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Broad impact of deleting endogenous cannabinoid hydrolyzing enzymes and the CB1 cannabinoid receptor on the endogenous cannabinoid-related lipidome in eight regions of the mouse brain

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Abstract

Background and purpose—The enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) hydrolyze endogenous cannabinoids (eCBs), *N*-arachidonoyl ethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG), respectively. These enzymes also metabolize eCB analogs such as lipoamines and 2-acyl glycerols, most of which are not ligands at CB₁. To test the hypothesis that deleting eCB hydrolyzing enzymes and CB₁ shifts lipid metabolism more broadly and impacts more families of eCB structural analogs, targeted lipidomics analyses were performed on FAAH KO, MAGL KO, and CB₁ KO mice and compared to WT controls in 8 brain regions.

Experimental approach—Methanolic extracts of discrete brain regions (brainstem, cerebellum, cortex, hippocampus, hypothalamus, midbrain, striatum and thalamus) were partially purified on C-18 solid-phase extraction columns. Over 70 lipids per sample were then analyzed with HPLC/MS/MS.

Key results—AEA and 2-AG were unaffected throughout the brain in CB₁ KO mice; however, there was an increase in the arachidonic acid (AA) metabolite, PGE₂ in the majority of brain areas. By contrast, PGE₂ and AA levels were significantly reduced throughout the brain in the MAGL KO corresponding to significant increases in 2-AG. No changes in AA or PGE₂ were seen throughout in the FAAH KO brain, despite significant increases in AEA, suggesting AA liberated by FAAH does not contribute to steady state levels of AA or PGE₂. Changes in the

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Conflict of interest

The author, Heather B Bradshaw, of this manuscript is on the Advisory Board for Phytects and consults on how endogenous cannabinoids function in the central nervous system. Phytects had no financial contribution to the current work. The other authors have no conflicts of interest to declare.

lipidome were not confined to the AA derivatives and showed regional variation in each of the eCB KO models.

Conclusions and implications—AEA and 2-AG hydrolyzing enzymes and the CB₁ receptor link the eCB system to broader lipid signaling networks in contrasting ways, potentially altering neurotransmission and behavior independently of cannabinoid receptor signaling.

Graphical Abstract

FAAH KO								
	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
Arachidonic acid derivatives								
<i>N</i> -arachidonoyl ethanolamine	↑↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑↑	↑↑	↑↑↑
<i>N</i> -arachidonoyl glycine	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
<i>N</i> -arachidonoyl taurine	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓↓		↓↓↓↓↓	↓↓↓
2-arachidonoyl- <i>sn</i> -glycerol			↓↓	↓	↓			
Arachidonic acid								
PGE ₂								
PGF _{2α}		↓	↑					
MAGL KO								
	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
Arachidonic acid derivatives								
<i>N</i> -arachidonoyl ethanolamine	↓↓		↓			↓↓	↓	↓↓
<i>N</i> -arachidonoyl glycine	↓↓		↓		↓		↓	↓↓
<i>N</i> -arachidonoyl taurine		↓	↓	↓			↓	↓
2-arachidonoyl- <i>sn</i> -glycerol	↑↑↑↑↑	↑↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑↑	↑↑↑↑↑	↑↑↑↑	↑↑↑↑
Arachidonic acid	↓↓↓	↓↓	↓↓	↓↓	↓↓	↓↓↓	↓↓↓	↓↓↓
PGE ₂	↓↓↓↓	↓↓↓	↓↓↓	↓↓↓↓	↓↓↓	↓↓↓	↓↓↓↓	↓↓↓↓
PGF _{2α}	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
CB ₁ KO								
	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
Arachidonic acid derivatives								
<i>N</i> -arachidonoyl ethanolamine								
<i>N</i> -arachidonoyl glycine		↑						↓
<i>N</i> -arachidonoyl taurine				↓				
2-arachidonoyl- <i>sn</i> -glycerol								
Arachidonic acid			↑					
PGE ₂		↑↑↑	↑	↑↑	↑↑	↑↑	↑↑↑	↑↑↑
PGF _{2α}	↑	↑↑		↑	↑		↑	↑

Keywords

Endogenous cannabinoid; lipidomics; FAAH; MAGL; CB₁; arachidonic acid

1. Introduction

1.1 CB₁ cannabinoid receptor

Although the behavioral effects of delta-9-tetrahydrocannabinol (THC), the primary psychoactive component of the cannabis plant, were documented upon its isolation, its main mechanism of action was elucidated more than 20 years later, when a specific cannabinoid receptor was identified in the rat brain [1]. Cloned by Matsuda and colleagues, this cannabinoid receptor is a G-protein coupled receptor (GPCR) frequently signaling via G_{i/o} G

proteins [2]. Subsequently, Munro and colleagues isolated a clone for a second cannabinoid receptor, also a GPCR, the highest levels of which were found in macrophage cell lines and spleen; they did not detect mRNA for this second cannabinoid receptor in the brain [3]. Later, the brain-enriched cannabinoid receptor was named CB₁ and the peripherally-enriched cannabinoid receptor was designated CB₂. THC acts as a low efficacy agonist at both of these receptors [4, 5]. The endogenous agonists for CB₁ and CB₂ receptors are lipids and are referred to as endogenous cannabinoids (eCBs) [6]. The eCBs, *N*-arachidonoyl ethanolamine (AEA) [7], also known as anandamide, and 2-arachidonoyl glycerol (2-AG) [8, 9] are derived from arachidonic acid (AA) containing lipids. The activation of cannabinoid receptors is implicated in many processes, such as the regulation of appetite [10, 11], pain perception [12], motivation [13, 14], inflammation [15], and learning and memory [16].

1.2 Fatty acid amide hydrolase (FAAH) is a ubiquitous enzyme with a wide range of substrates

AEA belongs to a larger structural family of *N*-acyl ethanolamines (NAEs); whereas AEA is formed from *N*-arachidonoyl phosphatidylethanolamine, in which its structure represents the conjugation of AA and ethanolamine, other NAEs are formed from *N*-acyl phosphatidylethanolamine with other fatty acids (*i.e.* acyl groups). The first NAE to be identified was *N*-palmitoyl ethanolamine (PEA), the conjugate of palmitic acid and ethanolamine. Kuehl and colleagues found PEA in soybeans, peanuts, and egg yolks and determined that PEA had anti-inflammatory effects [17].

Lipoamines, also known as *N*-acyl amides (*i.e.* fatty acid amides), are molecules derived from the conjugation of amines (*e.g.* amino acids) with fatty acids (Figure 1). Lipoamines are all structurally analogous to the NAEs [18]. A theoretical computation of 7 common fatty acids in mammalian systems (arachidonic, stearic, docosahexaenoic, oleic, palmitic, linolenic, and linoleic) with 20 amino acids, and 4 common amines (ethanolamine, dopamine, GABA, and taurine), yields 168 possible lipoamine combinations in mammals [19]. Lipoamines are a rapidly growing class of signaling molecules that are produced throughout the brain and body [20–22]. Most lipoamines do not activate cannabinoid receptors, so they are not categorized as eCBs; however, some do activate other receptors, such as GPR18 [23], GPR119 [24] as well as transient receptor potential (TRP) channels [25].

FAAH is hypothesized to be responsible for the vast majority of AEA hydrolysis, initially producing ethanolamine and AA [26, 27]. In the mammalian brain, FAAH is particularly highly expressed in the cerebellum, hippocampus and cortex. Even though FAAH is best known for its role in AEA hydrolysis, it can also hydrolyze other lipoamines [28]. Earlier work demonstrated that *N*-arachidonoyl glycine (NAGly) and *N*-arachidonoyl dopamine (NADA) were significantly reduced in the rat striatum when FAAH was inhibited, suggesting FAAH participates in their synthesis, and not solely as a degradative enzyme for NAEs [29, 30].

1.3 Monoacylglycerol lipase (MAGL) is the primary hydrolytic enzyme for 2-AG

Hydrolyzing 2-AG into AA and glycerol, monoacylglycerol lipase (MAGL) is hypothesized to hydrolyze 50–85% of the brain's 2-AG. Enzymes such as α/β hydrolase domain-6 (ABHD6) and α/β hydrolase domain-12 (ABHD12) are hypothesized to be responsible for most of the remaining hydrolysis in brain [31], with a small portion of 2-AG metabolism catalyzed by FAAH and cyclooxygenase (COX) enzymes [32]. In cultured hippocampal neurons, cerebellar slices, and hippocampal slices, MAGL helps set the duration of depolarization induced suppression of excitation/inhibition (DSE/DSI) [33–35], a classic property of 2-AG signaling [36], suggesting it is an important 2-AG hydrolyzing enzyme at many synapses. MAGL mRNA is widely expressed in the brain throughout the rat cortex, hippocampus, cerebellum, thalamus, striatum, and pontine nuclei [37]. Using immunohistochemistry, the same study revealed that MAGL protein was localized in axons and co-localized with CB₁ in the rat amygdala, hippocampus, and cerebellum. MAGL's localization in axons contrasts with the expression of FAAH, which is most abundant in dendrites and soma [38].

