TITLE: ROLE OF INTERLEUKIN 1-BETA IN THE INFLAMMATORY RESPONSE IN A FATTY ACID AMIDE HYDROLASE-KNOCKOUT MOUSE MODEL OF ALZHEIMER'S DISEASE

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ABSTRACT

The search for novel therapies for the treatment of Alzheimer's disease is an urgent need, due to the current paucity of available pharmacological tools and the recent failures obtained in clinical trials. Among other strategies, the modulation of amyloid-triggered neuroinflammation by the endocannabinoid system seems of relevance. Previous data indicate that the enhancement of the endocannabinoid tone through the inhibition of the enzymes responsible for the degradation of their main endogenous ligands may render beneficial effects. Based on previously reported data, in which we described a paradoxical effect of the genetic deletion of the fatty acid amide hydrolase, we here aimed to expand our knowledge on the role of the endocannabinoid system in the context of Alzheimer's disease. To that end, we inhibited the production of interleukin-1β, one of the main inflammatory cytokines involved in the neuroinflammation triggered by amyloid peptides, in a transgenic mouse model of this disease by using minocycline, a drug known to impair the synthesis of this cytokine. Our data suggest that interleukin-1β may be instrumental in order to achieve the beneficial effects derived of fatty acid amide hydrolase genetic inactivation. This could be appreciated at the molecular (cytokine expression, amyloid production, plaque deposition) as well as behavioral levels (memory impairment). We here describe a previously unknown link between the endocannabinoid system and interleukin-1β in the context of Alzheimer's disease that open new possibilities for the development of novel therapeutics.

Keywords

Fatty acid amide hydrolase, transgenic mice, amyloid, microglia, interleukin-1beta.

1. INTRODUCTION

The role of neuroinflammation in Alzheimer's disease (AD) is a subject of intense debate and analysis. The classical view dictates that the appearance of beta amyloid (Aβ) oligomers and the formation of amyloid-enriched neuritic plaques in the brain parenchyma trigger a myriad of deleterious effects and, among them, a powerful inflammatory response in which glial cells (and, remarkably, microglia) play a crucial role by secreting cytokines, activating membrane hemichannels, phagocytosing amyloid and cell debris, etc [1]. As this inflammatory process extends in time, the neuronal damage in surrounding areas gets increased and inflammation perpetuates the process [2]. In this context, the use of antiinflammatory drugs has been postulated, but clinical and preclinical data have not been conclusive so far [3].

Under this conceptual paradigm, the activation of neuroprotective endogenous mechanisms, such as the endocannabinoid system (ECS), seems of great interest. The ECS is comprised of receptors, endogenous ligands and synthetic and degradative enzymes that has been shown to provide neuroprotection under different types of brain insult [4]. Among other possible approaches, the enhancement of the EC tone through the inhibition of the main degrading enzymes for these endogenous ligands is presently considered a promising approach in AD, as several recent studies have shown the beneficial effects derived of the genetic or pharmacological inhibition of monoacylglycerol lipase (MAGL, the main degradative enzyme for 2-arachidonoylglycerol) or fatty acid amide hydrolase (FAAH, for *N*-arachidonoylethanolamine, AEA) [5–7].

Regarding FAAH, we recently reported that its genetic inactivation led to a pro-inflammatory phenotype in the context of AD, both in vitro as well as in vivo [7,8]. Astrocytes in primary culture obtained from neonatal $FAAH^{-/-}$ mice exhibited an enhanced secretion of inflammatory cytokines (IL1β, IL6, CCL-2, etc) that was accompanied by an increased expression of COX-2, iNOS or TNFα when exposed to the pathological form of the beta amyloid peptide. Further, several signaling cascades known to be directly involved in amyloid-triggered neuroinflammation (p38 MAPK, ERK 1/2 and NFkB) were also proved to be earlier, and more intensely, activated in $FAAH^{-/-}$ than in WT astrocytes after exposure to Aβ. Interstingly, this exacerbated pro-inflammatory response was only evident in FAAH-lacking astrocytes, but not present when this enzyme was subjected to pharmacological inhibition with the well-described irreversible inhibitor, URB597 [8].

These data were subsequently confirmed and expanded by using an animal model of AD [7]. Thus, samples from cortices and hippocampi of 5xFAD/$FAAH^{-/-}$ brains showed increased mRNA levels of several key cytokines (IL1β, IL6, and TNFα) that were associated to a significant decrease in APP, in amyloid-enriched neuritic plaques as well as in $A\beta_{1-40}$ and $A\beta_{1-42}$ levels. Finally, this enhanced inflammatory milieu was paradoxically accompanied by decreased astro- and micro-gliosis and by an improved memory-related behavioral performance. Altogether, these data led us to conclude that FAAH gene deletion had a profound effect in the cerebral inflammatory status in the context

of AD, involving several cytokines known to play a critical role in this disease, including IL1β [7].

IL1β is a cytosolic cytokine with pleiotropic functions in the brain, including neuronal proliferation, differentiation, apoptosis, and long-term potentiation [9]. The canonical synthetic route for this cytokine shows that it is synthesized in a precursor, biologically inactive form (pro-IL1β) that will be subsequently cleaved by an intracellular enzyme (IL1β-converting enzyme, also identified as caspase-1) to generate the mature, fully active peptide [10]. The mechanisms controlling the release of IL1β from cells in the CNS are still a matter of controversy, ranging from plasma membrane translocation, exosomes or other forms of secretory microvesicles, and being microglia the main source of this cytokine in the context of neuroinflammation [9][11]. Importantly, previous in vitro reports pointed out to a direct interaction between the ECS and the IL1β signaling pathways in the control of cell damage [12] and, more recently, neurogenesis [13].

