British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.01889.x
www.brjpharmacol.org

RESEARCH PAPER

β–Amyloid exacerbates inflammation in astrocytes lacking fatty acid amide hydrolase through a mechanism involving PPAR-α, PPAR-γ and TRPV1, but not CB_1 or CB_2 receptors

Cristina Benito[1]*, Rosa María Tolón[1], Ana Isabel Castillo[1†], Lourdes Ruiz-Valdepeñas[1], José Antonio Martínez-Orgado[2], Francisco Javier Fernández-Sánchez[1], Carmen Vázquez[1], Benjamin F Cravatt[3] and Julián Romero[1]

[1]*Laboratorio de Apoyo a la Investigación, Hospital Universitario Fundación Alcorcón, Alcorcón, Madrid, Spain,* [2]*Neonatología, Servicio de Pediatría, Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain, and* [3]*The Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, The Scripps Research Institute, La Jolla, CA, USA*

Correspondence
J. Romero, Laboratorio de Apoyo a la Investigación, Hospital Universitario Fundación Alcorcón, Alcorcón, 28922 Madrid, Spain. E-mail: jromerop@fhalcorcon.es

Present addresses: *Department of Biochemistry and Molecular Biology, Faculty of Medicine, Complutense University, 28040, Madrid, Spain; †Centro Nacional de Investigaciones Cardiovasculares (CNIC), 28029, Madrid, Spain.

Keywords
Endocannabinoids; beta-amyloid; astrocytes; PPAR; TRPV1; Neuroinflammation

Received
24 May 2011
Revised
4 January 2012
Accepted
16 January 2012

BACKGROUND AND PURPOSE

The endocannabinoid system may regulate glial cell functions and their responses to pathological stimuli, specifically, Alzheimer's disease. One experimental approach is the enhancement of endocannabinoid tone by blocking the activity of degradative enzymes, such as fatty acid amide hydrolase (FAAH).

EXPERIMENTAL APPROACH

We examined the role of FAAH in the response of astrocytes to the pathologic form of β-amyloid (Aβ). Astrocytes from wild-type mice (WT) and from mice lacking FAAH (FAAH-KO) were incubated with Aβ for 8, 24 and 48 h, and their inflammatory responses were quantified by ELISA, western-blotting and real-time quantitative-PCR.

KEY RESULTS

FAAH-KO astrocytes were significantly more responsive to Aβ than WT astrocytes, as shown by the higher production of pro-inflammatory cytokines. Expression of COX-2, inducible NOS and TNF-α was also increased in Aβ-exposed KO astrocytes compared with that in WTs. These effects were accompanied by a differential pattern of activation of signalling cascades involved in mediating inflammatory responses, such as ERK1/2, p38MAPK and NFκ B. PPAR-α and PPAR-γ as well as transient receptor potential vanilloid-1 (TRPV1), but not cannabinoid CB_1 or CB_2 receptors, mediate some of the differential changes observed in Aβ-exposed FAAH-KO astrocytes. The pharmacological blockade of FAAH did not render astrocytes more sensitive to Aβ. In contrast, exogenous addition of several acylethanolamides (anandamide, palmitoylethanolamide and oleoylethanolamide) induced an antiinflammatory response.

CONCLUSIONS

The genetic deletion of FAAH in astrocytes exacerbated their inflammatory phenotype against Aβ in a process involving PPAR-α, PPAR-γ and TRPV1 receptors.

Abbreviations

Aβ, beta amyloid; AEA, anandamide; CPZ, capsazepine; FAAH, fatty acid amide hydrolase; FAAH-KO, fatty acid amide hydrolase-knockout; GFAP, glial fibrillary acidic protein; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; SR1, SR141716A; SR2, SR144528; TG, troglitazone; TRPV1, transient receptor potential vanilloid-1; WY, WY-14643

Introduction

The endocannabinoid system comprises several elements including two G-protein–coupled membrane receptors (CB_1 and CB_2; receptor nomenclature follows Alexander *et al.*, 2011), the endogenous ligands for these receptors (so called 'endocannabinoids') and proteins with enzymic activity capable of regulating the synthesis and degradation of these endogenous molecules (such as fatty acid amide hydrolase, FAAH). FAAH is the primary catabolic enzyme for the family of *N*-acylethanolamines, which includes the endocannabinoid arachidonylethanolamide (anandamide, AEA). However, FAAH also regulates the contents of other *N*-acylethanolamines that do not activate cannabinoid receptors, including palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (see Pertwee, 2008). Non-cannabinoid targets of the *N*-acylethanolamine family of lipids include the transient receptor potential vanilloid-1 (TRPV1) receptor, PPAR-α and PPARγ, and GPR55 (see Pertwee *et al.*, 2010). It is thus clear that controlling the levels of the *N*-acylethanolamines could affect many physiological functions, including motor activity, immune function, body temperature, metabolic activity or inflammation.

In the present study, our aim was to explore the hypothesis that FAAH, through regulation of *N*-acylethanolamine concentrations, altered astrocyte function in the context of Alzheimer's disease. Immunohistochemical data show that FAAH is expressed in astrocytes (Romero *et al.*, 2002) as well as neurons (Tsou *et al.*, 1998), and that astrocytes in culture contain detectable amounts of several *N*-acylethanolamines, including AEA, PEA and OEA (Walter *et al.*, 2002; Stella, 2004). AEA has been shown to inhibit potassium and sodium conductances and modulate astrocytic homeostasis as well as excitatory amino acid transport in rat cortical astrocytes (Shivachar, 2007; Vignali *et al.*, 2009).

A role for FAAH in Alzheimer's disease was first suggested by data indicating that its expression and activity was increased in areas of inflammation in Alzheimer's disease (Benito *et al.*, 2003; Jung *et al.*, 2011) and also in Down's syndrome (Nuñez *et al.*, 2008). Increased FAAH expression occurs in β-amyloid (Aβ)-activated astrocytes, but not in microglia, located in the proximity of senile plaques. An astrocyte-specific increase in FAAH expression is markedly maintained in other neuroinflammatory conditions, such as multiple sclerosis or viral encephalitis (see Benito *et al.*, 2007) which suggests that FAAH may play a major role in the response of astrocytes to inflammation. Furthermore, recent studies have shown that the non-cannabinoid *N*-acylethanolamine, PEA, decreased inflammation in rat primary astrocytes exposed to Aβ (Scuderi *et al.*, 2011).

The purpose of the present studies was to determine the effect of the loss of FAAH activity on the response of astrocytes to the pathological form of the Aβ peptide. We measured a number of proinflammatory cytokines, analysed cell signalling pathways and monitored mRNA levels of a number of proinflammatory mediators using primary astrocytes from mice in which the FAAH gene had been deleted (FAAH-KO) and consequently have approximately 10 times higher levels of AEA and other *N*-acylethanolamines than those in the WT mice (Cravatt *et al.*, 2001).

Methods

Mice

All animal care and experimental procedures were performed in accordance with international and local guidelines (86/609/EEC). Mice homozygous for disruption of the gene that encodes FAAH (FAAH-KO) were backcrossed >6 generations to the wild type (C57BL/6J) genetic background (Cravatt *et al.*, 2001). Mice were maintained in a temperature-controlled, pathogen-free facility with a 12 h light/dark cycle and with free access to food and water.

Preparation of $A\beta_{1–42}$

$A\beta_{1–42}$ was purchased from American Peptide Company Inc. (Sunnyvale, CA, USA), solubilised in sterile water (1 $mg \cdot mL^{-1}$) and incubated for 7 days at 37°C, to allow fibrilization. This method has been described and validated previously (Burdick *et al.*, 1992; Lorenzo and Yankner, 1994; Coraci *et al.*, 2002; Bamberger *et al.*, 2003).

Cell culture

Primary astrocytes were obtained from neonatal mice brains (P1) and cultured as described (McCarthy and De Vellis, 1980; Molina-Holgado *et al.*, 2002), with slight modifications. Isolated cells were cultured on poly-L-ornithine–treated 75-cm^2 flasks with Dulbecco's modified Eagle's medium supplemented with L-glutamine (2.5 mM), penicillin (50 $U \cdot mL^{-1}$), streptomycin (50 $U \cdot mL^{-1}$) and 10% fetal bovine serum (FBS) (all from BioWhittaker, Walkersville, MD, USA) at 37°C in 5% CO_2. Medium was changed once per week and, after 14 days in culture, cells were subjected to shaking (240 rpm for 4 h) to separate astrocytes from oligodendrocytes and microglia. Purity of the cells in culture was tested with a monoclonal anti-glial fibrillary acidic protein (GFAP, Dako) antibody and only cultures with more than 95% GFAP-positive cells were employed for the experiments.

Exposure of primary astrocytes to $A\beta_{1–42}$

Astrocytes were trypsinized and seeded in p35 dishes at a density of 10^5 $cells \cdot cm^{-2}$, overnight. After washes with PBS, medium without FBS was added to the astrocytes. $A\beta_{1–42}$ (5 μM) or vehicle (distilled water) was added to the medium and incubated for different periods of time (8, 24 and 48 h). This concentration was chosen based on previously pub-

lished data (Koenigsknecht-Talboo and Landreth, 2005) and on the estimated concentrations of $A\beta_{1-42}$ in the Alzheimer's disease brain (Naslund *et al.*, 2000).

In other experiments, following the experimental procedure of the time course study, FAAH-KO primary astrocytes were treated 30 min before the 8-h exposure to $A\beta_{1-42}$ with selective cannabinoid and TRPV1 receptor antagonists. SR141716A (SR1) and SR144528 (SR2), the selective CB_1 and CB_2 receptor antagonists, respectively, kindly provided by Sanofi Recherche (Montpellier, France) were used at 100 nM. The final concentration of the selective TRPV1 receptor antagonist, capsazepine (Sigma, St Louis, MO, USA) was 1 μM. Some experiments also included the selective PPAR-α and PPAR-γ agonists, WY-14643 (WY; Sigma) and troglitazone (Cayman Chemical, Ann Arbor, MI, USA), respectively, at 10 μM. In some studies, wild-type (WT) astrocytes were treated with the selective, very slowly reversing FAAH inhibitor URB597 (Cayman Chemical) at a dose of 1 μM for 16 h before the addition and subsequent 8-h incubation of the astrocytes with $A\beta_{1-42}$ peptide. Additional experiments with URB597 also included 30-min incubation with SR1 or SR2 prior to exposure to Aβ. AEA, OEA and PEA (from Sigma, 10 μM each, alone or in combination) were added 24 h before exposure to the pathological peptide.

Analysis of cytokine secretion

After treatments, inflammation-related cytokines were quantified by ELISA in the culture supernatants. Levels of chemokines CCL2 and CCL5, and TNF-α were measured using specific ELISA kits provided by R&D Systems (Minneapolis, MN, USA) following the manufacturer's instructions. Assay sensitivities were 2 $pg \cdot mL^{-1}$ (CCL5, CCL2) and 5 $pg \cdot mL^{-1}$ (TNF-α). IL-1β and IL-6 were measured with ELISA kits purchased from Raybiotech (Norcross, GA, USA); limits of detection of these assays are less than 2 and 5 $pg \cdot mL^{-1}$ respectively.

