Alteration of the Endocannabinoid System in Human Alzheimer's Disease Brain

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Abstract

There is a growing interest in the endocannabinoid (EC) system, which includes the cannabinoid receptors 1 & 2 (CB1R & CB2R) and the enzymes that degrade/regulate endogenous ligands. These enzymes include Fatty Acid Amide Hydrolase (FAAH), which degrades anandamide, a ligand for the CB1R, and Monoacylglycerol Lipase (MAGL), which degrades 2-arachidonoylglycerol, a ligand for the CB2R. Yet the roles of the CBR and enzymes in Alzheimer's Disease (AD) remain inconclusive and controversial. Therefore, our project objective was to investigate changes, if any, in endocannabinoid signaling within human Alzheimer's disease brains. We hypothesized (1) FAAH expression would correlate with a decrease in CB1R expression in AD brains (2). Furthermore, we expected sex-dependent differences as such differences have been shown in the EC in brains. Two EC proteins were analyzed in three different brain areas in 60 AD brain samples. Western blots were assayed, and densitometry was performed to quantify the bands of interest using the LI-COR software. Our research demonstrated that the EC is indeed altered in AD brains, with CB1R expression increasing in the hippocampus. Furthermore, we found a sex-dependent difference in the EC alteration, with females having a greater expression of CB1R in the hippocampus compared to males. Finally, a strong correlation was observed between CB1R expression and FAAH expression in the cortex, hippocampus, and cerebellum of males, but no such correlation existed in the female data. Therefore, we refuted our first hypothesis but accepted the second one. Our data suggests that CB1R expression is regulated by FAAH activity in males but not females. Our research provides evidence of the possibility that AD follows distinct progressions in males and females with the same pathophysiological late-stage symptoms.

Keywords: Alzheimer's Disease, Endocannabinoid System

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Introduction

Alzheimer's Disease

AD is a chronic neurodegenerative disease characterized by severe cognitive disabilities, including learning and memory impairments, associated with neurodegeneration and neuroinflammation, leading to severe memory loss, dependence, and eventually death (1). Amyloid β (Aβ) plaques & neurofibrillary tangles are the two hallmarks of AD pathology (1). Aβ plaques are formed via the amyloidogenic pathway in which amyloid precursor protein is sequentially cleaved by the enzymes β-secretase and γ-secretase (2). Neurofibrillary tangles are formed when the protein tau is excessively acted upon by tau kinases, becoming hyperphosphorylated. Because hyperphosphorylated tau is unstable, it aggregates to other tau proteins within close vicinity, forming clumps or tangles (3).

Endocannabinoid System

The EC is a system of lipid signalling found throughout the body derived from arachidonic acid derivatives and is a key mediator in the development of the central nervous system and involvement in several cognitive and physiological processes such as synaptic plasticity, neurodevelopment, and immunity (4). The two primary EC system receptors are the CB1 receptor (CB1R) and CB2 receptor (CB2R) (5). These receptors exhibit sex-dependent localization and belong to a family of G-protein coupled receptors, which are first activated by the attachment of an extracellular ligand to the receptor (5). Attachment causes the heterotrimeric G protein to become activated (present within the cell), inhibiting the enzyme adenyl cyclase (5). Upon inhibition of this enzyme, the MAPK pathway is activated, leading to a large and long-lasting intracellular response (5). The CB1R is densely located in the central nervous system, including the hippocampus and cerebral cortex, and is involved in a variety of executive functions such as cognition and memory (5). In contrast, the CB2R is primarily located in immune cells such as B cells and microglia and plays a vital role in immunological activity by protecting healthy neuronal cells (5). The two main endogenous ligands found in the EC system are arachidonoylethanolamide (AEA; anandamide) and 2-arachidonoylglyceroland (2-AG), and their major functions are involved in retrograde signalling by releasing from the post-synaptic cell (5). AEA has a high affinity for CB1R, whereas 2-AG has a high affinity for CB2R (6). AEA is converted into arachidonic acid and incorporated into membrane phospholipids through the hydrolysis of ester and amide bonds, facilitated by a catalyst (5). The enzyme responsible for AEA degradation is fatty acid amide hydrolase (FAAH) (6). Similarly, 2-AG is converted into glycerol or ethanolamine and incorporated into membrane phospholipids through the hydrolysis of ester and amide bonds, facilitated by a catalyst (5). The enzyme responsible for 2-AG degradation is monoacylglycerol lipase (MAGL) (6).