The present study was designed to deeper explore the exacerbated inflammatory phenotype observed in the absence of FAAH as well as the interplay between the ECS and IL1β in the context of Aβ-induced neuroinflammation. To that end, we used minocycline to impair IL1β synthesis in a mouse model of AD and analyzed the molecular changes that could differentially develop in WT vs FAAH-lacking mice.

2. MATERIALS AND METHODS

2.1. Mice and minocycline treatments

Mice used in these studies were described in our previous study [7] and were housed and bred in the animal facilities of Universidad Rey Juan Carlos (Alcorcón, Madrid, Spain). Experimental protocols met the European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005 and 53/2013). 6-month old WT and 5xFAD mice (B6SJL-Tg (APPSwFlLon,PSEN1*M146L*L286V) 6799Vas/ Mmjax; Jackson Laboratories; Bar Harbor, ME, USA; [14]) were used for the present studies. 5xFAD mice overexpress the human APP gene with 3 Familiar Alzheimer's Disease (FAD) mutations, as well as the human PS1 gene containing two FAD mutations, both of them under the control of mouse *Thy1* promoter. 5xFAD mice were backcrossed with $FAAH^{-/-}$ mice (which have replaced the first FAAH exon; [15]) for at least 10 generations, so that to obtain the 5xFAD/$FAAH^{-/-}$ counterparts. Mice of each genotype were sub-divided in two groups: 5xFAD and 5xFAD/$FAAH^{-/-}$ vehicle (veh), treated with NaCl 0,9%, and 5xFAD and 5xFAD/$FAAH^{-/-}$ minocycline (mino), treated with 10mg/kg/day of minocycline hydrochloride (Sigma Aldrich, St Louis, MO, USA), as described by [16]. The experimental protocol consisted of injecting intraperitoneally either the drug or the vehicle once a day during 12 consecutively days. Two hours after the last dose, mice were sacrificed by cervical dislocation, and brains were quickly removed, and divided in hemispheres: one of them was embedded in paraffin for staining, and the other one was frozen in isopentane, and stored at -80^{o}C.

The procedure followed to prepare the minocycline solution comprise the reconstitution of the minocycline hydrochloride in NaCl 0,9% at a concentration of 1,5 mg/mL, and the resulted fresh solution was later adjusted at a pH of 7.4, and stored at -80^{o}C until use. Minocycline-treated mice received a dose of 200 µL per 30g weight, while vehicle animals were treated with the corresponding volume of NaCl 0,9%. Weight of animals was measured every day in order to inject the correct volume and dose, and for the detection of any symptom of toxicity [17].

2.2. Behavioral determinations

Memory performance was evaluated as described in our previous work [7] and based on the Morris water maze test (MWM) method described by Vorhees and Williams [18]. On day 8 of treatment with minocycline or vehicle (N=9-11 per group), spatial memory acquisition was tested for four consecutive days, four trials per day. Mice were released into opaque water facing the tank wall starting from different positions each day and were allowed to swim for a maximum of 60 seconds in order to reach a hidden platform. The parameter analysed in the present experiments was the time spent in finding the hidden platform (escape latency). Data were analysed by using the Smart 3.0 software (Panlab, Barcelona, Spain).

2.3. Isolation of microglial cells and flow cytometry

To facilitate the separation of microglia, brains were dissected and enzymatically digested. Then, the resulting suspension was mechanically dissociated and filtered through a 70-µm cell strainer. Cells were incubated

immediately with CD11b MicroBeads and were separated in a magnetic field using MACS Column (Miltenyi Biotec, Madrid, Spain). Both CD11b-negative and CD11b-positive effluent fractions were collected for further characterization. Additionally, cells were stained with CD11bPE (Miltenyi), CD45-APCVio770 (Miltenyi), CD16/32-PerCP-Cy5.5 and CD206-APC (both from Invitrogen, Carlsbad, CA, USA) antibodies. Samples were read on a MACSQuant Flow Cytometer and analysed with MACS Quantify (Miltenyi Biotec).

Fluorescence spillover compensation values were generated using pooled unseparated/enriched cell preparations rather than commercially available beads to account for the high autofluorescence of myeloid cells. Debris and aggregates were eliminated from the analysis by forward and side scatter characteristics, then microglia was identified as $CD11b^{+}$ $CD45^{lo}$. Polarization states of $CD11b^{+}CD45^{lo}$ microglia were evaluated by expression of the M1 marker CD16/32 and the M2 marker CD206 with gates established by fluorescence minus one (FMO) controls. For each hemisphere, approximately ten thousand $CD11b^{+}$ singlets were analyzed.

