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Pharmacological inhibition of FAAH modulates TLR-induced neuroinflammation, but not sickness behaviour: an effect partially mediated by central TRPV1

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Abstract

Aberrant activation of toll-like receptors (TLRs), key components of the innate immune system, has been proposed to underlie and exacerbate a range of central nervous system disorders. Increasing evidence supports a role for the endocannabinoid system in modulating inflammatory responses including those mediated by TLRs, and thus this system may provide an important treatment target for neuroinflammatory disorders. However, the effect of modulating endocannabinoid tone on TLR-induced neuroinflammation *in vivo* and associated behavioural changes is largely unknown. The present study examined the effect of inhibiting fatty acid amide hydrolyase (FAAH), the primary enzyme responsible for the metabolism of anandamide (AEA), *in vivo* on TLR4-induced neuroimmune and behavioural responses, and evaluated sites and mechanisms of action. Systemic administration of the FAAH inhibitor PF3845 increased levels of AEA, and related FAAH substrates N-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA), in the frontal cortex and hippocampus of rats, an effect associated with an attenuation in the expression of pro- and anti-inflammatory cytokines and mediators measured 2hrs following systemic administration of the TLR4 agonist, lipopolysaccaharide (LPS). These effects were mimicked by central i.c.v. administration of PF3845, but not systemic administration of the peripherally-restricted FAAH inhibitor URB937. Central antagonism of TRPV1 significantly attenuated the PF3845-induced decrease in *IL-6* expression, effects not observed following antagonism of CB_1, CB_2, PPARα, PPARγ or GPR55. LPS-induced a robust sickness-like behavioural response and increased the expression of markers of glial activity and pro-inflammatory cytokines over 24hrs. Systemic administration of PF3845 modulated the TLR4-induced expression of neuroimmune mediators and anhedonia without altering acute sickness behaviour. Overall, these findings support an important role for FAAH substrates

directly within the brain in the regulation of TLR4-associated neuroinflammation and highlight a role for TRPV1 in partially mediating these effects.

Abbreviations: 2-AG, 2-arachidonoyl glycerol; CB, cannabinoid; FAAH, fatty acid amide hydrolyase; i.c.v., intracerebroventricular; IL, interleukin; NFκB, nuclear factor kappa B; LPS, Lipopolysaccharide,; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PPAR, peroxisome proliferator-activated receptor; TLR, toll-like receptor; TNF, tumour necrosis factor; SOCS, suppressor of cytokine signalling; TRPV1, Transient receptor potential cation channel subfamily V member 1.

Introduction

Toll-like receptors (TLRs) are key players in host defense, homeostasis and response to injury. However, uncontrolled and aberrant TLR activation has been proposed to trigger the onset of certain psychiatric and neurodegenerative disorders and elicit detrimental effects on the progression and outcome of established disease [for reviews see (Arroyo *et al*, 2011; Bergink *et al*, 2014; Deleidi and Isacson, 2012; Reus *et al*, 2015)]. Furthermore, TLR-induced neuroinflammation results in a constellation of behavioural changes which include altered appetite, reduced mood, cognitive changes, anxiety and anhedonia. Accumulating evidence demonstrates potent immunoregulatory effects of the endogenous cannabinoid (endocannabinoid) system, suggesting that this system may represent an important therapeutic target in disorders with a neuroinflammatory component [for reviews see (Downer, 2011; Fitzgibbon *et al*, 2015; Henry *et al*, 2016)]. The most widely studied endocannabinoid, *N*-arachidonoyl ethanolamine (AEA, also referred to as anandamide), has been shown to modulate neuroimmune responses, including those induced following TLR activation, although the effects depend on conditions under investigation. For example, several *in vitro* studies have demonstrated that increasing AEA tone, directly or via inhibition of the primary enzyme responsible for its metabolism, the serine hydrolase fatty acid amide hydrolase (FAAH), is associated with attenuation of TLR4-induced production of pro-inflammatory cytokines and mediators such as TNFα, IL-1β, prostaglandins and nitric oxide (Facchinetti *et al*, 2003; Molina-Holgado *et al*, 1997; Ortega-Gutierrez *et al*, 2005; Puffenbarger *et al*, 2000; Tham *et al*, 2007), while concurrently increasing anti-inflammatory mediators such as IL-10 (Correa *et al*, 2010; Krishnan and Chatterjee, 2012). However, data also demonstrate an augmentation of TLR4-induced pro-inflammatory mediators such as IL-6 by AEA (Molina-Holgado *et al*, 1998; Ortega-Gutierrez *et al*, 2005). While some studies have demonstrated anti-inflammatory effects of AEA

on TLR4-induced inflammatory responses to be mediated by cannabinoid CB_1 and/or CB_2 receptor activation and consequential regulation of NFκB and MAPK activation (Correa *et al*, 2009a; Correa *et al*, 2010; Krishnan *et al*, 2012; Ortega-Gutierrez *et al*, 2005), non-CB_1/CB_2 receptor mediated effects of AEA on inflammatory processes *in vitro* have also been reported (Correa *et al*, 2008; Tham *et al*, 2007). AEA also has affinity for and activity at additional receptor targets to CB_1 and CB_2 receptors, namely the peroxisome proliferator-activated receptors (PPARs), the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) and also the novel cannabinoid receptor, G-protein coupled receptor (GPR)55 [for reviews see (Alexander and Kendall, 2007; Di Marzo *et al*, 2001; Madasu *et al*, 2015; O'Sullivan and Kendall, 2010)], activity at which may account for the variability in the effects of AEA on neuroinflammatory responses following TLR activation.

Similar to *in vitro* data, *in vivo* studies have also revealed modulation of TLR4-induced inflammatory responses by AEA. The proposed AEA reuptake inhibitor AM404 has been shown to attenuate TLR4-induced increases in plasma levels of IL-6 and IL-1β, the latter effect mediated by CB_1 receptor activation (Roche *et al*, 2008). Furthermore, AM404 or enhancing AEA tone via pharmacological inhibition of FAAH, augmented TLR4-induced increases in plasma TNFα levels, an effect at least partially mediated via activation of PPARγ (Roche *et al*, 2008). In the brain, AEA activation of hypothalamic CB_1 receptors has been shown to facilitate (De Laurentiis *et al*, 2010), while antagonism of the central CB_1 receptors attenuates (Steiner *et al*, 2011), TLR4-induced increases in plasma TNFα levels. In addition, work from our laboratory has demonstrated that enhancing AEA levels following FAAH inhibition was associated with attenuation of TLR4-induced increases in IL-1β, and increases in expression of suppressor of cytokine signalling (SOCS3), in the hypothalamus (Kerr *et al*, 2012). It should be noted that in addition to AEA, related fatty acid amides, *N*-oleoylethanolamide (OEA) and *N*-