In the same manner as NAEs, endogenous structural analogs of 2-AG likewise exist wherein additional fatty acids are conjugated to glycerols to make a range of 2-acyl glycerols, such as 2-oleoyl glycerol (2-OG) and 2-linoleoyl glycerol (2-LG) [39]. Although 2-LG and 2-OG do not directly activate CB₁ or CB₂ [40], they are agonists at some of the orphan GPCRs that are members of the extended cannabinoid receptor family [41], such as GPR119, the same receptor activated by *N*-oleoyl ethanolamine (OEA) to reduce appetite [42]. Another proposed mechanism of action of 2-LG is that it potentiates the binding of 2-AG to CB₁ and inhibits 2-AG degradation in neuronal cells. This effect has been called the “entourage effect” [40, 43], though molecular evidence for these interactions is sparse. Moreover, AA is a product of AEA and 2-AG degradation and is a substrate for prostaglandin (PG) biosynthesis, linking the eCB system to broader lipid signaling including signaling through PG receptors [44].

1.4 Mice lacking FAAH, MAGL and CB₁ have distinctive behavioral phenotypes

Illustrating an analgesic phenotype, FAAH knockout (KO) mice demonstrate prolonged response latency in the tail immersion and hot plate tests compared to wild-type (WT) mice. Showing that other types of pain sensitivity are also reduced in FAAH KO mice, pain behavior was attenuated in the formalin test [27]. Consequently, FAAH became a therapeutic target for chronic pain; however, limited analgesic efficacy of a FAAH inhibitor was found in a human clinical trial [45], which may be explained by the observation that FAAH deletion actually enhances some forms of pain [46]. Similarly to FAAH, MAGL is a potential drug target to treat pain. Although acute treatment with MAGL blockers was effective at reducing cold and mechanical allodynia in the chronic constriction injury mouse model of chronic pain, prolonged, complete blockade of MAGL desensitized CB₁, leading to analgesic tolerance [47, 48]. MAGL KO mice demonstrated improved performance over WT in a hippocampal-dependent learning task [49] and were somewhat protected from different models of neurodegeneration, independent of CB receptors and likely through alterations in AA levels and PG production [50].

Many studies have examined mice lacking cannabinoid receptors as an indirect approach to understanding the function of these receptors. The behavioral characteristics of CB₁ KO mice have been well-documented: one prominent facet involves a protective phenotype against addiction. For example, CB₁ KO mice had reduced voluntary alcohol consumption compared to WT littermates and appear to find drugs of abuse less rewarding. CB₁ KO mice failed to release dopamine, measured by cyclic voltammetry, into the nucleus accumbens (NAc) after consuming alcohol, a chemical event that is believed to drive the rewarding effect of a drug [51]. Further studies showed that CB₁ activation was required for dopamine release into the NAc in response to nicotine and cocaine, as well as alcohol [52].

Highlighting the effect of CB₁ deletion on metabolism, CB₁ KO mice are much leaner, resistant to diet-induced obesity, and demonstrated enhanced sensitivity to leptin compared to WT mice [11]. Signaling via CB₁ is hypothesized to inhibit the HPA-axis to reduce levels of stress hormones. Consistent with this fact, the HPA is overactive in CB₁ KO mice, under both basal and stressful conditions [53]. In addition, CB₁ KO mice are very resistant to the extinction of fear conditioning, as CB₁ activation in the basolateral amygdala seems to be crucial to extinguish aversive memories [16].

1.5 What are the effects of deleting eCB-hydrolyzing enzymes on the brain's eCB-related lipidome?

The biochemical changes contributing to the phenotypes of each of the eCB enzyme KO mice are not only likely increases in eCB signaling, but broader changes in levels of eCB analogs due to alterations in lipid metabolism. To test the hypothesis that deleting an enzyme that metabolizes AEA (FAAH), an enzyme that metabolizes 2-AG (MAGL), and the brain's main eCB receptor (CB₁) has differential effects on the eCB-related lipidome, we compared levels of NAEs and over 60 structurally analogous *N*-acyl amides, 2-AG and its structural analogs, and two species of PGs, PGE₂ and PGF_{2α}, in a targeted lipidomics screen in 8 distinct brain regions: brainstem, cerebellum, cortex, hippocampus, hypothalamus, midbrain, striatum and thalamus. Deleting these different enzymes or the CB₁ receptor impacted the production of a wide-range of lipids in ways that went well beyond changes in levels of an enzyme's substrate or a receptor's ligand. Reflecting the link between eCBs and PGs as AA derivatives, both deletions in MAGL and CB₁ affected levels of PGs, whereas deletions in FAAH affected levels of AA-derived lipoamines, but not AA or PGs. Therefore, disrupting the hydrolysis of eCBs and related lipids has potential consequences for signaling in the brain independent of cannabinoid receptor activation.

2 Methods

2.1 Mice and tissue collection

For the FAAH KO study, 6 WT mice were compared to 6 FAAH KO mice from the C57BL6/J strain. Similarly, for the MAGL KO study, 6 WT mice were compared to 6 FAAH KO mice from the C57BL6/J strain. To compare CB₁ KO to WT, 6 KO and 6 WT mice from the CD1 strain were used. The mice for the CB₁ KO versus WT screenings and the MAGL KO versus WT screenings were all male. The remaining groups were of mixed sex: FAAH KO 2 female/4 male with 2 male/4 female WT. MAGL KO and FAAH KO mice were a

generous gift from Ben Cravatt. CB₁ heterozygous mice used to found a CB₁ knockout colony were a generous gift from Catherine Ledent. The mice were 2 months old at the time of sacrifice. All protocols involving mice were approved by the Indiana University Institutional Animal Care and Use Committee and ARRIVE guidelines.

Brain tissue was collected and dissected as previously described [54]. In summary, all mice were sacrificed via rapid cervical dislocation on the same day and brains were immediately removed, flash frozen, and stored in a –80°C freezer until dissections were performed. Brains were dissected while semi-frozen on an ice-cold plate into the following regions: striatum (STR), hippocampus (HIP), cerebellum (CER), thalamus (THAL), cortex (CTX), hypothalamus (HYP), midbrain (MID), and brainstem (STEM). Each dissected area was immediately placed in liquid nitrogen and stored at –80°C until used for lipid extraction.

2.2 Lipid extraction

Tissue extracts were performed as previously described [22, 55]. In brief, to begin the lipid extraction, samples were shock frozen in liquid nitrogen, and weighed before being transferred to a centrifuge tube. The mass of the largest sample was multiplied by 50 to determine how many milliliters of HPLC-grade methanol (Avantor Performance Materials, Inc., Center Valley PA) to be added to the centrifuge tube. Then, samples were spiked with 500 picomols deuterium-labeled NAGly (d₈NAGly; Cayman Chemical, Ann Arbor, MI, USA) as an internal standard to determine extraction efficiency. Samples were placed on ice in darkness for 2 hours then individually homogenized. Homogenates were centrifuged at 19,000g for 20 minutes at 20°C. Supernatants were decanted and diluted with HPLC water (purified in house) to make a 75:25 water to supernatant solution. Partial purification was achieved using C-18 solid phase extraction columns (Agilent, Palo Alto, CA, USA). A series of 4 elutions with 1.5 mL of 60%, 75%, 85%, and 100% methanol were collected for analysis [54]. Vials of eluants were stored at –80°C until they were ready for analysis.

2.3 High pressure liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS)

Samples were analyzed using an Applied Biosystems API 3000 triple quadrupole mass spectrometer with electrospray ionization (Foster City, CA, USA). 20μL from each elution were chromatographed using XDB-C18 reversed phase HPLC analytical column (Agilent) and optimized mobile phase gradients. Mobile phase A: 20% methanol, 80% water (v/v) and 1 mM ammonium acetate (Sigma, St. Louis, MO, USA). Mobile phase B: 100% methanol, 1 mM ammonium acetate. Two Shimadzu 10ADvp pumps (Columbia, MD, USA) provided the pressure for gradient elution. Every method run began with 0% mobile phase B, reached a state of 100% mobile phase B flowing at 0.2 mL per minute, and gradually returned to 0% mobile phase B.

Levels of each compound were determined by running each sample using a multiple reactions monitoring method tailored for each amide family of compounds (Figure 2). Analysis of the HPLC/MS/MS data was performed using Analyst software (Applied Biosystems, Framingham, MA, USA) as previously described [22, 55]. Chromatograms

were generated by determining the retention time of analytes with a [M–1] or [M+1] parent peak and a fragmentation peak corresponding to the programmed values.

