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Female Mice are Resistant to *Fabp1* Gene Ablation-Induced Alterations in Brain Endocannabinoid Levels

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Abstract Although liver fatty acid binding protein (FABP1, L-FABP) is not detectable in the brain, *Fabp1* gene ablation (LKO) markedly increases endocannabinoids (EC) in brains of male mice. Since the brain EC system of females differs significantly from that of males, it was important to determine if LKO differently impacted the brain EC system. LKO did not alter brain levels of arachidonic acid (ARA)-containing EC, i.e. arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG), but decreased non-ARA-containing *N*-acylethanolamides (OEA, PEA) and 2-oleoylglycerol (2-OG) that potentiate the actions of AEA and 2-AG. These changes in brain potentiating EC levels were not associated with: (1) a net decrease in levels of brain membrane proteins associated with fatty acid uptake and EC synthesis; (2) a net increase in brain protein levels of cytosolic EC chaperones and enzymes in EC degradation; or (3) increased brain protein levels of EC receptors (CB1, TRVP1). Instead, the reduced or opposite responsiveness of female brain EC levels to loss of FABP1 (LKO) correlated with intrinsically lower FABP1 level in livers of WT females than males. These data show that female mouse brain endocannabinoid levels were unchanged (AEA, 2-AG) or decreased (OEA, PEA, 2-OG) by complete loss of FABP1 (LKO).

Keywords Female · Mouse · FABP1 · Gene ablation · Brain · Endocannabinoid

Abbreviations

ACBP	Acyl-CoA binding protein
ARA	Arachidonic acid
AEA, anandamide	Arachidonoylethanolamide
2-AG	2-Arachidonoylglycerol
CB1, *Cnr1*	Cannabinoid receptor-1
CB2, *Cnr2*	Cannabinoid receptor-2
DAGL-α, *Dagla*	Diacylglycerol lipase α
DHEA	Docosahexaenoylethanolamide
EPEA	Eicosapentaenoylethanolamide
EC	Endocannabinoid
FAAH, *Faah*	Fatty acid amide hydrolase
FABP1, L-FABP	Liver fatty acid binding protein-1
FABP-3, *Fabp3*	Fatty acid binding protein-3
FABP-5, *Fabp5*	Fatty acid binding protein-5
FABP-7, *Fabp7*	Fatty acid binding protein-7
FAT/CD36	Fatty acid translocase/thrombospondin receptor
FATP-1	Fatty acid transport protein-1
FATP-4	Fatty acid transport protein-4
LKO	*Fabp1* gene ablated mouse on C57BL/6NCr background
GPCR	G protein coupled receptor
GRK-2, *Adrbk2*	G protein coupled receptor kinase-2
LCFA	Long chain fatty acid
LCFA-CoA	Long chain fatty acyl-CoA

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2-MG	2-Monoacylglycerol
MGL, *Mgll*	2-Monoacylglycerol lipase
NAAA, *Naaa*	*N*-Acylethanolamide-hydrolyzing acid amidase
NAE	*N*-Acylethanolamide
NAPE	*N*-Acylphosphatidylethanolamide
NAPE-PLD, *Nape-pld*	*N*-Acylphosphatidylethanolamide phospholipase-D
OEA	Oleoylethanolamide
2-OG	2-Oleoylglycerol
PEA	Palmitoylethanolamide
2-PG	2-Palmitoyl glycerol
SCP-2, *Scp2*	Sterol carrier protein-2
TRVP-1, vanilloid receptor-1, *Trvp-1*	Transient receptor potential cation channel subfamily V member 1
WT	Wild-type C57BL/6NCr mouse

Introduction

The endogenous cannabinoid receptor (CB) agonists (i.e. endocannabinoids, EC) *N*-arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) are both synthesized from arachidonic acid (ARA)-esterified to phospholipids [1, 2]. Unlike other tissues, however, ability of the brain to synthesize ARA is not sufficient to meet needs and thus brain ARA is derived primarily from plasma [3, 4]. However, plasma ARA availability for brain uptake is limited by high hepatic clearance [5, 6]. Hepatic ARA clearance is associated with high hepatic levels of liver fatty acid binding protein (FABP1), a cytosolic protein that not only binds ARA with high affinity [7, 8] but also facilitates ARA uptake [9–12]. Recent findings with male mice have shown that ablation of FABP1, a protein not found in the brain [13–15], markedly increases serum ARA availability for brain uptake concomitant with increasing brain levels of ARA, AEA and 2-AG [16, 17].

Although most animal studies of the EC system have been performed with male rodents, increasing evidence indicates that the EC system of female humans and rodents differs significantly from that of their male counterparts [18–22]. For example, females have a higher pain sensitivity threshold and are more susceptible to cannabinoid antinociception [18, 21, 23, 24]. At the same time females are also more susceptible to developing cannabinoid abuse and dependence, while having more severe withdrawal, and are more likely to relapse than males [18]. Female brains have fewer CB1 receptor binding sites, but their CB1 receptors are more efficient as compared to those in males [21, 23]. Female rat brain hypothalamus and pituitary have higher AEA and 2-AG levels than those of males [21, 23], consistent with higher blood ARA levels in females as compared to blood ARA levels to males [25, 26].

Taken together, the above findings suggested that the brain EC system of females may respond differently to ablation of FABP1 from that observed with male *Fabp1* gene ablated mice. Therefore, this possibility was examined using female WT and *Fabp1* gene ablated mice to determine the potential impact of its ablation on brain: (1) levels of ARA-containing EC, i.e. AEA and 2-AG; (2) levels of non-ARA containing *N*-acylethanolamides and 2-monoacylglycerols; and (3) protein levels and expression of proteins in the endocannabinoid system. The data show that the brain EC system of female mice was not altered (i.e. AEA, 2-AG) or decreased (OEA, PEA, 2-OG) in response to *Fabp1* gene ablation that was opposite of changes previously shown in males [17]. This correlated with livers of WT female mice exhibiting significantly lower basal FABP1 levels than those of WT males.