Measurement of cell death

LDH released to the medium was quantified as an index of cell death, by means of the CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI, USA), following the instructions of the manufacturer.

Analysis of signalling cascades by Western blotting

Astrocytes were seeded in p60 dishes at a density of 10^5 $cells \cdot cm^{-2}$ (ERK1/2 and p38MAPK) or in p100 dishes at a density of 5 x 10^4 $cells \cdot cm^{-2}$ (NFκB) and were preincubated in serum-free medium for 24 h before addition of $A\beta_{1-42}$. Shorter periods of exposure were chosen, in concordance with previous literature on signalling cascades (Bamberger *et al.*, 2003; Lim *et al.*, 2007). After incubation with $A\beta_{1-42}$ for 5, 10, 15, 30, 60, 120 and 180 min (ERK1/2 and p38MAPK) or for 1, 3 and 6 h (NFκB), cells were collected and processed for Western blotting.

For kinase (ERK1/2 and p38MAPK) detection, cells were washed with PBS and lysed with 100 μL of lysis buffer (100 mM Tris-HCl pH.7.5, 2 mM EGTA, 2 mM EDTA, 20 mM NaF, 540 mM sucrose, 0.2 mM PMSF, 2% Triton X-100, 1 mM Na_3VO_4, 0.1% 2-mercaptoethanol and 10 $\mu g \cdot mL^{-1}$ protease inhibitors). Lysates were incubated on ice for 3 min before centrifuging at 10 000× *g* for 10 min at 4°C; protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). For Western blotting, 50 μg of protein was subjected to SDS-PAGE on a 10% gel under reducing conditions. Proteins were transferred onto a Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) for 1 h at 100 V. Membranes were blocked in tris-buffered saline containing 0.1% Tween 20 (TBS-T) with 5% dry non-fat skimmed milk powder (Sigma) for 1 h at 37°C before an overnight incubation with the primary antibody anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}) diluted 1:2500 in blocking solution or anti-phospho-p38 (Thr^{180}/Tyr^{182}) diluted 1:1000 in TBS-T with 5% BSA. Both of the antibodies were obtained from Cell Signalling Technology (Beverly, MA, USA). After extensive washing with TBS-T, membranes were incubated with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) (1:2500 in blocking solution) for 2 h at room temperature Finally, blots were rinsed and the peroxidase reaction was developed by enhanced chemiluminescence with Amersham ECLTM Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK). Afterward, the blots were stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS and 100 mM 2-mercaptoethanol) for 30 min at 50°C. Then the membranes were washed in TBS-T, blocked again with TBS-T containing 5% dry non-fat skimmed milk powder and incubated with primary antibodies for all forms of ERK1/2 (Cell Signalling Technology) and p38 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), both diluted 1:2500 in blocking solution. Secondary antibody incubation, membrane washing and detection was performed as described previously.

For NFκB determination, nuclear and cytoplasmic extracts were prepared using a nuclear and cytoplasmic extraction reagent (NPER; Pierce, Thermo Fisher Scientific, Freemont, CA, USA) following the manufacturer's instructions. Protein contents were measured using the bicinchoninic acid method (BCA protein assay; Pierce). Nuclear proteins (15 μg) or cytoplasmic fractions (20 μg) were resolved on a 10% SDS-PAGE and then transferred to an Immobilon-P membrane (Millipore). The membranes were blocked with TBS-T containing 5% dry non-fat skimmed milk powder. Incubation with the anti-NF-kB antibody (p65) (1:500; Santa Cruz Biotechnology, Inc.) in blocking solution was carried out overnight at 4°C. After three washes with TBS-T, the incubation with an anti-rabbit secondary antibody conjugated to HRP and the visualization of the bands were carried out as described earlier. Membranes were stripped and reblotted with a monoclonal antibodies against β-actin (1:2500; Sigma) and histone 3 (1 $\mu g \cdot mL^{-1}$; Abcam, Cambridge, MA, USA) for the cytoplasmic and nuclear extracts respectively. These proteins were used as an internal control for protein loading and transfer as well as to verify purity of the subcellular fractionation. Films were analysed using Quantity One Software (Bio-Rad).

Table 1
Primers used in the present study

Genes	Primer/probe	Sequences for primers and probes
18S	sense	AAATCAGTTATGGTTCCTTTGGTC
	antisense	GCTCTAGAATTACCACAGTTATCCAA
	Probe #55	Use universal ProbeLibrary, Roche Applied Science
iNOS	sense	GCTCCTCCCAGGACCACA
	antisense	GCTGGAAGCCACTGACACTT
	Probe TaqMan	6FAM-CACCTACCGCACCCGAGATGG–BBQ
COX-2	sense	TGACCCACTTCAAGGGAGTCT
	antisense	CTGTCAATCAAATATGATCTGGATGTC
	Probe TaqMan	6FAM-AACAACATCCCCTTCCTGCGAAGTT–BBQ
TNF-α	sense	GCCTATGTCTCAGCCTCTTCTCATT
	antisense	CCACTTGGTGGTTTGCTACGA
	Probe TaqMan	6FAM-CCATAGAACTGATGAGAGGGAGGCCATTT–BBQ
TRPV1	sense	GATTTTCCACAGCCGTAGTGACA
	antisense	GACATGTGGAATACAGGCTGTTGTA
	Probe TaqMan	FAM-AACTCACTGCCTGTGGAGTCCCCA-BBQ
PPAR-α	sense	ACTGAAAGCAGAAATTCTTACCTGTG
	antisense	AAGCGTCTTCTCGGCCATA
	Probe FRET	GGGCCTTGACCTTGTTCATGTTGAA-FL
		640-TTCTTCAGGTAGGCTTCGTGGATTCTCTTG P
PPAR-γ	sense	CCAGATCTTCCTGAACTTGACCT
	antisense	CCAGTGTCTCTGTGAGGACCG
	Probe TaqMan	FAM-ACAGACAGCTTTCTGGGTGGATTGAA-TMR
CB_1	sense	CATCATCATCCACACGTCAG
	antisense	ATGCTGTTGTCTAGAGGCTG
	No Probe	SYBR Green
CB_2	sense	GCGGCTGACAAATGACAC
	antisense	CGGGAGGACAGGATAATATAGAG
	Probe FRET	TTCTGTCTCCCGGCATCCCTCCA-FL
		640-GGATGGGCTTTGGCTTCTTCTACTGGAGCT P

RT quantitative–PCR (qRT-PCR) for type 2 COX (COX-2), inducible NOS (INOS), TNF-α, CB_1, CB_2, TRPV1, PPAR-α and PPAR-γ

Total RNA was isolated using Master PureTM Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the protocol of the supplier. RNA was dissolved in RNase-free water and quantified by absorption at 260 nm. Aliquots were subjected to 1% denaturing agarose gel electrophoresis and ethidium bromide staining to verify the quantity and quality of RNA. Single-stranded complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the Transcriptor First Strand cDNA Synthesis kit (AMV) (Roche Diagnostics, Mannheim, Germany). PCR primers and TaqMan probes were designed by Tib Molbiol (Berlin, Germany) as shown in Table 1. For normalization, 18S primers and probe number 55 from Universal ProbeLibrary (Roche) were utilized. Gene expression was quantified using LightCycler FastStart DNA Master HybProbe and LightCycler Taqman Master (Roche) and Quantimix Easy Probes kit (Biotools, Madrid, Spain) in a LightCycler thermocycler (Roche). The concentration of primers and probes were 0.5 and 0.2 μM respectively. PCR assays were performed using 2 μL of the cDNA reaction. All assays were carried out twice as independent PCR runs for each cDNA sample. Mean values were used for further calculation. A negative (no template) control was measured in each of the PCR runs. Standard curves were calculated for quantification purposes using fivefold serial dilutions of cDNA from mouse brain. The transcript amounts were calculated using the second derivative maximum mode of the LC-software version 4.0. The specific transcript quantities were normalized to the transcript amounts of the reference gene 18S. All further calculations and statistical analyses were carried out with these values referred to as relative expression ratios.