Hypothesis and Experimental Objectives

We hypothesized (1) FAAH expression would correlate with a decrease in CB1R expression in AD brains compared to neurologically normal controls. (2) We also hypothesized that there would be sex-dependent differences in the EC in AD brains.

The experimental objectives of this project were to (1) Understand the complex link between the EC and AD. (2) Investigate whether any changes in endogenous ligands AEA and 2-AG, measured indirectly using expression of degradation enzymes, would correlate with changes in CB1R expression in AD brains.

Relevance of Research

AD is one of the most prominent chronic neurodegenerative diseases, affecting over 46 million people worldwide (7). Because the aging population is at a higher risk of developing AD, AD is relevant now more than ever before (7). The United Nations predicts the number of elderly aged 60 and above to increase from 900 million in 2015 to over 2 billion by 2050 (7). Despite these alarming numbers, the origin, treatment, and prevention of AD are still largely unknown and yet to be fully characterized (7). A relatively recent area of research is the EC system; the link between the EC system and potential therapeutic targets in a variety of neurodegenerative diseases is an auspicious area of research (8). Gaining a better grasp of the involvement of the EC system signaling within

AD will provide promising information in the field of medicine and potential treatment or prevention opportunities (8).

Summary of Findings

We demonstrated that there is an increase in average CB1R expression for both early-onset Alzheimer's Disease (EOAD) and late-onset Alzheimer's Disease (LOAD) in the hippocampus, but not in the cortex or cerebellum, compared to controls. Additionally, the increase in hippocampal CB1R expression was detected primarily in females. Furthermore, while we did not observe any increase in CB1R expression in males, we did observe a very strong correlation between FAAH expression and CB1R expression in the cortex (EOAD & LOAD), hippocampus (LOAD), and cerebellum (EOAD) of males, but no correlation in females.

Materials and Methods

Materials

Two EC system proteins – i.e., CB1R and FAAH, and two housekeeping genes, GAPDH, and α-Tubulin – were analyzed in three different brain regions (the cortex, cerebellum, and hippocampus) in control and sex-matched AD brains. Sixty male and female samples were obtained from the Douglas Bell Canada Brain Bank, in which 24 were controls (neurologically normal), 16 were early-onset Alzheimer's Disease patients (EOAD – AD development before the age of 65), and 18 were late-onset Alzheimer's Disease patients (LOAD – AD development after the age of 65).

Methods

Western blots were assayed to measure levels of protein expression within the three different brain regions. Densitometry was then performed to quantify all six proteins of interest using a western blot quantification software known as LI-COR. Kruskal-Wallis (non-parametric), *post hoc* Dunn's multiple comparison test was performed to assess expression levels of proteins. Linear regression (Pearson's r) test was performed to investigate if any

correlation existed between the degradation enzymes and receptors.

Results

A. CB1R and FAAH Protein Expression Table

|			Cx:	Hippo:	Cereb:
CB1R	diag	P =	0.4830	0.0058	0.3951
	diagXsex	(M) P =	0.6593	0.5395	0.5022
		(F) P =	0.4656	0.0065	0.1658
FAAH	diag	P =	0.6319	0.5207	0.2344
	diagXsex	(M) P =	0.7913	0.8808	0.2337
		(F) P =	0.1727	0.1474	0.4582

Table 1: P values for CB1R and FAAH protein expression for pooled (diag) and sex separated (diagXsex) AD diagnosis groups stratified by brain region: Cx (cortex); Hippo (hippocampus); Cereb (cerebellum). (M): male data only; (F) female data only. Level of significance based on P < 0.05.

## B. CB1R and FAAH Pearson's r Correlation Table

	Cx:			Hippo:			Cereb:		
	CTL	EOAD	LOAD	CTL	EOAD	LOAD	CTL	EOAD	LOAD
(M) P=	0.1665	0.0051	0.0203	0.1903	0.6659	0.0329	0.0101	0.0026	0.9534
r2 =	0.1821	0.8182	0.6206	0.3829	0.0403	0.6309	0.4999	0.8607	0.0006
(F) P=	0.8077	0.1107	0.1294	0.6304	0.1302	0.7512	0.0218	0.8886	0.0432
r2 =	0.0056	0.3225	0.2632	0.0241	0.3386	0.0153	0.3665	0.0030	0.4186