Materials and Methods

Mice

Female inbred C57BL/6NCr mice were from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). Female *Fabp1* gene ablated (LKO) mice on the same C57BL/6NCr background were backcrossed to C57BL/6NCr to the N10 generation [27]. Mice were fed a standard rodent chow mix [5 % calories from fat; D8604 Teklad Rodent Diet, Teklad Diets (Madison, WI)] and water *ad libitum*. Mice were housed in barrier cages on ventilated racks at 12-h light/dark cycle in a temperature controlled facility (25 °C), sentinel monitored quarterly, and confirmed free of all known rodent pathogens. At age 8 week, WT and *Fabp1* gene ablated female mice were placed on a defined phytol-free [28–33], phytoestrogen-free [34, 35] control chow to avoid dietary complications due to their potential impact on hepatic FABP1 level and/or the EC system. After 4 weeks on the phytol-free, phytoestrogen-free diet the mice were fasted overnight followed by brain removal/flash freezing and storage at −80 °C. Mouse experimental protocols were approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Extraction and Liquid Chromatography-Mass Spectrometry (LC–MS) Analysis of Brain N-Acylethanolamide (NAE) and 2-Monoacylglycerol (2-MG)

Arachidonoylethanolamide (AEA), oleoylethanolamide (OEA), palmitoylethanolamide (PEA), n-3 docosahexaenoylethanolamide (DHEA), n-3 eicosapentaenoylethanolamide (EPEA), 2-arachidonoylglycerol (2-AG), 2-oleoylglycerol (2-OG), 2-palmitoylglycerol (2-PG), AEA-d_4, OEA-d_2, PEA-d_4, DHEA-d_4, EPEA-d_4, and 2-AG-d_8 were purchased from Cayman Chemical (Ann Arbor, MI). All solvents and reagents were highest grade available commercially. Frozen mouse brain (100–200 mg wet weight) was homogenized in 1.0 mL of ice-cold homogenization buffer containing 2000 pg each of AEA-d_4, OEA-d_2, PEA-d_4, DHEA-d_4, EPEA-d_4, and 2-AG-d_8. Lipids were extracted from mouse brain essentially as described in [36], reconstituted in 100 μL of ice-cold methanol, purged with nitrogen, and stored at -80 °C until analysis by liquid chromatography-mass spectrometry (LC–MS). The NAE (AEA, OEA, PEA, DHEA, EPEA) in the brain lipid extract were resolved, identified, and quantified in the Texas A&M University Protein Chemistry Laboratory (Dr. Larry Dangott, Director) essentially as described in [37] and modified as in [17]. Likewise, the 2-MG (2-AG, 2-OG, and 2-PG) in the brain lipid extract were resolved, identified and quantified in the Protein Chemistry Laboratory basically as in [38] and as modified in [17]. Brain NAE and 2-MG levels are expressed as pmol/g wet weight and nmol/g wet weight, respectively.

Antibodies and Proteins for Western Blotting

Rabbit polyclonal anti-SCP2 was prepared as described in [39]. Caveolin-1 (CAV1; 610060) polyclonal anti-rabbit antibody was from BD Transduction Laboratories (Lexington, KY). Fatty acid transport protein 1 (FATP-1; sc-25541) polyclonal anti-rabbit, fatty acid binding protein-3 (FABP3; sc-58275) monoclonal anti-mouse, fatty acid binding protein-7 (FABP7; sc-30088) polyclonal anti-rabbit, FABP1 (sc-16064) polyclonal anti-mouse, *N*-acylethanolamide hydrolyzing acid amidase (NAAA; sc-100470) monoclonal anti-mouse, and ß-Actin (sc-47778) monoclonal anti-mouse were from Santa Cruz Biotech (Santa Cruz, CA). Fatty acid binding protein-5 (FABP5; RD181060100) antibody was from BioVendor R&D (Asheville, NC). Fatty acid translocase/cluster of differentiation 36/thrombospondin receptor (FAT/CD36; RDI-M1537db) monoclonal anti-mouse antibody was from Research Diagnostics (Flanders, NJ). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; MAB374) monoclonal anti-mouse antibody was from Millipore (Billerica, MA). Diacylglycerol lipase α (DAGLα; 13626 Cell Signaling, Danvers, MA) and 2-monoacylglycerol lipase (MAGL; 310212) polyclonal antibodies were from Cayman Chemical Co (Ann Arbor, MI). Cytochrome C oxidase 4 (COX4, ab16056) polyclonal anti-rabbit, antibody to cannabinoid receptor-1 (CB1; AB172970), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, AB8245), fatty acid amide hydrolase (FAAH, AB54615), and *N*-acylphosphatidylethanolamide phospholipase D (NAPEPLD; AB95397) were from Abcam (Cambridge, MA). Antibody to transient receptor potential cation channel subfamily V member 1 (TRVP-1: 75j-254) was from Antibodies Inc. (Davis, CA). For quantitative Western blotting, recombinant protein standards were purified and delipidated as described in the following cited papers: murine FABP1 [7, 40], murine acyl-CoA binding protein (ACBP) [41, 42], and human sterol carrier protein-2 (SCP-2) [43–45].

Brain Protein Levels of Enzymes and Other Proteins in the Endocannabinoid System

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis was performed on brain post-nuclear supernatants (PNS) as described earlier [17, 46, 47]. Brain proteins were resolved by 12 % Tris-SDS-PAGE gel, transferred to 0.2 µm nitrocellulose membrane (162-0112, BioRad Laboratories, Hercules, CA), blocked with 3 % gelatin for 1 h, and incubated overnight with select primary antibodies followed by species-specific Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP) conjugated secondary antibodies for 1–2 h. After rinsing nitrocellulose membrane three times for 5 min in TBST (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.05 % Tween 20), the HRP conjugated antibodies were exposed to the Super Signal West Pico chemiluminescent substrate (34077, Pierce, Rockford, IL) or Immuno-star HRP substrate (Bio-Rad, Hercules, CA). Images were obtained with an Image Quant LAS 4000 mini (GE Healthcare Life Sciences, Marlborough, MA) or C-DiGiT scanner (Li-COR, Lincoln, NE). AP-conjugated antibodies were exposed to BCIP/NBT solution (B6404, Sigma Aldrich) and images obtained with an Epson Perfection V700 Photo scanner (Long Beach, CA). Proteins were quantified by densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD). Relative protein levels normalized to GAPDH or ß-actin internal gel-loading controls and representative cropped Western blot images are inserted into figure panels similarly as in earlier publications in which individual Western blots are separated by a white line/space [48–52]. Quantitative Western blotting of FABP1 was performed using a standard curve with recombinant murine FABP1 as in [53–56]. Images of the blots were taken by Epson Perfection V700 Photo scanner (Long Beach, CA)

and quantified by densitometric analysis with ImageJ software (NIH, Bethesda, MD) as described earlier [57].