palmitoylethanolamide (PEA), are also metabolised by (FAAH) and shown to be increased following FAAH inhibition. These *N*-acylethanolamines have been shown to exert potent biological effects on satiety, pain and inflammation (Esposito and Cuzzocrea, 2013; Mattace Raso *et al*, 2014; Sayd *et al*, 2015; Skaper *et al*, 2015; Suardiaz *et al*, 2007; Thabuis *et al*, 2008) and so it cannot be ruled out that some of the effects of FAAH inhibition may be due in part to activity of OEA or PEA, alone or in combination, with AEA. In addition to AEA, OEA has activity at the TRPV1 (Ahern, 2003; Almasi *et al*, 2008; Gonzalez-Aparicio and Moratalla, 2014; Movahed *et al*, 2005; Starowicz *et al*, 2013; Wang *et al*, 2005) and increasing evidence supports an important physiological role for TRPV1 in the brain (Edwards, 2014; Madasu *et al*, 2015; Martins *et al*, 2014). Furthermore, FAAH inhibition can lead to indirect activation/desensitization of TRPV1 and subsequent analgesic effects, anti-inflammatory effects and central effects on mood (Maione *et al*, 2007; Rubino *et al*, 2008; Starowicz *et al*, 2013). Taken together, data indicate that enhancing AEA (and related fatty acid amides) *in vivo* can modulate neuroinflammatory and behavioural responses. However, it remains to be determined if immunomodulatory effects occur due to indirect modulation of peripheral TLR4-induced immune responses, or directly at the level of the brain. Recent work from our group has demonstrated an important role for central FAAH substrates in attenuating the neuroinflammatory response to TLR3 activation (Henry *et al*, 2014). However, it is unknown if a similar effect is observed following activation of TLR4, or the receptor and molecular mechanisms involved. Furthermore, it remains to be determined if modulation of TLR4-induced neuroinflammatory responses results in concominent alterations in behaviour. As such the aim of the current study was to investigate if enhancing FAAH substrate, tone in the brain directly modulates TLR4-induced neuroinflammatory responses, sickness behaviour and anhedonia, and examine the potential receptor and molecular mechanism(s) mediating these effects.

Materials and Methods

Animals

Experiments were carried out on male Sprague-Dawley rats (weight, 250-300g; Charles River, UK) housed singly in plastic bottomed cages (45*25*20 cm) containing wood shavings as bedding. The animals were maintained at a constant temperature (21 ± 2°C) under standard lighting conditions (12:12 h light-dark, lights on from 0700 to 1900 h). All experiments were carried out during the light phase between 0800 h and 1800 h. Food and water were available *ad libitum.* Animals were habituated to handling and received i.p. injection of sterile saline (0.89% NaCl) for 3-4 days before experimentation to minimise the influence of the injection procedure on biological endpoints. The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under the licence from the Irish Department of Health and Children, and in compliance with the European Communities Council directive 2010/63/EU. All sections of the study adhered to the ARRIVE Guidelines for reporting in animal research (Kilkenny *et al*, 2010).

Experimental design

Experiment 1: The effect of systemic administration of the FAAH inhibitor PF3845 on TLR4-induced inflammation in the rat brain

Rats were randomly assigned into one of two treatment groups: Vehicle-LPS or PF3845-LPS (n = 8-10 per group) sacrificed 2 post LPS. The potent FAAH inhibitor PF3845 (NIMH drug synthesis programme; 10mg/kg, i.p.) or vehicle (ethanol:cremaphor:saline; 1:1:18) was administered 30 min prior to systemic administration of LPS (100μg/kg, i.p.). The dose of PF3845 was chosen on the basis of previous published work which demonstrated that systemic

administration of PF3845 increased brain levels of the FAAH substrates AEA, OEA and induced biological effects in several assays (Ahn *et al*, 2009; Booker *et al*, 2012; Nasirinezhad *et al*, 2015; Rock *et al*, 2015). Published and pilot work in our laboratory has also demonstrated that FAAH inhibitors do not modulate cytokine expression in the brain in the absence of an immune stimulus (Kerr *et al*, 2012). Furthermore, previous work from our group has demonstrated that LPS, at the dose used in this study, enhances cytokine expression in the brain and periphery at 2 hours post administration without altering levels of endocannabinoids or related fatty acids (Kerr *et al*, 2012; Roche *et al*, 2006; Roche *et al*, 2008). Animals were sacrificed by decapitation 2 hours post LPS administration, the brain rapidly removed, the frontal cortex and hippocampus excised, snap-frozen on dry ice and stored at -80°C until assayed for endocannabinoid and *N*-acylethanolamine levels and gene expression of markers of neuroinflammation.

Experiment 2: The effect of systemic administration of the FAAH inhibitor PF3845 on locomotor activity, sucrose preference and expression of inflammatory mediators 24hrs following TLR4 activation.

Rats were presented with 2 water bottles in their home cage, one containing tap water and the other 1% (w/v) sucrose solution, for 4 days prior to (to confirm sucrose preference prior to experiment), and for 24 hours following LPS. Homecage locomotor activity was also assessed during this time using the Opto M3 Dual Axis system (Columbus Instruments, Columbus, OH) as previously described (Bree *et al*, 2016; Bree *et al*, 2015). On the day of the experiment rats were randomly assigned into one of four treatment groups: Vehicle-Saline, PF3845-Saline, Vehicle-LPS or PF3845-LPS (n = 8-10 per group). PF3845 (10mg/kg, i.p.) or vehicle (ethanol:cremaphor:saline; 1:1:18) was administered 30 min prior to systemic administration of LPS (100μg/kg, i.p.) or Saline. Animals were returned to their home cage and horizontal activity

(total beam breaks) was recorded and presented as activity during the dark phase (10-22 hours post LPS). Bottles containing water and sucrose were weighed prior to and 24 hours following treatment, following which animals were immediately euthanised, the brain removed and the frontal cortex and hippocampus dissected and weighed, snap-frozen on dry ice, and stored at -80°C until assayed for the expression of markers of glial activation and cytokines. Sucrose preference over 24 hours was calculated as amount of sucrose solution consumed/total fluid intake x 100%.

Experiment 3: The effect of increasing FAAH substrate levels directly within the brain on TLR4-induced neuroinflammation

(a) The effect of i.c.v. administration of PF3845 on TLR4-induced increases in expression of neuroinflammatory mediators in the rat brain

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see 2.3 below), rats were randomly assigned into one of two treatment groups: Vehicle-LPS or PF3845-LPS (n = 8-10 per group). Rats received a single i.c.v. administration of PF3845 (500nmoles) or vehicle (100% DMSO) in an injection volume of 5µl/min followed 15 min later by systemic administration of LPS (100µg/kg, i.p.). The dose and time of PF3845 was chosen based on pilot work within our laboratory demonstrating increases in AEA, OEA and PEA in discrete rat brain regions following i.c.v. administration. Animals were returned to their home cages and euthanised 2 hours following LPS administration, the brain removed, frontal cortex excised, snap-frozen and stored at -80°C until assayed for endocannabinoid and *N*-acylethanolamine levels, and cytokine expression.

(b) The effect of systemic administration of the peripherally restricted FAAH inhibitor URB937 on TLR4-induced cytokine expression in the rat brain

Rats were randomly assigned to one of two treatment groups: Vehicle-LPS and URB937-LPS (n = 8 per group). The peripherally restricted FAAH inhibitor URB937 (Cayman Chemical; 1mg/kg, i.p.) or vehicle (ethanol: cremophor: saline; 1:1:18) were administered 1 hour prior to systemic administration of LPS (100ug/kg, i.p.). The dose of URB937 was chosen on the basis of previous published work demonstrating that at this dose, URB937 inhibits FAAH activity and increases AEA levels peripherally, but not in the rodent brain (Clapper *et al*, 2010). Animals were returned to their home cages and euthanised 2 hours following LPS administration, the frontal cortex and spleen excised, snap-frozen and stored at -80°C until assayed.

