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A Dysregulated Endocannabinoid-Eicosanoid Network Supports Pathogenesis in a Mouse Model of Alzheimer's Disease

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SUMMARY

Although inflammation in the brain is meant as a defense mechanism against neurotoxic stimuli, increasing evidence suggests that uncontrolled, chronic, and persistent inflammation contributes to neurodegeneration. Most neurodegenerative diseases have now been associated with chronic inflammation, including Alzheimer's disease (AD). Whether anti-inflammatory approaches can be used to treat AD, however, is a major unanswered question. We recently demonstrated that monoacylglycerol lipase (MAGL) hydrolyzes endocannabinoids to generate the primary arachidonic acid pool for neuroinflammatory prostaglandins. In this study, we show that genetic inactivation of MAGL attenuates neuroinflammation and lowers amyloid β levels and plaques in an AD mouse model. We also find that pharmacological blockade of MAGL recapitulates the cytokine-lowering effects through reduced prostaglandin production, rather than enhanced endocannabinoid signaling. Our findings thus reveal a role of MAGL in modulating neuroinflammation and amyloidosis in AD etiology and put forth MAGL inhibitors as a potential next-generation strategy for combating AD.

INTRODUCTION

Neuroinflammation is a fundamental underlying hallmark of Alzheimer's disease (AD), a debilitating neurodegenerative condition marked by accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles composed of aggregated amyloid β (Aβ) and hyperphosphorylated tau, respectively, leading to progressive cognitive impairment and dementia (Glass et al., 2010). Suppressing inflammation has been shown to reduce AD pathological hallmarks as well as cognitive and behavioral deficits in AD models (Choi and Bosetti, 2009; Liu et al., 2012). Recently, there has been considerable interest in exploring the therapeutic potential of anti-inflammatory agents to prevent, treat, or slow the progression of AD (Aïd and Bosetti, 2011; Cunningham and Skelly, 2011).

Ablation of cyclooxygenases (COX) 1 or 2 with nonsteroidal anti-inflammatory drugs (NSAIDs) or in COX1 or COX2 knockout mice reduces prostaglandins and suppresses neuroinflammation, concordant with significant improvements in cognitive, behavioral, and memory impairments as well as reductions in Aβ plaques and hyperphosphorylated tau in AD mouse models (Choi and Bosetti, 2009; Kotilinek et al., 2008; McKee et al., 2008). Retrospective human epidemiological studies have also demonstrated protective effects or delayed onset of AD upon prolonged NSAID treatment when initiated early or before disease initiation, respectively (Rogers et al., 1993; Szekely et al., 2008); however, NSAIDs have not shown efficacy in AD patients with mild to moderate cognitive impairment (Imbimbo et al., 2010). Other anti-inflammatory strategies have also shown efficacy at reducing pathology in animal models, including treatment with antitumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) antibodies (Kitazawa et al., 2011; Shi et al., 2011). Pharmacological intervention based on chronic treatment with COX inhibitors or treatment with anticytokine therapies, however, is not ideal for long-term use due to their respective gastrointestinal (COX1-selective), cardiovascular (COX2-selective), or immunosuppressive (anticytokine therapies) side effects (Ng and Chan, 2010; Raychaudhuri et al., 2009). Novel and safer anti-inflammatory strategies are thus required not only to gain a deeper understanding of the role that inflammation plays in AD disease progression, but also to investigate its therapeutic potential in combating AD.

We have recently discovered that monoacylglycerol lipase (MAGL), an enzyme that terminates the signaling of the anti-inflammatory endocannabinoid signaling lipid 2-arachidonoylglycerol (2-AG) (Long et al., 2009), also controls arachidonic acid (AA) release for the production of proinflammatory eicosanoids (which include lipids such as prostaglandins and thromboxanes) in the brain (Nomura et al., 2011). We found that genetic and pharmacological blockade of MAGL not only leads to enhanced endocannabinoid levels, but also reduced prostaglandins in the brain under both basal and inflammatory states.