QrtPCR Reagents for Analyzing Brain mRNA of Genes in the Endocannabinoid System

TaqMan® RNA-to-C_T™ 1-Step PCR Master Mix Reagent kit was purchased from Life Technologies™ (Carlsbad, CA). The following gene-specific TaqMan® PCR probes and primers were obtained from Life Technologies™ (Carlsbad, CA) to determine brain mRNA levels of: G protein coupled receptor kinase-2 (*Adrbk2*, Mm00622042_m1); cannabinoid receptor-1 (*Cnr1*, Mm01212171_s1); cannabinoid receptor-2 (*Cnr2*, Mm02620087_s1); diacylglycerol lipase α (*Dagla*, Mm00813830_m1); diacylglycerol lipase β (*Daglb,* Mm00523381_m1); fatty acid amide hydrolase (*Faah*, Mm00515684_m1); 2-monoacylglycerol lipase (*Mgll*, Mm00449274_m1); fatty acid binding protein-3 (*Fabp3*, Mm02342494_m1); fatty acid binding protein-5 (*Fabp5*, Mm00783731_s1); fatty acid binding protein-7 (*Fabp7*, Mm01246302_m1); *N*-acylethanolamide hydrolyzing acid amidase (*Naah*, Mm01341699_m1); *N*-acylphosphatidylethanolamide phospholipase D (*Napepld*, Mm00724596_m1); transient receptor potential cation channel subfamily V member 1 (*Trvp-1*, Mm01246302_m1).

mRNA Extraction and QrtPCR to Determine mRNA Levels of Genes in the Brain Endocannabinoid System

Brain total RNA was isolated and purified with the RNeasy mini kit (Qiagen, Valencia, CA) using the manufacturer's standard protocol. Concentration and quality of mRNA were determined by a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). Samples were stored at −80 °C. QrtPCR expression patterns were analyzed with an ABI PRISM 7000 sequence detection system (Applied Biosystems®, Foster City, CA) using TaqMan® RNA-to-C_T™ 1-Step PCR Master Mix Reagent kit, gene-specific TaqMan PCR probes and primers. The thermal cycler protocol was as follows: 48 °C for 30 min, 95 °C for 10 min, 95 °C for 0.15 min and 60 °C for 1.0 min, repeated a total of 40 cycles. TaqMan® gene expression assays to determine brain mRNA transcript levels of the genes listed above. Two replicates of each sample reaction (20 µL total volume each) were performed on 96 well plates (Applied Biosystems®, Foster City, CA). The threshold cycle from each well was established with ABI Prism 7000 SDS software (Applied Biosystems®, Foster City, CA) and QrtPCR data normalized to the housekeeping gene 18S RNA for mRNA. Expression of *Adrbk2*, *Arrb2, Cnr1*, *Cnr2*, *Dagla*, *Daglb, Faah*, *Mgll*, *Fabp3*, *Fabp5*, *Fabp7*, *Naah*, *Nape-pld*, and *Trvp-1* were relative to the control female mouse group.

Brain Cytokine Levels

Mouse LINCO*plex* kit (MADPK-71 K) and mouse LINCO*plex* kit (MADPCYT-72 K) from LINCO Research (St. Charles, MO) were used to determine brain levels of insulin, resistin, leptin, adiponectin, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6), and tumor necrosis factor α (TNFα) according to the manufacturer's instructions. Samples were detected with a Luminex 100IS microsphere analyzer (Luminex Corp., Austin, TX) and analyzed with Luminex 100 version 2.1 software supplied by the manufacturer using 5-parameter data reduction.

Statistical Analysis

Values represent the mean ± standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test. Statistical significance was assigned to values with $p < 0.05$.

Results

Fabp1-Gene Ablation (LKO) Differentially Impacts Brain Levels of Arachidonic Acid (ARA)containing *versus* non-ARA-containing Endocannabinoids (EC)

Brain contains three major classes of endocannabinoids: (1) ARA-containing (2-AG ≫ AEA) EC are the major endogenous ligand activators of cannabinoid (CB) receptors [4, 38, 58–62]; (2) non-ARA-containing 'potentiating' EC (OEA, PEA, 2-OG, and/or 2-PG) that enhance the activity of ARA-containing EC by increasing their affinities for CB receptors or decreasing their enzymatic degradation [63–68]; (3) non-ARA-containing antagonistic EC (DHEA, EPEA) that displace ARA from membrane phospholipids and decrease ARA containing phospholipid synthesis to thereby lower AEA and 2-AG production [69].

Arachidonoylethanolamide (AEA) levels were not different between groups (Fig. 1a), levels of potentiating endocannabinoids OEA and PEA were nearly 2-fold higher in the WT than LKO mice (Fig. 1b, c). LKO differentially impacted brain levels of potentiating, but not antagonistic non-ARA containing, EC. LKO did not significantly alter brain levels of AEA (Fig. 1a) or 2-AG (Fig. 2a). In contrast, brain levels of potentiating OEA and PEA (Fig. 1b, c) and 2-OG (Fig. 2b) were decreased. In contrast, LKO did not significantly alter the brain levels of antagonistic DHEA or EPEA (Fig. 1d, e).