2.4. mRNA isolation and RT-qPCR

One frozen hemisphere of each minocycline and vehicle-treated animal was dissected so that to recover the cortex and hippocampus areas. Then, Tripure Isolation Reagent (Roche Diagnostics, Manheim, Germany) was used so as to isolate total RNA for further qPCRs assays. After RNA isolation, its quality was ensured by a 1% agarose gel electrophoresis, while its quantity was measured by spectrophotometry at 260 nm. Single-strand complementary DNA (cDNA)

was synthesized from 1 µg of total RNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche). The amount of expression of the different genes and cytokines of interest was quantified by qPCR assays in a LightCycler® 480 Instrument II (Roche). PCR primers and TaqMan probes for TNFα and COX2 were designed by Tib Molbiol (Berlin, Germany), and were all employed at 0.5 µM. IL1r and IL1ra were analysed by using a RealTime ready Single Assays (assay IDs: 312175 and 318527, correspondingly, Roche), while IL1β, IL10 and IL4 were quantified by employing Predesigned qPCR Assays (assay IDs: Mm.PT.58.42962427, Mm.PT.58.13531087 and Mm.58.7882098, respectively, IDT®, Coralville, IA, USA). 18S was chosen for normalization. PCR assays were performed using LightCycler® TaqMan® Master (Roche) and Quantimix Easy Probes Kit (Biotools, Madrid, Spain). All assays, except the corresponding to IL10 and IL4, were carry out with 2 µL of cDNA, whereas IL10 and IL4 assays needed 4 µL. Because of the use of a 96-well plate, the qPCR reaction was run in duplicates for each sample, including the positive (a sample known for showing high expression of all the genes tested) and negative control (no-template wells). The transcript amounts were calculated using the second derivative maximum mode of LC-software, version 1.5 (Roche), and later analysed by the $2^{-\Delta\Delta Ct}$ algorithm, so that to obtain the Relative Quantification (RQ) values. Further calculations and statistical analysis were done with these RQ values.

2.5. Neuritic plaque quantification

Paraffin-embedded tissues (N=3-4 per group) were cut in 4µm slices and transferred to slides coated with Vectabond (Vector Laboratories, Burlingame,

CA, USA). Once deparaffined and rehydrated, amyloid aggregates were labelled with methoxy-X04 staining, as described [19]. Slides were studied and photographed with an upright microscope (Nikon 90i, Nikon, Tokyo, Japan) and using a DXM1200F camera. Quantification of the slides was performed with Metamorph (Molecular Devices, Sunnyvale, CA, USA) and ImageJ (NIH, Baltimore, MD, USA) software. Images were converted to 8-bit grayscale for analysis. Next, a threshold value was set (to highlight the signal corresponding to stained cells only) and the region of interest outlined by using the polygon selection tool (ROI tools). The area occupied by methoxy-X04-labelled plaques, was quantified and divided by the total area of the selected region.

2.6. $A\beta_{1-42}$ quantification by ELISA

To determine $A\beta_{1-42}$ levels in brain tissue, hippocampus and cortex were homogenized in 10 volumes of ice-cold guanidine buffer (5.0 M guanidine·HCl/50 mM Tris·Cl, pH 8.0) containing protease inhibitor cocktail (Roche). Protein concentrations were determined by BCA assay (Thermofisher, Waltham, MA, USA). Human ELISA kits (Invitrogen) were used according to manufacturer's specifications. Optical signals at 450 nm were read on a Sunrise microplate reader (Tecan, Männedorf, Switzerland) and sample concentrations were determined by comparison with the respective standard curves.

2.7. IL1β quantification by ELISA

Brain cortex was homogenized with ice-cold lysis buffer [25 mM Hepes (pH 7.5), 150 mM NaCl, 1% (vol/vol) IGEPAL CA-630, 10 mM MgCl2, 1 mM EDTA, and 2% (vol/vol) glycerol] containing protease inhibitor cocktail (Roche), and

clarified by centrifugation at 14,000 *g* at 4°C for 15 minutes. Protein concentrations were determined by BCA assay (Thermofisher). IL1β levels in the tissue lysates were measured using the RayBio® Mouse IL-1 beta ELISA kit (RayBiotech, Inc., Norcross, GA, USA) following the manufacturer's protocol. Optical signals at 450 nm were read on a Sunrise microplate reader (Tecan) and sample concentrations were determined by comparison with the respective standard curves.

2.8. Statistics

Results are expressed as mean ± SEM. Statistical analysis were made using 2-way analysis of variance (ANOVA) with Bonferroni's post-test for multiple comparisons. A p value <0.05 was considered as statistically significant. Data were analysed with Graph Pad Prism software version 6.0 (San Diego, CA, USA).

3. RESULTS

3.1. The phenotype associated to FAAH$^{-/-}$ mice is proinflammatory and affects microglial cells

We analyzed the M1/M2 profile of microglial cells in the brains of WT and FAAH-lacking mice by flow cytometry (figure 1). As expected, we found a significant increase in the M1 to M2 ratio in AD brain samples as compared to non-pathological ones, both in WT and FAAH$^{-/-}$ mice. Importantly, there was a significant difference in this ratio among 5xFAD vs 5xFAD/FAAH$^{-/-}$ mice (figure 1A), indicating an exacerbated pro-inflammatory state associated to FAAH gene deletion. Surprisingly, this increase in the M1/M2 ratio was also accompanied by a significant decrease in the number of microglial cells isolated from samples obtained from FAAH-lacking mice, suggestive of decreased microgliosis (figure 1B). We must then conclude that the absence of FAAH partially prevents the microgliosis that is prototypical of amyloid pathology but also triggers an imbalance of the microglial phenotype towards an exacerbated pro-inflammatory state, as revealed by the increased M1 over M2 markers.