Experiment 4: Investigating the role of central CB_1 and CB_2 receptors in the FAAH substrate-mediated modulation of neuroinflammation following TLR4 activation

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle, rats were randomly assigned into one of 5 treatment groups: Vehicle-vehicle-saline, Vehicle-vehicle-LPS, Vehicle-PF3845-LPS, AM251-PF3845-LPS, AM630-PF3845-LPS, (n = 10-13 per group). Rats received a single. administration of either AM251 (2.5µg/5µl), AM630 (5µg/5µl), or vehicle (100% DMSO) over 1 min, followed immediately by systemic administration of PF3845 (10mg/kg, i.p.) or vehicle (ethanol:cremaphor:saline; 1:1:18). Animals were returned to their home cage and received a systemic injection of LPS (100µg/kg, i.p.) or sterile saline (0.89%), 30 min post PF3845/vehicle injection. The doses of the antagonists were based on in-house pilot experiments and previous published work demonstrating antagonistic activity *in vivo* (Fernandez-Solari *et al*, 2006; Fraga *et al*, 2009; Rea *et al*, 2013). Two hours

following LPS or saline, animals were euthanised by decapitation, the frontal cortex excised, snap-frozen and stored at -80°C until analysis.

Experiment 5: Investigating the role of non-CB_1/CB_2 receptors in the FAAH substrate-mediated modulation of neuroinflammatory genes following TLR4 activation

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle, rats were randomly assigned into one of 7 treatment groups: Vehicle-vehicle-saline, Vehicle-vehicle-LPS, Vehicle-PF3845-LPS, MK886-PF3845-LPS, GW9662-PF3845-LPS, IRTX-PF3845-LPS and CID-16020046 (CID)-PF3845-LPS (n = 9-10 per group). Rats received a single i.c.v. administration of either the PPARα antagonist MK886 (300ng), the PPARγ antagonist GW9662 (50μg), the TRPV1 antagonist IRTX (1nmol), the GPR55 receptor antagonist CID (10μM) or vehicle (100% DMSO) in an injection volume of 5μl over 1 min, followed immediately by systemic administration of PF3845 (10mg/kg, i.p.) or vehicle (ethanol:cremaphor:saline; 1:1:18). Animals were returned to their home cage and 30 min later received a single injection of LPS (100μg/kg, i.p.) or sterile saline (0.89%). The doses of the antagonists were based on in-house pilot experiments and previous published work demonstrating antagonistic activity *in vivo* (de Novellis *et al*, 2011; Fakhfouri *et al*, 2012; Kargl *et al*, 2013; Morgenweck *et al*, 2010). Two hours following LPS or saline, animals were euthanised by decapitation, the frontal cortex excised, snap-frozen and stored at -80°C until assayed.

Experiment 6: The effect of FAAH inhibition and/or central TRPV1 antagonism on LPS-induced sickness behaviour over 24hrs

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle, rats were randomly assigned into one of 5 treatment groups: Vehicle-vehicle-saline,

Vehicle-vehicle-LPS, Vehicle-PF3845-LPS, IRTX-PF3845-LPS or IRTX-Vehicle-LPS (n = 8-9 per group). Rats were presented with 2 water bottles in their home cage, one containing tap water and the other containing 1% (w/v) sucrose solution, for 4 days prior to testing (to confirm sucrose preference prior to experiment). On the experimental day, rats received a single i.c.v. administration of either the TRPV1 antagonist IRTX (1nmol) or vehicle (100% DMSO) in an injection volume of 5ul over 1 min, followed immediately by systemic administration of PF3845 (10mg/kg, i.p.) or vehicle (ethanol:cremaphor:saline; 1:1:18). Animals were returned to their home cage and 30 min later received a single injection of LPS (100μg/kg, i.p.) or sterile saline (0.89%). Rectal temperature was recorded 2hrs post LPS administration. Food, water, sucrose and body weight were recorded prior to any experimental manipulation and 24hrs post LPS administration and used to calculate body weight gain, food, water and sucrose intake and sucrose preference over the 24hr period.

Intracerebroventricular (i.c.v.) guide cannula implantation

Intracerebroventricular (i.c.v.) guide cannulae were implanted into the rat brain as previously described (Henry *et al*, 2014). In brief, under isoflurane anaesthesia (1-3% in O_2; 0.5L/min), a guide cannula (5mm, Plastics One Inc., Roanoke, Virginia, USA) was stereotaxically implanted into the right lateral ventricle (coordinates: AP: -0.07mm; ML: -0.15mm, DV: -0.30mm; (Paxinos, 2006)). The cannula was permanently fixed to the skull using stainless steel screws and dental acrylic cement and the guide remained patent by the insertion of a stainless steel stylet (Plastics One Inc., USA). Animals received the broad spectrum antibiotic enrofloxacin (2.5mg/kg s.c.; Baytril, Bayer Ltd., Ireland) on the day of and for 3 days post surgery. Correct cannula placement was verified by the Angiotensin (Ang) II drinking test 3 days prior to the

experiment as previously described (De Fanti and Martinez, 2002). Animals were considered non-responders if they drank <3mls over 20 min post AngII infusion and were not included in the experiment. Over all experiments, the average number of non-responders was <5%. Animals were allowed to recover from surgery for at least 6 days prior to experimentation.

Quantification of endocannabinoids and N-acylethanolamine concentrations by liquid chromatography – tandem mass spectrometry (LC-MS/MS)

Quantitation of endocannabinoids and *N*-acylethanolamines was essentially as described previously (Ford *et al*, 2011; Henry *et al*, 2014; Kerr *et al*, 2012; Kerr *et al*, 2013a; Kerr *et al*, 2013b). In brief, tissue samples were homogenised in 400µL 100% acetonitrile containing deuterated internal standards (0.014 nmol anandamide-d8, 0.48nmol 2-AG-d8, 0.016nmol PEA-d4, 0.015nmol OEA-d2). Lyophilised samples were re-suspended in 40µL 65% acetonitrile and separated on a Zorbax® C18 column (150 × 0.5mm internal diameter) by reversed-phase gradient elution initially with a mobile phase of 65% acetonitrile and 0.1% formic acid which was ramped linearly up to 100% acetonitrile and 0.1% formic acid over 10 min and held at this for a further 20min. Under these conditions, anandamide, 2-AG, PEA and OEA eluted at the following retention times: 11.4 min, 12.9 min, 14.4 min and 15.0 min respectively. Analyte detection was carried out in electrospray-positive ionisation and multiple reaction monitoring (MRM) mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Quantification of each analyte was performed by ratiometric analysis and expressed as nmol or pmols g^{-1} of tissue. The limit of quantification was 1.3pmol g^{-1}, 12.1pmol g^{-1}, 1.5pmol g^{-1}, 1.4pmol g^{-1} for anandamide, 2-AG, PEA and OEA respectively.