Table 2: Correlation (Pearson's r) for CB1R and FAAH stratified by diagnosis: CTL (neurologically normal/control); EOAD (early-onset AD); LOAD (late-onset AD). (M): male data only; (F) female data
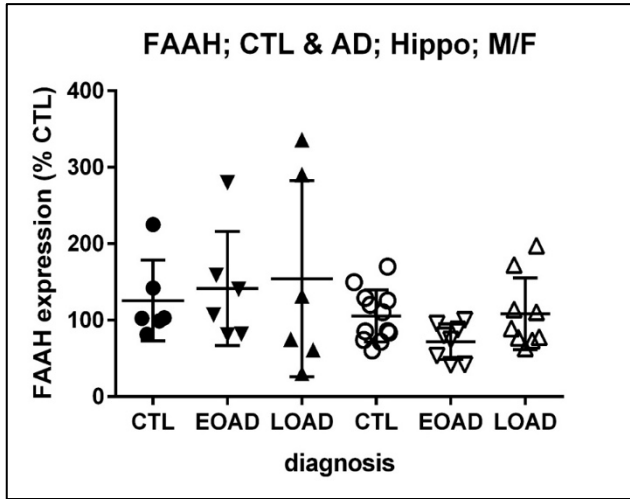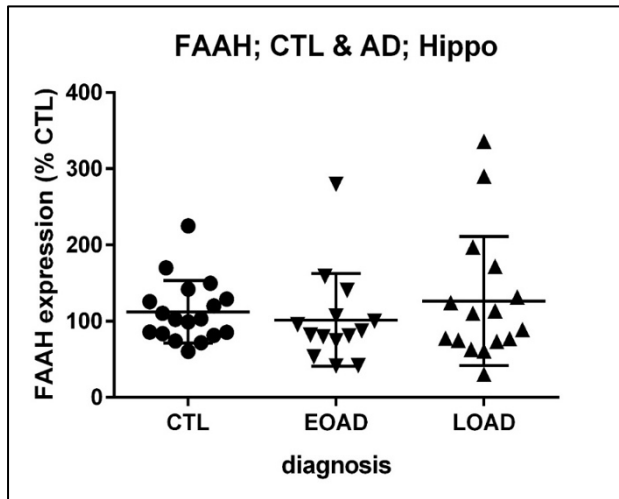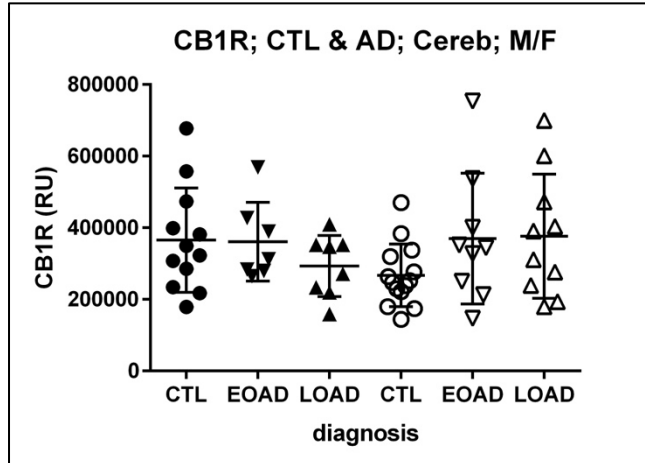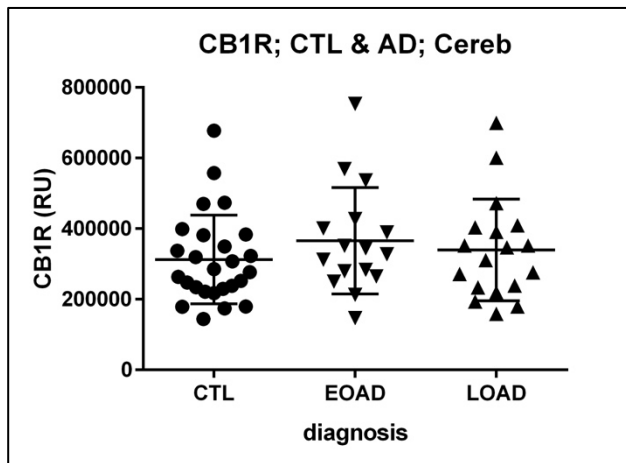
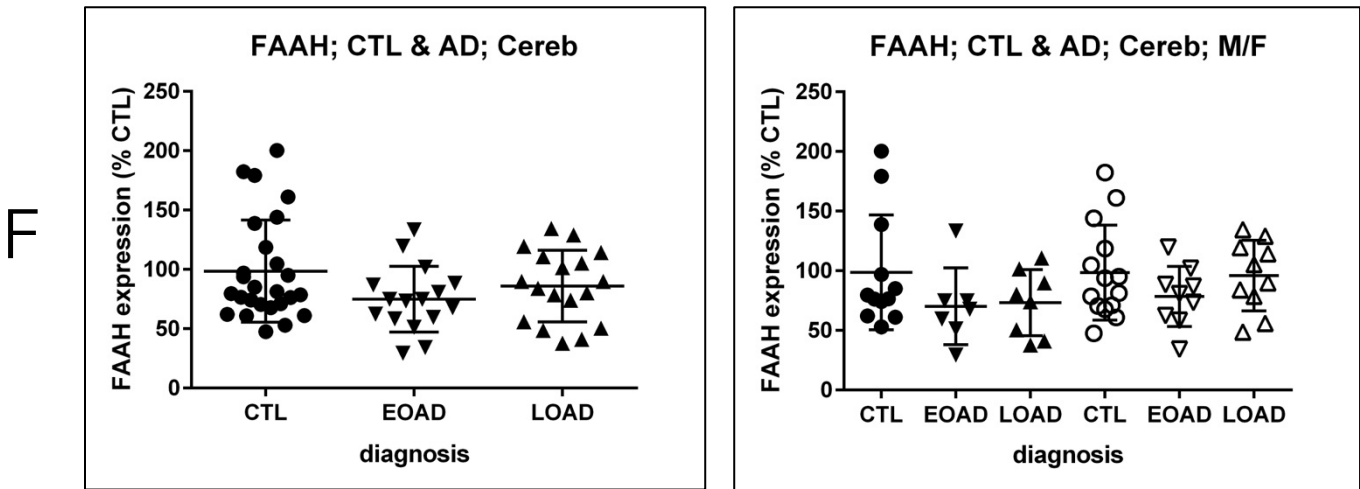## C. CB1R and FAAH Protein Expression Graphs