In addition, we focused on the pattern of expression of several pro- and anti-inflammatory cytokines known to play a relevant role in the pathogenesis of AD and produced mostly by glial cells, including IL1β, TNFα, IL10 and IL4 (figure 2). Our data show that the genetic deletion of FAAH has a differential effect on the expression levels of these cytokines, by promoting the expression of pro-inflammatory cytokines (IL1β and TNFα) while inducing a decrease in that of anti-inflammatory ones (IL10 and IL4). Thus, as expected, both IL1β and TNFα

mRNA levels were significantly elevated in the brains of AD mice but were further increased in samples from 5xFAD/FAAH$^{-/-}$ mice as compared to 5xFAD mice (figure 2A and B). On the other hand, IL10 and IL4 were also increased as a consequence of the pathology, but only in 5xFAD, not in 5xFAD/FAAH$^{-/-}$ mice (figure 2C and D). IL10 mRNA levels were significantly lower in the absence of FAAH as compared to 5xFAD. These data show that the genetic deletion of FAAH alters the expression pattern of glial cytokines and promotes inflammation.

3.2. IL1β system is altered in FAAH$^{-/-}$ mice and is partiatlly normalized by minocycline exposure

We next studied the status of the IL1β system in the brains of FAAH-lacking mice and analyzed the impact of the treatment with the IL1β synthesis inhibitor, minocycline. We thus quantified the expression levels of several elements of this system, including IL1β, IL1βr and IL1βra (figure 3). We found that mRNA levels of IL1β and IL1βr were significantly elevated in brain samples of 5xFAD/FAAH$^{-/-}$ mice as compared to 5xFAD mice (figure 3A and B), while those of the IL1βra showed no difference (figure 3C). Finally, IL1β protein levels were not modified by FAAH gene deletion (figure 3D). These data confirm previous observations indicating that the genetic deletion of FAAH leads to an exacerbated expression of some elements of the IL1β transmission system without reflect in protein levels.

In addition, we also measured the impact that the exposure to minocycline, a compound known to impair IL1β synthesis, may have on these observations.

We found that increased IL1β mRNA levels were normalized after treatment with minocycline (figure 3A) while those of IL1βr also showed similar levels to those observed in 5xFAD mice (figure 3B). IL1βra mRNA levels did not change after minocycline treatment (figure 3C). Finally, IL1β protein levels were significantly decreased after the treatment, confirming the efficacy of minocycline regarding the inhibition of IL1β synthesis (figure 3D).

3.3. Minocycline treatment triggers opposite effects in 5xFAD vs 5xFAD/FAAH$^{-/-}$ mice

We also studied the effects of the treatment with minocycline at the behavioral and molecular levels in respect to the amyloid pathology. Our data show that, in concordance with previous reports, minocycline treatment improved memory in 5xFAD mice (figure 4A) as showed in the MWM test. In contrast, 5xFAD/FAAH$^{-/-}$ mice showed a worsening in memory-related behavior in the same test after exposure to minocycline (figure 4A).

In addition, minocycline treatment induced a significant decrease in amyloid plaques (figure 4B) in 5xFAD but had the opposite effect on 5xFAD/FAAH$^{-/-}$ mice. In addition, cortical and hippocampal soluble A$\beta_{1\text{-}42}$ levels in 5xFAD mice were significantly higher in 5xFAD as compared to 5xFAD/FAAH$^{-/-}$ mice and remained unchanged under minocycline treatment (figures 4C and D). Finally, the same treatment regime led to significant increases in amyloid soluble levels in 5xFAD/FAAH$^{-/-}$ mice (figures 4B to D).

4. DISCUSSION

The present data confirm previous reports showing that the genetic inactivation of FAAH leads to a pro-inflammatory, yet beneficial, state in the context of AD and suggesting that IL1β may be instrumental in this phenomenon [7,20]. We first confirmed the exacerbated inflammatory status associated to the genetic deletion of FAAH in a mouse model of AD (5xFAD mice) by studying: i) the phenotypic profile of microglial cells by flow cytometry; and ii) the expression levels of pro- and anti-inflammatory cytokines. We then used the highly lipophilic derivative of tetracycline, minocycline, to interfere the synthesis of IL1β and analyzed the effects of this impairment on the effects associated to the genetic deletion of FAAH. The present results confirm that the blockade of IL1β synthesis prevents the beneficial effects observed in FAAH-lacking mice in terms of cytokine expression, amyloid deposition, and behavior.

Firstly, we found that FAAH-lacking mice exhibited a significantly higher M1/M2 ratio of microglial cells as compared to their WT counterparts. This observation is in agreement with previous data from our group [7,21] and from others [20] and confirm the exacerbated inflammatory state derived of the genetic inactivation of FAAH. Furthermore, microglial cells were found to be significantly less abundant in the brains of 5xFAD/FAAH$^{-/-}$ mice than in 5xFAD mice. This decreased microgliosis was quantified by flow cytometry and confirms our previous observation by means of Iba1^{+} cell densitometry [7], though is in contrast with another paper in which an increased microgliosis in FAAH-lacking mice was reported [20]. This paradoxical observation (less number of microglial

cells but exacerbated inflammatory M1 phenotype) matches with our previously suggested hypothesis that FAAH gene deletion has a profound, long-term effect on microglial-mediated neuroinflammation, and that the FAAH-lacking brain has a net pro-inflammatory status that is evidenced when an acute as well as chronic challenge takes place [7,21].