The retention time was then compared to the retention time of a standard for the suspected compound. If the retention times matched, then the concentration of the compound was determined by calculating the area under the curve for the unknown and comparing it to the calibration curve obtained from the standards. Therefore, unknown lipids are matched to known standards according to retention time from the analytical column and according to their mass fingerprint.

2.4 Data analysis and statistical procedures

Extraction efficiency was calculated with the d₈NAGly spiked recovery vial as a standard as previously described [22, 25, 54, 55]. For each individual lipid in each brain region, concentrations in moles per gram adjusted for percent recovery from the KO animals were compared to WT concentrations using a one-way ANOVA. All statistical tests were carried out using SPSS Statistics (IBM, Armonk, NY, USA). Statistical significance was defined as $p < .05$ and a trending effect was defined as $.05 < p < .10$.

Analysis of such large data sets continues to be problematic for both the implementation and the interpretation of such data. The hypothesis that we are testing is simply that the WT values for each individual lipid differs from the KO values in each brain region analyzed. There would be the expectation that the values would be evenly distributed and a T-type comparison would yield an analysis of the means by which this could be determined. However, theoretical constructs of using T-test analyses on large data sets suggest the potential to generate false positives with a series of T-tests; therefore, the use of corrective analyses allows for a more conservative approach to these types of calculations. To this end, we have grouped data for analyses by lipid species based on either the amine group for the *N*-acyl amides, free fatty acids, 2-acyl glycerols, or PGs. This separation also extends to discrete analyses being performed on these categories only within a brain region. In this configuration there is no expectation that one specific lipid category has an influence on the other; therefore, the post-hoc adjustments for multiple comparisons are limited to the specific subcategories listed. We do recognize that there may be potential predictive analyses that can be performed between brain areas and categories of lipid species; however, those types of analyses fall into a realm of modeling that is beyond the scope of the questions being asked of these initial data sets. An example of a categorical analysis is that of all NAE species (6 total) within a discrete brain region would be considered a selection of values with the most likelihood of having the same direction and magnitude of effects by the deletion of a gene given their strong structural homology and their regional specificity (i.e. a discrete brain region). Therefore, all of these lipids were analyzed as a unit with an overall ANOVA and then a corrected post-hoc analysis using Fishers Least Significant Difference was performed in the event that the overall ANOVA determined an interaction.

3 Results

3.1 HPLC/MS/MS signal detection of targeted lipidome in WT and KO mice

Of the 76 lipoamines in our screening library, over 60 were detected in most brain regions in the WT, FAAH, MAGL, and CB₁ KO mice, with more lipids being detected in the larger brain regions such as the cortex (CTX) and brainstem (STEM) and the fewest being detected in the hypothalamus (HYP). Each of the PGs, 2-acyl glycerol, and free fatty acid species analyzed here were detected in all regions of the brain. This level of analysis constitutes ~23000 data points; therefore, the detailed list of levels of analytes detected in WT and KO mice and the statistical analyses of each are available in the Supplemental Tables. In order to summarize the analyses, we have presented the data using a schematic representation of the following output: 1) significance from WT within specific brain regions, 2) magnitude of change and 3) direction of change (Figure 3).

3.2 FAAH deletion has contrasting effects on brain lipoamines

3.2.1 Levels of all NAEs in all brain regions are significantly higher in FAAH KO mice—Of the 6 NAEs in the screening library, all were significantly increased by FAAH deletion, including the eCB AEA, in all brain regions assayed (Figure 4). The increases in polyunsaturated fatty acid derivatives like *N*-linoleoyl ethanolamine (LEA), *N*-docosahexaenoyl ethanolamine (DEA) and AEA were of a lower magnitude than the increases in saturated derivatives, PEA and *N*-stearoyl ethanolamine (SEA), or the increase in levels of monounsaturated derivatives like OEA. Notably, the NAEs were the only family of lipids screened in FAAH KO mice whose every member increased in all regions.

3.2.2 AA derivative lipoamines are particularly sensitive to FAAH deletion—Of the lipids measured in this screen, those with AA as part of their molecular structure showed a wide-range in their levels following FAAH deletion (Figure 5). Although several AA-derived lipoamines were detected throughout the brain in WT and FAAH KO mice, only AEA was significantly increased in all brain regions. The only other AA-derived lipoamine whose levels *increased* in FAAH KO was *N*-arachidonoyl serine (A-Ser), with region-specific increases in the striatum (STR), thalamus (THAL), and STEM. By contrast, levels of 7 additional *N*-arachidonoyl amides were *decreased* in FAAH KO mice. In all 8 brain regions, NAGly and *N*-arachidonoyl GABA (A-GABA) were significantly reduced in FAAH KO mice. The next most prominent decrease was in *N*-arachidonoyl taurine (A-Taur), whose levels fell in all areas except the HYP. The concentration of *N*-arachidonoyl alanine was significantly lower in the hippocampus (HIP), cerebellum (CER), and THAL of FAAH KO. In FAAH KO mice, *N*-arachidonoyl methionine levels were trending lower in CER and significantly lower in the CTX. *N*-arachidonoyl phenylalanine levels (A-Phe) were significantly lower in the CER and trending lower in the HIP, THAL, and midbrain (MID), and *N*-arachidonoyl tyrosine (A-Tyr) levels were trending lower in the THAL and CTX of FAAH KO mice. NADA levels were drastically reduced in the STR of FAAH KO mice, which was the only region where NADA was reliably detected.

3.2.3 Levels of 2-AG change in a region-specific manner in FAAH KO mice—Compared to WT, levels of 2-AG were significantly lower in the CER, THAL, and CTX but

were unchanged in the STR, HIP, HYP, MID, and STEM in FAAH KO mice. The magnitude of the decrease in 2-AG was largest in the CER. The 2-AG analog 2-OG was changed in some areas of the FAAH KO mice: it was increased in the STR, CER, and MID, but the changes in 2-OG was not correlated with those of 2-AG. 2-LG was the only 2-acyl glycerol screened that was not affected in any brain region by FAAH deletion.

3.2.4 Free AA and PG levels are insensitive to FAAH deletion—Given that the primary function of FAAH is reported to be the hydrolysis of fatty acid amides such as AEA into free AA and ethanolamine [26], it was predicted that levels of AA should be significantly reduced in FAAH KO mice. However, there were no changes in the amount of free AA in any brain region of FAAH KO (Figure 5). Therefore, none of the changes in AA derivatives were accompanied by a significant change in free AA, suggesting FAAH feeds into a metabolic pathway that does not lead to accumulation of free AA. Likewise, no changes in PGE₂ levels were measured in FAAH KO mice.

3.3 MAGL deletion has regionally specific effects on lipoamines

3.3.1 Increases in 2-acyl glycerols and reductions in PGs and AA after MAGL deletion—Replicating previous finding and supporting the hypothesis that MAGL is a hydrolytic enzyme that releases (and increases) free AA [56], MAGL KO mice had higher levels of 2-AG in all brain regions and corresponding decreases in AA (Figures 5 and 6). Levels of 2-AG were 10-fold greater than those seen in WT in the STR, HIP, CTX, and HYP and were over 5 times WT levels in the CER, THAL, HYP and MID. 2-LG increased to a similar extent in MAGL KO mice. Unlike 2-AG and 2-LG, levels of 2-OG did not increase in every region with MAGL KO. Instead, 2-OG levels were higher than WT in the STR, HIP, CER, THAL, and CTX but were the same as WT in the HYP, MID and STEM. Even where 2-OG increased, the increases were of a smaller magnitude than those of 2-AG and 2-LG. In contrast to increases in levels of 2-AG, levels of PGE₂, PGF_{2α}, and free AA were significantly lower in all 8 brain regions of MAGL KO mice. Levels of PGs were at least fifty percent lower in the MAGL KO mice relative to WT.

3.3.2 AA-derived lipoamines decrease in MAGL KO mice—Similarly to FAAH KO mice, levels of AA-derived lipoamines were decreased throughout the brain (Figure 5). In contrast to the FAAH KO mice, MAGL deletion caused decreases in AA-derived lipoamines that were very region-specific. In MAGL KO mice, levels of the eCB AEA were significantly lower than WT in the STR, CER, MID and STEM and trending lower than WT in the HYP. Levels of the AEA metabolite NAGly were significantly lower in the STR, CER, and STEM and trending lower in the CTX and MID of MAGL KO mice. Levels of A-GABA decreased relative to WT in the CER and MID and were unaffected in the STR, HIP, CTX, HYP and STEM of MAGL KO mice. MAGL KO mice had lower levels of A-Phe in the CTX only and lower levels of A-Tyr in the CER only. A-Taur concentrations were significantly lower in the HIP, CER, THAL, MID, and STEM of MAGL KO mice.