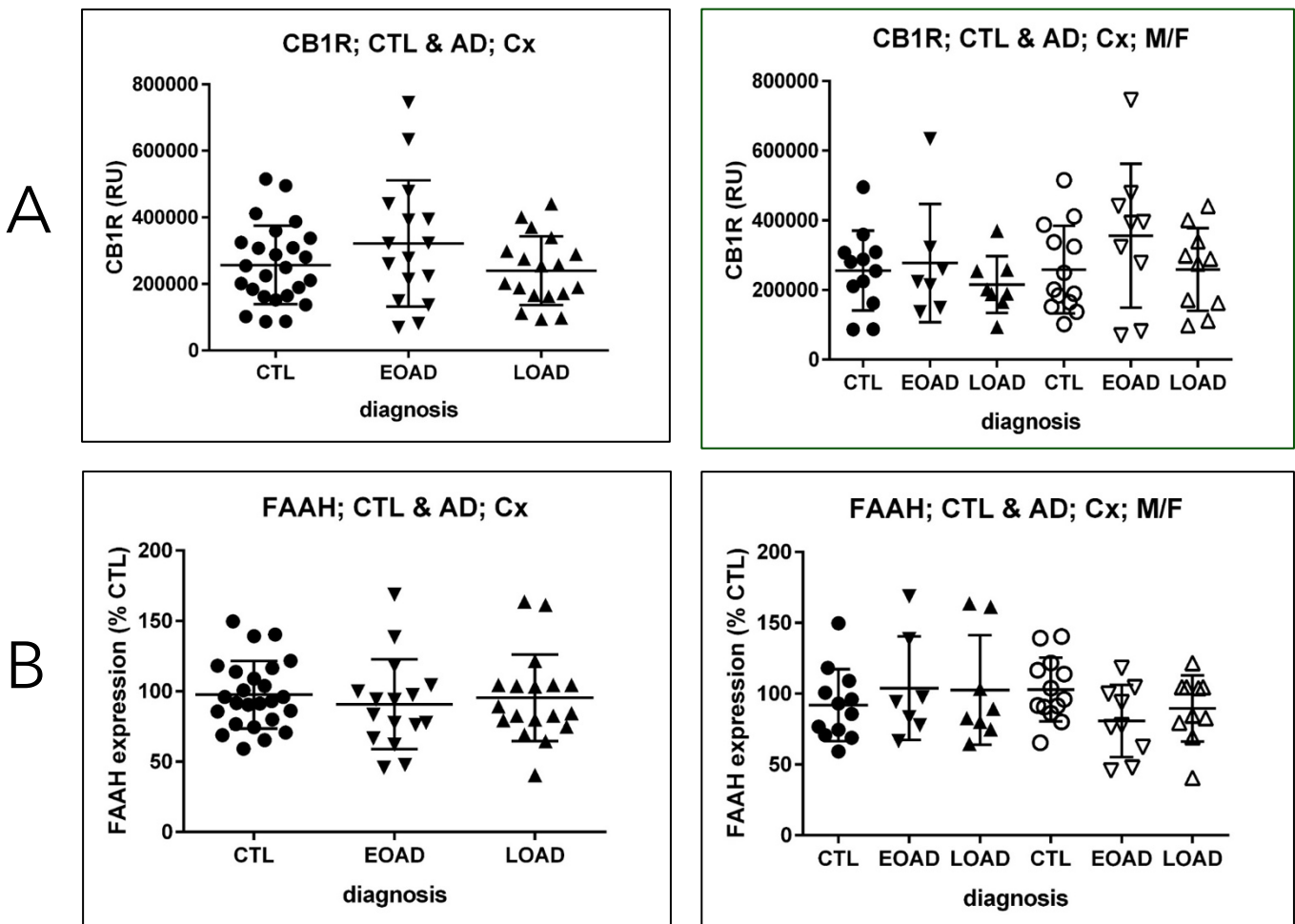
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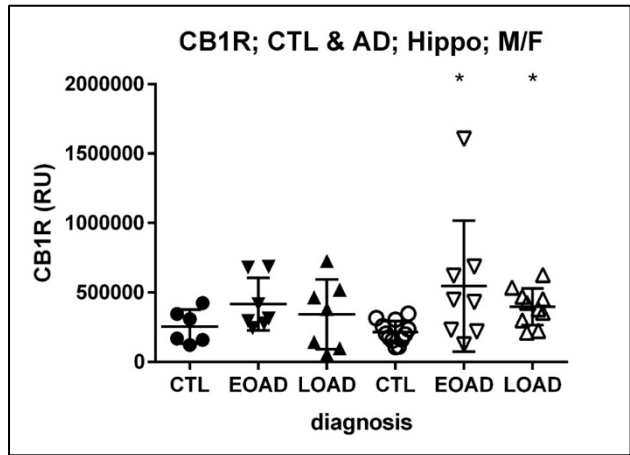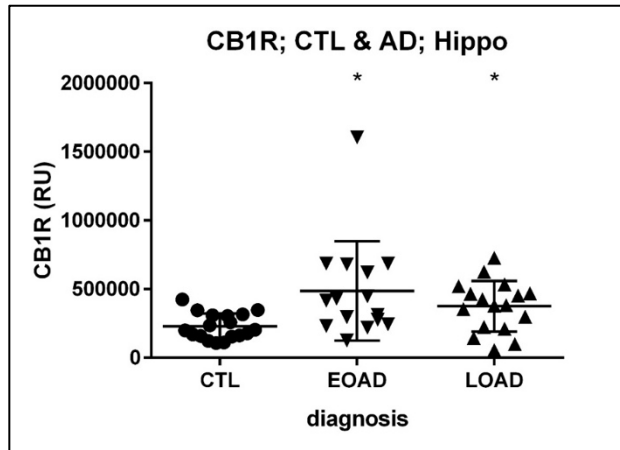
D

