of cannabinoid exposures causing synaptic and memory impairments (Lichtman and Martin, 1996; Hoffman et al., 2007; Puighermanal et al., 2009; Fan et al., 2010; Han et al., 2012). Previous studies show that the endocannabinoid 2-AG suppresses COX-2 via a CB1R-depedent mechanism in response to proinflammatory and excitotoxic insults (Zhang and Chen, 2008). Surprisingly, we found in the present study that the exogenous cannabinoid Δ^9-THC increases COX-2 activity and expression, which are also mediated via CB1R. We demonstrate that COX-2 induction by Δ^9-THC is mediated via Gβγ subunits, whereas COX-2 suppression by 2-AG is mediated via the Gαi1 subunit, suggesting that activation of the same CB1 receptor may induce opposite biological effects. Indeed, previous studies showed that endogenous cannabinoids and exogenous Δ^9-THC exhibit different behavioral responses via CB1R (Long et al., 2009). However, it is still not clear how activation of CB1R and its coupled Gi/o by the endogenous cannabinoid 2-AG results in Gαi-mediated suppression of COX-2 in response to proinflammatory insults but by the exogenous cannabinoid Δ^9-THC leads to Gβγ-mediated induction of COX-2. Activation of CB1R/Gi/o either by 2-AG or Δ^9-THC should induce both Gαi- and Gβγ-mediated effector responses through different downstream signaling events. For example, inhibition of N type calcium channel currents by 2-AG appears to be mediated via Gβγ (Guo and Ikeda, 2004), suggesting that 2-AG is also capable of triggering Gβγ-mediated responses in