3.3.3 Levels of linoleic acid and specific linoleic acid-derived lipoamines increase in MAGL KO mice—Reductions in AA and AA-derived lipoamines appeared to be contrasted by increases in linoleic acid and its lipoamine derivatives. Importantly, the

AEA analog LEA was increased in all 8 regions of MAGL KO. Increases in levels of other linoleic acid lipoamine derivatives were region dependent. For example, levels of *N*-linoleoyl GABA were higher in the CTX, levels of *N*-linoleoyl glycine were significantly higher in the THAL, CTX, MID and trending higher in the CER, and levels of *N*-linoleoyl serine were significantly higher in THAL, STEM and trending higher in the HYP of MAGL KO mice relative to WT.

3.3.4 Changes in specific lipoamine subfamilies are highly region dependent in the MAGL KO—Specific brain regions appeared to be more sensitive to changes in lipoamines in the MAGL KO mice. For example, the THAL had increases in 5 out of the 6 members of the *N*-acyl GABA and *N*-acyl serine families, whereas, other regions only had changes in at most a couple of members of these families. The THAL stands out because it contained many more significant increases in levels of lipoamines than decreases (the only *N*-acyl amides to be decreased in the THAL of MAGL KO were *N*-oleoyl methionine and A-Taur). In contrast, other regions like MID or STR contained more decreases than increases in levels of *N*-acyl amides in MAGL KO mice.

Of all 8 brain regions assayed, the HYP was the least affected by MAGL deletion in terms of the number of lipids affected and the magnitudes of the changes.

3.4 Effects of CB₁ KO on a targeted brain lipidome

3.4.1 Deletion of CB₁ does not affect eCB levels—Notably, in all 8 brain regions assayed there were no significant changes in either AEA or 2-AG in the CB₁ KO mice (Figure 5 and Figure 7). Also, NADA, a ligand at CB₁ whose expression is restricted to the STR [30], levels did not change in CB₁ KO mice. Only 2 NAE AEA analogs were regionally affected by CB₁ deletion, in that SEA increased in the THAL and STEM and in LEA in the CER. The AEA metabolite NAGly was both regionally and differentially affected by CB₁ deletion, with a significant decrease in the STEM of KO mice and with a trending increase in the HIPP.

3.4.2 All differential changes in the lipidome in CB₁ KO mice were regionally specific—There was no single lipid in the screening library that changed in all 8 brain regions of the CB₁ KO mice thereby highlighting the importance of examining individual brain regions (Figure 7). Interestingly, in the CER, none of the significant or trending changes involved decreases relative to WT but in the other 7 regions, levels of at least one lipid decreased relative to WT. In fact, all changes in the STR were decreases with the exception of the change in PGF_{2α}. Likewise, all changes in HYP were decreases except the increase in PGE₂. In general, *N*-acyl serines were one of the more impacted families of lipids in CB₁ KO with every member of the family changing concentration in at least one region. However, levels of any *N*-acyl serine failed to change in the STR and HYP.

Contrast this with the THAL, where levels of *N*-palmitoyl serine, *N*-stearoyl serine, *N*-linoleoyl serine and *N*-docosahexaenoyl serine rose in CB₁ KO, whereas there was a decrease in A-Ser. Therefore, effects of CB₁ deletion are highly region-dependent, which suggests region-specific adaptations in lipid biosynthesis and metabolism as a result of a lack of CB₁ signaling.

3.4.3 Prostaglandin levels are elevated in many brain regions of CB₁ KO mice

—Compared to WT, levels of PGE₂ were significantly elevated in the HIPPI, THAL, CTX, HYP, MID, and STEM of CB₁ KO mice (Figure 5 and Figure 7). Levels of PGE₂ were additionally trending higher in the CER. In STR, the concentration of PGE₂ did not differ significantly between KO and WT mice. Levels of PGF_{2α} were also increased relative to WT; however, often in different regions. PGF_{2α} levels were significantly higher in the HIPPI, THAL, CTX, MID and STEM and trending higher in the STR of CB₁ KO mice and were unchanged in the CER and HYP. The magnitude difference in PG levels between WT and CB₁ KO was highest in the HIPPI, with PGE₂ levels being twice as high in the KO mice and PGF_{2α} levels being over 1.5 times higher than WT levels. Despite the increases in PGs, free AA levels increased in only one region, which is in contrast to the dynamics of AA and PG reported for the MAGL KO [50]. AA levels were higher in the CER and were unchanged in the other 7 regions.

4 Discussion and conclusions

Cannabinoid research has tended to focus on the eCB ligand substrates AEA and 2-AG, overlooking a host of related compounds that are often co-produced or proximal metabolites of these lipids. Data here provide a broader window into the long-term effects of deletion of each of these eCB-related proteins. Our findings highlight the importance of considering the lipidome as a dynamic, interconnected system. Unraveling how these connections play a role in cell signaling will allow for a deeper understanding of cellular communication.

4.1 Functional specialization of lipid signaling in specific brain regions

Our results demonstrate the importance of evaluating multiple brain areas. For instance, a previous study of FAAH KO mice relying on whole-brain analysis revealed no changes in 2-AG [27]. In this multi-area study, we show that there are region-dependent decreases in 2-AG in FAAH KO mice. Similarly, our finding of decreased AEA levels in 5 brain regions of MAGL KOs is novel, as whole brain studies did not observe any changes in AEA [50] (Figure 5). Although a study by Imperatore and colleagues did not find any differences in AEA in the PFC, amygdala, HIPPI or CER of MAGL KO, they did find that desensitization of CB₁ was brain region dependent. The desensitization of CB₁ in the HIPPI and amygdala of MAGL KO was of particular importance, because it was linked to anxiety-like behavior [57]. This means that adaptations to a missing enzyme are not uniform across the brain, and elevations in 2-AG signaling have different consequences for neurotransmission and behavior depending on which neural circuitry is affected.

Most studies that use CB₁ KO mice looked at either the whole brain, or focus on brain areas where CB₁ receptor expression was high, like the HIPPI [58]. Here, by examining effects of CB₁ deletion in 8 different brain regions, the STR appears to be most resistant to the consequences of receptor deletion that result in changes in lipid levels. For example, PGE₂ levels are the same as WT in the STR of CB₁ KO, whereas, levels of that lipid were higher in CB₁ KO in the other 7 regions. Interestingly, the STR was the last region to show desensitization of CB₁ after chronic THC treatment in the rat brain, whereas CB₁ in HIPPI quickly desensitized and to a much larger degree [59–60]. The HIPPI was one of the only

regions where deleting the 2-AG synthesizing enzyme diacylglycerol lipase affected the activity of CB₁ [61]. Indeed, the HIPP was one of the most affected areas in CB₁ KO mice. The disruption of CB₁ signaling in the HIPP is hypothesized to impair discriminative memory and is associated with anxiety-like behaviors [57]. Therefore, it appears that the STR is a more resilient brain area when it comes to maintaining homeostatic lipid metabolism after challenges to CB₁ signaling.

4.2 Synergistic effects of deleting eCB hydrolyzing enzymes on *N*-acyl amides

Notably, AEA can also activate TRPV1, a receptor involved in pain perception [62]. Other NAEs such as OEA [63], LEA and DEA also activate TRPV1 [25]. NAEs are not the only TRPV agonists in the screening library: A-Taur activates TRPV1 and TRPV4 receptors [64]. We have also demonstrated a wider variety in TRPV activation with lipoamines [25]. At TRPV1, in addition to LEA and DEA, *N*-linoleoyl GABA, A-GABA, *N*-docosaheptaenoyl GABA, *N*-docosaheptaenoyl aspartic acid, *N*-docosaheptaenoyl glycine, and *N*-docosaheptaenoyl serine all demonstrated agonist activity at low micromolar concentrations. These ligands are also synergistic when applied in combination, perhaps through allosterism, since their EC₅₀ declines into the nanomolar range when they are applied together [19, 25]. This may be especially important in the case of FAAH KO, where all members of the NAE family are robustly elevated across all brain regions, or in the case of acute pharmacological FAAH inhibition [65].