In this study, we asked whether MAGL inactivation modulates AD pathogenesis in a mouse model of Aβ deposition. We present

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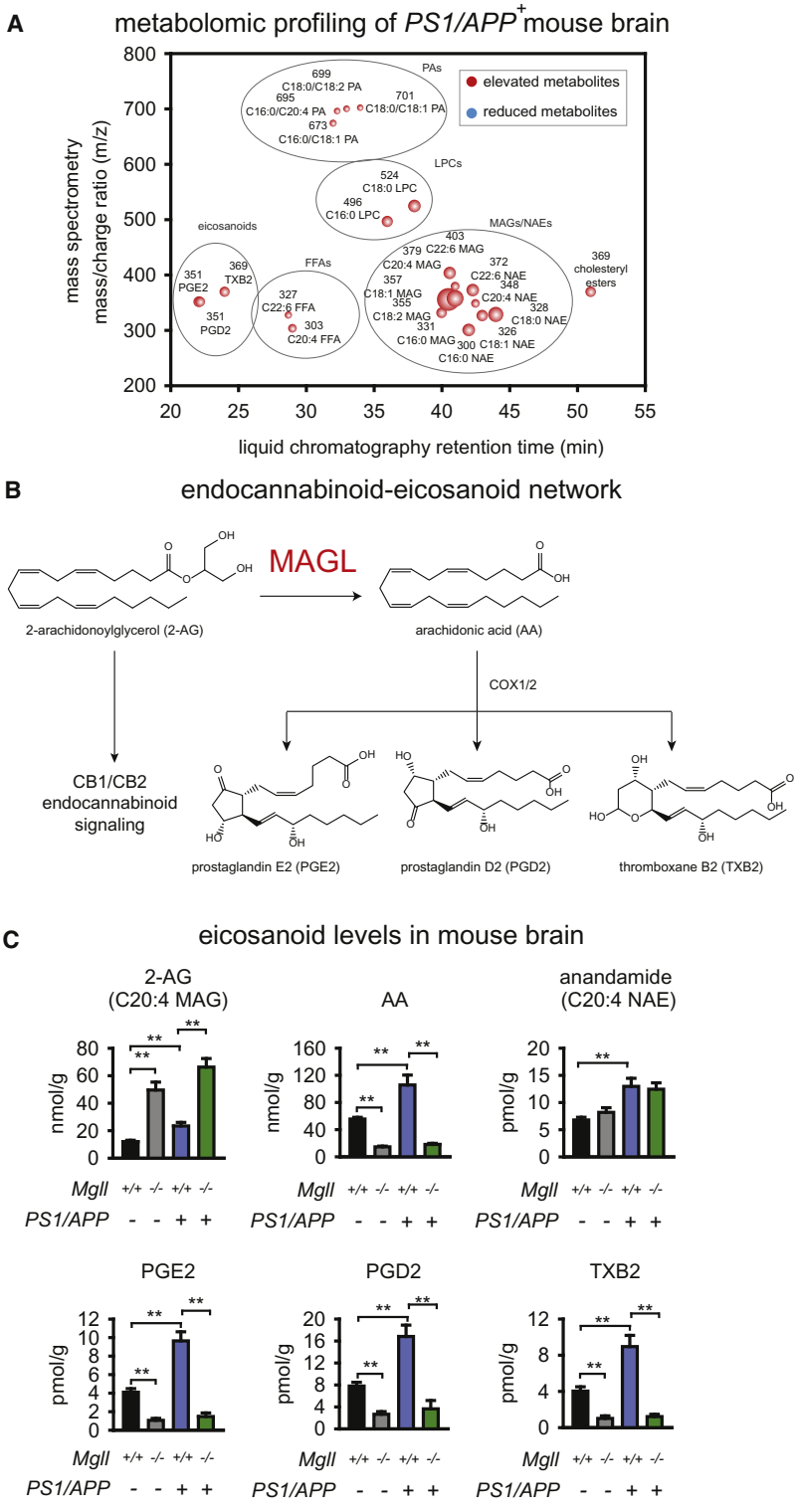

Figure 1. A Dysregulated Endocannabinoid-Eicosanoid Network in a Mouse Model of Alzheimer's Disease

(A) Comparative metabolomic profiling of *PS1/APP*$^{+}$ compared to *PS1/APP*$^{-}$ mouse brain lipidomes measuring levels of endocannabinoids; 2-AG (C20:4 monoacylglycerol (MAG)), anandamide (C20:4 *N*-acylethanolamines (NAEs)), arachidonic acid (C20:4 free fatty acids [FFAs]), and eicosanoids (prostaglandin E2 (PGE2), PGD2, and thromboxane B2 [TXB2]). Elevations in other MAGs, NAEs, and FFAs, as well as phosphatidic acids (PAs), lysophosphatidyl cholines (LPCs), and cholesteryl esters are also depicted. LPCs, MAGs, and NAEs mass/charge ratios (m/z) are represented as [M+H]. PAs, eicosanoids, and FFAs are represented as [M-H]. Cholesteryl esters are represented as loss of fatty acid and [M+H-H2O]+. Data were obtained from untargeted and multiple reaction monitoring (MRM)-based targeted LC/MS analysis. The data shown in Figure 1A represent metabolites that are significantly altered ($p < 0.05$) by greater than 1.5-fold in the *PS1/APP*$^{+}$ compared to *PS1/APP*$^{-}$ mouse brain (n = 4–5 mice/group) by two-tailed t test (raw data in Table S1). The y axis denotes mass over charge ratio (m/z), the x axis represents retention time on liquid chromatography, and the size of the circles represent relative fold-change of the metabolite between *PS1/APP*$^{+}$ compared to wild-type *PS1/APP*$^{-}$ mouse brain lipidomes. There were no metabolites that were reduced >1.5-fold in *PS1/APP*$^{+}$ brains. Data represent only the metabolites that were consistently altered in two separate experiments each including n = 4–5 mice/group.