Consistent with the literature, WT brain levels of the antagonistic DHEA (Fig. 1d) and even more so EPEA

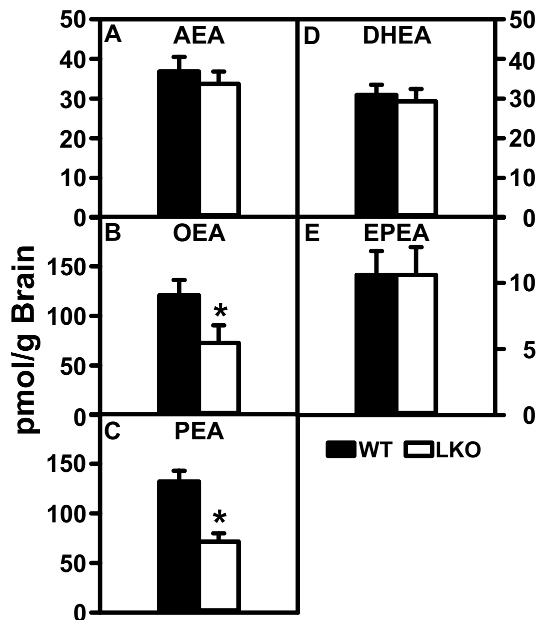

Fig. 1 Impact of FABP1 gene ablation (LKO) on brain *N*-acylethanolamide (NAE) levels. Female WT and LKO (8 week old) were fed phytol-free, phytoestrogen-free control diet for 4 weeks, fasted overnight, brains removed/flash frozen and stored at −80 °C, and NAE extracted for resolution, identification and quantitation by LC–MS analysis as described in Materials and Methods to determine content of: **a** arachidonoylethanolamide (AEA), **b** oleoylethanolamide (OEA), **c** palmitoylethanolamide (PEA), **d** docosahexaenoylethanolamide (DHEA), and **e** eicosapentaenoylethanolamide (EPEA). Data represent the mean ± SEM ($n = 8$); $^*p < 0.05$ for LKO *vs* WT

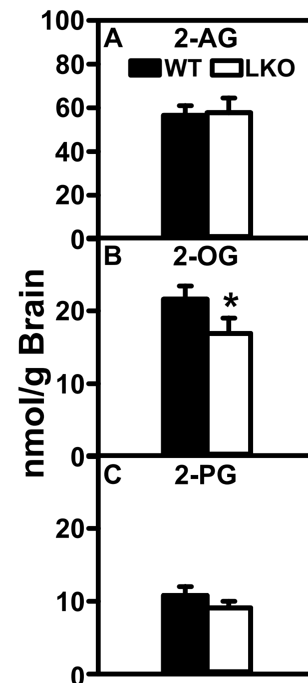

Fig. 2 Effect of FABP1 gene ablation (LKO) on brain 2-monoacylglycerol (2-MG) levels. All conditions were as in legend to Fig. 1 except that LC–MS analysis was used to quantify 2-monoacylglycerols as described in "Materials and Methods": **a** 2-arachidonoylglycerol (2-AG), **b** 2-oleoylglycerol (2-OG), and **c** 2-palmitoylglycerol (2-PG). Data represent the mean ± SEM ($n = 8$); $^*p < 0.05$ for LKO *vs* WT

(Fig. 1e) were lower. WT brain levels of the other major ARA-containing EC, i.e. 2-arachidonoylglycerol (2-AG) (Fig. 2a) were 3 orders of magnitude higher than those observed for AEA, but WT brain levels of the potentiating 2-monoacylglycerols (2-MGs) 2-OG and 2-PG (Fig. 2b, c) were 2-4 fold lower than those of 2-AG (Fig. 2a). Nevertheless the WT brain levels of 2-OG and 2-PG (Fig. 2b, c) were still markedly higher than those of AEA (Fig. 1a).

Fabp1 Gene Ablation (LKO) Does Not Affect Brain Protein Levels of Membrane Fatty Acid Transport/Translocase Proteins

WT brain contains several membrane associated proteins (CD36/FAT, CAV1, FATP1 and FATP4) that facilitate translocation/uptake of long chain fatty acids such as ARA as well as other non-ARA fatty acids (e.g. palmitic or oleic acid) [3]. As shown by Western blotting, LKO did not alter expression of CD36/FAT, CAV1, FATP1, or FATP4 (Fig. 3a–d). The lower levels of OEA, PEA, and 2-OG in LKO brain (Figs. 1, 2) did not correlate with decreased levels of membrane fatty acid uptake proteins.

Impact of *Fabp1* Gene Ablation (LKO) on Brain Levels of Proteins Involved in NAE and 2-MG Synthesis and Degradation

Brain levels of NAE and 2-MG are determined in part both by synthetic enzymes in the plasma membrane (NAPEPLD and DAGLα) and degradative membrane enzymes (FAAH, NAAA, MAGL) localized in intracellular sites [70–72]. Thus, it was important to examine if LKO-induced alteration in brain EC levels was attributable to altered levels of these key enzymes.

Western blotting showed that *Fabp1* gene ablation did not alter expression of the NAE synthetic enzyme NAPE-PLD (Fig. 4a) or the 2-MG synthetic enzyme DAGLα (Fig. 4b). With regards to the NAE degradative enzymes, LKO did not alter that of the major one, i.e. FAAH (Fig. 4c), but decreased that of NAAA (Fig. 4d). Protein levels of the 2-MG degradative enzyme MAGL were not altered by LKO (Fig. 4e). Finally, Western blotting showed that LKO did not alter protein levels of the AEA and 2-AG receptor CB1 (Fig. 4f), but did reduce protein levels of TRVP1 in the brain (Fig. 4g).

