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


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.

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.

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

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 lipodomes measuring levels of endocannabinoids; 2-AG (C20:4 monoacylglycerol (MAG)), anandamide (C20:4 N-acyl ethanolamines (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-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 lipodomes. 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+ and 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

Metabolic 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 1A). Using a combination of targeted and untargeted liquid chromatography/mass spectrometry (LC/MS)-based metabolic 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-acyl ethanolamines (NAEs), free fatty acids (FFAs), eicosanoids, phosphatidic acids (PAs), lysophosphatidyl cholines (LPCs), and cholesteryl esters. Among this metabolic
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 substrates 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+ expression, revealed no detectible changes in human APP (Figure 3B). Quantitative RT-PCR, conducted to ensure that 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 prostanoid 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 combatting 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 promotor and is upregulated upon inflammatory stimuli (Chen et al., 2011; Sastre et al., 2008). Anti-inflammatory...
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...
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 eicosanoids or cholesterol in the brain (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 cholesterol 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 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+, and 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).

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.

**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, Aβ1-40, and Aβ1-42 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.
**A** endocannabinoids and eicosanoids

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**B** inflammatory cytokines

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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.

Metabolic Analysis

Metabolic 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 ELISA Array 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.

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