(B) MAGL hydrolyzes the endocannabinoid 2-AG to release the dominant source of AA for COX-mediated eicosanoid (e.g., PGE2, PGD2, TXB2) production in the brain. TXB2 is a nonbioactive breakdown product of TXA2.

(C) Brain endocannabinoid and eicosanoid levels determined by multiple reaction monitoring (MRM) using a QQQ-LC/MS from *Mgll*$^{+/+}$/*PS1/APP*$^{-}$, *Mgll*$^{-/-}$/*PS1/APP*$^{-}$, *Mgll*$^{+/+}$/*PS1/APP*$^{+}$, *Mgll*$^{-/-}$/*PS1/APP*$^{+}$ mouse brains.

Data are means ± SEM, n = 4–8 mice/group for (A) and n = 7–8 mice/group for (C). Significance is shown as $^{*}p < 0.05$, $^{**}p < 0.01$ using a linear model with ANOVA.

RESULTS

Metabolomic Profiling of *PS1/APP*$^{+}$ AD Mouse Model

To identify dysregulated metabolic networks that underlie AD pathophysiology, we profiled the lipidome of the *PS1/APP*$^{+}$ mouse brains, which exhibit an age dependent elevation in Aβ levels and plaque deposition (Figure S1). Using a combination of targeted and untargeted liquid chromatography/mass spectrometry (LC/MS)-based metabolomic profiling platforms, we identified several classes of lipids that were elevated in the *PS1/APP*$^{+}$ mouse brains compared to their wild-type counterparts (Figure 1A), including monoacylglycerols (MAGs), *N*-acylethanolamines (NAEs), free fatty acids (FFAs), eicosanoids, phosphatidic acids (PAs), lysophosphatidyl cholines (LPCs), and cholesteryl esters. Among this metabolic

direct evidence that MAGL inactivation reduces proinflammatory prostaglandins and cytokine signaling machinery, and produces profound suppression of neuroinflammation and reductions in Aβ levels and plaque burden.

signature, we were particularly intrigued by the heightened endocannabinoid and eicosanoid levels in *PS1/APP*$^+$ brains (Figure 1A; Table S1). We postulated that the elevated eicosanoid levels in *PS1/APP*$^+$ mouse brains were driven by MAGL-mediated 2-AG hydrolysis and AA release (Figure 1B).

Genetic Ablation of MAGL Reduces Neuroinflammatory Eicosanoid Levels in the *PS1/APP*$^+$ AD Mouse Model

To determine whether MAGL contributes to eicosanoid production, neuroinflammation, and pathophysiology in the *PS1/APP*$^+$ mouse brain, we bred MAGL-disrupted (*Mgll*$^{-/-}$) (Chanda et al., 2010) and *PS1/APP*$^+$ transgenic mice (Samaroo et al., 2012) to generate four genotypes for our studies: *Mgll*$^{+/+}$/*PS1/APP*$^-$, *Mgll*$^{+/+}$/*PS1/APP*$^+$, *Mgll*$^{-/-}$/*PS1/APP*$^-$, and *Mgll*$^{-/-}$/*PS1/APP*$^+$. We found that *Mgll*$^{-/-}$/*PS1/APP*$^-$ *and Mgll*$^{-/-}$/*PS1/APP*$^+$ mice presented higher levels of the MAGL substrate 2-AG (C20:4 MAG) and dramatically reduced levels of AA (C20:4 FFA), prostaglandins (PGE2 and PGD2), and thromboxane B2 (TXB2) in their brains compared to their *Mgll*$^{+/+}$/*PS1/APP*$^-$ and *Mgll*$^{+/+}$/ *PS1/APP*$^+$ counterparts, respectively (Figure 1C; Table S1). In contrast, the second major endocannabinoid, anandamide (C20:4 NAE), although elevated in *PS1/APP*$^+$ mouse brains, was not altered upon genetic deficiency of MAGL (Figure 1C; Table S1). These results show that *PS1/APP*$^+$ mice have heightened levels of proinflammatory prostaglandins that are generated primarily by MAGL-mediated hydrolysis of 2-AG, and that blocking this enzyme can restore prostaglandins to basal or subbasal levels.