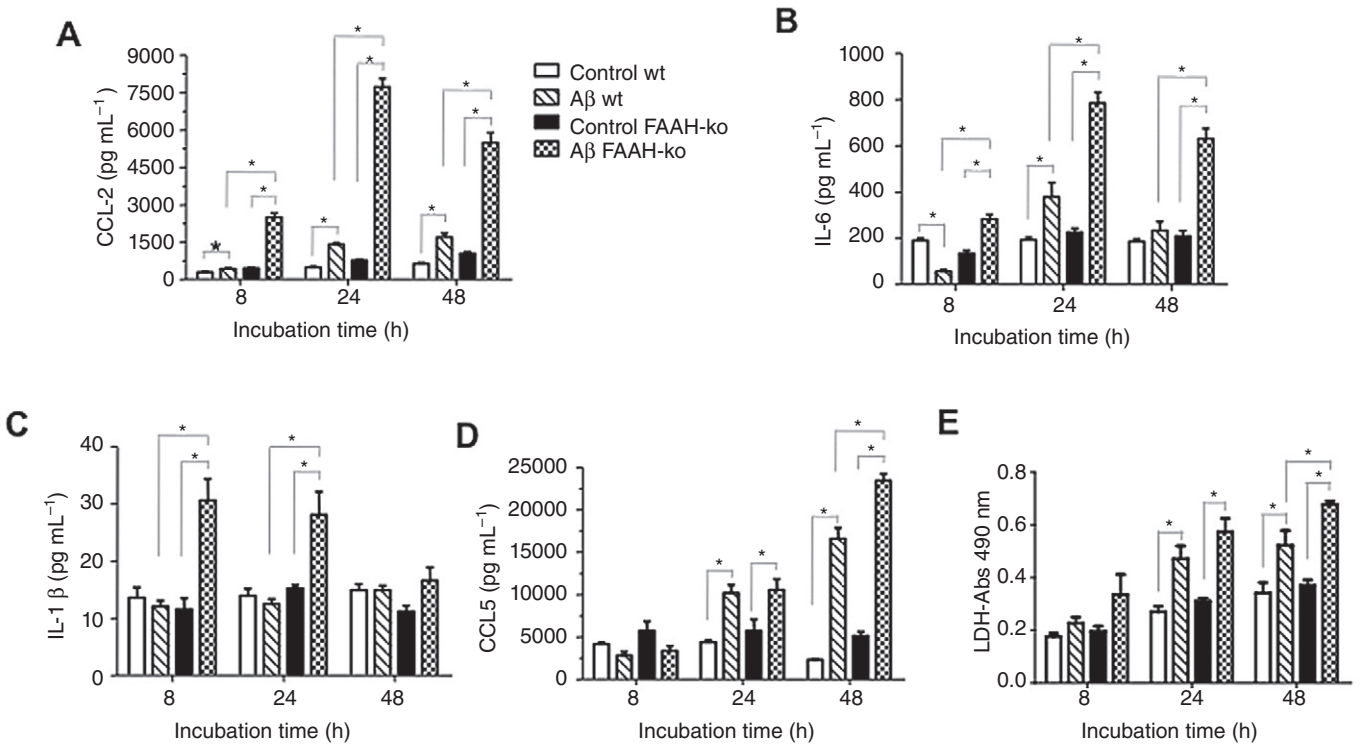


Figure 1

The absence of FAAH in astrocytes modifies the secretion of inflammatory cytokines after exposure to $A\beta_{1-42}$ and makes them more sensitive to its deleterious effects, as revealed by a time course quantitative analysis of cell supernatants. Cytokines studied included CCL2 (A), IL-6 (B), IL-1β (C) and CCL5 (D). Cell death was measured by LDH activity (E). Results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by two-way ANOVA (*$P < 0.05$). CCL-2: genotype F (75.2/4), $P = 0.00001$; time: F (30.4/3.1), $P = 0.0001$. IL-6: genotype F (85.1/4), $P = 0.00001$; time: F (25.1/3.1), $P = 0.0001$. IL-1β: genotype F (30.4/4), $P = 0.00001$; time: F (8.25/3.2), $P = 0.008$. CCL5: genotype F (1.1/4), $P = 0.3$; time: F (18.5/3.1), $P = 0.00011$. LDH: genotype F (12.9/4.2), $P = 0.001$; time: F (6.2/3.3), $P = 0.005$.

Statistical analysis

Results are expressed as mean ± SEM of three experiments performed in triplicate. Statistical analysis were made using Student's *t*-test for comparisons between two groups, ANOVA with Newman–Keuls test for multiple comparisons of results from a single group (FAAH-KO or WT) and repeated measures two-way ANOVA for multiple comparisons between both groups or when including time course. A P value < 0.05 was considered as statistically significant. Statistical analysis was performed using the 11.0.0 version of SPSS software (SPSS Inc., Chicago, IL, USA).

Results

FAAH-KO astrocytes produce increased amounts of proinflammatory cytokines than WTs when exposed to $A\beta_{1-42}$

In a first set of experiments, we explored the effects of exposure to $A\beta_{1-42}$ for different periods of time on the profile of cytokine secretion by astrocytes. The basal concentrations of all four cytokines were similar in supernatants from FAAH-KO and WT astrocytes (Figure 1). Exposure to $A\beta_{1-42}$ increased CCL2 concentrations in both WT and FAAH-KO astrocytes, but $A\beta_{1-42}$–triggered CCL2 production was significantly higher in FAAH-KO than in WTs at all time points measured (Figure 1A). Similarly, $A\beta_{1-42}$ exposure induced an increase in IL-6 concentration that was significantly greater in FAAH-KO than in WTs at all time points (Figure 1B). Interestingly, IL-6 levels were significantly lower in WT astrocytes at 8 h after the treatment (Figure 1B). In addition, exposure to $A\beta_{1-42}$ did not induce an increase in IL-1β concentration in WT but, in contrast, $A\beta_{1-42}$ exposure for 8 and 24 h increased IL-1β concentrations in FAAH-KO (Figure 1C). CCL5 concentrations increased 24 and 48 h after $A\beta_{1-42}$ exposure both in WTs and FAAH-KO, with the increase being significantly greater in FAAH-KO (Figure 1D). TNF-α levels were undetectable under basal conditions in all groups (data not shown).

FAAH-KO astrocytes are more sensitive to the deleterious effects of $A\beta_{1-42}$

In addition to the effects on the cytokine profile, we analysed whether $A\beta_{1-42}$ was also able to induce cell death. As expected, the presence of the pathogenic peptide induced astrocyte death, as measured by LDH activity in the cell media 24 and 48 h after exposure. Interestingly, LDH activity was elevated more in samples from FAAH-KO astrocytes than from WTs, suggesting that the absence of FAAH makes astrocytes more sensitive to the damaging effects of $A\beta_{1-42}$ and, subsequently, more prone to death (Figure 1E).

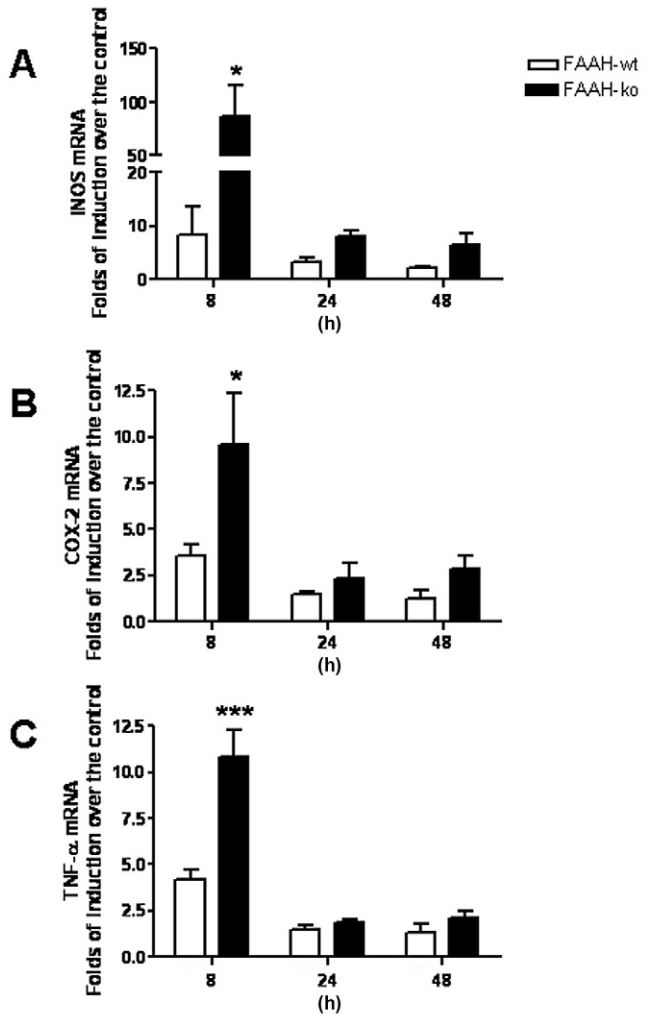


Figure 2

mRNA expression of iNOS, COX-2 and TNF-α is significantly higher in primary FAAH-KO astrocytes than in WT after 8 h of exposure to $A\beta_{1-42}$. Graphs show the induction of iNOS (A), COX-2 (B) and TNF-α (C) mRNA levels expressed as fold of control, (not exposed to $A\beta_{1-42}$), in astrocytes treated with $A\beta_{1-42}$ (5 μM). Results shown are the mean ± SEM of three independent experiments performed by triplicate. Data were analysed by two-way ANOVA (*$P < 0.05$ and ***$P < 0.001$ vs. WT). COX-2: genotype F (7.6/4.6), $P = 0.015$; time: F (4.7/3.8), $P = 0.041$. iNOS: genotype F (19.5/4.7), $P = 0.0008$; time: F (22.1/3.8), $P = 0.0001$. TNF-α: genotype F (9.9/4.6), $P = 0.007$; time: F (25.1/3.8), $P = 0.00003$.

$A\beta_{1-42}$-triggered increases in mRNA expression of key inflammatory mediators are higher in FAAH-KO than WT astrocytes

We measured mRNA expression of two key enzymes known to participate in inflammatory processes of the CNS: iNOS and COX-2 (Figure 2). iNOS mRNA expression was increased following an 8-h exposure to $A\beta_{1-42}$, increasing approximately eightfold over basal levels in WT astrocytes and more than 80-fold in FAAH-KO astrocytes (Figure 2A). This induction was lower at 24 and 48 h in both groups, but was more pronounced in FAAH-KO than in WTs.

With respect to COX-2 (Figure 2B), exposure to $A\beta_{1-42}$ induced a three-fold increase in mRNA expression over basal in WT astrocytes. Astrocytes from FAAH-KO mice treated with $A\beta_{1-42}$ exhibited a significantly greater increase (10-fold) in COX-2 mRNA expression than similarly treated WT after 8 h of exposure. There were no significant differences in expression between the genotypes after 24 and 48 h of treatment.

The response of TNF-α mRNA expression to $A\beta_{1-42}$ treatment was also determined. Results of the time course exposure to $A\beta_{1-42}$ showed a similar cytokine pattern of expression to the enzymes studied above. TNF-α mRNA was significantly increased in both types of astrocytes and was significantly greater in FAAH-KO cells than in WT after the 8-h incubation with the peptide (Figure 2C). At 24 and 48 h, the differences between the two groups were not significant.

Exogenous AEA, PEA and OEA have anti-inflammatory effects on $A\beta_{1-42}$–induced inflammation

We next tested if the exogenous addition of several *N*-acylethanolamines (concentrations of which are putatively affected by FAAH deletion) triggers the same effect observed in FAAH-KO astrocytes. Therefore WT astrocytes were incubated with AEA, PEA or OEA (alone or combined) and their effects on the inflammation induced by $A\beta_{1-42}$ were quantified. We found that all of the three *N*-acylethanolamines partially prevented the increase in TNF-α, COX-2 and iNOS induced by the pathological peptide (Figure 3). No additive effect was found when the three *N*-acylethanolamines were added together to the cell cultures.

The absence of FAAH modifies the time course and degree of activation of ERK1/2, p38MAPK and NFκB by $A\beta_{1-42}$

In order to determine the signalling cascades involved in the differential effect of $A\beta_{1-42}$ in astrocytes, we analysed the time course of the phosphorylation of several kinases known to participate in cell survival and inflammatory processes following $A\beta_{1-42}$ exposure in WT and FAAH-KO astrocytes. ERK1/2 were phosphorylated at shorter incubation periods and to a greater extent in astrocytes from FAAH-KO, compared with WT astrocytes (Figure 4A). Significant amounts of ERK1/2 phosphorylation were detected 5 min after $A\beta_{1-42}$ addition to the cells in FAAH-KO astrocytes with increases of 608% and 350% (ERK 1 and 2, respectively). In astrocytes from WT mice, significant increase in phosphorylation occurred 10 min after $A\beta_{1-42}$ incubation and the increases were smaller 298% and 176%, ERK1 and 2 respectively. Conversely, no significant difference in p38MAPK phosphorylation induced by $A\beta_{1-42}$ was seen between FAAH-KO and WTs astrocytes at shorter times (i.e. 0–15 min) after $A\beta_{1-42}$ addition (Figure 4B). Interestingly, the amount of phosphorylated p38 steadily decreased in WTs at longer incubation times, whilst FAAH-KO exhibited a significant second peak in phosphorylation of p38MAPK at 2 h after $A\beta_{1-42}$ addition (Figure 4B). These patterns of responses are in agreement with previously published *in vitro* data (Lim *et al.*, 2007).