Expression of inflammatory mediators assessed using quantitative real-time PCR

RNA was extracted from frontal cortex and hippocampal tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany). RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, UK). Taqman gene expression assays (Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probe were used to quantify the gene of interest and real-time PCR was performed using an ABI Prism 7500 instrument (Applied Biosystems, UK), as previously described (Burke *et al*, 2013a; Burke *et al*, 2013b; Henry *et al*, 2014; Kerr *et al*, 2012; Kerr *et al*, 2013a; Kerr *et al*, 2013b). Assay IDs for the genes were as follows: TNFα (Rn99999017_m1), IL-1β (Rn00580432_m1), IL-6 (Rn00561420_m1), IL-10 (Rn00563409_m1), IL-1ra (Rn00573488_m1), IκBα (Rn01473658_g1), SOCS3 (Rn00585674_s1), NOS2 (iNOS) (Rn00561646_m1), COX-2 (Rn01483828_m1), m-PGE-s (Rn00572047_m1), CD11b (Rn00709342_m1), GFAP (Rn00566603_m1), MRC2 (Rn01456616_m1), CD68 (Rn01495634_g1) and BDNF (Rn01441749_m1). CD11b is a widely used microglial/monocyte activation marker while CD68 and MRC2 are often used as a marker for actively phagocytic M1 and M2 microglial activation, respectively (Burke *et al*, 2014; Murphy *et al*, 2012; O'Sullivan *et al*, 2009; Reus *et al*, 2015). PCR was performed using Bioline one master mix (Medical Supply Company, Dublin, Ireland) and samples were run in duplicate. The cycling conditions were 90$_{o}$C for 10 min and 40 cycles of 90$_{o}$C for 15 min followed by 60$_{o}$C for 1 min. β-actin was used as an endogenous control to normalise gene expression data. Relative gene expression was calculated using the ΔΔCT method.

Western Immunoblotting

Western immunoblotting was carried out essentially as previously described (Butler *et al*, 2008; Roche *et al*, 2012). In brief, approximately 30mg of cortical tissue was homogenised in 400µl of RIPA lysis buffer followed by centrifugation at 13,000g at 4°C for 20 min. Following protein equalization (30µg/15µl) and the addition of sample buffer containing 20% 2-mercaptoethanol (5µl), lysates were heated at 95◦C for 5 minutes and proteins separated by SDS–PAGE electrophoresis on a 12% polyacrylamide gels and transferred onto PVDF membrane (Sigma Aldrich, Ireland). Following the incubation of membranes in blocking solution (5% milk, 0.1% Tween 20 in TBS) for 1 hour, membranes were incubated in primary antibody diluents (1:1000 dilution in 5% milk, 0.1% Tween 20 in TBS) containing the relevant primary diluted antibody (ERK1/2, p-ERK1/2, p38, p-p38, SAPK/JNK, p-SAPK/JNK: Cell Signaling Technologies, USA) overnight at 4$_0$C. Following washing, membranes were incubated in secondary antibody solution (1:10,000 dilution: LI-COR Biosciences UK) for 1hr, washed and analysed using the fluorescence ODYSSEY CLx scanner (LI-COR Biosciences UK). Membranes were subsequently reprobed for β-actin (Sigma; cat no A5441). Densitometry analysis was carried out using Image Studio Lite software V5.0 (LI-COR Biosciences UK). The optical density for each band was calculated, normalised to their respective density for β-actin (endogenous control), expressed as a change from the average of the control group (vehicle-LPS) and relative phosphorylated:total-protein ratio calculated.

Statistical analysis

SPSS statistical package (IBM SPSS v17.0 for Microsoft Windows; SPSS Inc., Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilks and Levene test, respectively. Data were analysed using unpaired *t*-test for 2 groups (Vehicle-LPS vs. PF3845-LPS) or one-way ANOVA for more than two groups on one

factor. A two-way ANOVA with the factors of FAAH inhibitor and LPS treatment were used to assess the impact of LPS and/or PF3845 on nocturnal activity, sucrose preference and the expression of inflammatory mediators 24hrs post LPS (Fig 2). *Post-hoc* analysis was performed using Student Newman Keuls test where appropriate. Data were considered significant when $p < 0.05$. Results are expressed as group means + SEM.

Results

Systemic administration of the FAAH inhibitor PF3845 enhances brain levels of AEA and related *N*-acylethanolamines, and attenuates TLR4-induced expression of NF-κB-inducible inflammatory genes in the frontal cortex and hippocampus

Systemic administration of the FAAH inhibitor PF3845 increased AEA [$t_{(16)} = 27.84$, $p < 0.001$], OEA [$t_{(16)} = 41.34$, $p < 0.01$] and PEA [$t_{(16)} = 23.06$, $p < 0.01$] (Fig 1A-C), but not 2-AG levels (suppl data Fig S1A) in the frontal cortex of LPS-treated rats, when compared to vehicle-treated controls. PF3845 administration significantly attenuated the expression of *IL-1β* [$t_{(15)} = 5.203$, $p < 0.01$], *IL-6* [$t_{(15)} = 2.23$, $p = 0.04$], *TNFα* [$t_{(15)} = 5.93$, $p < 0.01$], *IL-10* [$t_{(13)} = 2.15$, $p = 0.05$], *IκBα* [$t_{(14)} = 2.85$, $p = 0.01$] and *SOCS3* [$t_{(16)} = 2.88$, $p = 0.01$] but not *IL-1ra*, in the frontal cortex 2 hours post LPS administration (Fig 1D). Systemic administration of PF3845 elicited a similar attenuation in TLR4-induced cytokine expression in the hippocampus (suppl data Fig S2).

In addition to examining the effect of FAAH inhibition on cytokine expression, the effect of systemic administration of PF3845 on frontal cortical expression of the inflammatory mediators *COX2, iNOS* and *mPGE-s* was also examined. Statistical analysis revealed that systemic administration of PF3845 attenuated the LPS-induced increases in the expression of *COX2* [$t_{(22)} = 2.27$, $p = 0.03$] and *iNOS* [$t_{(21)} = 2.89$, $p = 0.01$] in the frontal cortex, 2 hours following LPS administration (Fig 1E). This treatment regime failed to alter frontal expression of *mPGE-s* (Fig 1E).

FAAH inhibition attenuates LPS-induced anhedonia and markers of glial activation over 24hrs

In order to evaluate if enhancing FAAH-substrate levels modulates TLR4-induced behavioural responding and/or alterations in glial activity, the effect of systemic PF3845 on locomotor activity, anhedonia and mRNA expression of markers of glial activation, the neurotrophin BDNF and cytokines following LPS were examined. A 2 way ANOVA revealed a significant effect of LPS administration on nocturnal homecage activity [$F_{(1,28)} = 105.08$, $p < 0.01$] and fluid intake [$F_{(1,26)} = 25.03$, $p < 0.01$] and an effect of LPS [$F_{(1,26)} = 4.94$, $p = 0.04$] and PF3845 [$F_{(1,26)} = 4.16$, $p = 0.05$] on sucrose preference. Post hoc analysis revealed that LPS reduced homecage locomotor activity and fluid intake, an effect not altered by prior PF3845 administration (Fig 2A-B). LPS decreased sucrose preference, an effect not observed in rats administered PF3845, indicating that FAAH inhibition may prevent LPS-induced anhedonia (Fig 2C).