MAGL Inactivation Attenuates Neuroinflammation and Aβ Levels and Plaque Burden in the *PS1/APP*$^+$ Mouse Brain

We next addressed whether genetic ablation of MAGL reduces neuroinflammation in *PS1/APP*$^+$ mice. We found that *PS1/APP*$^+$ mice exhibit significant astrocyte and microglial activation concordant with elevations in proinflammatory cytokines and cytokine receptors (Figures 2A–2C). Intriguingly, this observed microglial and astrocyte activation was almost completely attenuated in the *Mgll*$^{-/-}$/*PS1/APP*$^+$ mouse brains assessed both by GFAP and Iba-1 immunohistochemical staining and quantitative RT-PCR of GFAP and CD11b, respectively (Figures 2A and 2B). Consistent with an antiinflammatory effect of MAGL inactivation, we also observed dramatic reductions in the inflammatory cytokines IL-1β, IL-6, TNF-α, and TNF-α receptor II (TNFRII) at the mRNA or active protein level (Figure 2C). We next assessed whether disruption of MAGL and reduction of neuroinflammation impacts Aβ levels and deposition in the transgenic mouse brain. We found that MAGL inactivation drastically reduced numbers of Aβ plaques (Figure 3A) and brain levels of total Aβ, as well as the $A\beta_{40}$, and $A\beta_{42}$ amyloidogenic peptides (Figure 3B). Quantitative RT-PCR, conducted to ensure that inactivation of MAGL did not lead to alteration in the transgene expression, revealed no detectible changes in human APP gene expression in the *Mgll*$^{-/-}$/*PS1/APP*$^+$ mice compared to the *PS1/APP*$^+$ mice (Figure S2). Collectively, our results show that MAGL inactivation suppresses neuroinflammation in the *PS1/APP*$^+$ AD mouse model, leading to a substantial reduction in amyloid plaque burden.

Pharmacological Blockade of MAGL in the *PS1/APP*$^+$ AD Model

We next asked whether pharmacological inactivation of MAGL in *PS1/APP*$^+$ mice, with treatment initiated at 6 months well after the onset of elevated Aβ levels (Figure S1) and formation of amyloid plaques, recapitulates the metabolic and anti-inflammatory effects observed with genetic deficiency of MAGL. We found that subchronic MAGL blockade with the selective inhibitor JZL184 (40 mg/kg intraperitoneally [i.p.], once per day over 16 days) in *PS1/APP*$^+$ mice, produced elevations in 2-AG and substantial reductions in AA, proinflammatory prostaglandins, and thromboxanes (Figure 4A). We found that JZL184 also significantly reduced proinflammatory cytokine levels in *PS1/APP*$^+$ mouse brains (Figures 4B and 4C). To investigate whether these eicosanoid and cytokine-lowering anti-inflammatory effects of the MAGL inhibitors were due to enhancements in 2-AG signaling versus an attenuation of eicosanoid production, we included a treatment arm in which JZL184 was coadministered with cannabinoid receptor type 1 and 2 (CB1 and CB2) antagonists, rimonabant and AM630 (3 mg/kg i.p.), respectively. We found that the reductions in both proinflammatory prostaglandins and cytokines were not reversed upon treatment with cannabinoid receptor antagonists (Figure 4B), suggesting that the anti-inflammatory effects observed are mediated primarily by reductions in AA levels and prostaglandin signaling, rather than heightened endocannabinoid action.

DISCUSSION

One of the common hallmarks of neurodegenerative diseases, including AD, is chronic and persistent neuroinflammation, which in most cases is involved in exacerbating neuronal loss and disease pathology (Glass et al., 2010). Anti-inflammatory strategies have thus been proposed as an attractive approach toward combating AD (Glass et al., 2010). In this study, we reveal a heretofore unrecognized neuroprotective role of MAGL inactivation as a strategy for quelling neuroinflammation and amyloidogenesis that subserves AD.