4.3 FAAH-mediated AEA hydrolysis is an important source of AA for other *N*-arachidonoyl amides but not for free AA

Data here suggest that effects of FAAH deletion on NAEs are not translatable to all other lipoamines in that many other members of this family of lipids were not upregulated in response to this deletion. In fact, levels of most AA-derived lipoamines measured here were lower in FAAH KO mice, such as NAGly, which was lower in all 8 regions. NAGly is an agonist at GPR18 [23, 66], drives microglial migration [67], and is antinociceptive and anti-inflammatory in several animal models of pain [18, 68, 69]. In mammalian cells, NAGly can be formed from AEA via two distinct pathways. The first is a direct oxidation of AEA by alcohol dehydrogenase, and the second is via the conjugation of AA and glycine using AA released by the hydrolysis of AEA by FAAH [29]. If the second pathway is more important, then we would predict that as AEA levels increase in the absence of FAAH, then NAGly levels will decrease, as less AEA is being metabolized as a source of AA for NAGly. Consistent with the latter pathway, levels of NAGly were lower in all 8 brain regions of FAAH KO mice. Furthermore, FAAH-mediated hydrolysis of AEA is a source for NADA production [30], and we found that FAAH KO mice had lower levels of NADA in STR. Our reports of lower NAGly and A-GABA levels in FAAH KO mice are consistent with another study examining the consequences of FAAH blockade on AA-derived signaling molecules, although that study used URB597 to inhibit FAAH [70]. These results suggest that the FAAH-mediated metabolism of AEA is necessary to maintain levels of other AA-derived lipoamines. Importantly, contrary to our current understanding of the metabolic pathways of AEA through FAAH, levels of free AA in the FAAH KO mice remained unchanged throughout the brain. The meaning of these data are unclear; however, it does suggest that the current understanding of the direct hydrolyses of AEA into free AA and ethanolamine is

in question. It is possible that any AA freed by FAAH is so rapidly conjugated into another lipid that the flux of AA through FAAH is much higher than suggested by the levels of AA.

4.4 2-AG as a stable storage form of AA

Free AA is very easily oxidized by environmental and enzymatic influences. For example, AA is oxidized by COX enzymes to form PGs [44]. Therefore, one could make an evolutionary argument that it is advantageous to “store” AA in a more stable form, until it can be liberated for oxidation as part of the AA cascade. As noted above, a previous study of MAGL deletion saw dramatically lowered levels of AA alongside rises in 2-AG [50], a finding consistent with the hypothesis that 2-AG is a stable precursor hydrolyzed on-demand, after which the unstable AA is promptly metabolized. Replicating and expanding upon these previous findings we found that levels of AA were significantly lower in all brain regions of MAGL KO mice. Moreover, the reduction in free AA has consequences for other AA-derivative lipid signaling molecules, as demonstrated by a brain-wide decrease in PG production in MAGL KO, presumably because less AA is available for COX-2. The magnitude of the drop in PGs was relatively consistent across the 8 regions, suggesting that 2-AG hydrolysis by MAGL is a major source of metabolic liberation for AA throughout the brain. Therefore, it is likely that MAGL hydrolysis of 2-AG is a significant source of AA for PG synthesis. In contrast, our work suggests that AEA is not a major storage form for free AA: despite a greater than 5 fold increases in AEA in FAAH KO mice, levels of AA and PGs are not significantly altered. Instead, these data suggest that AEA serves as a more directed substrate for AA-derived lipoamines.

4.5 Opposing effects of MAGL and CB₁ on PGs

PGE₂ and PGF_{2α} are potent mediators of inflammation [44]; however, PGE₂ in the brain has also been shown to be an important signaling molecule involved in the development of dendritic spines in some brain regions [71–73]. In addition, activation of PG receptors on microglia has been implicated as important for protection against neurodegenerative diseases [74, 75]. Therefore, a change in brain PGs may alter key aspects of neurodevelopment and effect both neuronal and immune functions in the brain. In MAGL KO mice, levels of PGE₂ and PGF_{2α} were strongly attenuated in all brain regions, replicating findings of lowered PGs in whole-brain of MAGL KOs [50]. Behaviorally, MAGL KO mice perform better than WT littermates on HIPPA-dependent tasks that test spatial memory. The improved memory in these animals is hypothesized to be due to desensitization of CB₁ in the HIPPA that occurs after prolonged exposure to elevated 2-AG [49]. However, PG production in the HIPPA has been recently linked to learning and memory. In one study, upregulated COX-2 expression in the HIPPA was correlated with memory deficits in rats. Treatment with COX inhibitors ‘rescued’ the phenotype of memory impairment [76]. Therefore, lowering PG levels via COX-2 inhibition is hypothesized to improve memory performance after CB₁ activation, raising the possibility that reduced PG levels are contributing to heightened spatial memory performance in MAGL KO.

Although MAGL KO is hypothesized to desensitize CB₁, genetically deleting CB₁ produced opposing effects on levels of PGs, with increases in PGs in many brain regions of CB₁ KO. Thus, differences may exist in how the brain’s lipid metabolism responds to desensitization

versus deletion. Our study is not the first to report a basal neuroinflammatory state in CB₁ KO mice: Cutando and colleagues found increased mRNA levels of COX-2 in the cerebellum on CB₁ KO mice. This was accompanied by upregulation of the cytokine interleukin 1 beta (IL-1 β), which by itself can induce COX-2 expression, and upregulation of mRNA for tumor necrosis factor alpha and chemokine (C-X-C motif) ligand 2 (CXCL2). Furthermore, mRNA levels for CB₂ were significantly higher in the cerebellum of CB₁ KO. The same study also reported evidence of microglial activation in the cerebellum of CB₁ KO: the microglial marker CD11b was upregulated and the number of CD11b positive cells was higher in CB₁ KO. Reducing the number of microglia with minocycline treatment significantly reduced the neuroinflammation, and levels of mRNA for COX-2, IL-1 β , CXCL2 and CB₂ all significantly fell in CB₁ KO after minocycline treatment [77]. A follow-up study will examine changes in COX-2 expression as a potential mechanism for the increased PG levels in CB₁ KO. These studies will further detail interactions between eCB and PG signaling.

The link between reducing CB₁ expression and increasing inflammation may be relevant for cannabis use in humans. Tolerance to THC's effects develops with chronic administration, which is hypothesized to be due to downregulation of CB₁ and CB₁ signaling [78, 79]. PET studies showed a decrease in CB₁ density in the brains of cannabis smokers, especially in cortical regions, that returned to the same levels as healthy controls after 4 weeks of supervised abstinence [80]. In mice, attenuations in CB₁ expression were found 5 days after THC treatment [77], as well as upregulated expression of the markers of microglial activation CD11b and IL-1 β in the cerebellum [77]. The specific effects of THC on CB₁ levels are dose and brain-region dependent, but in general THC reduces CB₁ levels [60]. Thus, reducing CB₁ expression, THC possibly dysregulates PG signaling.

Given the role of eCBs in brain development, the effects of THC may be longer lasting and more severe in adolescence [81]. After 3 weeks of escalating THC treatment in adolescent rats, CB₁ binding was reduced in all 4 brain regions assayed: the hippocampus, caudate putamen, substantia nigra and the cingulate gyrus [82]. A previous study highlighted a persistent neuroinflammatory state after chronic THC treatment in adolescent female rats that correlated with depressive-like behaviors, especially in the pre-frontal cortex, accompanied by upregulation of markers of inflammation such as COX-2 and a downregulation of the anti-inflammatory marker IL-10 [83]. Taken together our data suggest that disrupting CB₁ drives up PG production which may contribute to an inflammatory phenotype and which could be causing a dysregulation of microglial activity.

4.6 Conclusion

The lipids profiled in this study are not static, and instead can be considered as part of a complex fluvial network. Through metabolism, they 'flow' from one form to another. Deprived of upstream precursors, the downstream pathways dry up, while a dammed pathway (as in enzymatic blockade) raises the levels of upstream lipids, perhaps to the point of overflowing into other, perhaps unexpected, pathways. These dynamic processes are inherently difficult to study because we are viewing snapshots in time. By systematically comparing deletion mutants of two enzymes that hydrolyze eCBs and one receptor protein

we have been afforded windows into these dynamic relationships. We have observed dramatic shifts in CNS lipid metabolism that vary considerably both by enzyme, receptor, and by brain region. FAAH-mediated hydrolysis of AEA appears to be important for maintaining levels of AA-derived lipoamines but not for free AA. In contrast, 2-AG hydrolysis by MAGL appears to be important for maintaining levels of free AA and downstream synthesis of PGs. The lipid profiles of FAAH KO, MAGL KO, and CB₁ KO mice are a great model to demonstrate the interconnectedness of biochemical pathways that metabolize eCBs with the broader AA cascade. They also offer insight into the compartmentalization of lipid signaling in specific brain regions that likely have functional implications. Altered lipid profiles may have consequences for other receptor classes such as the TRP family since several of the lipids studied here are agonists at those receptors. The consequences of FAAH and MAGL deletion for the lipid profile were more striking, often impacting lipids that were far ‘from the tree’. Illuminating the distant consequences of deleting a receptor for eCBs on lipid signaling, That CB₁ deletion upregulates PG signaling but does not affect levels of eCBs is another example of an “off target” effect that is not easily predicted. Our results highlight the benefits of systematic side-by-side comparisons such as those undertaken in this study. These results also point to the sometimes unexpected consequences of perturbing the flow of the CNS lipid stream, consequences that must be considered in development and clinical use of blockers for these eCB proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2-AG	2-arachidonoyl glycerol
2-LG	2-linoleoyl glycerol
2-OG	2-oleoyl glycerol
AA	arachidonic acid
ABHD6	α/β hydrolase domain-6
ABHD12	α/β hydrolase domain-12
AEA	<i>N</i> -arachidonoyl ethanolamine
A-GABA	<i>N</i> -arachidonoyl GABA
A-Phe	<i>N</i> -arachidonoyl phenylalanine
A-Ser	<i>N</i> -arachidonoyl serine
A-Taur	<i>N</i> -arachidonoyl taurine