Post mortem analysis of gene expression (2 way ANOVA) revealed a significant effect of LPS [$F_{(1,28)} = 90.39$, $p < 0.01$], PF3845 [$F_{(1,28)} = 19.56$, $p < 0.01$] and LPS x PF3845 interaction [$F_{(1,28)} = 12.10$, $p <0.01$] on the expression of the microglial marker *CD11b* in the frontal cortex (Fig 2D) and hippocampus (Suppl data Fig S3A). Post hoc analysis revealed that LPS increased the expression of *CD11b*, an effect attenuated by prior PF3845 administration. Analysis also revealed a significant effect of LPS on the expression of the astrocytic activation marker GFAP [$F_{(1,287} = 10.37$, $p < 0.01$], the M1 microglial marker *CD68* [$F_{(1,27)} = 17.89$, $p <0.01$], the M2 microglial marker MRC2 [$F_{(1,28)} = 13.70$, $p < 0.01$], the neurotrophin *BDNF* [$F_{(1,28)} = 14.31$, $p < 0.01$], and the pro-inflammatory cytokines *IL-1β* [$F_{(1,26)} = 26.92$, $p < 0.01$], *IL-6* [$F_{(1,27)} = 8.23$, $p < 0.01$] and a LPS x PF3845 interaction effect for *TNFα* [$F_{(1,27)} = 4.53$, $p = 0.04$] in the frontal

cortex (Fig 2E-K). Post hoc analysis revealed that LPS increased the expression of the M1 microglial marker *CD68* (Fig 2E) and reduced the expression of the M2 marker *MRC2* (Fig 2F) and the neurotrophin *BDNF* (Fig 2H), an effect not altered by prior administration of PF3845. In comparison, LPS increased the expression of *GFAP* (Fig 2G), an effect attenuated by prior administration of PF3845. Furthermore, LPS increased the expression of *IL-1β* (Fig 2I) in the frontal cortex 24hrs post administration. Prior systemic administration of PF3845 augmented the LPS-induced increase in *IL-1β* and increased the expression of *TNFα* (Fig 2K) in the frontal cortex of LPS treated animals. LPS and/or PF3845 elicited similar but not identical effect on the expression of inflammatory mediators in the hippocampus (data presented in Suppl FigS3).

Increasing FAAH substrate levels directly within the brain attenuates TLR4-induced expression of NF-κB-inducible inflammatory genes in the frontal cortex

Direct administration (i.c.v.) of PF3845 into the brain resulted in increased levels of AEA [$t_{(25)}$ =6.49, $p <$ 0.01], OEA [$t_{(25)}$ =12.82, $p < 0.01$] and PEA [$t_{(25)}$ =10.92, $p < 0.01$], but not 2-AG (Suppl data Fig S1B), in the frontal cortex of LPS-treated rats, when compared to vehicle-LPS-treated counterparts (Fig 3A-C). Furthermore, this increase in FAAH substrate levels was associated with a robust attenuation in the expression of *IL-1β* ($t_{(15)}$ =2.38, p = 0.03), *TNFα* ($t_{(15)}$ = 3.68, $p < 0.01$), *IL-6* ($t_{(13)}$ = 4.51, p = 0.01), *IL-10* ($t_{(10)}$ = 2.43, p = 0.03] and *SOCS3* ($t_{(15)}$ = 3.31, p = 0.01) following LPS administration, when compared to vehicle-treated counterparts (Fig 3D). Central (i.c.v.) administration of PF3845 elicited a similar attenuation in TLR4-induced cytokine expression in the hippocampus (suppl data Fig S4).

The peripherally restricted FAAH inhibitor URB937 does not alter TLR4-induced increases in NF-κB-responsive genes in the frontal cortex

In order to confirm that the effects of increasing FAAH substrate levels on TLR4-induced cytokine expression in the brain were mediated directly within the brain, the effect of systemic administration of the peripherally restricted FAAH inhibitor URB937 was examined. Statistical analysis revealed that when compared with vehicle-treated counterparts, URB937 did not alter AEA or 2-AG levels in the frontal cortex, however both OEA [$t(_{14}) = 2.41$, $p = 0.03$] and PEA [$t(_{14}) = 2.39$, $p = 0.03$] levels were increased when compared to vehicle-LPS-treated counterparts (Fig 4A-C). It was also confirmed that this treatment regime increased AEA (8.27pmol/g vs. 15.71 pg/g tissue, $p < 0.001$), OEA (101.14pmol/g vs. 169.16pmol/g, $p < 0.001$) and PEA (147.27pmol/g vs. 233.01pmol/g, $p < 0.001$) levels peripherally (spleen). It should be noted that the magnitude of the increase in OEA and PEA levels in the brain following systemic URB937 was significantly less than that observed following systemic or i.c.v. administration of the centrally acting FAAH inhibitor PF3845 (Fig 1 and 3). Despite the increases in OEA and PEA levels within the brain, systemic administration of URB937 failed to significantly alter LPS-induced expression of the NF-κB-responsive genes *IL-1β, IL-6 and TNFα* within the frontal cortex (Fig 4D).

FAAH substrate modulation of TLR4-induced neuroinflammation is not mediated via central CB_1 or CB_2 receptors

Given that FAAH inhibition increases AEA levels in the brain and potently attenuates TLR4-induced increases in neuroinflammatory mediators, this study aimed to examine the role of central CB_1 and CB_2 receptors in eliciting this response. Statistical analysis (one-way ANOVA) revealed a significant treatment effect on the expression of the NF-κB-inducible genes *IL-1β*

[$F_{3,56}$=4.97, $p < 0.01$], *IL-6* [$F_{3,56}$=5.49, $p < 0.01$] and *TNFα* [$F_{3,56}$=4.48, $p < 0.01$], in the frontal cortex at 2 hours post systemic LPS administration (Fig 5A-C). Systemic administration of PF3845 significantly attenuated frontal cortical expression of *IL-1β* and *IL-6*, but not *TNFα*, when compared to vehicle-vehicle-LPS-treated counterparts (Fig 5). Central (i.c.v.) administration of the selective CB_1 or CB_2 antagonists AM251 or AM630 respectively, did not alter the decrease in frontal cortical expression of *IL-1β* or *IL-6* in PF3845 treated animals [AM251-PF-LPS vs. Vehicle-PF-LPS $p>0.05$; AM630-PF-LPS vs. Vehicle-PF-LPS $p>0.05$] (Fig 5A-B). However, the expression of TNFα was reduced in PF3845-LPS-treated animals following i.c.v. administration of AM251 or AM630, when compared to vehicle-vehicle-LPS treated counterparts (Fig 5C).

Systemic administration of PF3845 does not alter MAPK activation in the rat frontal cortex following TLR4 activation

Activation of CB_1 and CB_2 receptors is positively coupled to MAPK activation which in turn is known to be important in modulating NF-κB-inducible cytokines in activated microglia (Correa *et al*, 2010; Krishnan *et al*, 2012). Thus, MAPK signalling was assessed to further determine if the reported immunomodulatory effects of PF3845 in the brain are dependent or independent of $CB_{1/2}$ activation of MAPK signalling pathways. Statistical analysis revealed that PF3845 did not alter ERK1/2 or JNK phosphorylation in the presence of LPS when compared to vehicle-treated counterparts (Fig 5D-E). Phosphorylated levels of p38 in the frontal cortex were undetectable at 2 hours post systemic LPS administration (data not shown).