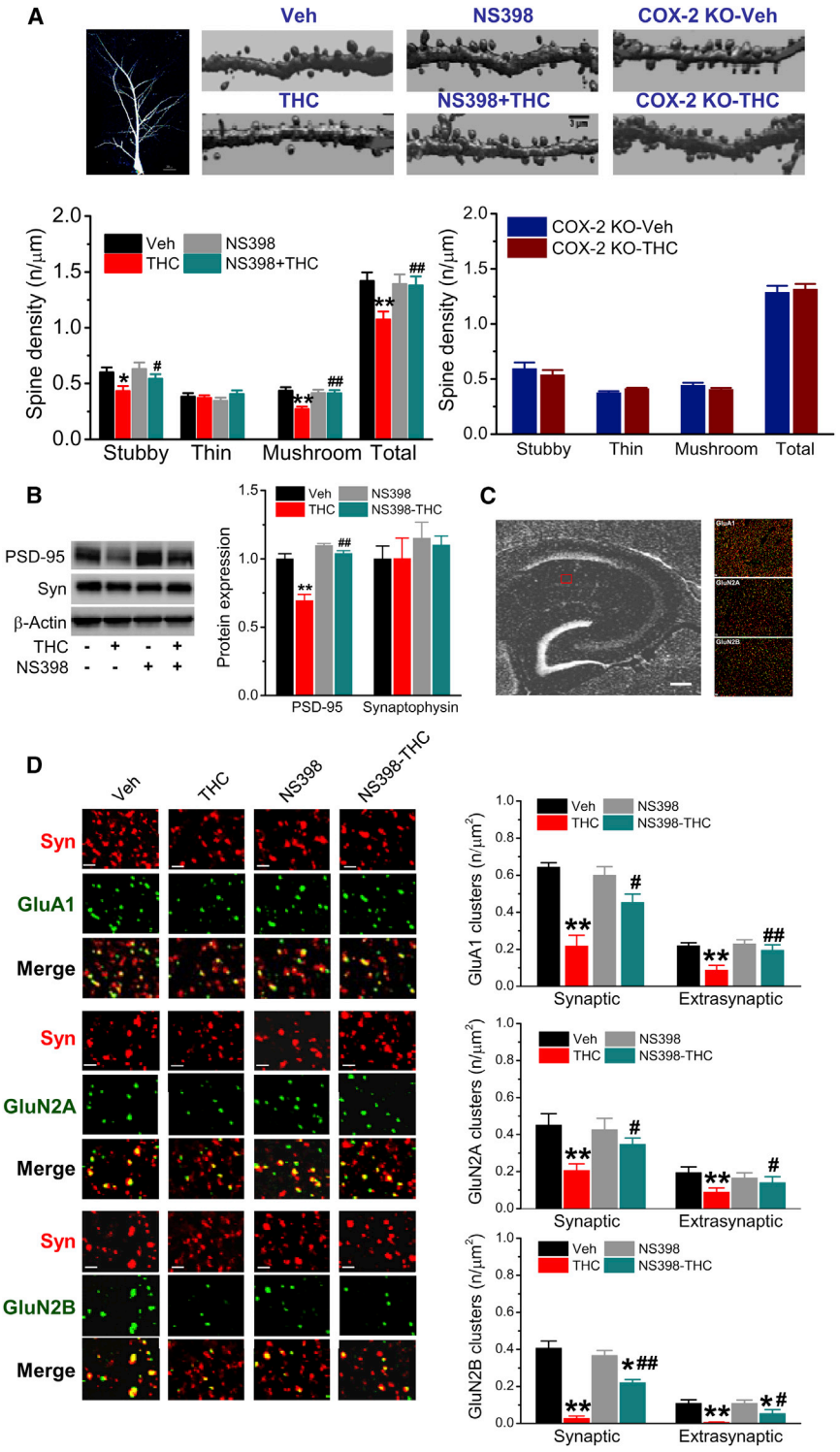

Figure 5. Decreases in Dendritic Spine Density and Glutamate Receptor Expression by Δ^9-THC Are Prevented by Inhibition of COX-2

(A) Two-photon imaging of dendritic spines in CA1 hippocampal pyramidal neurons expressing GFP of transgenic mice. Top left: representative image of a CA1 pyramidal neurons. Scale bar, 20 μm. Top right: representative images of dendritic spine segments from animals received different treatments. Scale bars, 3 μm. Lower left: spine density in WT animals, and lower right: in COX-2 knockout (KO) mice (n = 5 animals/group).

(B) Expression of PSD-95 and synaptophysin (Syn) in animals treated with Δ^9-THC or NS398 for 7 days (n = 3 animals).

(C) Immunostaining analysis of synaptic and extrasynaptic glutamate receptor subunits. Left: schematic of a hippocampal section. The red dashed-line box marks the sampling field of immunostaining analysis. Scale bar, 200 μm. Right: representative GluA1, GluN2A, GluN2B, and Syn immunoreactivities (scale bar, 5 μm).

(D) Left: enlarged immunosignals of GluA1, GluN2A, GluN2B, Syn, and their overlay. Scale bars, 1.5 μm. Right: quantification of synaptic (colocalized with Syn) and extrasynaptic (nocolocalized) GluA1, GluN2A, and GluN2B (n = 5 animals/group).

Error bars represent ± SEM; $^{**}p < 0.01$ compared with the vehicle control, $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ compared with Δ^9-THC (ANOVA with Fisher's PLSD or Bonferronni post hoc tests).

addition to Gαi-mediated responses. In the case of COX-2 induction, the Gβγ-mediated COX-2 induction by Δ^9-THC may be predominant, which may mask Gαi-mediated COX-2 suppression. In addition, our results showing that the beneficial effects of Δ^9-THC are retained in the presence of COX-2 inhibition further suggest that activation of CB1R by Δ^9-THC may have both Gαi- and Gβγ-mediated effector responses. It is likely that COX-2 induction by Δ^9-THC may be just one of several Gβγ-mediated effects, and we cannot exclude the possibility that other biological effects are mediated via Gβγ. The divergent roles of G protein subunits in mediating endogenous and exogenous cannabinoids may be a consequence the intrinsic mechanisms of CB1R/G protein coupling, such as the agonist binding sites in the receptor, the efficacy of binding, or different conformational changes in the receptor/G protein upon binding with different agonists.

Synaptic and cognitive impairments by Δ^9-THC are apparently associated with alterations in glutamatergic synaptic transmission and functional expression of glutamate receptor subunits (Fan et al., 2010; Han et al., 2012; Monory et al., 2007; Tonini et al., 2006). It has been demonstrated that cannabinoid exposure leads to downregulation, internalization, and endocytosis of glutamate receptor subunits (Fan et al., 2010; Han et al., 2012; Suárez et al., 2004). In this