Several reports have shown that select patterns of microglial activation can lead to phagocytosis and clearance of Aβ plaques in mouse models of AD (Jantzen et al., 2002; Lemere and Masliah, 2010; Stahl et al., 2006). In contrast, our results show that reducing neuroinflammation in the *PS1/APP*$^+$ transgenic AD mouse model leads to a dramatic decrease in brain amyloid plaques. Our results are supported by multiple studies showing that inactivation of the cyclooxygenases or directly blocking cytokine action with antibodies improves memory function and reduces neuroinflammation and amyloid plaques in mouse AD models (Choi and Bosetti, 2009; Kitazawa et al., 2011; McKee et al., 2008; Shi et al., 2011).

Despite considerable evidence associating neuroinflammation to AD pathophysiology (Glass et al., 2010), the precise mechanisms by which it affects Aβ plaques or AD disease progression are not as well understood. One possible mechanism is the inflammatory regulation of β-secretase (BACE1), the protease that cleaves APP to form Aβ, which possesses an NFκB site in its promoter and is upregulated upon inflammatory stimuli (Chen et al., 2011; Sastre et al., 2008). Anti-inflammatory

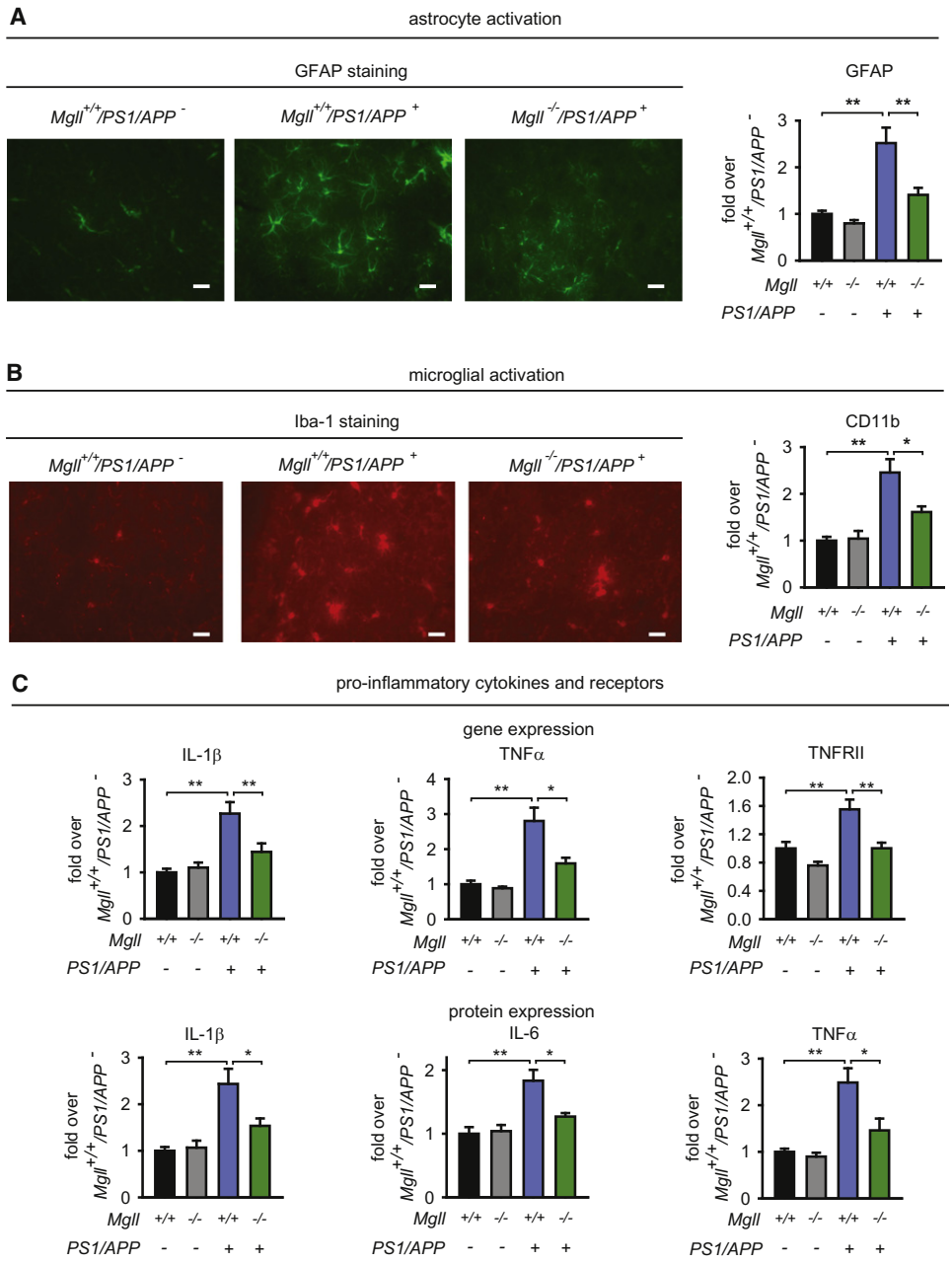

Figure 2. MAGL Inactivation Attenuates Gliosis and Neuroinflammation in *PS1/APP*$^+$ Mouse Brains