A-Tyr	<i>N</i> -arachidonoyl tyrosine
CER	cerebellum
COX	cyclooxygenase
CTX	cortex
CXCL2	chemokine (C-X-C motif) ligand 2
DEA	<i>N</i> -docosaheptaenoyl ethanolamine
DSE/I	depolarization induced suppression of excitation/inhibition
eCB	endogenous cannabinoid
FAAH	fatty acid amide hydrolase
GPCR	G-protein coupled receptor
HIPP	hippocampus
HPLC/MS/MS	high pressure liquid chromatography coupled with tandem mass spectrometry
HYP	hypothalamus
IL-1β	interleukin 1 beta
KO	knockout
LEA	<i>N</i> -linoleoyl ethanolamine
MAGL	monoacylglycerol lipase
MID	midbrain
NADA	<i>N</i> -arachidonoyl dopamine
NAc	nucleus accumbens
NAE	<i>N</i> -acyl ethanolamine
NAGly	<i>N</i> -arachidonoyl glycine
OEA	<i>N</i> -oleoyl ethanolamine
PEA	<i>N</i> -palmitoyl ethanolamine
PG	prostaglandin
SEA	<i>N</i> -stearoyl ethanolamine
STEM	brainstem
STR	striatum

THAL	thalamus
THC	delta-9-tetrahydrocannabinol
TRP	transient receptor potential
WT	wild-type

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R = fatty acid R₁ = amine

Figure 1.

Generic structure of a lipoamine (also known as an *N*-acyl amide), the conjugation of a fatty acid and an amine via an amide bond.

<i>N</i> -acyl alanine	[M – H] [–]	Fragment	<i>N</i> -acyl proline	[M – H] [–]	Fragment
<i>N</i> -palmitoyl alanine	326.5	88.09	<i>N</i> -palmitoyl proline	352.53	114.12
<i>N</i> -stearoyl alanine	354.55	88.09	<i>N</i> -stearoyl proline	380.59	114.12
<i>N</i> -oleoyl alanine	352.53	88.09	<i>N</i> -oleoyl proline	378.31	114.12
<i>N</i> -linoleoyl alanine	350.52	88.09	<i>N</i> -linoleoyl proline	376.56	114.12
<i>N</i> -arachidonoyl alanine	374.5	88.09	<i>N</i> -arachidonoyl proline	400.58	114.12
<i>N</i> -docosahexaenoyl alanine	398.56	88.09	<i>N</i> -docosahexaenoyl proline	424.6	114.12
<i>N</i> -acyl dopamine	[M – H] [–]	Fragment	<i>N</i> -acyl serine	[M – H] [–]	Fragment
<i>N</i> -oleoyl dopamine	416.3	123.2	<i>N</i> -palmitoyl serine	342.3	74
<i>N</i> -arachidonoyl dopamine	438.4	123.2	<i>N</i> -stearoyl serine	370.3	74
<i>N</i> -acyl ethanolamine	[M + H] ⁺	Fragment	<i>N</i> -oleoyl serine	368.3	74
<i>N</i> -palmitoyl ethanolamine	300.29	62.1	<i>N</i> -linoleoyl serine	366.27	74
<i>N</i> -stearoyl ethanolamine	328.3	62.1	<i>N</i> -arachidonoyl serine	390.3	74
<i>N</i> -oleoyl ethanolamine	326.3	62.1	<i>N</i> -docosahexaenoyl serine	414.3	74
<i>N</i> -linoleoyl ethanolamine	324.3	62.1	<i>N</i> -acyl taurine	[M – H] [–]	Fragment
<i>N</i> -arachidonoyl ethanolamine	348.29	62.1	<i>N</i> -arachidonoyl taurine	410.6	124
<i>N</i> -docosahexaenoyl ethanolamine	372.6	62.1	<i>N</i> -acyl tryptophan	[M – H] [–]	Fragment
<i>N</i> -acyl GABA	[M – H] [–]	Fragment	<i>N</i> -palmitoyl tryptophan	441.63	203.1
<i>N</i> -palmitoyl GABA	340.54	102.1	<i>N</i> -stearoyl tryptophan	469.68	203.1
<i>N</i> -stearoyl GABA	368.58	102.1	<i>N</i> -oleoyl tryptophan	467.67	203.1
<i>N</i> -oleoyl GABA	366.57	102.1	<i>N</i> -linoleoyl tryptophan	465.65	203.1
<i>N</i> -linoleoyl GABA	364.54	102.1	<i>N</i> -arachidonoyl tryptophan	489.67	203.1
<i>N</i> -arachidonoyl GABA	388.57	102.1	<i>N</i> -docosahexaenoyl tryptophan	513.69	203.1
<i>N</i> -docosahexaenoyl GABA	412.59	102.1	<i>N</i> -acyl tyrosine	[M – H] [–]	Fragment
<i>N</i> -acyl glycine	[M – H] [–]	Fragment	<i>N</i> -palmitoyl tyrosine	418.59	180.18
<i>N</i> -palmitoyl glycine	312.26	74.2	<i>N</i> -stearoyl tyrosine	446.65	180.18
<i>N</i> -stearoyl glycine	340.3	74.2	<i>N</i> -oleoyl tyrosine	444.63	180.18
<i>N</i> -oleoyl glycine	338.3	74.2	<i>N</i> -linoleoyl tyrosine	442.61	180.18
<i>N</i> -linoleoyl glycine	336.3	74.2	<i>N</i> -arachidonoyl tyrosine	466	180.18
<i>N</i> -arachidonoyl glycine	360.3	74.2	<i>N</i> -docosahexaenoyl tyrosine	490.66	180.18
<i>N</i> -docosahexaenoyl glycine	384.3	74.2	<i>N</i> -acyl valine	[M – H] [–]	Fragment
<i>N</i> -acyl leucine	[M – H] [–]	Fragment	<i>N</i> -palmitoyl valine	354.31	116.31
<i>N</i> -palmitoyl leucine	368.58	130.1	<i>N</i> -stearoyl valine	382.6	116.14
<i>N</i> -stearoyl leucine	396.63	130.1	<i>N</i> -oleoyl valine	380.59	116.14
<i>N</i> -oleoyl leucine	394.61	130.1	<i>N</i> -nervonoyl valine	464.75	116.14
<i>N</i> -linoleoyl leucine	392.6	130.1	<i>N</i> -linoleoyl valine	378.58	116.14
<i>N</i> -docosahexaenoyl leucine	440.64	130.1	<i>N</i> -docosahexaenoyl valine	426.62	116.14
<i>N</i> -acyl methionine	[M – H] [–]	Fragment	Free Fatty Acids	[M – H] [–]	Fragment
<i>N</i> -palmitoyl methionine	386.62	148.2	Linoleic Acid	279.5	261
<i>N</i> -stearoyl methionine	414.64	148.2	Arachidonic Acid	303.5	285
<i>N</i> -oleoyl methionine	412.65	148.2	2-acyl- <i>sn</i> -glycerol	[M + H] ⁺	Fragment
<i>N</i> -linoleoyl methionine	410.64	148.2	2-arachidonoyl- <i>sn</i> -glycerol	379.3	287.5
<i>N</i> -arachidonoyl methionine	434.66	148.2	2-linoleoyl- <i>sn</i> -glycerol	355.5	245
<i>N</i> -docosahexaenoyl methionine	458.68	148.2	2-oleoyl- <i>sn</i> -glycerol	357.5	265.2
<i>N</i> -acyl phenylalanine	[M – H] [–]	Fragment	Prostaglandins	[M – H] [–]	Fragment
<i>N</i> -palmitoyl phenylalanine	402.59	164.1	PGE ₂	351.2	315
<i>N</i> -stearoyl phenylalanine	430.65	164.1	PGF _{2α}	353.3	309.2
<i>N</i> -oleoyl phenylalanine	428.63	164.1			
<i>N</i> -linoleoyl phenylalanine	426.61	164.1			
<i>N</i> -arachidonoyl phenylalanine	450.64	164.1			
<i>N</i> -docosahexaenoyl phenylalanine	474.66	164.1			

Figure 2.