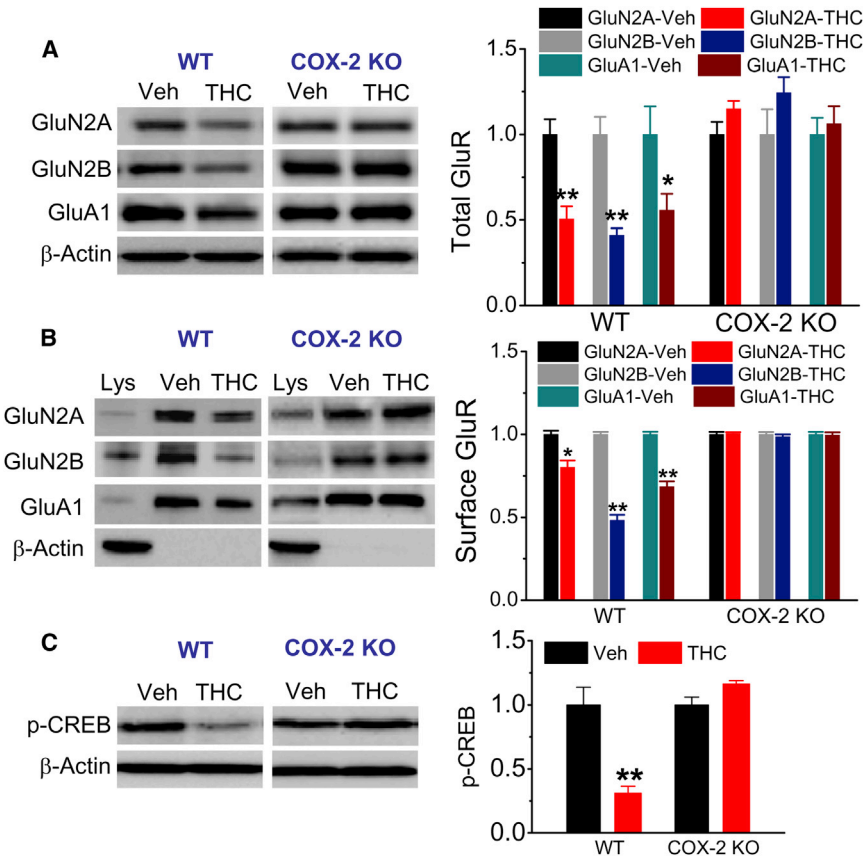

Figure 6. Reduced Expression of Glutamate Receptor Subunits and Phosphorylation of CREB by Δ^9-THC Is Rescued by COX-2 Inhibition

(A) Immunoblot analysis of hippocampal expression of GluR1, NR2A, and NR2B subunits in WT and COX-2 KO mice treated with vehicle or Δ^9-THC for 7 days (n = 3).

(B) Surface expression of GluR1, NR2A, and NR2B in WT and COX-2 KO mice treated with vehicle or Δ^9-THC for 7 days (n = 4).

(C) Phosphorylation of hippocampal CREB in WT and KO mice treated with vehicle or Δ^9-THC for 7 days (n = 3).

Error bars represent ± SEM; *p < 0.05 and **p < 0.01 compared with the vehicle control (ANOVA with Fisher's PLSD). See also Figures S6 and S7.

study, we also demonstrate that density of dendritic spines in hippocampal neurons is reduced in animals that received Δ^9-THC for 7 days. The reduced expressions of synaptic and extrasynaptic of glutamate receptor subunits as well as PSD-95 by Δ^9-THC are likely associated with elevated extracellular glutamate levels. Indeed, it has been shown that cannabinoids elevate extracellular glutamate levels, which may result from increased synaptic and astrocytic release of glutamate or reduced uptake of glutamate by glutamate transporters (Fan et al., 2010; Ferraro et al., 2001; Han et al., 2012; Navarrete and Araque, 2008; Tomasini et al., 2002; Suárez et al., 2004; Tonini et al., 2006). We detected that expression of glutamate transporters is significantly decreased in Δ^9-THC exposed animals, and this decrease is attenuated by COX-2 inhibition (Figure S6). These previous studies, together with our results, suggest that accumulation of glutamate in the extracellular apartment by repeated Δ^9-THC exposure contributes to reductions in total and surface expression of the glutamate receptors and the density of dendritic spines.