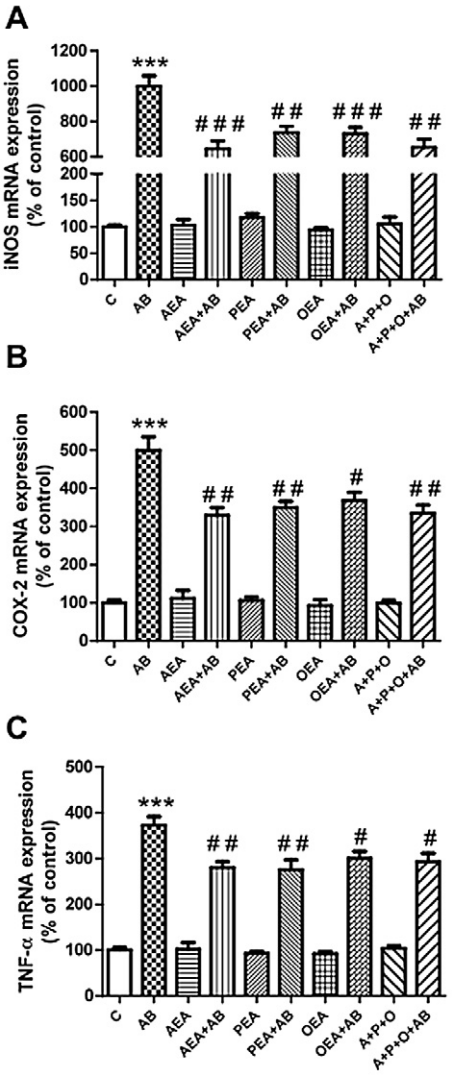


Figure 3

Exogenously added *N*-acylethanolamines partially reverse Aβ-induced inflammation in WT astrocytes by reducing the increase in the expression levels of iNOS (A), COX-2 (B) and TNF-α (C). Astrocytes were incubated with AEA, PEA or OEA (10 μM each), alone or in combination, for 24 h and then exposed to Aβ (AB) for 8 h. iNOS, COX-2 and TNF-α mRNAs were quantified by qRT-PCR. Results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by one-way ANOVA (***$P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. Aβ). COX-2: F (31.2/2), $P = 0.00001$. iNOS: F (54.7/2.1), $P = 0.00001$. TNF-α: F (19.9/2.1), $P = 0.00001$.

NFκB is a critical mediator in a wide variety of inflammation-related processes, as it regulates the expression of several key genes. In order to exhibit such cellular actions, it must first translocate into the nucleus; thus, the ratio of nuclear and cytoplasmic NFκB is considered an index of its activation. To observe such changes, longer periods of observation are required. The ratio of nuclear/cytoplasmic NFκB was significantly higher in FAAH-KO than in WT astrocytes after 3- and 6-h exposure to $A\beta_{1-42}$ (Figure 4C).

Changes observed in FAAH-KO astrocytes when exposed to $A\beta_{1-42}$ are not mediated by cannabinoid receptors

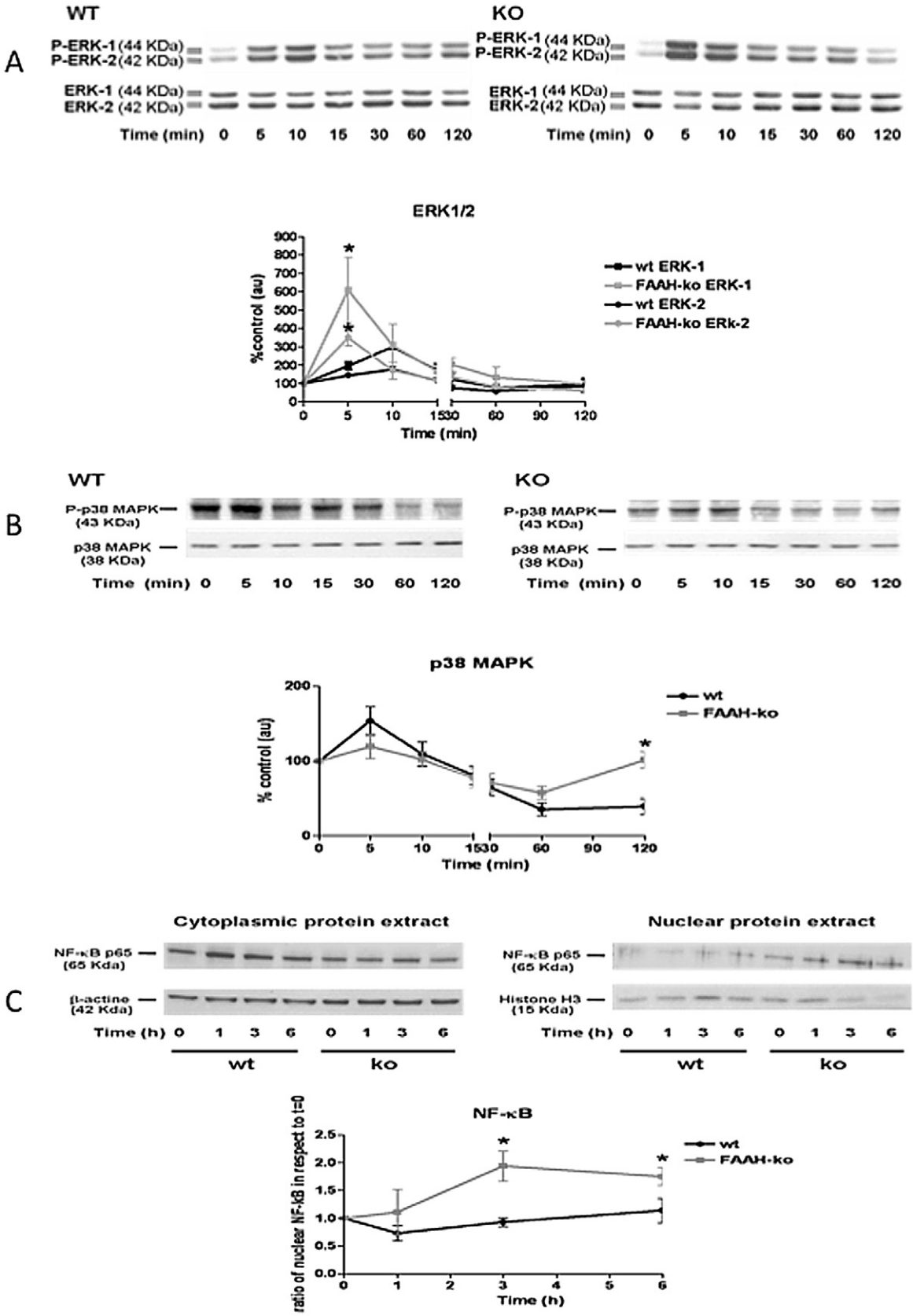
To explore whether the proinflammatory effects triggered by the pathological peptide in FAAH-KO astrocytes were mediated by CB_1 or CB_2 receptors, cells were co-treated with $A\beta_{1-42}$ and CB_1 and the CB_2 receptor selective antagonists, rimonabant (SR1) and SR144528 (SR2) respectively. Astrocytes in culture expressed both CB_1 and CB_2 receptors and no differences were found in their expression levels between WT and FAAH-KO astrocytes (not shown). The incubation time in this experiment was 8 h since the most significant differences between FAAH-KO and WT astrocytes in the $A\beta_{1-42}$-induced expression of the cytokines and enzymes studied were most consistently observed at this time (Figure 1 and 2). Results from these experiments are shown in Figures 5 and 6. We found that blockade of the cannabinoid receptors could not prevent the enhancement of $A\beta_{1-42}$-induced increases in IL-1β production or in the increased expression of iNOS, COX-2 and TNF-α mRNA in FAAH-KO astrocytes.

FAAH-KO astrocytes express lower levels of PPAR-α and PPAR-γ mRNAs and increased levels of TRPV1 mRNA

Some *N*-acylethanolamines degraded by FAAH are agonists of PPARs (seePertwee *et al.*, 2010) and PPARs are potential anti-inflammatory targets in Alzheimer's disease (Jiang *et al.*, 2008). We determined whether deletion of FAAH influences the expression of these nuclear receptors in astrocytes. Our results show that PPAR-α and PPAR-γ mRNA levels were significantly decreased in FAAH-KO astrocytes as compared with WTs under basal conditions (Figure 7A and B). mRNA levels of TRPV1 receptors were also quantified because AEA is capable of activating TRPV1 receptors (Starowicz *et al.*, 2007). In addition, AEA levels, as well as affinity and potency at TRPV1 receptors, can be enhanced by other *N*-acylethanolamines such as PEA through an 'entourage' effect (De Petrocellis *et al.*, 2001; Starowicz *et al.*, 2007). We found that the basal expression of TRPV1 receptors in

Figure 4

Representative Western blots and quantification of the signalling cascades involved in the differential activation of FAAH-KO astrocytes against $A\beta_{1-42}$. (A) $A\beta_{1-42}$-induced phosphorylation of ERK1 and 2 is earlier (5 min) and significantly more robust (maximum of 600%) in FAAH-KO than in WT astrocytes. (B) The phosphorylation levels of p38 protein are similar between FAAH-KO and WT astrocytes at shorter times, but are significantly higher in FAAH-KO at 120 min after exposure to the pathological peptide. (C) The $A\beta_{1-42}$-induced translocation of NFκB is earlier (1 h) and significantly stronger (maximum of 100% increase at 3 h) in FAAH-KO than in WT astrocytes. Densitometric analysis results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by two-way ANOVA (*$P < 0.05$ for differences from WT and for interaction). ERK1/2: genotype F (14.9/2.2), $P = 0.00001$; time: F (17.7/4.1), $P = 0.0001$. p38: genotype F (14.1/2.1), $P = 0.00001$; time: F (2.8/3.9), $P = 0.09$; interaction: F (3.1/2.7), $P = 0.001$. NFκB: genotype F (5.11/4.2), $P = 0.03$; time: F (.41/3), $P = 0.7$.