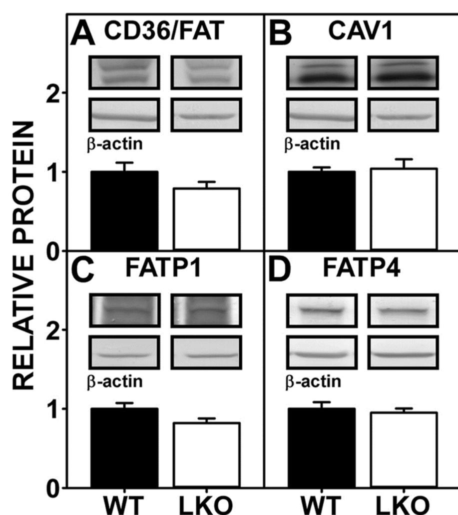

Fig. 3 FABP1 gene ablation (LKO) impact on protein levels of brain membrane proteins involved in fatty acid uptake. Female WT and LKO mice (8 week old) were fed phytol-free, phytoestrogen-free control chow for 4 weeks, overnight fasted, brains removed/flash frozen and stored at −80 °C, and aliquots of brain homogenate proteins examined by SDS-PAGE and subsequent Western blot analysis as described in Materials and Methods. **a** CD36/FAT, **b** CAV1, **c** FATP1, and **d** FATP4. Insets show representative Western blot images of the respective protein (*upper blot*) and the gel-loading control protein β-Actin (*lower blot*). Relative protein levels were normalized to gel-loading control protein; values were compared to WT set to 1. Data represent the mean ± SEM ($n = 7$); $^*p < 0.05$ for LKO *vs* WT

Impact of *Fabp1* Gene Ablation (LKO) on Brain Levels of Cytosolic NAE and 2-MG 'Chaperone' Proteins

Due to their highly hydrophobic nature, not only ARA but even more so NAE and 2-MG, require cytosolic 'chaperone' proteins for intracellular transport/targeting to metabolic organelles. These roles are served by the brain cytosolic FABPs 3, 5, and 7 [13, 46, 73–79] and SCP-2 [17, 45, 80, 81]. Therefore, it was important to determine the impact of LKO on brain proteins levels of these lipidic ligand 'chaperones'.

As shown by Western blotting, LKO differentially impacted the expression of the cytosolic 'chaperone' proteins. Brain protein level of FABP3 was significantly increased by LKO (Fig. 5a). Concomitantly, brain protein levels of the other 'chaperones' were either significantly decreased, e.g. SCP-2 (Fig. 5d) or did not change (FABP5, FABP7) (Fig. 5b, c).

Ablation/inhibition of cytosolic 'chaperones' is known to decrease NAE and 2-MG targeting for degradation which in turn increases their level [38, 47, 79]. Since LKO decreased brain levels of non-ARA NAE and 2-MG, this would suggest that the concomitant upregulation of FABP3 may have exerted a larger impact than downregulation of the other cytosolic 'chaperones' which were either decreased or unchanged.

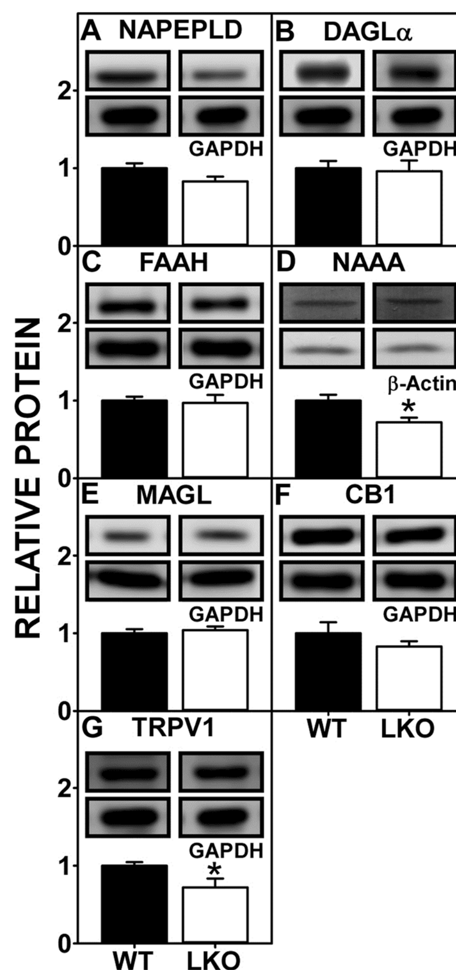

Fig. 4 Impact of FABP1 gene ablation (LKO) on protein levels of brain proteins involved in endocannabinoid synthesis anInsets show representatived degradation and associated receptors. All conditions were as in legend to Fig. 3 except that Western blot analysis was performed to determine protein levels of **a** NAPEPLD, **b** DAGLα, **c** FAAH, **d** NAAA, **e** MAGL, **f** CB1, and **g** TRVP1. Insets show representative Western blot images of the respective protein (*upper blot*) and the gel-loading control protein (GAPDH or β-Actin, *lower blot*). Relative protein levels were normalized to the gel-loading control protein; values were compared to WT set to 1. Data represent the mean ± SEM ($n = 7$); $^*p < 0.05$ for LKO *vs* WT

Role of Transcriptional Regulation on the Impact of *Fabp1* Gene Ablation (LKO) on Brain Protein Levels of Proteins And Enzymes in the Endocannabinoid System

LKO-induced changes in protein levels of some, but not most, brain proteins were attributable in part to altered mRNA levels. The decreased protein level of the NAE degradative enzyme NAAA (Fig. 4d) was consistent with decreased *Naaa* mRNA level (Fig. 6e). LKO-induced decreased or unaltered protein levels of brain cytosolic 'chaperones' such as FABP5 and SCP2 (Fig. 5b, d) was

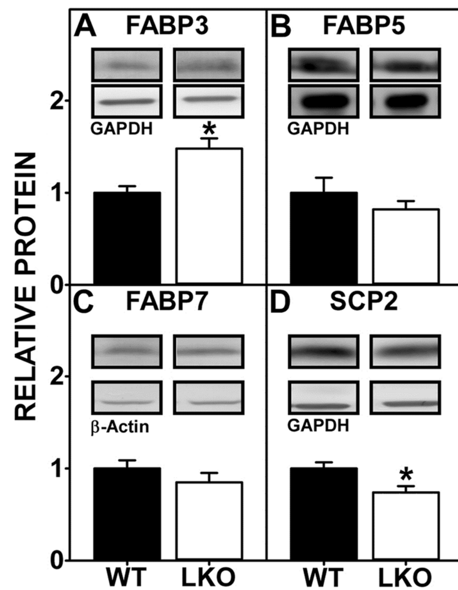

Fig. 5 FABP1 gene ablation (LKO) alters protein levels of brain cytosolic 'chaperone' endocannabinoid binding proteins. All conditions were as in legend to Fig. 3 except that Western blot analysis was performed to determine protein levels of **a** FABP3, **b** FABP5, **c** FABP7, and **d** SCP-2. Insets are representative Western blot images of the respective protein (*upper blot*) and gel-loading control (GAPDH or β-Actin, *lower blot*). Relative protein levels were normalized to the gel-loading control protein; values were compared to WT set to 1. Data represent the mean ± SEM ($n = 7$); $*p < 0.05$ for LKO *vs* WT