(A and B) Immunohistochemical analysis of astrocytes (A) and microglia (B) in *Mgll*$^{+/+}$*/PS1/APP*$^-$, *Mgll*$^{+/+}$*/PS1/APP*$^+$, *and Mgll*$^{-/-}$*/PS1/APP*$^+$ mouse brains determined by GFAP (A) and Iba-1 staining (B), respectively, and by quantitative RT-PCR for GFAP (A) or CD11b (B) mRNA expression in cortical brain. Representative images of n = 4–5 mice/group are shown. Scale bars for images are 20 μm.

(C) Measurements of proinflammatory cytokines and receptors in *Mgll*$^{-/-}$*/PS1/APP*$^-$, *Mgll*$^{+/+}$*/PS1/APP*$^-$, *Mgll*$^{+/+}$*/PS1/APP*$^+$, *and Mgll*$^{-/-}$*/PS1/APP*$^+$ mouse brains determined by quantitative RT-PCR of IL-1β, TNF-α, and TNFRII gene expression from hippocampus or cortex and ELISA-based protein quantitation for IL-1β, IL-6, and TNF-α from soluble proteomes of whole mouse brain. Data are means ± SEM, n = 4–5 mice/group. Significance is shown as *$p < 0.05$, **$p < 0.01$ using ANOVA with Tukey-Kramer post-hoc test.

agents such as NSAIDs and peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists have been shown to reduce the expression and activity of β-secretase and lower Aβ secretion (Sastre et al., 2003).

Earlier studies have implicated phospholipases, such as cPLA2, as the primary driver for AA release for prostaglandin synthesis (Bonventre et al., 1997). We recently discovered that, although cPLA2 does contribute to a significant portion

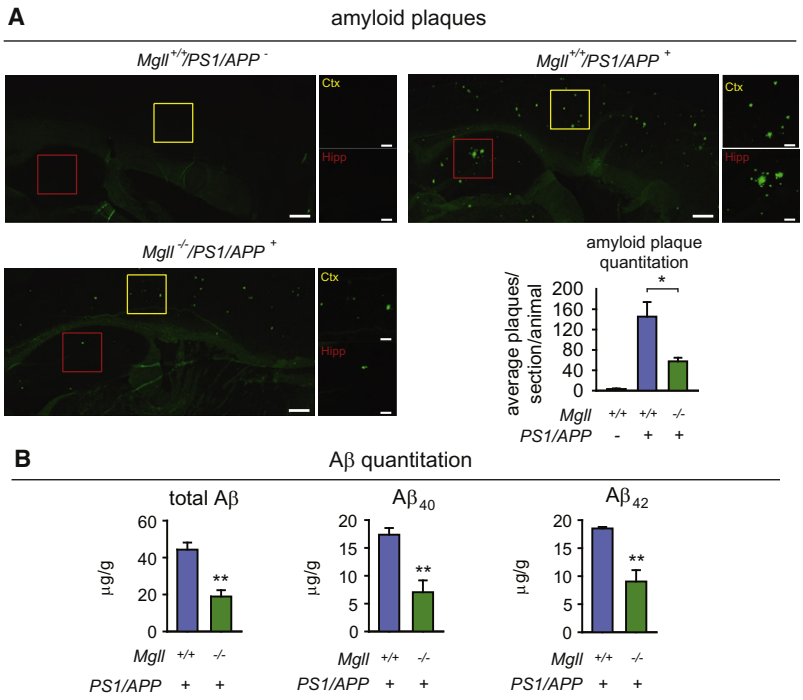

Figure 3. MAGL Ablation Reduces Amyloid Plaques in *PS1/APP*$^+$ Mouse Brain

(A) Quantification of amyloid plaques in *Mgll*$^{+/+}$/*PS1/APP*$^-$, *Mgll*$^{+/+}$/*PS1/APP*$^+$, *and Mgll*$^{-/-}$/*PS1/APP*$^+$ mouse brains determined by thioflavin S staining (Ctx: Cortex; Hipp: Hippocampus). Quantitation of the number of amyloid plaques per section per mouse is shown. Thioflavin S images on the left panel are representative. Scale bars for images represent 400 and 100 μm, respectively, for the left and right panels.