Earlier studies showed that the levels of the eicosanoid PGE_2 in circulation and the brain are elevated in humans and animals exposed to marijuana or Δ^9-THC, and the elevation could be antagonized by indomethacin, a nonsteroidal anti-inflammatory drug (NSAID) (Burstein et al., 1989; Fairbairn and Pickens, 1979, 1980; Perez-Reyes et al., 1991). NSAIDs are nonselective inhibitors for both COX-1 and COX-2. This suggests that COX-1 and/or COX-2 may be involved in marijuana- or Δ^9-THC-induced increase in PGE_2. Although both COX-1 and COX-2 are capable of converting arachidonic acid (AA) into five primary prostanoids and prostaglandins (PGD_2, PGE_2, $PGF_2\alpha$, PGI_2, and TXA_2), they exhibit preferences in synthesizing these substances. It is evident that PGE_2 is primarily derived from the COX-2 pathway (Brock et al., 1999; Sang et al., 2005). Because COX-1 expression is not affected by Δ^9-THC (Figure S1) and because COX-2 is expressed both in constitutive and inducible forms in the brain, it is likely that COX-2 is responsible for the marijuana- or Δ^9-THC-induced elevation of PGE_2. Our data showing that Δ^9-THC increases PGE_2 in the brain and that this increase is blocked by COX-2 inhibition support this speculation. Interestingly, Δ^9-THC-induced cataleptic response can be eliminated by NSAIDs and mimicked by direct administration of PGE_2 (Burstein et al., 1989; Fairbairn and Pickens, 1979). We also provide convincing evidence that pharmacological or genetic inhibition of COX-2 prevents or attenuates cataleptic and locomotor depressive responses by Δ9-THC. Importantly, synaptic and cognitive deficits following repeated Δ^9-THC exposure are eliminated or attenuated by COX-2 inhibition.

The elevated levels of extracellular glutamate by Δ^9-THC result likely from induction of COX-2, which makes PGE_2. It has been shown that PGE_2 stimulates or facilitates both synaptic and astrocytic release of glutamate (Bezzi et al., 1998; Chen et al., 2002; Dave et al., 2010; Sang et al., 2005; Sanzgiri et al., 1999). In fact, COX-2 and PGE_2 signaling have been shown to regulate glutamatergic synaptic transmission and plasticity via EP2 or EP3 receptors (Akaneya and Tsumoto, 2006; Chen et al., 2002; Cowley et al., 2008; Sang et al., 2005). It is possible that Δ^9-THC exposure stimulates COX-2 expression and activity through CB1R-coupled $G\beta\gamma$ subunits and the downstream Akt-ERK/MAPK-NF-κB signaling pathway, resulting in increase of COX-2 transcription, expression, and activity, which in turn enhance the release of PGE_2 from neurons and astroglial cells. Our results show that Δ^9-THC-induced COX-2 expression in