Central TRPV1 antagonism blocks FAAH substrate-mediated attenuation of TLR4-induced *IL-6* expression

In addition to CB_1 and CB_2 receptors, AEA is known to directly activate additional receptor targets including GPR55, while FAAH substrates (AEA, OEA and PEA) have affinity for and activity at TRPV1 and PPARs. Thus, the role of central PPARα, PPARγ, TRPV1 and GPR55 in FAAH substrate-mediated modulation of TLR4-induced increases in the expression of neuroinflammatory cytokines was examined. Statistical analysis (one-way ANOVA) revealed a significant treatment effect on the expression of *IL-1β* [$F_{5,74}$=4.02, $p < 0.01$], *IL-6* [$F_{5,73}$=2.27, $p < 0.05$] and *TNFα* [$F_{5,73}$=2.50, $p < 0.05$], in the frontal cortex (Fig 6). Systemic administration of PF3845 significantly attenuated LPS-induced increases in the expression of *IL-1β* and *IL-6*, but not *TNFα* [Vehicle-PF3845-LPS vs. Vehicle-vehicle-LPS] (Fig 6A-C). Central (i.c.v.) administration of IRTX (TRPV1 antagonist), but not MK886 (PPARα antagonist), GW9662 (PPARγ antagonist) or CID (GPR55 antagonist), prevented the PF3845-induced decrease in cortical expression of *IL-6* following LPS administration [IRTX-PF3845-LPS vs. Vehicle-PF3845-LPS] (Fig 5E). In a separate experiment, it was noted that i.c.v. administration of IRTX alone did not significantly alter frontal cortical expression of *IL-6* following LPS [vehicle-vehicle-LPS 100 ± 34% vs. IRTX-Vehicle-LPS 67 ± 29%; $p>0.05$].

LPS-induced sickness behaviour is not altered by systemic administration of PF3845 in the presence or absence of central TRPV1 antagonism

In order to determine if the immuno-modulatory effects of increasing FAAH substrate levels in the brain, and the role of TRPV1, is associated with altered behavioural responding, the effects of PF3845, in the presence and absence of central TRPV1 blockade, were examined on a range of sickness behavioural parameters over a 24hr period. Statistical analysis revealed that LPS significantly increased body temperature 2hr post administration [$F_{4,41}$=2.46, $p = 0.05$], and reduced body weight [$F4_{,4,41}$=6.05, $p < 0.01$] and food intake [$F_{4,41}$=6.05, $p < 0.01$] over the 24hrs period post administration. These effects were not altered by prior systemic administration

of PF3845 and/or central administration of the TRPV1 antagonist IRTX (Fig 7A-C). Similar to earlier findings, LPS tended to reduce sucrose preference, an effect not observed in PF3845 treated animals, however this failed to reach statistical significance (Fig 7F).

Discussion

The data presented herein have demonstrated that the inhibition of FAAH and subsequent increases in levels of AEA and related *N*-acylethanolamines in the brain, is associated with modulation of neuroinflammatory responses following TLR4 activation. Furthermore, the data demonstrate that the effect of FAAH inhibition on neuroinflammation is mediated directly within the brain and does not involve CB_1 or CB_2 receptor activation. Rather, a role for central TRPV1 receptors in mediating, at least partially (IL-6), the effects of increased FAAH substrate levels on TLR4-induced neuroinflammation was observed. Furthermore, despite the pronounced effects of increasing FAAH substrate levels on TLR4-induced neuroinflammation, this was not associated with a change in sickness behaviour but rather tended to attenuate anhedonic-like behaviour. Overall these findings demonstrate an important role for FAAH substrates within the brain in the modulation of TLR4-induced inflammatory responses which may have implications in the treatment of neuroinflammatory disorders.

Although several studies have demonstrated that enhancing AEA tone modulates TLR4-induced neuroinflammatory responses, it remain unknown if effects are directly mediated at the level of the brain, the receptor and molecular mechanisms involved and the behavioural consequences of such a response. The current study demonstrated that systemic administration of the FAAH inhibitor PF3845 increases levels of AEA, OEA and PEA in both the frontal cortex and hippocmpus. This was associated with a robust attenuation in the early expression of a wide array of pro-and anti-inflammatory cytokines and mediators and the expression of markers of glial activity. However, the expression of TLR4-induced pro-inflammatory cytokines (IL-1β and TNFα) was augmented in PF3845 treated animals 24hrs post LPS. Thus, increasing FAAH substrate levels in the brain may shift the TLR4-induced cytokine response to a later timepoint.

Furthermore, although the PF3845-induced suppression of early TLR4-induced inflammatory genes was similar in both cortical and hippocampal tissue, differences were noted in the two brain regions at later (24hr) time points. Specifically, PF3845 treated animals exhibited an attenuation of TLR4-indued microglial (CD11b) and astrocyte (GFAP) activation markers in the cortex while in the hippocampus an attenuations of M1 microglial activation (CD11b and CD68) was noted. It is possible that the discrepancy between effects in the different regions may relate to resting state of neuronal, glial or endocannabinoid activity in these different regions. Furthermore, region specific modulation of neuroinflammatory responses may underlie the effects of PF3945 on certain behaviours (anhedonia) and not others (sickness behaviour). Despite such differences, taken together the data demonstrate that enhancing FAAH substrate levels in the brain potentially modulates TLR4-induced neuroinflammatory responses.

It is well known that TLR4-induced inflammation is associated with behavioural alterations including fever, hypolocomotion, altered appetite, anxiety and anhedonia (Dantzer, 2006; Dantzer *et al*, 2008). The current data demonstrate that while PF3845 potently attenuates TLR4-induced cytokines in the brain, this does not alter the associated sickness behaviour (hypolocomotion, fever, reduced body weight and food intake). Similarly, recent data have demonstrated that systemic administration of PF3845 does not alter LPS-induced hypothermia in mice (Nass *et al*, 2015), although effects of FAAH inhibition on other sickness-related behaviours have not been reported prior to this study. However, the data presented herein also indicate that LPS reduces sucrose intake, an effect not observed in PF3845-treated rats. This effect is modest and although not repeated in the second behavioural study, a trend for a similar effect was observed. This lack of robust anhedonic effects following LPS is likely due to the low dose of LPS used herein compared with previous studies (Biesmans *et al*, 2016; Sayd *et al*, 2015) and the confounding effect of prior surgery and icv vehicle administration. Despite this, the data

suggest that FAAH inhibition may modulate immune-mediated anhedonia, a core symptom of psychiatric disorders such as depression. A recent study has demonstrated that OEA, but not PEA, attenuates LPS-induced anhedonia, an effect associated with attenuation of cytokine and inflammatory mediators in the brain (Sayd *et al*, 2015). Thus, it remains to be determined if one or a combination of the FAAH substrates enhanced following administration of PF3845 is responsible for the anti-anhedonic effects observed here-in, or modulates other immune-related behavioural and physiological responses.