(B) Quantitation of total Aβ, $A\beta_{40}$, and $A\beta_{42}$ protein levels by DELFIA. Data are means ± SEM, n = 4–5 mice/group. Significance is shown as $^*p < 0.05$, $^{**}p < 0.01$ using ANOVA with Tukey-Kramer post-hoc test for (A) and two-tailed t test for (B).

See also Figures S1 and S2.

of proinflammatory prostaglandins, MAGL contributes the majority of AA for eicosanoid synthesis in the brain both basally and under the lipopolysaccharide-paradigm of neuroinflammation (Nomura et al., 2011). Moreover, genetic deletion of diacylglycerol lipase (DAGL), the enzyme that synthesizes 2-AG, leads to reduction in brain AA levels (Gao et al., 2010), further supporting a role for endocannabinoid metabolism as a source for brain AA. Nevertheless, genetic and pharmacological ablation of cPLA2 has also been shown to ameliorate cognitive deficits and reduce brain eicosanoid levels in *APP* transgenic mice (Sanchez-Mejia et al., 2008). These results collectively suggest that both MAGL and cPLA2 may contribute to the generation of AA for prostaglandin production in AD.

We show here that pharmacological blockade of MAGL over a 16 day dosing regimen raises endocannabinoid levels and lowers prostaglandin production, recapitulating the eicosanoid and cytokine-lowering effects observed with genetic deletion of MAGL. We found, however, that cannabinoid receptor signaling is likely not involved in lowering MAGL-driven neuroinflammation in *PS1/APP*$^+$ mice, despite previous studies showing neuroprotective benefits derived from both enhancing cannabinoid effects or lowering eicosanoid levels in AD (Aïd and Bosetti, 2011; Bisogno and Di Marzo, 2010; Martín-Moreno et al., 2012). The lack of cannabinoid-mediated effects in our study may be due to functional desensitization of the cannabinoid system that results from chronic and complete MAGL blockade (Schlosburg et al., 2010). It would therefore be of future interest to test whether partial MAGL blockade can elicit additive or synergistic benefits against AD by maintaining CB1 signaling and suppressing prostaglandin pathways simultaneously.

Despite their well-documented protective effects when initiated early and taken over prolonged periods (Rogers et al., 1993; Vlad et al., 2008), an issue with NSAIDs has been their lack of efficacy in AD clinical studies (Imbimbo et al., 2010; McGeer and McGeer, 2007). We believe that anti-inflammatory agents may be most beneficial when treatment is initiated during amyloidogenesis but before neuronal loss and synaptic failure, since anti-inflammatory agents will not likely reverse neurodegeneration.

Beyond its anti-inflammatory roles, enhancing endocannabinoid signaling has also been shown to promote neurogenesis and improve memory (Aguado et al., 2005; Galve-Roperh et al., 2007; Pan et al., 2011). In this study, we have not yet formally demonstrated the efficacy of MAGL inhibitors at lowering amyloid plaques or improving memory or behavior. It will thus be of future interest to test whether MAGL inhibitors provide therapeutic efficacy toward these parameters.

Our metabolomic profiling showed altered endocannabinoid and eicosanoid brain metabolism in the *PS1/APP*$^+$ AD mouse model, alongside alterations in certain phospholipids and cholesteryl esters. Our findings are consistent with previous studies showing that lowering eicosanoids or cholesterol in the brain may have therapeutic benefit in AD (Di Paolo and Kim, 2011; Sanchez-Mejia et al., 2008).

In this study, we find that blocking MAGL substantially reduces neuroinflammation that underlies AD pathogenesis and attenuates amyloidosis, likely through suppressing proinflammatory eicosanoid production. Our results thus highlight MAGL inhibitors as an attractive therapeutic strategy for treating AD.

EXPERIMENTAL PROCEDURES

Mice and Chemicals

Mgll$^{-/-}$ mice (Chanda et al., 2010) were crossed with *PS1/APP*$^+$ transgenic mice (Samaroo et al., 2012) to create the four genotypes used in these studies: *Mgll*$^{+/+}$/*PS1/APP*$^-$, *Mgll*$^{+/+}$/*PS1/APP*$^+$, *Mgll*$^{-/-}$/*PS1/APP*$^-$, and *Mgll*$^{-/-}$/*PS1/APP*$^+$. All experiments were conducted in accordance with Pfizer's IACUC guidelines. The compounds JZL184 and rimonabant were synthesized in the laboratory (Pfizer Inc.). AM 630 was purchased from Tocris Bioscience (Minneapolis, MN).