Lipids in HPLC/MS/MS screening library with parent ion and fragment ion masses. Lipids are grouped by amide family and are screened in a multiple reactions monitoring (MRM) method. Negative ionization mode, resulting in a [M – H][–] parent ion, is used for all methods except the *N*-acyl ethanolamine and 2-acyl glycerol methods, which uses positive ionization and generates a [M + H]⁺ parent ion. The parent ion is then fragmented into the collision chamber and an abundant fragment can be selected as the fragment ion.

Key					
Statistical inference		Increases		Decreases	
Significant decrease relative to WT ($p \leq .05$)		↑↑↑↑↑	10 or more times higher than WT	↓	1-1.49 times lower than WT
Trending decrease relative to WT ($.05 < p \leq .10$)		↑↑↑↑	3-9.99 times higher than WT	↓↓	1.50-1.99 times lower than WT
Trending increase relative to WT ($.05 < p \leq .10$)		↑↑↑	2-2.99 times higher than WT	↓↓↓	2-2.99 times lower than WT
Significant increase relative to WT ($p \leq .05$)		↑↑	1.50-1.99 times higher than WT	↓↓↓↓	3-9.99 times lower than WT
Below detection limit	<i>BDL</i>	↑	1-1.49 times higher than WT	↓↓↓↓↓	10 or more times lower than WT

Figure 3.

Key for Figures 4–7. The arrow color (left column) indicates the direction of a significant result relative to wild-type. Green colors represent increases and orange color represents decreases in a lipid's concentration. The number of arrows (right columns) indicates the magnitude of the difference between the wild-type and KO mice. To determine the magnitude change and therefore the number of arrows to assign each significant or trending difference, the mean level of a particular lipid in a specific region of the KO mice was divided by that same lipid's mean level in the same brain region of the corresponding WT mice. For decreases the process was very similar: the mean level in the KO was divided by the mean level in the WT; however, the reciprocal of the decimal was taken to express a fold decrease (if the level in the KO mouse is $\frac{1}{2}$ of the WT level then that is a 2 fold decrease, if the level in the KO mouse is $\frac{1}{4}$ of the WT level then that is a 4 fold decrease, etc.).

Lipid species	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
N-acyl alanine								
N-palmitoyl alanine	↑↑↑	↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑	↑↑	↑↑↑↑	↑↑↑↑
N-stearoyl alanine	↑↑		↑↑	↑↑	↑		↑↑	↑↑↑
N-oleoyl alanine	↑↑↑↑	↑↑↑	↑↑↑	↑↑↑↑			↑↑↑	↑↑↑
N-arachidonoyl alanine		↓↓↓↓	↓↓		↓↓↓			
N-acyl dopamine								
N-arachidonoyl dopamine	↓↓↓↓↓	BDL	BDL	BDL	BDL	BDL	BDL	BDL
N-acyl ethanolamine								
N-palmitoyl ethanolamine	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑
N-stearoyl ethanolamine	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑
N-oleoyl ethanolamine	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑
N-linoleoyl ethanolamine	↑↑↑	↑↑↑	↑↑↑	↑↑↑↑	↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑
N-arachidonoyl ethanolamine	↑↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑↑	↑↑	↑↑↑
N-docosahexaenoyl ethanolamine	↑↑↑	↑↑↑	↑	↑↑	↑	↑↑	↑	↑↑
N-acyl GABA								
N-palmitoyl GABA		↓↓		↓	↓	↓↓	↓↓	↓
N-stearoyl GABA	↑↑↑↑	↑↑↑	↑↑	↑↑↑	↑↑	↑↑↑	↑↑↑	↑
N-oleoyl GABA	↑↑	↓			↓	↓	↓	
N-linoleoyl GABA			↑					
N-arachidonoyl GABA	↓↓↓	↓↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
N-docosahexaenoyl GABA	↑↑↑↑	↑↑↑	↑↑	↑↑↑↑	↑↑	↑↑↑↑	↑↑↑	↑↑
N-acyl glycine								
N-palmitoyl glycine	↑↑	↑	↑	↑↑	↑	↑	↑	
N-stearoyl glycine	↑↑↑↑	↑↑↑	↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑
N-oleoyl glycine	↑↑↑	↑	↑↑	↑↑↑	↑	↑↑↑	↑↑	↑
N-linoleoyl glycine			↑↑		↓			
N-arachidonoyl glycine	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
N-docosahexaenoyl glycine		↓		↓	↓		↓	↓
N-acyl leucine								
N-palmitoyl leucine					↓			
N-stearoyl leucine			↑					
N-oleoyl leucine	↓							
N-docosahexaenoyl leucine	↓							
N-acyl methionine								
N-palmitoyl methionine					↓			↓
N-arachidonoyl methionine	BDL		↓↓		↓↓			
N-acyl phenylalanine								
N-arachidonoyl phenylalanine		↓	↓↓↓	↓			↓↓	
N-acyl proline								
N-palmitoyl proline		↓↓				↑↑	↓↓	
N-stearoyl proline			↑					
N-oleoyl proline						↓		
N-acyl serine								
N-palmitoyl serine	↑	↑		↑	↑↑	↑	↑	↑
N-stearoyl serine	↑	↑	↑↑		↑↑		↑	
N-oleoyl serine	↑			↑	↑			
N-arachidonoyl serine	↑↑↑			↑↑				↑↑
N-acyl taurine								
N-arachidonoyl taurine	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓	↓↓↓↓↓		↓↓↓↓	↓↓↓
N-acyl tryptophan								
N-palmitoyl tryptophan							↓	
N-stearoyl tryptophan		↓						
N-acyl tyrosine								
N-stearoyl tyrosine							↑↑	
N-arachidonoyl tyrosine		BDL		↓↓	↓↓↓	BDL		BDL
N-acyl valine								
N-palmitoyl valine					↓	↓		
N-stearoyl valine		↓					↓↓	↓
N-oleoyl valine							↓	
Free Fatty Acids								
Linoleic acid			↑					↑
2-acyl-sn-glycerol								
2-arachidonoyl-sn-glycerol			↓↓	↓	↓			
2-oleoyl-sn-glycerol	↑		↑				↑	
Prostaglandins								
PGF _{2α}		↓	↑					

Figure 4.

Significant differences in brain lipidome in FAAH KO compared to WT. Only those lipids with changes from WT are shown here. Up arrows and green shading denote significant increases; whereas down arrows and orange shading denote significant decreases. See Figure 3 and Methods for more detailed description of analysis.

FAAH KO								
	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
Arachidonic acid derivatives								
<i>N</i> -arachidonoyl alanine		↓↓↓↓	↓↓		↓↓↓			
<i>N</i> -arachidonoyl dopamine	↓↓↓↓↓	BDL	BDL	BDL	BDL	BDL	BDL	BDL
<i>N</i> -arachidonoyl ethanolamine	↑↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑↑	↑↑	↑↑↑
<i>N</i> -arachidonoyl GABA	↓↓↓	↓↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
<i>N</i> -arachidonoyl glycine	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
<i>N</i> -arachidonoyl methionine	BDL		↓↓		↓↓			
<i>N</i> -arachidonoyl phenylalanine		↓	↓↓↓	↓			↓↓	
<i>N</i> -arachidonoyl proline	BDL					BDL		
<i>N</i> -arachidonoyl serine	↑↑↑			↑↑				↑↑
<i>N</i> -arachidonoyl taurine	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓↓	↓↓↓↓	↓↓↓↓↓		↓↓↓↓	↓↓↓
<i>N</i> -arachidonoyl tryptophan	BDL	BDL	BDL	BDL	BDL	BDL		
<i>N</i> -arachidonoyl tyrosine		BDL		↓	↓	BDL		BDL
2-arachidonoyl- <i>sn</i> -glycerol			↓↓	↓	↓			
Arachidonic acid								
PGE ₂								
PGF _{2α}		↓	↑					
MAGL KO								
	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
Arachidonic acid derivatives								
<i>N</i> -arachidonoyl alanine								
<i>N</i> -arachidonoyl dopamine		BDL	BDL	BDL	BDL	BDL	BDL	BDL
<i>N</i> -arachidonoyl ethanolamine	↓↓		↓			↓↓	↓	↓↓
<i>N</i> -arachidonoyl GABA			↓	↑			↓	
<i>N</i> -arachidonoyl glycine	↓↓		↓		↓		↓	↓↓
<i>N</i> -arachidonoyl methionine	BDL	BDL				BDL		BDL
<i>N</i> -arachidonoyl phenylalanine					↓↓			
<i>N</i> -arachidonoyl proline	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
<i>N</i> -arachidonoyl serine								
<i>N</i> -arachidonoyl taurine		↓	↓	↓			↓	↓
<i>N</i> -arachidonoyl tryptophan	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
<i>N</i> -arachidonoyl tyrosine			↓↓			BDL		BDL
2-arachidonoyl- <i>sn</i> -glycerol	↑↑↑↑↑	↑↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑↑	↑↑↑↑↑	↑↑↑↑	↑↑↑↑
Arachidonic acid	↓↓↓	↓↓	↓↓	↓↓	↓↓	↓↓↓	↓↓↓	↓↓↓
PGE ₂	↓↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
PGF _{2α}	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
CB ₁ KO								
	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
Arachidonic acid derivatives								
<i>N</i> -arachidonoyl alanine		↑↑				BDL		
<i>N</i> -arachidonoyl dopamine		BDL	BDL	BDL	BDL	BDL	BDL	BDL
<i>N</i> -arachidonoyl ethanolamine								
<i>N</i> -arachidonoyl GABA				↓			↓	↓
<i>N</i> -arachidonoyl glycine		↑						↓
<i>N</i> -arachidonoyl methionine	BDL							
<i>N</i> -arachidonoyl phenylalanine						↓↓		
<i>N</i> -arachidonoyl proline	BDL					BDL		
<i>N</i> -arachidonoyl serine		↓		↓	↓			
<i>N</i> -arachidonoyl taurine				↓				
<i>N</i> -arachidonoyl tryptophan	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
<i>N</i> -arachidonoyl tyrosine		↓↓		↓		BDL		
2-arachidonoyl- <i>sn</i> -glycerol								
Arachidonic acid			↑					
PGE ₂		↑↑↑	↑	↑↑	↑↑	↑↑	↑↑↑	↑↑↑
PGF _{2α}	↑	↑↑		↑	↑		↑	↑