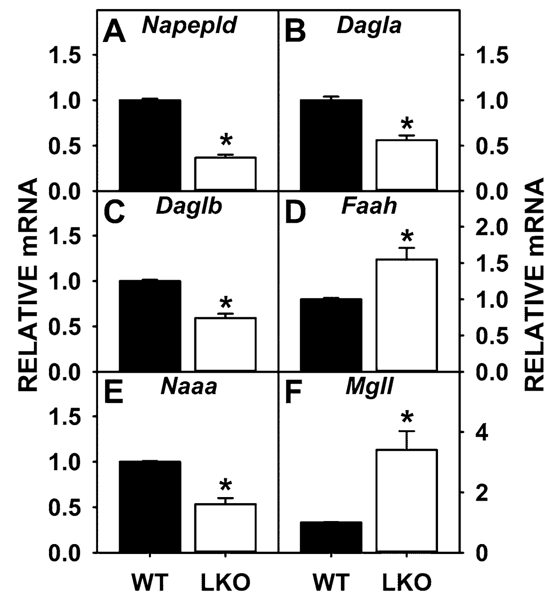

Fig. 6 Effect of FABP1 gene ablation (LKO) on brain levels of mRNAs encoding proteins for endocannabinoid synthesis and degradation. Female WT and LKO mice (8 week old) were fed phytol-free, phytoestrogen-free control chow for 4 weeks, overnight fasted, brains removed/flash frozen and stored at −80 °C, and aliquots of brain homogenate used for qrtPCR to determine mRNA levels of **a** *Napepld*, **b** *Dagla*, **c** *Daglb*, **d** *Faah*, **e** *Naaa*, and **f** *Mgll* as described in "Materials and Methods". Levels of mRNA were normalized to an internal control (18S RNA); values were compared to WT set to 1. Data represent the mean ± SEM ($n = 7$); $*p < 0.05$ for LKO *vs* WT

consistent with decreased or unaltered *Fabp5* and *Scp2* mRNAs (Fig. 7b, d).

In contrast, other brain EC system protein levels did not correlate with the respective mRNAs in LKO mice. The protein levels of the synthetic enzymes NAPEPLD and DAGLα were unaltered (Fig. 4a, b) despite significantly decreased *Napepld* and *Dagl* mRNA levels (Fig. 6a–c). The protein levels of the degradative enzymes FAAH and MAGL were unaltered (Fig. 4c, e) despite increased *Faah* and *Mgll* mRNA levels (Fig. 6d, f). The protein level of FABP3 was increased (Fig. 5a) but the *Fapb3* mRNA decreased (Fig. 7a). Finally, the protein level of FABP7 was unchanged (Fig. 5c); however, *FABP7* mRNA level was decreased in LKO (Fig. 7c).

Hepatic FABP1 Expression is Sexually Dimorphic

Since LKO did not alter brain AEA and 2-AG levels in females (Figs. 1a, 2a), but significantly increased that in males (AEA: MWT = 15 ± 2 pmol/g brain, MLKO = 24 ± 2 pmol/g brain; 2-AG: MWT = 16 ± 2 nmol/g brain, MLKO = 44 ± 3 nmol/g brain) [17], the possibility that this might be attributed at least in part to sex-differences in hepatic FABP1 expression

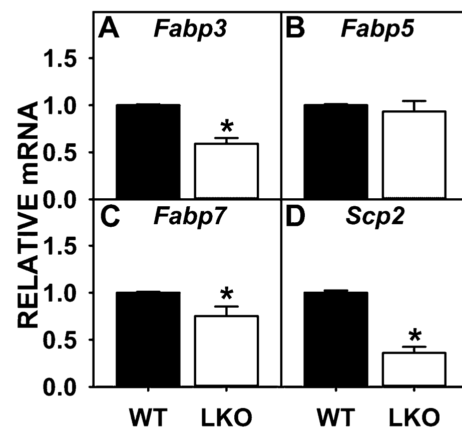

Fig. 7 FABP1 gene ablation (LKO) alters brain levels of mRNAs encoding cytosolic 'chaperone' endocannabinoid binding proteins. All conditions were as in legend to Fig. 6 except that qrtPCR was performed to determine mRNA levels of **a** *Fabp3*, **b** *Fabp5*, **c** *Fabp7*, and **d** *Scp-2* as described in "Materials and Methods". Levels of mRNA were normalized to an internal control (18S RNA); values were compared to WT set to 1. Data represent the mean ± SEM ($n = 6$); $*p < 0.05$ for LKO *vs* WT

in WT mice was examined by quantitative Western blotting using a standard curve with purified recombinant murine FABP1 as described in Materials and Methods. As shown

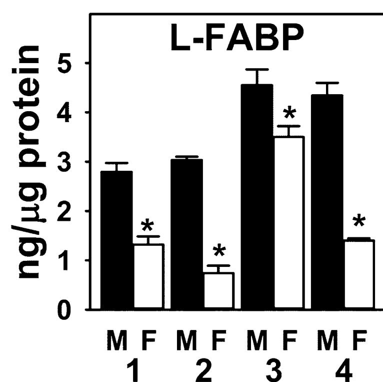

Fig. 8 Hepatic FABP1 expression is sexual dimorphic. C57BL/6 N male and female mice (8 week old) were fed phytol-free, phytoestrogen-free control chow for 4 weeks, overnight fasted, livers removed and frozen at −80 °C. Quantitative Western blotting was performed on livers to determine FABP1 protein level compared to standard curve of pure recombinant FABP1 as described [53–56]. FABP1 levels (ng L-FABP/µg total protein) are shown from four separate experiments, each presented as mean ± SEM ($n = 3$–10); *$p < 0.05$ for LKO *vs* WT

in multiple separate experiments, FABP1 was more highly expressed in livers of male than female mice fed a phytol-free, phytoestrogen-free diet (Fig. 8).