Furthermore, we here report changes in the cytokine and amyloid levels of the AD-inflammed brain that can be associated to the absence of FAAH activity. Thus, while mRNA levels of the pro-inflammatory cytokines IL1β and TNFα were significantly elevated in FAAH-lacking mice, those of anti-inflammatory cytokines were decreased (IL10), or at least not elevated (IL4), as a consequence of the amyloid pathology in these mice. These changes were also accompanied by modifications in the expression pattern of another element of the IL1β system (IL1βr). Taken together, these data clearly point out to a pro-inflammatory milieu in the brains of $FAAH^{-/-}$ mice in the context of AD. It is of special relevance to note that IL10 has been recently attributed a critical role in amyloid pathology [22–24]. Surprisingly, these reports indicated that increasing IL10 levels had a significant negative impact on amyloid pathology in terms of behavior, amyloid plaques formation or Aβ phagocytosis [22]; in line with this, IL10 deficiency had a significant positive influence on amyloid pathology in APP/PS1 mice [23]. These observations allow us to speculate that the relative decrease in IL10 mRNA levels in 5xFAD/$FAAH^{-/-}$ mice herein reported may be one of the causes of the decreased amyloid pathology observed in these mice.

The role of inflammation, and specifically of IL1β, in the context of AD is a subject of intense debate [25]. Though the classic perspective pointed out to a neuroprotective role of the early inflammatory response, and attributed deleterious effects to the sustainably increased production of pro-inflammatory cytokines [2,26], recent findings have paved the way for new approaches to this topic. Elegant studies by Shaftel et al [27] and Matousek et al [28] showed that the hippocampal overexpression of IL1β in the brains of APPswe/PS1dE9 mice drives a beneficial effect, reflected mainly at the molecular level. Paradoxically, the same group reported a COX-1-mediated impairment of memory in this paradigm of exacerbated IL1β hippocampal activity [29].

Our present data show that IL1β mRNA levels were significantly higher in 5xFAD/FAAH$^{-/-}$ mice while those of the protein remained unaltered, as already observed in our previous study [7]. We do not have a plausible explanation for this discrepancy, other than differences in the sensitivity of the methods employed or the existence of putative post-translational mechanisms that might dampen the increase in the expression levels of the cytokine. It is worth to mention that IL1β concentration has been shown critical in order to enhance or inhibit hippocampal LTP through NMDARs, having an impact on memory processing [30]. It can thus be speculated that the increase in mRNA levels observed in 5xFAD/FAAH$^{-/-}$ mice could lead to a subtle increase in IL1β protein at the synaptic level (though not significant when measured in tissue samples), that in turn could enhance LTP in these mice. The putative increase in IL1βr could also collaborate in this effect. Preliminary data obtained in our laboratory

suggest a recovery of LTP in the CA1 region of 5xFAD/FAAH$^{-/-}$ mice as compared to 5xFAD mice (Ruiz-Pérez et al, in preparation).

The interaction between the ECS and IL1β has been studied for the last few years in in vitro experiments [12] as well as in the context of multiple sclerosis [31–33] and point to an intimate interplay between the ECS and the IL1β transmission system in the CNS. Cannabinoid CB_1 and TRPV1 receptors have been involved in this interaction that has been mainly observed by electrophysiological and behavioral methods. In addition, blockade of cannabinoid CB_1 receptors was shown to prevent the beneficial effects of minocycline in a mouse model of brain edema [34]. Due to the paucity of available pharmacological tools capable of modifying the activity of the IL1β-controlled transmission and, more specifically, the inability of current IL1r antagonists to cross the BBB when administered systemically, we decided to explore the putative consequences of impairing IL1β synthesis by using the well-known drug minocycline [35]. This compound is widely used in different experimental paradigms because of its neuroprotectant properties; in the context of AD, minocycline has been shown to provide neuroprotection, prevent Aβ deposition, and improve cognitive functions in mice models of AD [17,36]. Importantly, minocycline has been also shown to block the activity of the caspase-1, also called IL1β-converting enzyme, that plays a key role in the process of maturation of this cytokine from its precursor form (pro-IL1β) to its fully functional, mature, variant (IL1β) [16]. Thus, minocycline is capable of decreasing the levels of mature IL1β. Our data showed that minocycline was effective in impairing IL1β synthesis in both 5xFAD and 5xFAD/FAAH$^{-/-}$ mice

and that this effect was also accompanied by an improvement in 5xFAD mice and a worsening in 5xFAD/FAAH$^{-/-}$ mice in the WM memory test. These observations match with previously published data [37] and suggest that mice lacking FAAH exhibit an exacerbated sensitivity to minocycline treatment and, more importantly, that this impairment is detrimental in the context of AD.

In summary, our present findings corroborate that the genetic deletion of FAAH has a pro-inflammatory effect in the brain of a transgenic mouse model of AD that, paradoxically, has beneficial consequences. In addition, we describe a putative link between IL1β and the ECS in the mediation of these effects. Further experiments should unveil the precise role of IL1β signaling in the effects triggered by an enhancement of the endocannabinoid tone in the inflamed mouse brain.

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Conflicts of interest

The authors declare no competing financial interests.