A
WT
KO
P-ERK-1 (44 KDa)
P-ERK-2 (42 KDa)
ERK-1 (44 KDa)
ERK-2 (42 KDa)
Time (min) 0 5 10 15 30 60 120
ERK1/2
% control (au)
Time (min)
wt ERK-1
FAAH-ko ERK-1
wt ERK-2
FAAH-ko ERk-2
B
WT
KO
P-p38 MAPK (43 KDa)
p38 MAPK (38 KDa)
Time (min) 0 5 10 15 30 60 120
p38 MAPK
% control (au)
Time (min)
wt
FAAH-ko
C
Cytoplasmic protein extract
Nuclear protein extract
NF-κB p65 (65 Kda)
β-actine (42 Kda)
Histone H3 (15 Kda)
Time (h) 0 1 3 6 0 1 3 6
wt
ko
NF-κB
ratio of nuclear NF-kB in respect to t=0
Time (h)
wt
FAAH-ko

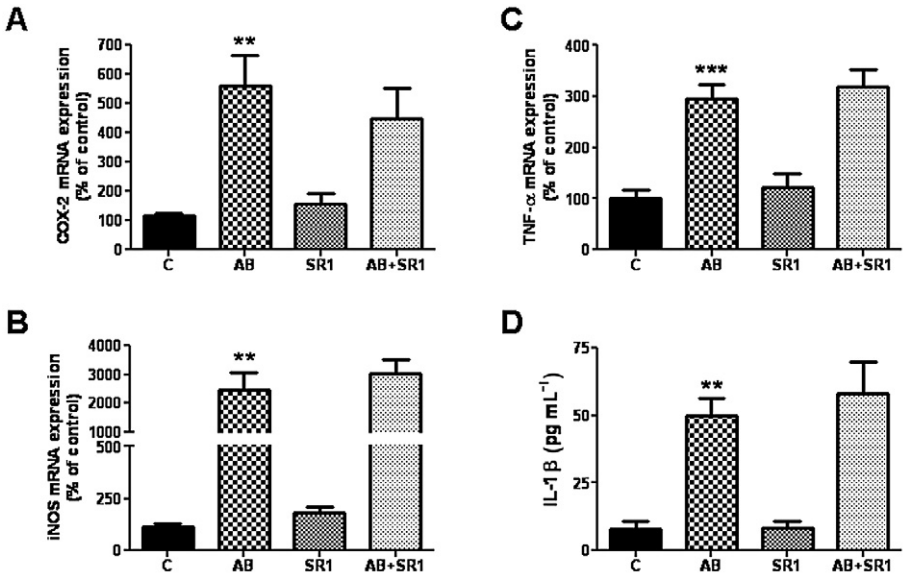


Figure 5

Incubation with the CB_1 receptor antagonist, SR141716A (100 nM), did not prevent the increased mRNA levels of COX-2 (A), iNOS (B) and TNF-α (C) in FAAH-KO astrocytes stimulated by $A\beta_{1-42}$ (AB). IL-1β production was also not modified by the antagonist (D). The results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by one-way ANOVA (**$P < 0.005$ and ***$P < 0.0001$ vs. control). COX-2: F (3.16/2.9), $P = 0.03$. iNOS: F (5.83/2.9), $P = 0.002$. TNF-α: F (8.15/2.7), $P = 0.0001$. IL1-β: F (14.3/4), $P = 0.001$.

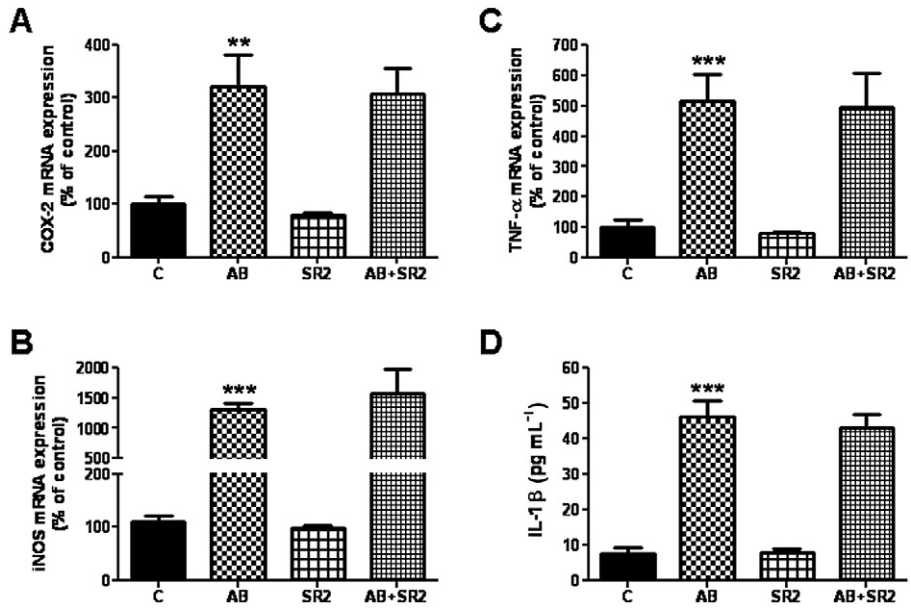


Figure 6

Blockade of the CB_2 cannabinoid receptor with its selective antagonist, SR144528 (100 nM), did not alter the pattern of expression of the enzymes (COX-2, Figure A; iNOS, Figure B) and the cytokines (TNF-α, Figure C; IL-1β, Figure D) studied in FAAH-KO astrocytes exposed during 8 h to $A\beta_{1-42}$ (AB; 5 μM). The results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by one-way ANOVA (**$P < 0.005$ and ***$P < 0.0001$ vs. control). COX-2: F (9.5/3), $P = 0.0003$. iNOS: F (6.2/2.9), $P = 0.002$. TNF-α: F (4.9/3), $P = 0.008$. IL1-β: F (41.8/3.6), $P = 0.00001$.

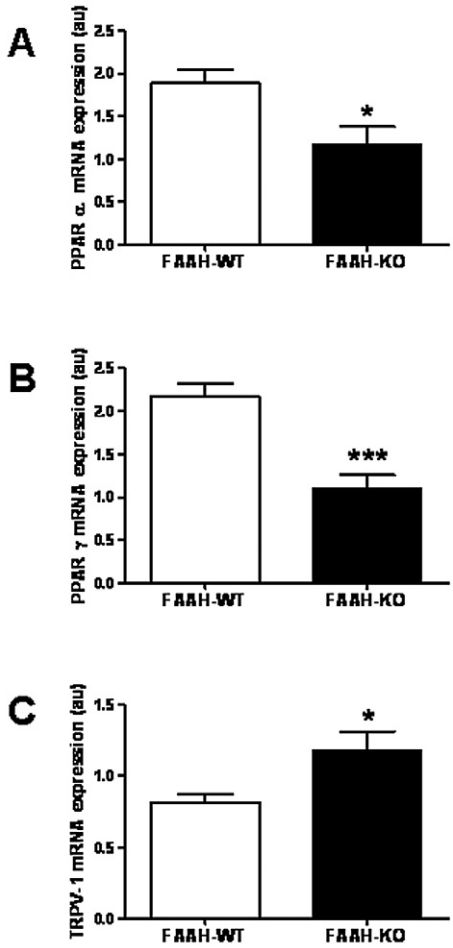


Figure 7

Genetic deletion of FAAH modifies the expression of PPARs and TRPV1 receptors in primary astrocytes. The basal levels measured by qRT-PCR of PPAR-α alpha (A) and PPAR-γ (B) were significantly decreased in FAAH-KO cells meanwhile TRPV1 receptor expression (C) was significantly higher than in WT astrocytes. The results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by Student's *t*-test (*$P < 0.05$; ***$P < 0.001$).

FAAH-KO astrocytes was significantly higher than in WT (Figure 7C). Neither PPARs nor TRPV1 receptor mRNA expression were modified by the addition of the β-amyloid peptide (data not shown). Thus, the deletion of FAAH enzyme modifies the expression of these receptors in a differential way.

PPAR-α and PPAR-γ and TRPV1 mediate the exacerbated proinflammatory changes observed in FAAH-KO astrocytes when exposed to Aβ$_{1–42}$

To evaluate the possibility that changes in PPAR and/or TRPV1 receptor expression or activity mediate the exacerbation of proinflammatory changes induced by Aβ$_{1–42}$ exposure in FAAH-KO astrocytes, we performed a set of pharmacological treatments. PPAR-α and PPAR-γ were activated using selective agonists, WY-14643 and troglitazone, respectively, during exposure to Aβ$_{1–42}$ (Figure 8). An 8-h exposure to Aβ$_{1–42}$ in the presence of the PPAR ligands resulted in a significant decrease in the expression of the inflammatory mediators studied (Figure 8). On the other hand, the blockade of TRPV1 receptors with the selective antagonist capsazepine, prevented the increase in mRNA levels of the enzymes iNOS and COX-2 and the cytokine TNF-α; (Figure 9A–C). However, the presence of the antagonist had no effect on the levels of IL-1β protein released into the medium of FAAH-KO astrocytes (Figure 9D). These results suggest that PPARs and TRPV1 receptors may be involved in the proinflammatory changes observed when FAAH-KO astrocytes are exposed to the pathologic peptide Aβ$_{1–42}$.

The pharmacological blockade of FAAH does not lead astrocytes to a pro-inflammatory phenotype

Pharmacological blockade of FAAH in WT astrocytes with 1 μM URB597 for 16 h did not mimic the response of FAAH-KO astrocytes against Aβ$_{1–42}$ (Figure 10). These data suggest that the observed changes could reflect a compensatory change in astrocytes in response to genetic deletion of FAAH rather than a lack of FAAH activity in the presence of Aβ. In addition, we tested whether co-incubation with URB597 and SR1 or SR2 modified the astrocytic response. We found (Figure 10) that URB597 did not introduce any significant change in the presence of any of both antagonists, thus suggesting that: (i) the pharmacological blockade of FAAH does not fully mimic the situation in FAAH-KO; and (ii) that CB_1 and CB_2 receptors do not play a major role in the reported observations.