Figure 5.

Comparison of effects of FAAH, MAGL and CB₁ deletion on levels of arachidonic acid derivatives

Lipid species	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
N-acyl alanine								
N-palmitoyl alanine				↑				
N-oleoyl alanine							↓	
N-acyl ethanolamine								
N-palmitoyl ethanolamine	↓		↑					↓
N-stearoyl ethanolamine				↑				
N-linoleoyl ethanolamine	↑	↑↑	↑↑	↑↑↑	↑↑	↑↑	↑↑	↑
N-arachidonoyl ethanolamine	↓↓		↓			↓↓	↓	↓↓
N-docosahexaenoyl ethanolamine	↓↓						↓	↓↓
N-acyl GABA								
N-palmitoyl GABA	↓		↓	↑			↓	
N-stearoyl GABA				↑↑		↑	↓	
N-oleoyl GABA				↑↑				
N-linoleoyl GABA					↑↑↑	BDL		
N-arachidonoyl GABA			↓	↑			↓	
N-docosahexaenoyl GABA				↑↑				
N-acyl glycine								
N-palmitoyl glycine				↑				
N-stearoyl glycine		↑		↑				
N-linoleoyl glycine			↑↑	↑↑↑	↑↑↑	BDL	↑↑↑	
N-arachidonoyl glycine	↓↓		↓		↓		↓	↓↓
N-docosahexaenoyl glycine				↑				
N-acyl leucine								
N-palmitoyl leucine							↓	
N-oleoyl leucine							↓	
N-docosahexaenoyl leucine				↑			↓	
N-acyl methionine								
N-palmitoyl methionine		↑↑					↑	
N-stearoyl methionine				↑				
N-oleoyl methionine				↓↓				↓↓↓
N-acyl phenylalanine								
N-palmitoyl phenylalanine		↓			↓			
N-stearoyl phenylalanine					↓			
N-oleoyl phenylalanine	↓				↓		↓	
N-arachidonoyl phenylalanine					↓↓			
N-acyl proline								
N-palmitoyl proline	↓↓↓							
N-stearoyl proline				↑↑	↑			
N-oleoyl proline	↑	↑↑		↑↑				
N-acyl serine								
N-palmitoyl serine			↑	↑				
N-stearoyl serine				↑				
N-oleoyl serine				↑				↑↑
N-linoleoyl serine				↑		↑		↑↑
N-docosahexaenoyl serine				↑↑				
N-acyl taurine								
N-arachidonoyl taurine		↓	↓	↓			↓	↓
N-acyl tryptophan								
N-palmitoyl tryptophan								↑
N-stearoyl tryptophan								↑
N-acyl tyrosine								
N-stearoyl tyrosine				↑↑				
N-arachidonoyl tyrosine			↓↓			BDL		BDL
N-acyl valine								
N-oleoyl valine							↓	
Free Fatty Acids								
Linoleic acid	↑↑	↑↑	↑	↑↑	↑↑			
Arachidonic acid	↓↓↓	↓↓	↓↓	↓↓	↓↓	↓↓↓	↓↓↓	↓↓↓
2-acyl-sn-glycerol								
2-arachidonoyl-sn-glycerol	↑↑↑↑↑	↑↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑↑	↑↑↑↑↑	↑↑↑↑	↑↑↑↑
2-linoleoyl-sn-glycerol	↑↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑	↑↑↑↑
2-oleoyl-sn-glycerol	↑↑↑	↑↑↑↑	↑↑↑	↑↑	↑↑↑			
Prostaglandins								
PGE ₂	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
PGF _{2α}	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓

Figure 6.

Significant differences in brain lipidome in MAGL KO compared to WT. Only those lipids with changes from WT are shown here. Up arrows and green shading denote significant increases; whereas down arrows and orange shading denote significant decreases. See Figure 3 and Methods for more detailed description of analysis.

Lipid species	STR	HIPP	CER	THAL	CTX	HYP	MID	STEM
N-acyl alanine								
N-palmitoyl alanine							↑	
N-stearoyl alanine						↓		
N-oleoyl alanine	↓							
N-arachidonoyl alanine		↑↑				BDL		
N-acyl ethanolamine								
N-stearoyl ethanolamine				↑				↑
N-linoleoyl ethanolamine			↑					
N-acyl GABA								
N-palmitoyl GABA	↓		↑					
N-stearoyl GABA					↓			
N-oleoyl GABA				↓	↓			
N-arachidonoyl GABA				↓			↓	↓
N-docosahexaenoyl GABA	↓↓							
N-acyl glycine								
N-palmitoyl glycine		↑	↑	↑				↑
N-oleoyl glycine								↓
N-linoleoyl glycine		↑↑						
N-arachidonoyl glycine		↑						↓
N-docosahexaenoyl glycine								↓
N-acyl leucine								
N-palmitoyl leucine				↑			↑	
N-stearoyl leucine		↑↑	↑	↑	↑		↑	
N-oleoyl leucine			↑					
N-linoleoyl leucine	BDL				↓			
N-docosahexaenoyl leucine		↑↑	↑		↑			
N-acyl methionine								
N-palmitoyl methionine		↑	↑↑		↑↑↑			
N-oleoyl methionine		↑↑↑			↑			
N-acyl phenylalanine								
N-palmitoyl phenylalanine		↑↑			↑			↑
N-oleoyl phenylalanine					↑	↓		↑
N-arachidonoyl phenylalanine						↓↓		
N-docosahexaenoyl phenylalanine		↑						
N-acyl proline								
N-palmitoyl proline		↑↑						
N-oleoyl proline			↑↑	↑↑				
N-acyl serine								
N-palmitoyl serine				↑			↑	
N-stearoyl serine			↑				↑	↑
N-oleoyl serine		↑	↑	↑			↑	↑
N-linoleoyl serine		↑	↑	↑	↑			↑↑
N-arachidonoyl serine		↓		↓	↓			
N-docosahexaenoyl serine				↑				
N-acyl taurine								
N-arachidonoyl taurine				↓				
N-acyl tryptophan								
N-palmitoyl tryptophan		↑		↑	↑↑			
N-stearoyl tryptophan			↑	↑				↑
N-acyl tyrosine								
N-oleoyl tyrosine								↓
N-arachidonoyl tyrosine		↓↓		↓		BDL		
N-acyl valine								
N-palmitoyl valine		↑↑						
N-stearoyl valine	↓↓	↑↑				↓↓		
N-oleoyl valine				↓				
Free Fatty Acids								
Arachidonic acid			↑					
2-acyl-sn-glycerol								
2-oleoyl-sn-glycerol								↓
Prostaglandins								
PGE ₂		↑↑↑	↑	↑↑	↑↑	↑↑	↑↑↑	↑↑↑
PGF _{2α}	↑	↑↑		↑	↑		↑	↑

Figure 7.

Significant differences in brain lipidome in CB₁ KO compared to WT. Only those lipids with changes from WT are shown here. Up arrows and green shading denote significant increases; whereas down arrows and orange shading denote significant decreases. See Figure 3 and Methods for more detailed description of analysis.