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LEGENDS TO FIGURES

Figure 1: Flow cytometry data reveal the pro-inflammatory status of the FAAH-lacking microglia cells and a decreased number of these cells as a consequence of FAAH gene deletion. Ratio of M1 (defined as CD11+CD45lo/CD16/32+) to M2 (defined as CD11+CD45lo/CD206+) in Mean Fluorescence Intensity (MFI) units **(A)** was elevated in 5xFAD mice and significantly increased in 5xFAD/FAAH$^{-/-}$ mice. measured by flow cytometry analysis. Quantification of microglial cells **(B)** (expressed as the percentage of total singlet cells that are CD11+CD45lo cells) increased significantly in 5xFAD mice while this number were significantly lower in the brains of FAAH-lacking AD mice. **(C)** Histogram comparing expression by MFI in microglia (CD11+CD45low gate) from mice 5xFAD (red line) or 5xFAD/FAAH$^{-/-}$ (magenta line) representative of 5 independent experiments. N=5 mice per group. *$p<0.05$ (2-way ANOVA followed by Bonferroni's post hoc test).

Figure 2: The genetic deletion of FAAH increases the expression of pro-inflammatory cytokines while decreases that of anti-inflammatory cytokines in the context of AD. IL1β and TNFα mRNA levels **(A and B)** were significantly elevated as a consequence of the amyloid pathology; in addition, 5xFAD/FAAH$^{-/-}$ mice showed an exacerbation in the increased levels in respect to 5xFAD mice. The mRNA levels of the anti-inflammatory cytokines IL10 **(C)** and IL4 **(D)** were differentially regulated, with significant increases in 5xFAD mice but non-significant in the case of 5xFAD/FAAH$^{-/-}$ mice. Further, IL10 were significantly lower in the brains of FAAH-lacking AD mice. A.u. stands for "arbitrary units".

N=8 mice per group. *p<0.05 (2-way ANOVA followed by Bonferroni's post hoc test).

Figure 3: Effects of the genetic deletion of FAAH and of the treatment with minocycline on several elements of the IL1β signaling system in the context of AD. IL1β **(A)** and IL1βr **(B)** mRNA levels were significantly higher in 5xFAD/FAAH$^{-/-}$ than in 5xFAD mice. Minocycline treatment decreased mRNA levels. IL1βra mRNA levels **(C)** remained unchanged. **(D)** The levels of IL1β protein were significantly decreased by the minocycline treatment. A.u. stands for "arbitrary units". N=8 (A to C) or N=5 (D) mice per group. *p<0.05 (2-way ANOVA followed by Bonferroni's post hoc test).

Figure 4: Exposure to minocycline induces opposite effects in AD mice, depending on FAAH activity. **(A)** MWM data reflect a significant improvement in 5xFAD mice treated with minocycline on day 3 of test while showing a significant worsening in memory performance in 5xFAD/FAAH$^{-/-}$ mice on days 3 and 4. **(B)** Relative amyloid plaque area was significantly reduced in 5xFAD mice and significantly increased in 5xFAD/FAAH$^{-/-}$ mice. Cortical **(C)** and hippocampal **(D)** soluble amyloid levels were significantly lower in 5xFAD/FAAH$^{-/-}$ mice and experienced a significant increase as a consequence of the treatment with minocycline. A.u. stands for "arbitrary units". N=8-11 (A) or N=5 (B to D) mice per group. *p<0.05 (2-way ANOVA followed by Bonferroni's post hoc test).

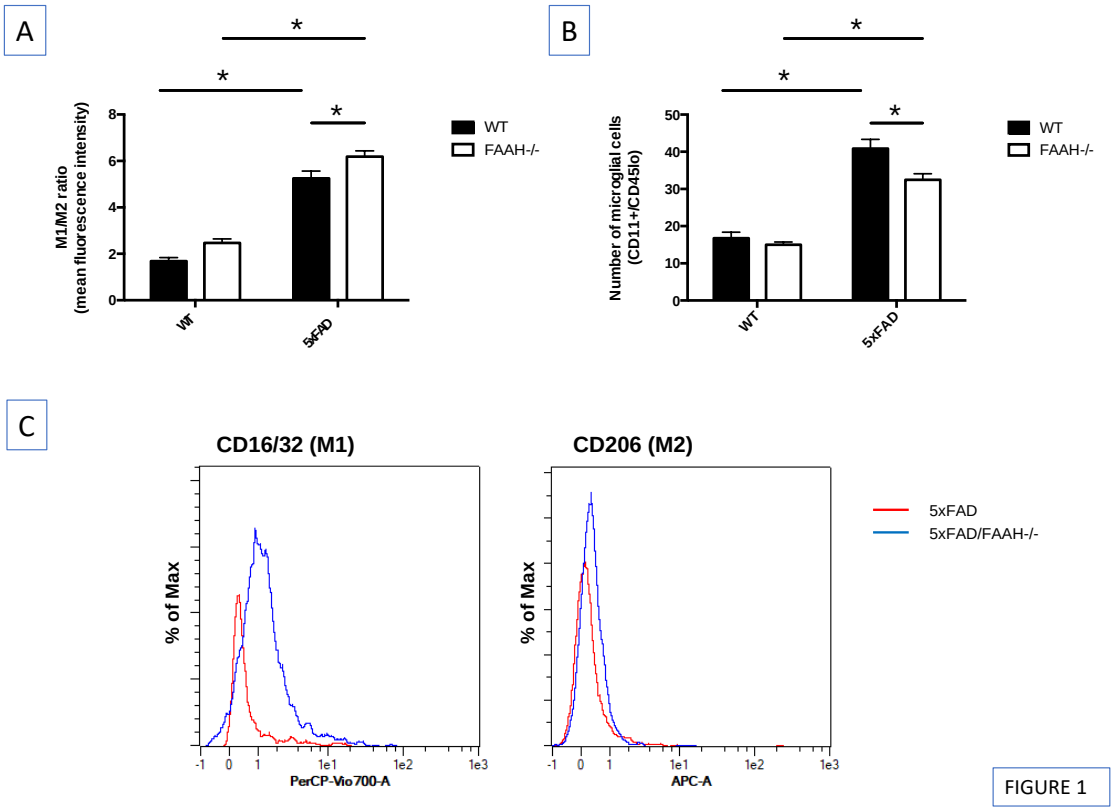
A
B
C
M1/M2 ratio
(mean fluorescence intensity)
WT
FAAH-/-
5xFAD
Number of microglial cells
(CD11+/CD45lo)
CD16/32 (M1)
CD206 (M2)
% of Max
PerCP-Vio700-A
APC-A
5xFAD
5xFAD/FAAH-/-