Discussion

Our data reveal a potentially harmful effect on cellular function due to the lack of FAAH activity after genetic deletion. Although recent reports support a protective role derived of the enhancement of endocannabinoid tone (Hwang *et al.*, 2010), we found that cortical mouse astrocytes lacking FAAH exhibit a pro-inflammatory phenotype when exposed to the pathogenic form of Aβ. The absence of FAAH potentiated the effects of Aβ$_{1–42}$ to increase: (i) the concentration of secreted cytokines and cell death; (ii) the rate of activation (in terms of time course of phosphorylation and nuclear translocation) of specific signalling cascades; and (iii) the expression levels of mediators of inflammation in astrocytes. Pharmacological blockade of FAAH activity for several hours *in vitro* did not mimic these effects, suggesting that the observed effects could be due to compensatory changes that result from the potentially prolonged enhancement of *N*-acylethanolamines known to occur in FAAH-KO mice (Long *et al.*, 2011). The latter response may be due to more profound alterations linked to FAAH gene deletion. However, the exogenous administration of AEA, PEA and OEA to WT astrocytes did not mimic the observed effects of the exposure to Aβ$_{1–42}$ on FAAH-KO cells. Indeed, pre-incubation with these *N*-acylethanolamines could partially prevent the increases in the expression levels of several markers of inflammation induced by the peptide. In our opinion, this suggests that

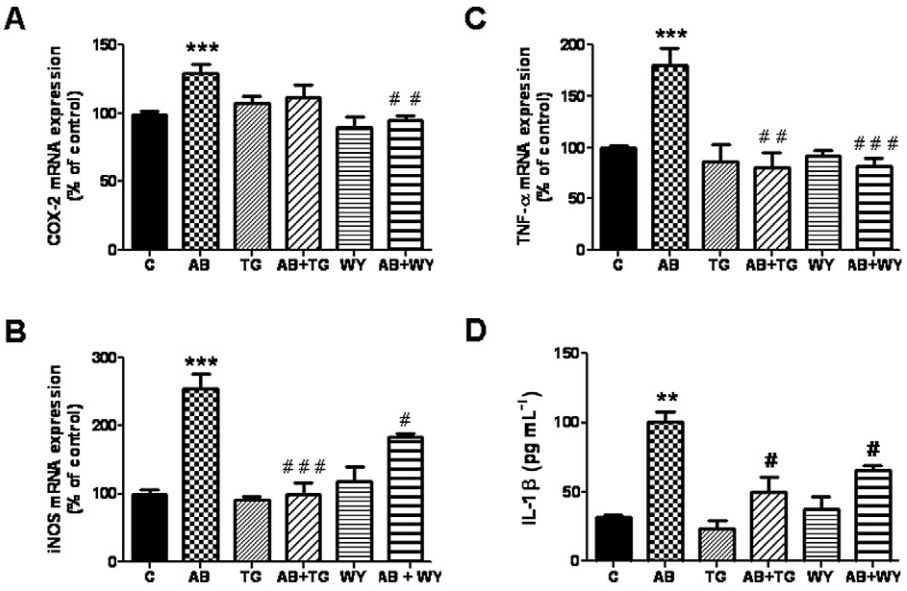


Figure 8

Treatment with the selective PPPAR-α and PPAR-γ agonists, WY-14643 (WY) and troglitazone (TG; both 10 μM), respectively, prevented the enhanced expression of the inflammatory mediators COX-2 (A), iNOS (B), TNF-α (C) and production of IL-1β (D), triggered by the exposure to $A\beta_{1-42}$ (AB) in FAAH-KO astrocytes. Results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by one-way ANOVA (**$P < 0.05$ and ***$P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. $A\beta_{1-42}$). COX-2: F (6.2/2.3), $P = 0.00009$. iNOS: F (8/2.4), $P = 0.00002$. TNF-α: F (7.1/2.5), $P = 0.0001$. IL-1β: F (14.8/3.1), $P = 0.00009$.

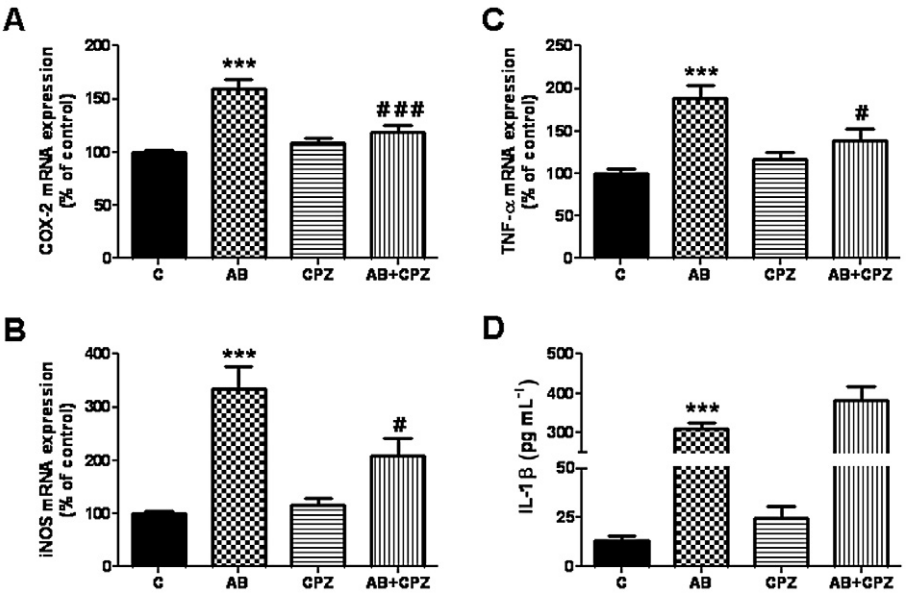


Figure 9

The selective TRPV1 antagonist, capsazepine (CPZ; 1 μM), significantly decreased mRNA expression of COX-2 (A), iNOS (B) and TNF-α (C) in FAAH-KO astrocytes treated with the pathological peptide (AB) but the quantity of IL-1β protein (D) measured by ELISA did not change. The results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by one-way ANOVA (***$P < 0.0001$ vs. control; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.0005$ vs. $A\beta_{1-42}$). COX-2: F (5.1/2.7), $P = 0.003$. iNOS: F (9.9/2.9), $P = 0.0001$. TNF-α: F (4.2/2.8), $P = 0.01$. IL1-β: F (100/4), $P = 0.000001$.

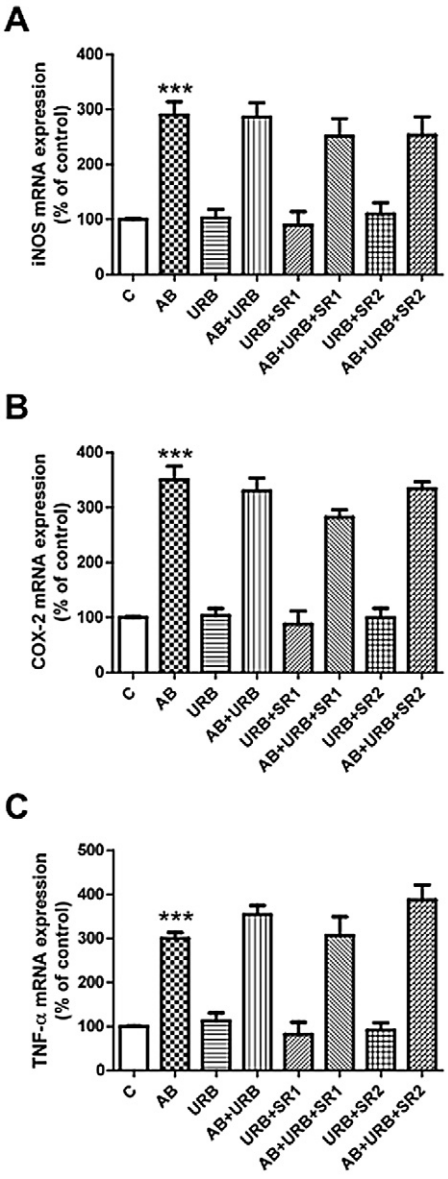


Figure 10

Pharmacological blockade of FAAH with URB597 (URB) does not mimic the effects derived of FAAH gene deletion. Increases in expression levels of iNOS (A), COX-2 (B) and TNF-α (C) were not modified by pre-incubation with URB597 alone or in combination with SR1 or SR2. The results shown are the mean ± SEM of three independent experiments performed in triplicate. Data were analysed by one-way ANOVA (***$P < 0.001$ vs. control). COX-2: F (39.9/2.2), $P = 0.00001$. iNOS: F (11.7/2.2), $P = 0.00001$. TNF-α: F (22.3/2.2), $P = 0.00001$.

critical phenotypic differences due to the lack of FAAH occur in KO astrocytes in agreement with previously published data obtained *in vivo* (Siegmund *et al.*, 2006; Mukhopadhyay *et al.*, 2011).

Our data also indicated that the pro-inflammatory effects of FAAH-KO did not involve increased CB_1 or CB_2 receptor activation. Recent data reported by van der Stelt *et al.* (2006) and Jung *et al.* (2011) showed that AEA levels are decreased as a consequence of Aβ administration or enhanced Aβ expression in human samples as well as in rat brains and also in murine Neuro-2a cells, which could account for the lack of cannabinoid receptor mediation in our model. Interestingly, reduced activation of PPARα and PPARγ and increased activation of TRPV1 receptors, appeared to participate in these effects. In particular, FAAH-KO astrocytes exhibited significant decreases in the mRNA for both PPAR-α and PPAR-γ and direct activation of PPARs by potent and selective agonists reduced the Aβ-triggered pro-inflammatory effects in FAAH-KO astrocytes without affecting their expression levels (data not shown). On the other hand, TRPV1 receptor expression was significantly increased at the level of mRNA transcription and capsazepine treatment of astrocytes from FAAH-KO mice reduced the effects of $A\beta_{1-42}$ to increase COX-2, iNOS and TNF-α mRNA expression.

There is substantial evidence for a neuroprotective role of the endocannabinoid system in acute and chronic neurodegenerative conditions (see Fernandez-Ruiz *et al.*, 2005), including Alzheimer's disease (Ramírez *et al.*, 2005; Noonan *et al.*, 2010). In particular, blockade of FAAH is considered as a promising therapeutic approach for the treatment of neurodegeneration (Hwang *et al.*, 2010) and both pharmacological and genetic inactivation of this enzyme have been shown to provide *in vivo* neuroprotection in experimental models (Karanian *et al.*, 2005). We previously suggested that FAAH inhibition could theoretically be a promising strategy to decrease Aβ-triggered inflammatory processes (Benito *et al.*, 2003). In fact, Van der Stelt *et al.* (2006) showed that the early, but not the late, enhancement of the endocannabinoid tone with the AEA uptake inhibitor VDM11 improved the pathological state in an animal model of Alzheimer's disease. Our present results however, depict a different situation, as the genetic deletion of FAAH exacerbated Aβ-linked inflammatory responses in astrocytes. Thus, it seems that the effects of a potential long-term increase in *N*-acylethanolamine concentration differs from the effects of the increase in *N*-acylethanolamine concentration that occurs in response to pharmacological blockade (Patel *et al.*, 2005) or, as we have shown here, by the addition of exogenous *N*-acylethanolamines. It must be emphasized, however, that the present data correspond to an *in vitro* model of *acute* exposure to $A\beta_{1-42}$ and thus the overall picture may differ from that in living animals subjected to chronic, increasingly higher, levels of Aβ. Ongoing experiments will clarify the putative pro-inflammatiory effect of FAAH deletion *in vivo* in animal models of Alzheimer's disease.