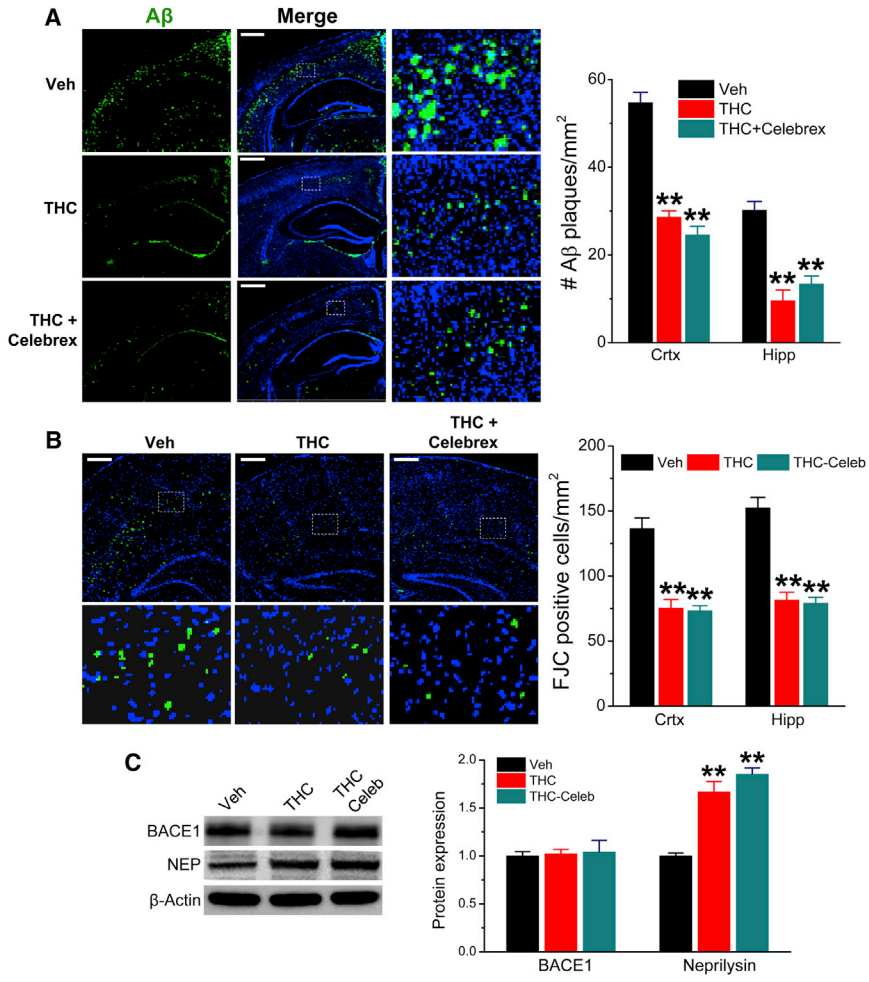

Figure 7. The Beneficial Effects of Reducing Aβ and Neurodegeneration by Δ^9-THC Are Preserved in the Presence of COX-2 Inhibition

(A) Δ^9-THC significantly reduces Aβ plaques detected using anti-4G8 antibody in 4-month-old 5XFAD APP transgenic (TG) mice in the absence and presence of COX-2 inhibition. TG mice received Δ^9-THC (3 mg/kg) or Celebrex (1 mg/kg) once daily for 4 weeks starting at 3 months of age.

(B) Δ^9-THC significantly reduces degenerated neurons detected by Fluoro-Jade C (FJC) staining in 6-month-old TG mice treated with/out Celebrex. TG mice received Δ^9-THC (3 mg/kg) or Celebrex (1 mg/kg) once daily for 4 weeks starting at 5 months of age.

(C) Δ^9-THC increases expression of neprilysin (NEP), but not β-site amyloid precursor protein cleaving enzyme 1 (BACE1) in TG mice.

Error bars represent ± SEM; **p < 0.01 compared with the vehicle control (n = 3 to 5 animals/group; one-way ANOVA, Bonferroni post hoc tests). Scale bars in (A) and (B), 400 μm.

astroglial cells is more pronounced than in neurons. A recent study also shows that CB1R expressed in astroglial cells is responsible for LTD and working memory impairment in animals exposed to cannabinoids (Han et al., 2012). This suggests that glutamate released from astroglial cells triggered by COX-2-derived PGE_2 and reduced uptake of glutamate by glutamate transporters in astrocytes resulting from repeated Δ^9-THC exposure may play an important role in extracellular glutamate accumulation. Sustained elevation and accumulation of extracellular glutamate upon repeated exposure to Δ^9-THC induce downregulation and internalization of glutamate receptor subunits and reduction in the density of dendritic spines in hippocampal neurons, leading to the deficits in long-term synaptic plasticity and cognitive function (Figure S7).

It has been well recognized that cannabinoids possess antioxidant, anti-inflammatory, and neuroprotective properties (Bahr et al., 2006; Campbell and Gowran, 2007; Centonze et al., 2007; Chen et al., 2011; Du et al., 2011; Gowran et al., 2011; Marchalant et al., 2008; Marsicano et al., 2003; Zhang and Chen, 2008). Also, cannabis has been used for thousands of years as medical treatments. However, neuropsychiatric and cognitive side effects limit medical use of marijuana, especially for a long-term treatment. The results presented here suggest that the unwanted side effects of cannabis could be eliminated or reduced—while retaining its beneficial effects—by administering a COX-2 inhibitor or NSAID along with Δ^9-THC for treatments of intractable medical conditions such as AD. In the present study, we did observe that brain Aβ and neurodegeneration in 5XFAD transgenic mice are significantly reduced by Δ^9-THC, and these beneficial effects are preserved in the presence of COX-2 inhibition. We also discovered that Δ^9-THC significantly elevates expression of neprilysin, an important endopeptidase for Aβ degradation. This suggests that Δ^9-THC is capable of reducing Aβ and neurodegeneration in an animal model of AD and that the Aβ-reducing effect is likely through elevating expression of neprilysin. This suggests that Δ^9-THC (brand name: Marinol) may have therapeutic potential for prevention and treatment of AD if its undesirable side effects (e.g., synaptic and cognitive impairments) can be eliminated by COX-2 inhibition. In particular, there are no effective medications currently available for preventing and treating AD or halting disease progression. Our results also suggest that selective COX-2 inhibitors or NSAIDs may be useful for treating the neuropsychological and cognitive side effects of cannabis abuse.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6, CB1 knockout, Thy1-EGFP transgenic, COX-2 knockout, and 5XFAD APP transgenic mice were used in the present study.