FIGURE 1

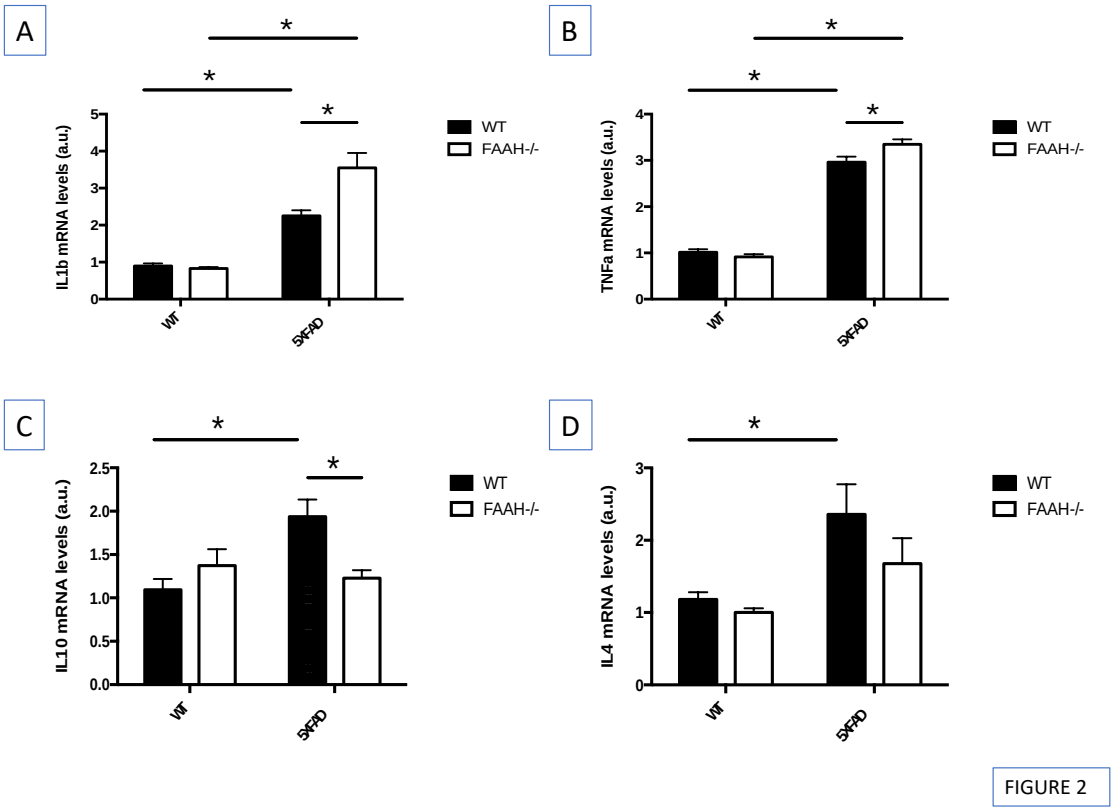
A
B
C
D
IL1b mRNA levels (a.u.)
TNFa mRNA levels (a.u.)
IL10 mRNA levels (a.u.)
IL4 mRNA levels (a.u.)
WT
FAAH-/-
5XFAD
*