Inflammation plays a vital role in both acute and chronic neurodegeneration (Strohmeyer and Rogers, 2001; Wyss-Coray *et al.*, 2001; Allan and Rothwell, 2003; Wyss-Coray, 2006). Cytokine production and release in response to inflammatory stimuli, including Aβ, induces direct damage and enhances the deleterious effects of oxidative stress and excitotoxicity. In Alzheimer's disease, astrocytes are known to participate in the inflammatory processes linked to the pathological deposition of Aβ (Wyss-Coray, 2006). Although some studies suggest that astrocytes play a role in Aβ processing, it also seems clear that astrocytes are a source of proinflammatory molecules in Alzheimer's disease (Tuppo and Arias, 2005). It must be noted that not all cytokines studied in the present work followed identical patterns of expression

after Aβ treatment. For instance, WT astrocytes showed a significant decrease in IL-6 expression at 8 h after the treatment, followed by increases at 24 and 48 h. As recently reported (Semple *et al.*, 2011), the interaction among different cytokines may lead to complex changes in the production and expression of others, including IL-6. These authors have found some discrepancies in the expression levels of IL-6 in mouse astrocytes exposed to LPS for 8 h, dependent on the levels of CCL2 and IL-1β, and they hypothesize that CCL2 may act during early immunoactivation in an inhibitory manner, both *in vivo* and *in vitro*. We can thus speculate that, in our experimental model, early increases in CCL2 (together with the non-significant change in IL-1β levels in wild-type astrocytes) may be responsible for the decrease in IL-6 expression observed at 8 h after treatment. This action would not be observed in FAAH-KO astrocytes probably due to the robust increase in IL-1β levels, capable of overcoming the CCL2-induced decrease of IL-6 expression.

AEA has been reported to exhibit both cytotoxic and pro-apoptotic properties (Maccarrone and Finazzi-Agró, 2003) as well as neuroprotective effects (see Correa *et al.*, 2009). It is possible that the effects of increased concentrations of AEA differ depending upon the glial cell type. Thus, Maccarrone and Finazzi-Agró (2003) and De Lago *et al.* (2006) found that AEA (whether administered directly or by increasing its levels through uptake blockade) induced cell toxicity in C6 glioma cells. In particular, De Lago *et al.* (2006) suggested a potentially harmful effect of chronically increased AEA concentrations. In contrast, several reports indicate that AEA may have anti-inflammatory properties in astrocytes (Molina-Holgado *et al.*, 1997; 1998; Ortega-Gutiérrez *et al.*, 2005) and microglia (Correa *et al.*, 2010) as well as being protective for neurons exposed to amyloid (Noonan *et al.*, 2010).

Our results confirm and expand recent observations made by Scuderi *et al.* (2011) showing that PEA (one of the substrates of FAAH) decreases inflammation in rat primary astrocytes exposed to Aβ. We have found that pharmacological manipulation of cortical astrocytes with PEA (as well as with AEA and OEA) produces significantly different results, which suggest that the balance of *N*-acylethanolamines, affected by FAAH deletion, together with other phenotypic changes induced by the prolonged absence of the enzyme, might account for these differences. In support of this idea, we found that the blockade of FAAH with URB597 did not mimic the changes observed in KO astrocytes, suggesting that a long lasting, chronic potentiation of the *N*-acylethanolamine tone may be required in order to modify the astrocytic phenotype.

The anti-inflammatory effects derived from PPAR activation have been well described over the past few years (Luna-Medina *et al.*, 2005; Drew *et al.*, 2006). PPAR activation decreased neuronal iNOS expression and cell death (Heneka *et al.*, 2000), and reduced Aβ-induced proinflammatory responses in microglial cells (Combs *et al.*, 2001). Recently, Xu *et al.* (2006) have shown that PPAR activation inhibited LPS-induced production of several proinflammatory molecules (including IL-1b, IL-6, CCL2, NO and TNF-α) by primary mouse astrocytes. It is important to note that LPS and Aβ are known to activate common proinflammatory pathways, such as the CD14 receptor (Liu *et al.*, 2005).

Our results also confirm a relationship between the *N*-acylethanolamines and PPARs, as could be expected from previous reports showing that some *N*-acylethanolamines act as PPAR ligands (see O'Sullivan, 2007). In particular, we observed a significant decrease in the mRNA expression levels of both PPAR-α and PPAR-γ in FAAH-KO astrocytes. As activation of these nuclear receptors has been shown to inhibit inflammatory responses in mouse astrocytes (Xu *et al.*, 2006; Xu and Drew, 2007; Wang *et al.*, 2010) we hypothesized that this decrease could limit their anti-inflammatory activity, which in turn could contribute to the profound inflammatory phenotype of the astrocytes from FAAH-KO mice. In agreement with this reasoning, the presence of potent and specific agonists of both PPAR receptors decreased Aβ–induced overexpression of COX-2, iNOS and TNF-α, as well as in the overproduction of IL-1β, in FAAH-KO astrocytes.

As several *N*-acylethanolamines including AEA, PEA and OEA have been reported to act as PPAR agonists, the genetic deletion of FAAH may lead to a continuous activation of these receptors by these endocannabinoids or by structurally related metabolites (Kozak *et al.*, 2002). However, our results contradict previous reports obtained with the 3T3-L1 cell line and in primary adipocytes suggesting that AEA induces the transcriptional activation of PPARγ (Bouaboula *et al.*, 2005; Karaliota *et al.*, 2009). Finally, *N*-acylethanolamines could also activate alternative receptors that could in turn affect PPAR transcription and which long-term activation could result in a loss of PPAR activity.

TRPV1 receptors also participated in the FAAH-dependent astrocytic response. Previous reports indicate that murine astrocytes express TRPV1 receptors *in vivo* and *in vitro* (Doly *et al.*, 2004; Tóth *et al.*, 2005; Amantini *et al.*, 2007) and that AEA may have cytotoxic effects by activating this membrane channel (Maccarrone and Finazzi-Agró, 2003; Maccarrone *et al.*, 2010). To our knowledge, this is the first report indicating that potentially elevated levels of NAEs can modify TRPV1 receptor expression. The molecular mechanisms for this regulation have not been the object of the present study and deserve further analysis. FAAH-KO mice produce significantly lower amounts of the endovanilloid N-arachidonoyl dopamine, as its synthesis depends on FAAH activity (Hu *et al.*, 2009). We can thus speculate that the absence of the enzyme may lead to a decreased N-arachidonoyl dopamine, signalling, which in turn might lead to an up-regulation of the expression levels of TRPV1 receptors.

In summary, we have found a proinflammatory effect of FAAH deletion in astrocytes, which adds complexity to our understanding of the role that the endocannabinoid system may play in neuroprotection (see Esposito *et al.*, 2007; Fowler *et al.*, 2010). Considering the well-known anti-inflammatory effects of some of the substrates of FAAH, these data suggest that an excessively prolonged enhancement of the endocannabinoid tone may have harmful consequences by, for instance, down-regulating other anti-inflammatory cellular mechanisms, such as those mediated by PPARs, or by exacerbating the cytotoxic effects derived from TRPV1 receptor activation.

Acknowledgements

The authors wish to thank the administrative work of Ms Julia Molina and the technical support of Ms Marta Moreno.

These experiments were financed by CIBERNED (CB06/05/1109), Ministerio de Ciencia e Innovación (SAF2010/16706), Fondo de Investigaciones Sanitarias (FIS PS09/01900) and Comunidad de Madrid (S2010/BMD-2308). LRV and CV are recipients of a FPI predoctoral fellowship (BES2011-093393) from the Spanish Ministry of Science (MICINN). Authors are indebted to Sanofi-Aventis for the kind gift of the antagonists for cannabinoid receptors and to Dr Cecilia J Hillard (Medical College of Wisconisn, Milwaukee, WI, USA) and Dr Manuel Guzmán (Complutense University, Madrid, Spain) for critical reading of the manuscript and helpful discussions.

Conflict of interest

The authors declare no conflict of interest.

References

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Allan SM, Rothwell NJ (2003). Inflammation in central nervous system injury. Philos Trans R Soc London B Biol Sci 358: 1669–1677.

Amantini C, Mosca M, Nabissi M, Lucciarini R, Caprodossi S, Arcella A *et al*. (2007). Capsaicin-induced apoptosis of glioma cells is mediated by TRPV1 vanilloid receptor and requires p38 MAPK activation. J Neurochem 102: 977–990.

Bamberger ME, Harris ME, McDonald DR, Husemann J, Landreth GE (2003). A cell surface receptor complex for fibrillar β-amyloid mediates microglial activation. J Neurosci 23: 2665–2674.

Benito C, Nunez E, Tolon RM, Carrier EJ, Rabano A, Hillard CJ *et al*. (2003). Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. J Neurosci 23: 11136–11141.

Benito C, Tolón RM, Núñez E, Pazos MR, Romero J (2007). Neuroinflammation and the glial endocannabinoid system. In: Koffalvi A (ed.). Cannabinoids and the Brain. Springer Verlag: Berlin, pp. 314–334.

Bouaboula M, Hilairet S, Marchand J, Fajas L, Le Fur G, Casellas P (2005). Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation. Eur J Pharmacol 517: 174–181.

Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M, Henschen A *et al*. (1992). Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. J Biol Chem 267: 546–554.

Combs CK, Bates P, Karlo C, Landreth GE (2001). Regulation of beta-amyloid stimulated proinflammatory responses by peroxisome proliferator-activated receptor alpha. Neurochem Int 39: 449–457.

Coraci IS, Husemann J, Berman JW, Hulette C, Dufour JH, Campanella GK *et al*. (2002). CD36, a class B scavenger receptor, is expressed on microglia in Alzheimer's disease brains and can mediate production of reactive oxygen species in response to β–amyloid fibrils. Am J Pathol 160: 101–112.

Correa F, Mestre L, Docagne F, Borrell J, Guaza C (2009). The endocannabinoid anandamide from immunomodulation to neuroprotection. Implications for multiple sclerosis. Vitam Horm 81: 207–230.

Correa F, Hernangómez M, Mestre L, Loría F, Spagnolo A, Docagne F *et al*. (2010). Anandamide enhance IL-10 production in activated microglia by targeting CB(2) receptors: roles of ERK1/2, JNK, and NF-kappaB. Glia 58: 135–147.

Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR *et al*. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc Natl Acad U S A 98: 9371–9376.

De Lago E, Gustafsson SB, Fernández-Ruiz J, Nilsson J, Jacobsson SOP, Fowler CJ (2006). Acyl-based anandamide uptake inhibitors cause rapid toxicity to C6 glioma cells at pharmacologically relevant concentrations. J Neurochem 99: 677–688.

De Petrocellis L, Davis JB, Di Marzo V (2001). Palmitoylethanolamide enhances anandamide stimulation of human vanilloid VR1 receptors. FEBS Lett 506: 253–256.

Doly S, Fischer J, Salio C, Conrath M (2004). The vanilloid receptor-1 is expressed in rat spinal dorsal horn astrocytes. Neurosci Lett 357: 123–126.

Drew PD, Xu J, Storer PD, Chavis JA, Racke MK (2006). Peroxisome proliferator-activated receptor agonist regulation of glial activation: relevance to CNS inflammatory disorders. Neurochem Int 49: 183–189.

Esposito G, Iuvone T, Savani C, Scuderi C, De Filippis D, Papa M *et al*. (2007). Opposing control of cannabinoid receptor stimulation on amyloid-beta-induced reactive gliosis: *in vitro* and *in vivo* evidence. J Pharmacol Exp Ther 322: 1144–1152.