FABP1 Gene Ablation (LKO) Impact on Brain Inflammatory Cytokine Levels

LKO had no significant impact on brain concentrations of insulin (Fig. 9a). LKO did modestly increase brain levels of inflammatory cytokines MCP-1 (Fig. 9e), PAI-1 (Fig. 9f), IL-6 (Fig. 9g), and TNFα (Fig. 9H). LKO also increased brain levels of adiponectin (Fig. 9b), resistin (Fig. 9c), and leptin (Fig. 9d); however, these cytokines are not normally associated with inflammation in the brain. Taken together, the lack of major changes in inflammatory cytokine levels correlated with the lack of change in brain AEA and 2-AG levels in LKO mice.

Discussion

Behavioral and other studies suggest considerable sexual dimorphism in the brain endocannabinoid (EC) system of both humans and rodents [18–23]. However, little is known concerning the molecular details on which these differences are based—especially with regards to factors outside the brain that influence brain endocannabinoid levels. For example, liver fatty acid binding protein (FABP1) is not detectable in the brain [13–15], but its ablation (LKO) in male mice markedly increases brain levels of arachidonic acid (ARA)-containing EC (e.g. AEA: MWT = 15 pmol/g

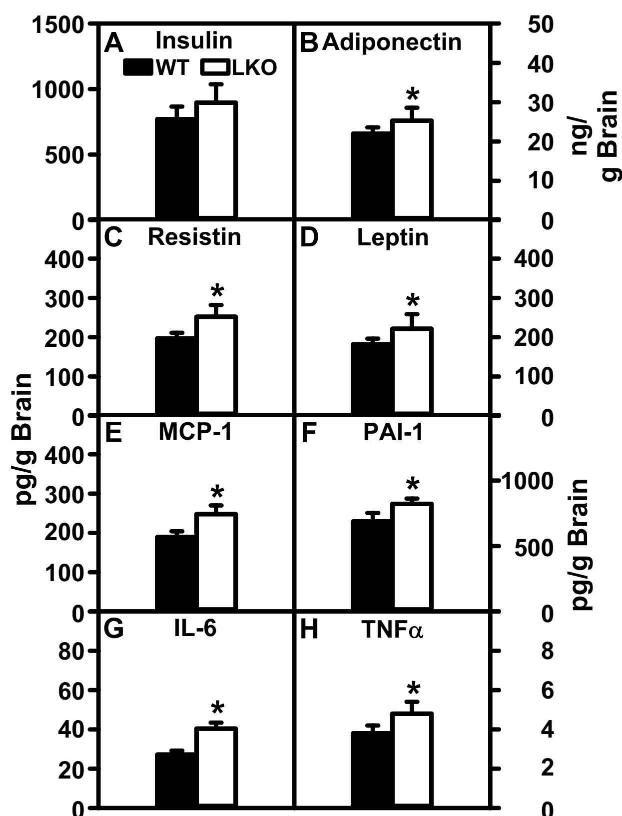

Fig. 9 Impact of FABP1 gene ablation on brain cytokine levels. Brain homogenate levels of **a** insulin, **b** adiponectin, **c** resistin, **d** leptin, **e** MCP-1, **f** PAI-1, **g** IL-6, and **h** TNFα were quantified as described in "Materials and Methods". Data represent the mean ± SEM ($n = 8$); *$p < 0.05$ for LKO *vs* WT

brain vs MLKO = 24 pmol/g; 2-AG: MWT = 16 nmol/g vs MLKO = 44 nmol/g) and non-ARA-containing EC (e.g. OEA: MWT = 72 pmol/g brain vs MLKO = 190 pmol/g; PEA: MWT = 80 pmol/g vs MLKO = 150 pmol/g; 2-OG: MWT = 5 nmol/g vs MLKO = 18 nmol/g) [16, 17]. Whether a similar effect is seen in the female brain EC system is unknown. The studies presented herein with female LKO mice presented new insights into the impact of sexual dimorphic FABP1 expression on the brain EC system.

First, there are a number of known differences in the brain EC system between males and females. For example, ARA-containing EC (AEA, 2-AG) levels were near 40 pmol/g and 60 nmol/g brain, respectively, in female brains (shown herein)—several fold higher than those observed in the brains of male mice (AEA, 15 pmol/g brain; 2-AG, 16 nmol/g brain) [16, 17]. Consistent with these findings, female rat brain hypothalamus and pituitary have higher AEA and 2-AG levels than those of males [21, 23]. In the rat, the higher AEA and 2-AG level in female brain is attributed to the higher plasma availability of ARA in females [25, 26]. This is important because most brain

ARA is derived from plasma for uptake into brain and rapid esterification into phospholipids from which AEA and 2-AG are derived [3, 4]. Finally, the markedly higher levels of AEA and 2-AG in brains of female vs male mice correlated with the significantly lower basal FABP1 levels in livers of female vs male mice (shown herein). The possibility that lower hepatic FABP1 levels in females contributed to higher brain EC levels is supported by earlier studies showing that: (1) FABP1 has high affinity for ARA [7, 9, 17, 82]; (2) native FABP1 isolated from liver is preferentially enriched with endogenously-bound ARA [8]; (3) hepatic FABP1 concentration is at least 20-fold higher than that of all the FABP (FABP 3, 5, 7) in the brain combined [83–90]; (4) FABP1 overexpression enhances ARA uptake [9–12].