E

Figure 1. Expression levels of (A) CB1R and (B) FAAH in cortex, and (C) CB1R and (D) FAAH in the hippocampus, and (E) CB1R and (F) FAAH in the cerebellum of controls (CTL) and cases of early-onset AD (EOAD) or late-onset AD (LOAD). The left panel in each column represents pooled data; the right panel depicts the data by sex, *e.g.*, first three sets in each right graph are males and the second three sets are females. RU denotes relative units. Summary of statistics: (A) pooled: $P = 0.4830$; M: $P = 0.6597$; F: $P = 0.4656$; (B) pooled: $P = 0.6319$; M: $P = 0.7913$; F: $P = 0.1727$; (C) pooled: $P = 0.0058$; M: $P = 0.5395$; F: $P = 0.0065$; (D) pooled: $P = 0.5207$; M: $P = 0.8808$; F: $P = 0.1474$; (E) pooled: $P = 0.3951$; M: $P = 0.5022$; F: $P = 0.1658$; (F) pooled: $P = 0.2344$; M: $P = 0.2337$; F: $P = 0.4582$. Statistics: Kruskal-Wallis (non-parametric), *post hoc* Dunn's multiple comparisons test.

D. CB1R and FAAH Pearson's r Correlation Graphs
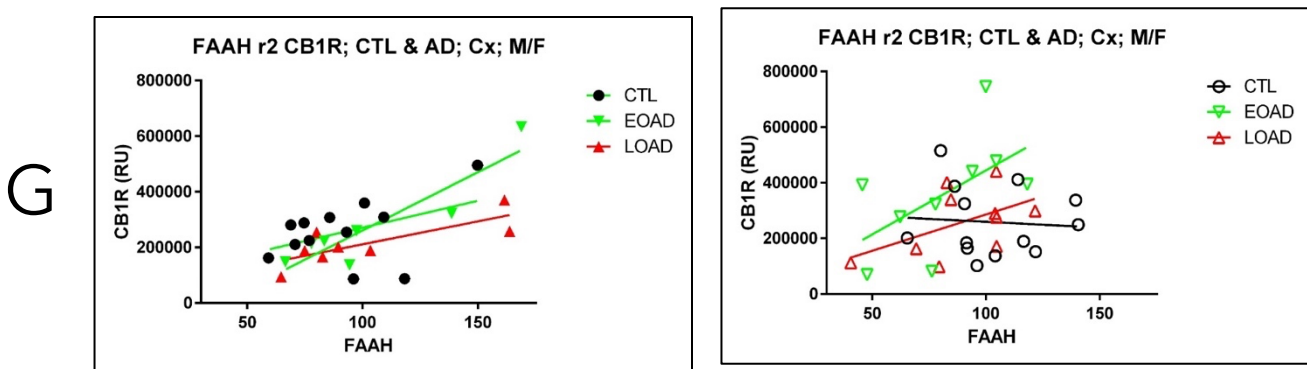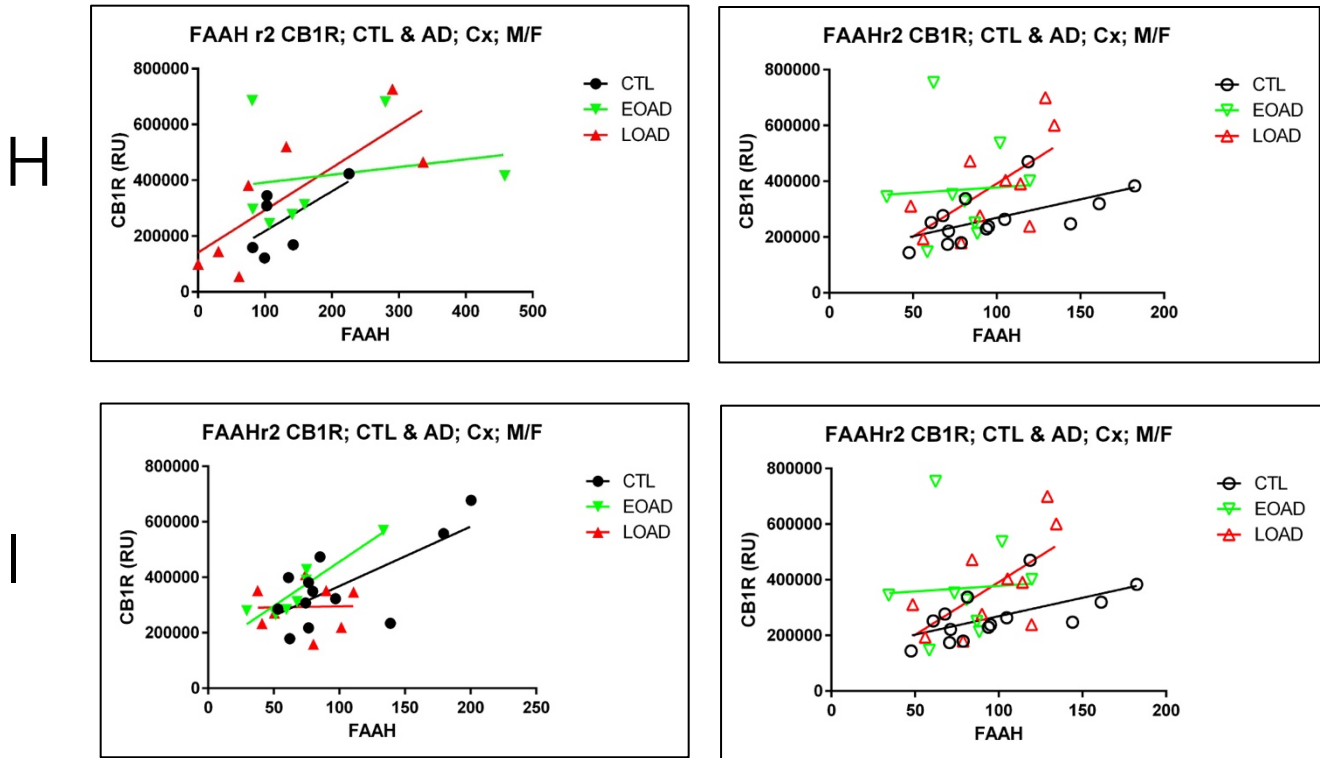
Figure 2. Linear regression (Pearson's r) showing correlation between CB1R expression and FAAH expression in males (left panel), and females (right panel), with AD in (G) the cortex, (H) the hippocampus, and (I) the cerebellum. RU denotes relative units. In cortex: males CTL [P = 0.1665, r = 0.1821], EOAD [P = 0.0051, r = 0.8182], LOAD [P = 0.0203, r = 0.6206]. In cortex: females CTL [P = 0.8077, r = 0.0056], EOAD [P = 0.1107, r = 0.3225], LOAD [P = 0.1294, r = 0.2632]. In hippocampus: males CTL [P = 0.1903, r = 0.3829], EOAD [P = 0.6659, r = 0.0403], LOAD [P = 0.0329, r = 0.6309]. In hippocampus: females CTL [P = 0.6304, r = 0.0241], EOAD [P = 0.1302, r = 0.3386], LOAD [P = 0.7512, r = 0.0153]. In cerebellum: males CTL [P = 0.0101, r = 0.4999], EOAD [P = 0.0026, r = 0.8607], LOAD [P = 0.9534, r = 0.0006]. In cerebellum: females CTL [P = 0.0218, r = 0.3665], EOAD [P = 0.8886, r = 0.0030], LOAD [P = 0.0432, r = 0.4186].