Cell Culture

Relative pure hippocampal neurons (astroglial cells < 2%), mixed neurons and astroglial cells (astroglial cells ~10%), astroglial cell-enriched (astroglial

cells > 95%), and NG108-15 cell cultures were made as described previously (Sang et al., 2005; Zhang and Chen, 2008).

Electrophysiological Recordings

Hippocampal LTP both at CA3-CA1 and perforant path synapses were recorded in acutely hippocampal slices and induced by a θ-burst stimulation (TBS) as described previously (Hoffman et al., 2007).

Immunoblots

Western blot assay was conducted using specific antibodies (Table S1) to determine expressions of COX-2, glutamate receptor subunits, PSD-95, G protein subunits, phosphoproteins, BACE1, and neprilysin in hippocampal tissue and/or in cultured cells as described previously (Chen et al., 2012). Surface biotinylation assays were performed to determine surface expression of glutamate receptor subunits in hippocampal slices as described previously (Fan et al., 2010).

Transfection of Plasmid and Lentiviral Vectors

NG108-15 cells were used for transfection of the pcDNA3.1 plasmid encoding Gβ1 and Gγ2 subunits or the pLL3.7 vector expressing scramble, Gβ1 and Gγ2 shRNA, and shRNA-resistant Gβ1γ2. Mixed culture of neurons and astroglial cells was used for transfection of the pLL3.7 lentiviral vector expressing scramble, Gαi1 shRNA, and shRNA-resistant Gαi1.

qRT-PCR

The iScript complementary (cDNA) synthesis kit (BioRad) was used for the reverse transcription reaction. Real-time RT-PCR specific primers for COX-2, β1, γ2, and GAPDH were synthesized by IDT. Samples were compared using the relative CT method as described previously (Zhang and Chen, 2008).

ChIP Analysis

ChIP analysis was performed to determine the binding activity of NF-κB in the promoter of the COX-2 gene.

PGE_2 Assay

PGE_2 in hippocampal tissue was detected using PGE_2 enzyme immunoassay kit (Cayman Chemical) according to the procedure described by the manufacturer (Zhang and Chen, 2008).

Immunostaining and Histochemistry

Aβ plaques, degenerated neurons, and glutamate receptor subunits in cryostat sectioning brain slices were performed as described previously (Chen et al., 2012; Li et al., 2011).

Two-Photon Imaging

Morphology of dendritic spines in hippocampal CA1 pyramidal neurons was determined in GFP-expressing transgenic mice using a two-photon laser scanning microscope as described previously (Chen et al., 2012). Shape, size, and density of spines were measured from the three-dimensional reconstructions using NeuronStudio version 0.9.92.

Behavioral Tests

The classic Morris water maze and fear conditioning tests were performed to determine spatial and fear memory as described previously (Chen et al., 2006a, 2012). The "open field" test was conducted to detect the locomotor activity, and the bar test was used to detect catalepsy (Egashira et al., 2007).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.10.042.

AUTHOR CONTRIBUTIONS

R.C. designed and performed the following experiments and analyzed the data: (1) LTP recordings in the hippocampal CA1 region; (2) two-photon imaging of dendritic spines in WT animals; and (3) water maze behavioral tests. J.Z. designed and performed the following experiments and analyzed the data: (1) immunoblot and qPCR; (2) immunohistochemistry; (3) overexpression of Gβγ subunits, shRNA knockdown, and rescue; (4) behavioral tests; and (5) cell culture, genotyping, and animal care. N.F. designed and performed the following experiments and analyzed the data: (1) LTP recordings in the hippocampal dentate gyrus area; (2) LTP recordings in animals treated with LPS; and (3) PGE2 assay. Z.T. designed and performed the following experiments and analyzed the data: (1) ChIP; (2) shRNA-resistant rescue; (3) immunohistochemistry; and (4) fear conditioning behavioral tests. Y.W. designed and performed the following experiments and analyzed the data: (1) two-photon imaging of dendritic spines in COX-2 knockout mice; (2) cataleptic and open field behavioral tests; and (3) immunoblot. H.Y., H.S., and Y.S. performed some experiments; Y.T. provided the behavioral testing setups; and C.C. conceived the project and wrote the manuscript.

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