An addition aim of the current study was to examine if FAAH substrates directly within the brain are responsible for the modulation of TLR4-induced neuroinflammation. The data here-in demonstrate that increasing AEA, OEA and PEA brain levels following central administration of PF3845 was association with an attenuation of TLR4-induced inflammatory mediators in both the frontal cortex and hippocampus. Furthermore, administration of the peripherally restricted FAAH inhibitor URB937 increased FAAH substrate levels peripherally, with slight increases in OEA and PEA, but not AEA, levels in the brain; this treatment regime did not alter TLR4-induced increases in the expression of pro-inflammatory cytokines. These data suggest that either high levels of FAAH substrates in the brain are required to modulate TLR4-induced neuroinflammatory responses or alternatively, that the increase in levels of AEA within the brain, rather than OEA or PEA, is primarily responsible for mediating the effects on TLR-induced neuroinflammatory responses. Thus, taken together the data indicate that FAAH substrates act to modulate TLR4-, in addition to TLR3- (Henry et al., 2014), induced neuroinflammatory responses directly within the brain, rather than via modulation of peripheral immune responses.

An important aim of this work was to attempt to uncover the mechanisms underlying FAAH substrate-induced modulation of neuroinflammation following TLR4 activation. AEA has been proposed to mediate some of its anti-inflammatory activity by increasing glial production of the anti-inflammatory cytokine IL-10 (Correa *et al*, 2010; Krishnan *et al*, 2012). Furthermore, chronic administration of PF3845 was associated with a shift in M1 to an M2 microglia activation phenotype, a primary source of IL-10, an effect associated with a reversal of TBI-induced impairments in functional outcomes and neurodegenerative processes (Tchantchou *et al*, 2014). However, the current data do not support this as a mechanism of action of PF3845 on TLR4-induced neuroinflammation given a lack of effect on the expression of the M2 microglial marker MRC2 and the attenuation of TLR4-induced IL-10. Similarly, previous work from our lab has demonstrated that systemic administration of an alternative FAAH inhibitor URB597 and subsequent increases in AEA, OEA and PEA, also attenuated TLR4-induced increases in hypothalamic expression of IL-10 (Kerr *et al*, 2012). In addition, our findings demonstrated that FAAH substrate-mediated effects on TLR4-induced neuroinflammation are not likely mediated via increases in the IL-1β receptor antagonist, IL-1ra or the negative regulator of IL-6, SOCS3. Thus, a likely explanation for the current findings is that increasing FAAH substrates in the brain prevents or delays inflammation-induced microglial activation, consequently reduces activation of NF-κB, and downstream transcription of pro- and anti-inflammatory genes. Further support for this hypothesis comes from the finding that PF3845 attenuated the TLR4-induced expression of the markers of astrocyte and microglial activation, *GFAP* and *CD11b*, and *IκBα*, often used as an indirect measure of NF-κB activation (Read *et al*, 1994). Thus, FAAH substrates may act to down-regulate or delay glial activation under TLR4-induced neuroinflammatory conditions

There has been conflicting evidence on the possible receptor mechanisms that mediate the neuro-immuno-modulatory effects of FAAH substrates. Evidence from *in vitro* studies indicates that

AEA-mediated modulation of TLR4-induced neuroinflammation may be mediated by CB_1/CB_2 receptor dependent (Correa *et al*, 2010; Correa *et al*, 2009b; Hernangomez *et al*, 2012) or independent (Correa *et al*, 2008; Tham *et al*, 2007) mechanisms. However, *in vivo* studies have been limited to the demonstration of a role for hypothalamic CB_1 receptors in LPS-induced increases in plasma TNFα (De Laurentiis *et al*, 2010; Steiner *et al*, 2011) and fever (Duncan *et al*, 2013). The current findings demonstrate that FAAH substrate-mediated attenuation of TLR4-induced increases in IL-1β and IL-6, is likely not mediated by central CB_1 or CB_2 receptors, given the lack of effect of i.c.v. administration of selective antagonists for these receptors on the immunosuppressive effect of PF3845. It should be noted that only in the presence of central CB_1 or CB_2 antagonism did PF3845 attenuate LPS-induced TNFα expression in this study. The discrepancy between this and the earlier data may be due to the added confound of prior i.c.v surgery and administration of vehicle i.c.v.. It is possible that by blocking central CB_1 or CB_2 under the conditions of this study, the FAAH substrates may act at other receptor targets (or a combination of such) resulting in an immunosuppressive effect of PF3845 on LPS-induced TNFα. Despite this discrepancy, the present data support the conclusion that increasing central FAAH substrate levels can attenuate early pro-inflammatory cytokine responses following LPS administration, an effect not mediated by central CB_1 or CB_2 receptors. This is further supported by the finding that phosphorylation of the MAPK signalling proteins ERK1/2 or JUN, which are positively coupled to, and an indirect marker of CB_1/CB_2 receptor activation, were unaltered by PF3845 administration. However, AEA and FAAH substrates are known to also mediate effects via alternative receptors including the PPARs, TRPV1 and GPR55. For example, AEA-induced activation of PPAR-γ has been shown to inhibit IL-2 release (Rockwell and Kaminski, 2004) and PPAR-γ was shown to mediate, at least in part, the effect of the putative AEA reuptake inhibitor AM404, on increases in plasma TNFα, induced following systemic TLR4 activation (Roche *et*

al, 2008). However, the present findings demonstrated that blockade of either PPAR-α or PPAR-γ, or the newly classified cannabinoid receptor GPR55, directly within the brain, does not alter FAAH substrate-mediated attenuation of *IL-1β* or *IL-6* following LPS. In comparison, TRPV1 antagonism prevented the FAAH substrate-mediated attenuation of TLR4-induced increases in frontal cortical expression of *IL-6*, highlighting an important role for central TRPV1 in mediating, at least some of the effects, of FAAH substrates on TLR4-induced neuroinflammation. Several lines of evidence indicate that TRPV1 activation exerts anti-inflammatory effects under a variety of experimental conditions (Demirbilek *et al*, 2004; Kissin *et al*, 2005; Tsuji *et al*, 2010; Ueda *et al*, 2008). However, to our knowledge this is the first study to report effects of TRPV1 in the modulation of TLR4-induced neuroinflammatory responses. It should be noted that in addition to AEA (Toth *et al*, 2009), OEA is a potent TRPV1 agonists/desensitizer (Ahern, 2003; Almasi *et al*, 2008; Gonzalez-Aparicio *et al*, 2014; Movahed *et al*, 2005; Starowicz *et al*, 2013; Wang *et al*, 2005). Furthermore, several studies have demonstrated that FAAH inhibition can lead to indirect activation/desensitization of TRPV1, and thus shunting of the effect of AEA and other FAAH substrates onto other receptor targets which in turn mediate analgesic and anti-inflammatory effects (Maione *et al*, 2007; Starowicz *et al*, 2013). The current data demonstrate that although FAAH inhibition attenuates early neuroinflammatory responses to TLR4 activation, an effect partially mediated by TRPV1, this is not accompanied by alterations in sickness behaviour or anhedonia. Thus, while one or a combination of FAAH substrates may be responsible for the TRPV1-mediated decrease in LPS-induced IL-6 following PF3845, multiple receptors, mechanisms and circuitries are involved in mediating the behavioural responses. Thus, further studies are require to determine the role of specific FAAH substrates in mediating the effects observed and the likely multitude of receptor and molecular mechanisms involved.