Fernandez-Ruiz JJ, Gonzalez S, Romero J, Ramos JA (2005). Cannabinoids in neurodegeneration and neuroprotection. In: Mechoulam R (ed.). Cannabinoids as Therapeutics. Birkhäuser Verlag: Basel, Switzerland, pp. 79–109.

Fowler CJ, Rojo ML, Rodríguez-Gaztelumendi A (2010). Modulation of the endocannabinoid system: neuroprotection or neurotoxicity? Exp Neurol 224: 37–47.

Heneka MT, Klockgether T, Feinstein DL (2000). Peroxisome proliferator-activated receptor-gamma ligands reduce neuronal inducible nitric oxide synthase expression and cell death *in vivo*. J Neurosci 20: 6862–6867.

Hu SSJ, Bradshaw HB, Benton VM, Chen JSC, Huang SM, Minassi A *et al*. (2009). The biosynthesis of N-arachidonoyl dopamine (NADA), a putative endocannabinoid and endovanilloid, via conjugation of arachidonic acid with dopamine. Prostaglandins Leukot Essent Fatty Acids 81: 291–301.

Hwang J, Adamson C, Butler D, Janero DR, Makryiannis A, Bahr BA (2010). Enhancement of endocannabinoid signalling by fatty acid amide hydrolase inhibition: a neuroprotective therapeutic modality. Life Sci 86: 615–623.

Jiang Q, Heneka M, Landreth GE (2008). The role of peroxisome proliferator-activated receptor-gamma (PPARgamma) in Alzheimer's disease: therapeutic implications. CNS Drugs 22: 1–14.

Jung KM, Astarita G, Yasar S, Vasilevko V, Cribbs DH, Head E *et al*. (2011). An amyloid b(42)-dependent deficit in anandamide mobilization is associated with cognitive dysfunction in Alzheimer's disease. Neurobiol Aging. doi: 10.1016/j.neurobiolaging.2011.03.012.

Karaliota S, Siafaka-Kapadai A, Gontinou C, Psarra K, Mavri-Vavayanni M (2009). Anandamide increases the differentiation of rat adipocytes and causes PPARgamma and CB1 receptor upregulation. Obesity 17: 1830–1838.

Karanian DA, Brown QB, Makryiannis A, Kosten TA, Bahr BA (2005). Dual modulation of endocannabinoid transport and fatty acid amide hydrolase protects against excitotoxicity. J Neurosci 25: 7813–7820.

Koenigsknecht-Talboo J, Landreth G (2005). Microglial phagocytosis induced by fibrillar β-amyloid and IgGs are differentially regulated by proinflammatory cytokines. J Neurosci 25: 8240–8249.

Kozak KR, Crews BC, Morrow JD, Wang LH, Ma YH, Weinander R *et al.* (2002). Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. J Biol Chem 277: 44877–44885.

Lim JH, Gibbons HM, O'Carroll SJ, Narayan PJ, Faull RLM, Dragunow M (2007). Extracellular signal-regulated kinase involvement in human astrocyte migration. Brain Res 1164: 1–13.

Liu Y, Walter S, Stagi M, Cherny D, Letiembre M, Schulz-Schaeffer H *et al.* (2005). LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. Brain 128: 1778–1789.

Long JZ, LaCava M, Jin X, Cravatt BF (2011). An anatomical and temporal portrait of physiological substrates for fatty acid amide hydrolase. J Lipid Res 52: 337–344.

Lorenzo A, Yankner BA (1994). Beta-amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. Proc Natl Acad Sci U S A 91: 12243–12247.

Luna-Medina R, Cortes-Canteli M, Alonso M, Santos A, Martinez A, Perez-Castillo A (2005). Regulation of inflammatory response in neural cells *in vitro* by thiadiazolidinones derivatives through peroxisome proliferator-activated receptor gamma activation. J Biol Chem 280: 21453–21462.

Maccarrone M, Finazzi-Agró A (2003). The endocannabinoid system, anandamide and the regulation of mammalian cell apoptosis. Cell Death Differ 10: 946–955.

Maccarrone M, Dainese E, Oddi S (2010). Intracellular trafficking of anandamide: new concepts for signalling. Trends Biochem Sci 35: 601–608.

McCarthy KD, de Vellis J (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85: 890–902.

Molina-Holgado F, Lledó A, Guaza C (1997). Anandamide suppresses nitric oxide and TNF-alpha responses to Theiler's virus or endotoxin in astrocytes. Neuroreport 8: 1929–1933.

Molina-Holgado F, Molina-Holgado E, Guaza C (1998). The endogenous cannabinoid anandamide potentites interleukin-6 production by astrocytes infected with Theiler's murine encephalomyelitis virus by a receptor-mediated pathway. FEBS Lett 433: 139–142.

Molina-Holgado F, Molina-Holgado E, Guaza C, Rothwell NJ (2002). Role of CB1 and CB2 receptors in the inhibitory effects of cannabinoids on lipopolysaccharide-induced nitric oxide release in astrocyte cultures. J Neurosci Res 67: 829–836.

Mukhopadhyay P, Horváth B, Rajesh M, Matsumoto S, Saito K, Bátkai S *et al.* (2011). Fatty acid amide hydrolase is a key regulator of endocannabinoid-induced myocardial tissue injury. Free Radic Biol Med 50: 179–195.

Naslund J, Haroutunian V, Mosh R, Davis KL, Davies P, Greengard P *et al.* (2000). Correlation between elevated levels of amyloid-beta peptide in the brain and cognitive decline. JAMA 283: 1571–1577.

Noonan J, Tanveer R, Klompas A, Gowran A, McKiernan J, Campbell VA (2010). Endocannabinoids prevent beta-amyloid-mediated lysosomal destabilization in cultured neurons. J Biol Chem 285: 38543–38554.

Nuñez E, Benito C, Tolón RM, Hillard CJ, Griffin WS, Romero J (2008). Glial expression of cannabinoid CB(2) receptors and fatty acid amido hydrolase are beta amyloid-linked events in Down's syndrome. Neuroscience 151: 104–110.

O'Sullivan SE (2007). Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. Br J Pharmacol 152: 576–582.

Ortega-Gutiérrez S, Molina-Holgado E, Guaza C (2005). Effect of anandamide uptake inhibition in the production of nitric oxide and in the release of cytokines in astrocyte cultures. Glia 52: 163–168.

Patel S, Carrier EJ, Ho WS, Rademacher DJ, Cunningham S, Reddy DS *et al.* (2005). The postmortal accumulation of brain N-arachidoylethanolamine (anandamide) is dependent upon fatty acid amide hydrolase activity. J Lipid Res 46: 342–349.

Pertwee R (2008). Ligands that target cannabinoid receptors in the brain: from THC to anandamide and beyond. Addict Biol 13: 147–159.

Pertwee R, Howlett AC, Abood ME, Alexander SPH, Di Marzo V, Elphick MR *et al.* (2010). International Union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. Pharmacol Rev 62: 588–631.

Ramírez BG, Blazquez C, Gomez del Pulgar T, Guzman M, De Ceballos ML (2005). Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. J Neurosci 25: 1904–1913.

Romero J, Hillard CJ, Calero M, Rabano A (2002). Fatty acid amide hydrolase localization in the human central nervous system: an immunohistochemical study. Mol Brain Res 100: 85–93.

Scuderi C, Esposito G, Blasio A, Valenza M, Arietti P, Steardo L *et al.* (2011). Palmitoylethanolamide counteracts reactive astrogliosis induced by beta-amyloid peptide. J Cell Mol Med 15: 2664–2674.

Semple BD, Frugier T, Morganti-Kossmann MC (2011). CCL2 modulates cytokine production in cultured mouse astrocytes. J Neuroinflammation 7: 67.

Shivachar AC (2007). Cannabinoids inhibit sodium-dependent, high affinity excitatory amino acid transport in cultured rat cortical astrocytes. Biochem Pharmacol 73: 2004–2011.

Siegmund SV, Seki E, Osawa Y, Uchinami H, Cravatt BF, Schwabe RF (2006). Fatty acid amide hydrolase determines anandamide-induced cell death in the liver. J Biol Chem 281: 10431–10438.

Starowicz K, Nigam S, Di Marzo V (2007). Biochemistry and pharmacology of endovanilloids. Pharmacol Ther 114: 13–33.

Stella N (2004). Cannabinoid signalling in glial cells. Glia 48: 267–277.

Strohmeyer R, Rogers J (2001). Molecular and cellular mediators of Alzheimer's disease inflammation. J Alzheimers Dis 3: 131–157.

Tóth A, Boczán J, Kedei N, Lizanecz E, Bagi Z, Papp Z *et al.* (2005). Expression and distribution of vanilloid receptor 1 (TRPV1) in the adult rat brain. Brain Res Mol Brain Res 135: 162–168.

Tsou K, Nogueron MI, Muthian S, Sañudo-Peña MC, Hillard CJ, Deutsch DG *et al*. (1998). Fatty acid amide hydrolase is located preferentially in large neurons in the rat central nervous system as revealed by immunohsitochemistry. Neurosci Lett 254: 137–140.

Tuppo EE, Arias HR (2005). The role of inflammation in Alzheimer's disease. Int J Biochem Cell Biol 37: 289–305.

Van der Stelt M, Mazzola C, Esposito G, Matias I, Petrosino S, De Filippis D *et al*. (2006). Endocannabinoids and beta-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels. Cell Mol Life Sci 63: 1410–1424.

Vignali M, Benfenati V, Caprini M, Anderova M, Nobile M, Ferroni S (2009). The endocannabinoid anandamide inhibits potassium conductance in rat cortical astrocytes. Glia 57: 791–806.

Walter L, Franklin A, Witting A, Möller T, Stella N (2002). Astrocytes in culture produce anandamide and other acylethanolamides. J Biol Chem 277: 20869–20876.

Wang HM, Zhao YX, Zhang S, Liu GD, Kang WY, Tang HD *et al*. (2010). PPARgamma agonist curcumin reduces the amyloid-beta-stimulated inflammatory responses in primary astrocytes. J Alzheimers Dis 20: 1189–1199.

Wyss-Coray T (2006). Inflammation in Alzheimer's disease: driving force, bystander or beneficial response? Nat Med 12: 1005–1015.

Wyss-Coray T, McConlogue L, Kindy M, Schmidt AM, Du Yan S, Stern DM (2001). Key signalling pathways regulate the biological activities and accumulation of amyloid-beta. Neurobiol Aging 22: 967–973.

Xu J, Drew PD (2007). Peroxisome proliferator-activated receptor-gamma agonists suppress the production of IL-12 family cytokines by activated glia. J Immunol 178: 1904–1913.

Xu J, Chavis JA, Racke MK, Drew PD (2006). Peroxisome proliferator-activated receptor-alpha and retinoid X receptor agonists inhibit inflammatory responses of astrocytes. J Neuroimmunol 176: 95–105.