E. GAPDH and α-Tubulin Data

Housekeeping genes are consistently expressed protein and being used as the internal loading control in Western blotting experiments. The expression level of our target proteins was normalized to the band intensity of the housekeeping proteins (GAPDH, and tubulin).

Discussion

Experimental Findings and Interpretations

1. CB1R Expression Levels

   i. Cortex: The pooled data shows no statistically significant differences, although slightly greater variability is seen in the EOAD group compared to the CTL and LOAD groups. The data separated by sex does not show any statistically significant differences, although slightly greater variation is seen for the EOAD groups for both males and females.

   ii. Hippocampus: The pooled data shows a statistically significant difference, with both EOAD and LOAD groups showing higher expression levels of CB1R compared to the CTL. The data separated by sex also shows statistically significant differences, with females having higher expression levels of CB1R in both EOAD and LOAD groups compared to males.

   iii. Cerebellum: The pooled data shows no statistically significant differences, with slight variability in all three experimental groups. The data separated by sex does not show any statistically significant differences although males have more variability in the CTL group while females have more variability in the EOAD and LOAD groups.

2. FAAH Expression Levels

   i. Cortex: The pooled data does not show any statistically significant differences although there is much less variability seen in the CTL group. The data separated by sex does not show any statistically significant differences although males have more variability in the EOAD and LOAD groups while females have the least variability in the CTL group.

   ii. Hippocampus: The pooled data does not show any statistically significant differences, although slightly greater variability is seen in the LOAD group. The data separated by sex does not show any statistically significant differences, although males have significantly more variability in every experimental group compared to females.

   iii. Cerebellum: The pooled data does not show any statistically significant difference, with the most variability in the CTL group. The data separated by sex does not show any statistically significant differences, although both males and females have more variability in the CTL groups.

3. CB1R and FAAH Correlation

   i. Cortex: The linear regression (Pearson's r) revealed the data show a statistically significant positive correlation in the males EOAD ($r = 0.551$) and LOAD ($r = 0.5104$) groups, but no such correlation exists for the females.

   ii. Hippocampus: The linear regression (Pearson's r) revealed the data show a statistically significant positive correlation in the males LOAD ($r = 0.6070$) group, but no such correlation exists for the females.

   iii. Cerebellum: The linear regression (Pearson's r) revealed the data show a statistically significant positive correlation in the males EOAD ($r = 0.8607$) group, but no such correlation exists for the females.