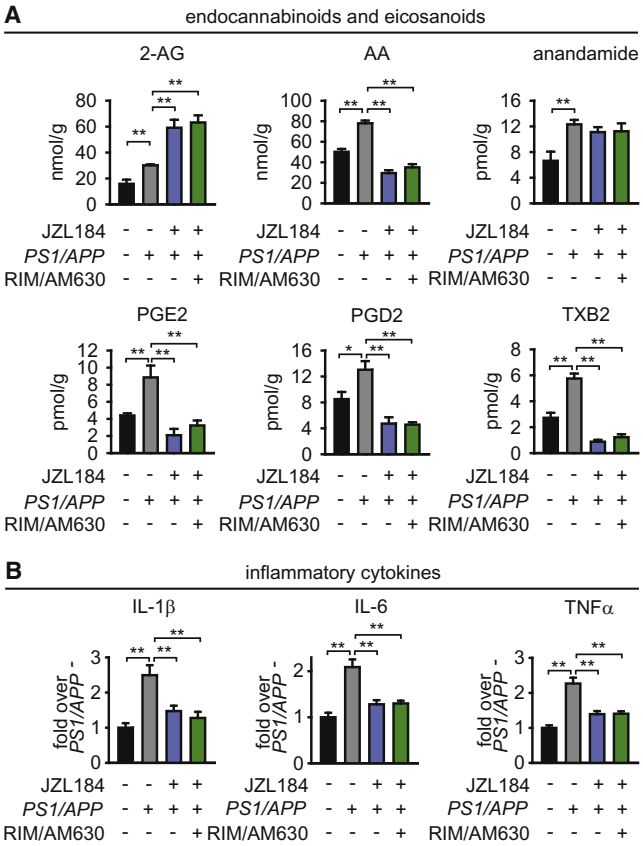

Figure 4. Pharmacological Inactivation of MAGL Reduces Eicosanoids and Lowers Inflammatory Cytokine Levels

(A and B) Endocannabinoid and eicosanoid (A) and inflammatory cytokine (B) levels in the brains of *PS1/APP*$^{-}$ and *PS1/APP*$^{+}$ mice treated, starting at 6 months, with either vehicle, JZL184 (40 mg/kg i.p.), or coadministration of the CB1 and CB2 antagonists, rimonabant (RIM) (3 mg/kg), and AM 630 (3 mg/kg i.p.), respectively, once per day over 16 days. Endocannabinoid and eicosanoid levels were determined by MRM-based targeted LC/MS analysis and raw data are presented in Table S1. Inflammatory cytokines were quantitated using ELISA from soluble proteomes from hemi-brains. Data are means ± SEM, n = 4–8 mice/group. Significance is shown as *p < 0.05, **p < 0.01 using a linear model ANOVA with no correction for multiplicity because of small sample sizes (A) and an ANOVA with Tukey-Kramer post-hoc test for (B).

mRNA Expression of Proinflammatory Cytokines and Receptors

Brains from transgenic or pharmacologically treated animals were dissected and rapidly frozen in liquid nitrogen. Total RNA was isolated from frozen tissues and gene expression was assessed by quantitative RT-PCR.

Metabolomic Analysis

Metabolomic analysis was performed as previously described (Nomura et al., 2011). Briefly, mice were sacrificed by cervical dislocation and brains were removed rapidly (≤30 s) and snap frozen in liquid nitrogen. Metabolites were extracted in 3 ml of 1:1 ethyl acetate:hexane and 1 ml PBS. An aliquot of the organic extract was injected onto a triple quadrupole-LC/MS/MS for untargeted and targeted metabolite analysis. We combined data obtained from both of these analytic procedures and have tabulated relative levels of metabolites in Table S1.

Measurement of Proinflammatory Cytokine Levels

Cytokines were measured from soluble brain proteome (25 μg) using the Mouse Inflammatory Cytokines Multi-Analyte ELISArray kit (QIAGEN, Valencia, CA) per a modified version of the manufacturer's instructions. More details are in Supplemental Information.

Immunohistochemistry and Plaque Quantitation

Four representative slides from each animal were incubated with anti-GFAP (Millipore, USA) or anti-Iba1 (Wako, Japan) to determine the activation of astrocytes or microglia respectively. Sections were also stained with thioflavin S. Multiple equivalent sections across each sample were imaged and thioflavin S^{+} plaques counted using a computer algorithm developed at Pfizer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, two figures, and one table and can be found with this article online at doi:10.1016/j.celrep.2012.05.001.

LICENSING INFORMATION

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