In contrast, very little is known with respect to differences in the non-ARA containing EC between males and females. Although brain can synthesize sufficient non-ARA fatty acids such as oleic acid and palmitic acid needed for incorporation into phospholipids from which non-ARA-containing EC are derived [3, 4], brain can take up non-ARA fatty acids from the blood [3, 74, 91]. Thus, it was difficult to predict *a priori* the net impact of sex on brain levels of non-ARA-containing EC. The data presented herein showed that female brain basal levels of non-ARA containing EC (OEA, PEA, 2-OG, and 2-PG near 125 pmol/g brain, 135 pmol/g, 22 nmol/g, and 11 nmol/g, respectively) were significantly higher than those in male brains (OEA, 70 pmol/g brain; PEA, 80 pmol/g; 2-OG, 5 nmol/g; 2-PG, 6 nmol/g) [16, 17]. This may be attributed at least in part by: (1) females' lower hepatic FABP1 level; (2) FABP1 also binding non-ARA fatty acids with high affinity, albeit less than that for ARA [7, 92–94]; (3) non-ARA fatty acids palmitic acid and oleic acid comprising the most common, i.e. 10 and 30 %, respectively, endogenously-bound fatty acids in native FABP1 isolated from liver [8]; (4) enhancement of non-ARA fatty acid uptake by FABP1 overexpression and in direct proportion to FABP1 level in cloned human HepG2 liver cells [10, 12, 95–98].

Second, although *Fabp1* gene ablation (LKO) markedly increased brain levels of both ARA-containing (AEA: WT = 15 pmol/g brain, LKO = 24 pmol/g; 2-AG: WT = 16 nmol/g brain, LKO = 44 nmol/g) and non-ARA containing (OEA: WT = 72 pmol/g, LKO = 190 pmol/g; PEA: WT = 80 pmol/g, LKO = 150 pmol/g, 2-OG: WT = 5 nmol/g, LKO = 18 nmol/g; 2-PG: WT = 7 nmol/g, LKO = 9 nmol/g) EC in males [16, 17], its impact in the female brain was not known. The data presented herein showed for the first time that (LKO) did not alter brain levels of AEA or 2-AG in females, while the levels of the non-ARA containing EC (OEA, PEA, 2-OG) were decreased by 20–50 %. While this was consistent with the already much lower level of hepatic FABP1 in WT females as compared to WT males, there is a paucity of literature regarding the impact of hepatic FABP1 level on sex differences in the brain EC system.

Third, the *Fabp1* gene ablation induced decreases in brain EC were not attributable to marked alteration in proteins levels of: (1) plasma membrane proteins for fatty acid uptake in the brain; (2) membrane enzymes in synthesis/degradation of non-ARA-containing EC; or (3) protein levels of cytosolic chaperones that would enhance non-ARA-containing EC cytosolic transport and targeting for degradation. Furthermore, the lack of compensatory changes in the brain EC system proteins in response to FABP1 gene ablation was not attributable to lack of changes in respective mRNA levels—many of which were significantly altered. While the lack of correlation between brain EC system protein levels and mRNA transcripts is not known, a similar lack of correlation in liver EC system protein levels and mRNAs has been attributed to specific micro RNA (miRNA) that inhibit mRNA translation [99–101].

With regards to physiological impact of these findings on brain function, an important function of endocannabinoids such as AEA is on analgesia [38]. Lower hepatic FABP1 level in females (shown herein) vs males [16, 17] correlate with females having higher brain levels of AEA and potentiating OEA and PEA (enhancers of AEA activity on CB receptors). Higher AEA level in brains of females is associated with lower sensitivity to pain as compared to males [18, 21, 23, 24]. Conversely, elevated liver FABP1 levels in human lipid disorders such as obesity [102], alcoholic fatty liver disease (AFLD) [103, 104], and nonalcoholic fatty liver disease (NAFLD) [105–108] are associated with increased pain sensitivity in obesity [118–120], AFLD [121, 122], and NAFLD [123] reported in these lipid disorders. While expression of a SNP in the human *Fabp1* gene coding region results in a T94A substitution also increases hepatic total FABP1 and is associated with NAFLD [109–111], another relatively common SNP in the human *Fabp1* gene promoter region (rs2919872) decreases FABP1 promoter transcriptional activity to decrease FABP1 [110]. However, the impact of these SNP on pain sensitivity is not known. Resolving this issue is important, especially since the SNP leading to the *Fabp1* T94A variant is highly prevalent in the human population, occurring with 26–38 % minor allele frequency and 8.3 ± 1.9 % homozygosity (MAF for 1000 genomes in NCBI dbSNP database; ALFRED database) [109, 112–117]. Taken together these studies would suggest that FABP1 reduction or *Fabp1* gene ablation may impact pain sensitivity much less in females than males—a possibility to be tested in future studies beyond the scope of the present investigation.

Another major physiological effect regulated by brain endocannabinoids is the desire for food intake. Elevated AEA increases desire for food intake [124], while increased

OEA, PEA, or 2-OG decrease the desire for food intake [69, 125, 126]. Thus, the female brain's higher AEA level (shown herein) as compared to that in the brain of males [16, 17] would suggest higher food intake by females. Conversely, the female brain's higher OEA, PEA and less so 2-OG content would tend to decrease food intake. The overall net effect led to less food intake in females *versus* males in control chow fed mice [127–131]. With regards to the impact of loss of FABP1, LKO did not alter female brain AEA level, but decreased OEA and PEA by about 50 %, and less so 2-OG (shown herein). As a result of the unaltered AEA and much smaller difference in potentiating EC, the LKO female mice had unaltered or only slightly altered control chow food intake [127, 128, 131, 132].

In summary, wild-type mouse brain EC levels in females (shown herein) differed significantly from those of males [16, 17]. This differential level of endocannabinoids adds a new level of understanding of our previously published studies demonstrating a reduction in food intake in female mice compared to males [127–131]. Our studies further extend the impact of sex-differences on the content of endocannabinoids in the brain, demonstrating a higher level in female mice as compared to male mice. Finally, female brain EC levels were much less responsive to *Fabp1* gene ablation (shown herein) as compared to their male FABP1 gene ablated counterparts [16, 17]. This diminution of responsiveness of female brain EC levels to loss of FABP1 was associated with intrinsically lower FABP1 level in livers of WT females than males. This was in marked contrast to males wherein lower brain EC levels correlated with higher liver FABP1 such that loss of FABP1 upon ablation markedly increased brain EC levels [16, 17], approaching the levels observed in female brains.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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