Limitations

Although the samples were sex matched and categorized based on CTL, EOAD, and LOAD, they were not age matched (i.e., a sample of EOAD donors could have more age variability than CTL donors). Also, the female samples could be, on average, of greater age compared to the male samples. We suspect that older individuals would display more aggressive pathology due to a greater period of disease progression, which could potentially account for the differences seen in our data. Additionally, analyzing other endogenous ligands would be a stronger approach because although AEA is the main ligand

for the CB1R, other ligands such as N-arachidonoyl-dopamine (NADA) have affinity as well which could account for the changes in receptor expression observed (9). These ligands do, however, degrade rapidly post-mortem (within hours), so access to brain samples would be needed immediately upon death. Furthermore, the western blots analyzed with LI-COR would occasionally have minor stains or marks obstructing the bands of interest, affecting the quantification levels (i.e., a dark stain covering a portion of the band would cause the value recorded to be extremely high which could potentially skew the data in one direction).

Findings in Relationship to Literature

Published literature shows evidence of post-mortem samples of AD brains displaying a decrease in CB1 receptor expression compared to neurologically normal brains (10). These findings contradict our observations of increases in CB1R expression in the hippocampus of AD brains compared to neurologically normal controls. The evidence supporting the decrease in CB1R expression comes from the fact that CB1Rs are involved in cognition, emotion, memory, and a variety of other higher-order functioning (5). Many of these functions are declined in individuals suffering from AD and might be due to a decrease in CB1R levels (11). One theory, however, that has attracted recent attention and supports our findings of increased CB1R expression is decreased DNA methylation at the FAAH gene promotor (12). Decreased DNA methylation means increased transcription of the FAAH gene and ultimately increased levels of FAAH protein (12). The FAAH protein is responsible for the breakdown of AEA and increase in CB1R as seen in our data. Furthermore, published literature on CB1 receptor expression shows females having greater expression in the basal ganglia, hippocampus, and areas of the limbic system (13). These findings support our research data of sex-dependent differences of the EC in AD, with females having greater CB1 receptor expression in the hippocampus compared to males.

Conclusions

Based on the above findings, we refuted our first hypothesis that FAAH expression would correlate with a decrease in CB1R expression (we cannot comment on the relation between CB2R and MAGL as the samples are still being analyzed), but we accepted our second hypothesis that there are sex-dependent differences. This is because we observed an increase in CB1R expression in the hippocampus which was primarily due to females and a strong correlation between an increase in CB1R expression and FAAH expression in males with AD in all three brain regions. The CB1R expression increase likely reflects an increase in FAAH expression and loss of endogenous ligand AEA, the endogenous ligand for CB1R. Our data suggest that in males but not females, CB1R expression is regulated by FAAH activity. Our research also provides evidence of the possibility that AD manifests differently in males and females and follows distinct disease progression based on sex, but with the same pathophysiological late-stage symptoms observed by the time one goes to see a physician.

Future Directions

Our study utilized the degradation enzymes FAAH to indirectly assess levels of endogenous ligands; however, synthesis enzymes N-acyl-phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) for AEA and diacylglycerol lipase (DAGL) for 2-AG is a potential area of future investigation (14). Examining whether levels of synthesis enzyme expression complement the expected changes in endogenous ligands and EC receptors is a future avenue to explore. Additionally, no concrete explanation exists as to why females have higher expression of CB1R in AD compared to males; further research needs to be done to investigate the physiology behind these observed sex-dependent differences. Moreover, taking previously published data on the Aβ processing in AD and comparing it to the EC system changes shown from our research to investigate if any correlation exists is another potential area of future research. If a correlation exists, it is important to examine whether this correlation is confined to the hippocampus, cortex, or extends to all three brain regions, and whether it is a negative or positive correlation, involving CB1R, FAAH, or both. Finally, it is crucial to investigate new pharmacological therapeutics and their efficacy in a sex-dependent manner, initially in animal models and, if proven successful, subsequently in human clinical trials. Our research provides evidence that AD disease

pathology manifests differently in males and females; thus, tailoring treatment plans on a sex-dependent basis and examining their results is a promising area of future research.

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