FIGURE 2

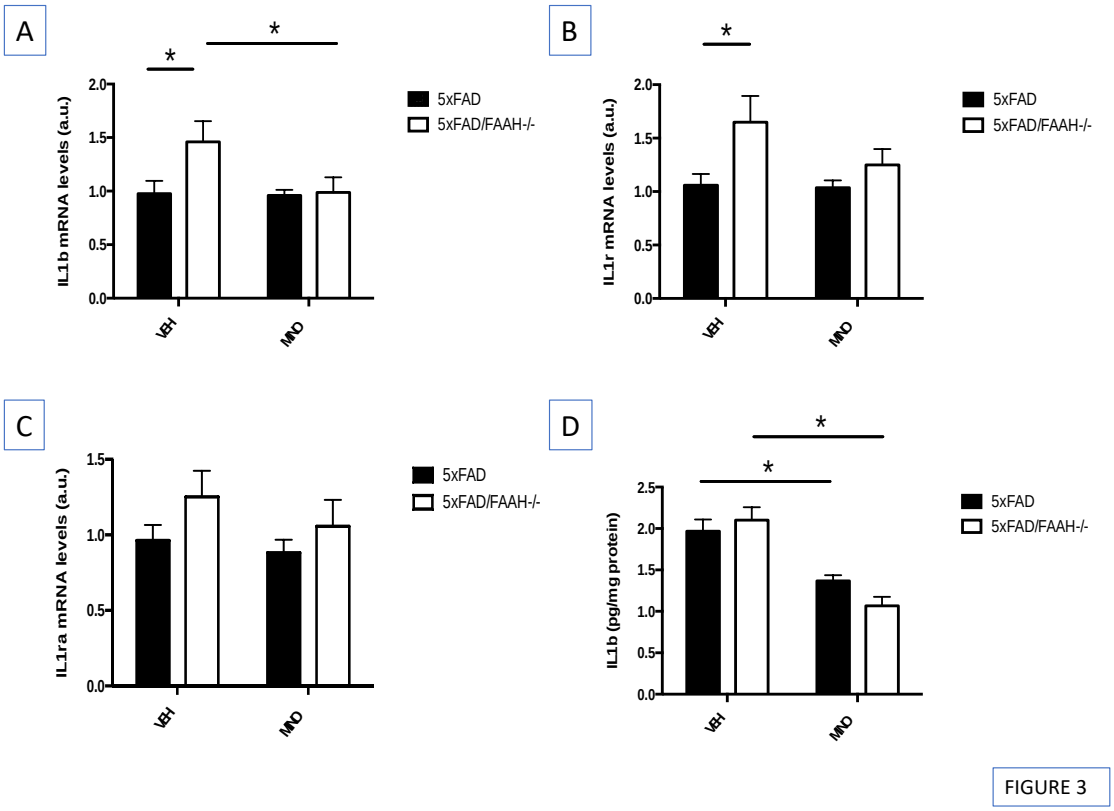
A
B
C
D
IL1b mRNA levels (a.u.)
IL1r mRNA levels (a.u.)
IL1ra mRNA levels (a.u.)
IL1b (pg/mg protein)
5xFAD
5xFAD/FAAH-/-
VEH
MMD
*

FIGURE 3

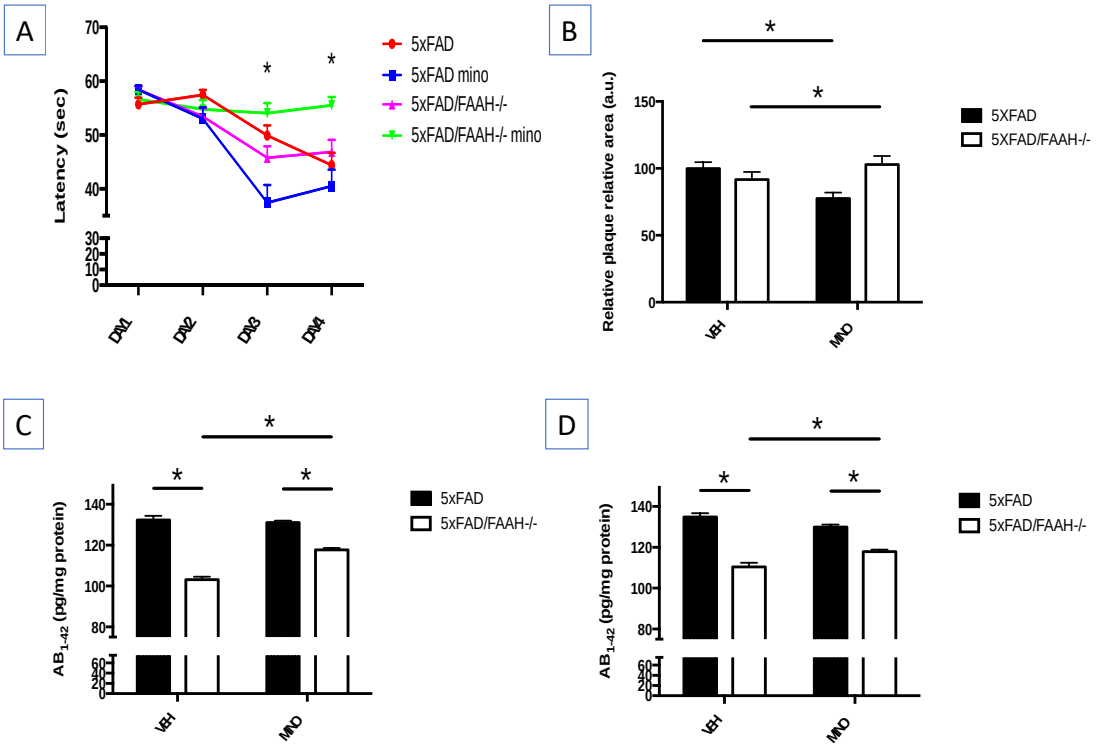
A
Latency (sec)
70
60
50
40
30
20
10
0
DAY1
DAY2
DAY3
DAY4
*
*
5xFAD
5xFAD mino
5xFAD/FAAH-/-
5xFAD/FAAH-/- mino
B
Relative plaque relative area (a.u.)
150
100
50
0
*
*
VEH
MND
5XFAD
5XFAD/FAAH-/-
C
AB1-42 (pg/mg protein)
140
120
100
80
60
40
20
0
*
*
*
VEH
MND
5xFAD
5xFAD/FAAH-/-
D
AB1-42 (pg/mg protein)
140
120
100
80
60
40
20
0
*
*
*
VEH
MND
5xFAD
5xFAD/FAAH-/-

FIGURE 4