In conclusion, the data reported in this study have demonstrated an important role for FAAH substrates in the brain in the modulation of TLR4-induced neuroinflammatory and associated anhedonic responses, but not acute sickness behaviour. Such potent neuroimmunomodulatory effects were shown to be likely due to the inhibition or delayed activation of inflammation-induced glial activation and subsequent NFκB activation. Evaluating the possible receptor mechanisms revealed that the effects are independent of central cannabinoid receptors (CB_1, CB_2, and GPR55) or PPARs (PPAR-α/γ) but rather demonstrate for the first time a role for central TRPV1 in partially mediating FAAH substrate-mediated modulation of TLR4-induced neuroinflammation. Overall, these findings support an important immunomodulatory role for FAAH substrates within the brain, and may have implications for the development of novel treatments for neuroinflammatory disorders

Conflicts of interest

The authors declare no conflict of interest

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References

Ahern GP (2003). Activation of TRPV1 by the satiety factor oleoylethanolamide. *J Biol Chem* **278**(33): 30429-30434.

Ahn K, Johnson DS, Mileni M, Beidler D, Long JZ, McKinney MK*, et al* (2009). Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. *Chem Biol* **16**(4): 411-420.

Alexander SP, Kendall DA (2007). The complications of promiscuity: endocannabinoid action and metabolism. *Br J Pharmacol* **152**(5): 602-623.

Almasi R, Szoke E, Bolcskei K, Varga A, Riedl Z, Sandor Z*, et al* (2008). Actions of 3-methyl-N-oleoyldopamine, 4-methyl-N-oleoyldopamine and N-oleoylethanolamide on the rat TRPV1 receptor in vitro and in vivo. *Life Sci* **82**(11-12): 644-651.

Arroyo DS, Soria JA, Gaviglio EA, Rodriguez-Galan MC, Iribarren P (2011). Toll-like receptors are key players in neurodegeneration. *Int Immunopharmacol* **11**(10): 1415-1421.

Bergink V, Gibney SM, Drexhage HA (2014). Autoimmunity, inflammation, and psychosis: a search for peripheral markers. *Biol Psychiatry* **75**(4): 324-331.

Biesmans S, Matthews LJ, Bouwknecht JA, De Haes P, Hellings N, Meert TF*, et al* (2016). Systematic Analysis of the Cytokine and Anhedonia Response to Peripheral Lipopolysaccharide Administration in Rats. *Biomed Res Int* **2016**: 9085273.

Booker L, Kinsey SG, Abdullah RA, Blankman JL, Long JZ, Ezzili C*, et al* (2012). The fatty acid amide hydrolase (FAAH) inhibitor PF-3845 acts in the nervous system to reverse LPS-induced tactile allodynia in mice. *Br J Pharmacol* **165**(8): 2485-2496.

Bree D, Moriarty O, Broom DC, Kelly JP, Roche M, Finn DP (2016). Characterization of the Affective Component of Acute Postoperative Pain Associated with a Novel Rat Model of Inguinal Hernia Repair Pain. *CNS Neurosci Ther* **22**(2): 146-153.

Bree D, Moriarty O, O'Mahony CM, Morris B, Bannerton K, Broom DC*, et al* (2015). Development and characterization of a novel, anatomically relevant rat model of acute postoperative pain. *J Pain* **16**(5): 421-435 e421-426.

Burke NN, Geoghegan E, Kerr DM, Moriarty O, Finn DP, Roche M (2013a). Altered neuropathic pain behaviour in a rat model of depression is associated with changes in inflammatory gene expression in the amygdala. *Genes Brain Behav* **12**(7): 705-713.

Burke NN, Kerr DM, Moriarty O, Finn DP, Roche M (2014). Minocycline modulates neuropathic pain behaviour and cortical M1-M2 microglial gene expression in a rat model of depression. *Brain Behav Immun* **42**: 147-156.

Burke NN, Llorente R, Marco EM, Tong K, Finn DP, Viveros MP*, et al* (2013b). Maternal deprivation is associated with sex-dependent alterations in nociceptive behavior and neuroinflammatory mediators in the rat following peripheral nerve injury. *J Pain* **14**(10): 1173-1184.

Butler RK, Rea K, Lang Y, Gavin AM, Finn DP (2008). Endocannabinoid-mediated enhancement of fear-conditioned analgesia in rats: opioid receptor dependency and molecular correlates. *Pain* **140**(3): 491-500.

Clapper JR, Moreno-Sanz G, Russo R, Guijarro A, Vacondio F, Duranti A*, et al* (2010). Anandamide suppresses pain initiation through a peripheral endocannabinoid mechanism. *Nat Neurosci* **13**(10): 1265-1270.

Correa F, Docagne F, Clemente D, Mestre L, Becker C, Guaza C (2008). Anandamide inhibits IL-12p40 production by acting on the promoter repressor element GA-12: possible involvement of the COX-2 metabolite prostamide E(2). *The Biochemical journal* **409**(3): 761-770.

Correa F, Docagne F, Mestre L, Clemente D, Hernangomez M, Loria F*, et al* (2009a). A role for CB2 receptors in anandamide signalling pathways involved in the regulation of IL-12 and IL-23 in microglial cells. *Biochem Pharmacol* **77**(1): 86-100.

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

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

Dantzer R (2006). Cytokine, sickness behavior, and depression. *Neurol Clin* **24**(3): 441-460.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* **9**(1): 46-56.

De Fanti BA, Martinez JA (2002). Central urocortin activation of sympathetic-regulated energy metabolism in Wistar rats. *Brain Res* **930**(1-2): 37-41.

De Laurentiis A, Fernandez-Solari J, Mohn C, Burdet B, Zorrilla Zubilete MA, Rettori V (2010). The hypothalamic endocannabinoid system participates in the secretion of oxytocin and tumor necrosis factor-alpha induced by lipopolysaccharide. *J Neuroimmunol* **221**(1-2): 32-41.

de Novellis V, Vita D, Gatta L, Luongo L, Bellini G, De Chiaro M*, et al* (2011). The blockade of the transient receptor potential vanilloid type 1 and fatty acid amide hydrolase decreases symptoms and central sequelae in the medial prefrontal cortex of neuropathic rats. *Molecular pain* **7**: 7.

Deleidi M, Isacson O (2012). Viral and inflammatory triggers of neurodegenerative diseases. *Sci Transl Med* **4**(121): 121ps123.

Demirbilek S, Ersoy MO, Karaman A, Gurbuz N, Bayraktar N, Bayraktar M (2004). Small-dose capsaicin reduces systemic inflammatory responses in septic rats. *Anesth Analg* **99**(5): 1501-1507; table of contents.

Di Marzo V, Bisogno T, De Petrocellis L (2001). Anandamide: some like it hot. *Trends Pharmacol Sci* **22**(7): 346-349.

Downer EJ (2011). Cannabinoids and innate immunity: taking a toll on neuroinflammation. *ScientificWorldJournal* **11**: 855-865.

Duncan M, Galic MA, Wang A, Chambers AP, McCafferty DM, McKay DM*, et al* (2013). Cannabinoid 1 receptors are critical for the innate immune response to TLR4 stimulation. *Am J Physiol Regul Integr Comp Physiol* **305**(3): R224-231.

Edwards JG (2014). TRPV1 in the central nervous system: synaptic plasticity, function, and pharmacological implications. *Prog Drug Res* **68**: 77-104.

Esposito E, Cuzzocrea S (2013). Palmitoylethanolamide is a new possible pharmacological treatment for the inflammation associated with trauma. *Mini Rev Med Chem* **13**(2): 237-255.

Facchinetti F, Del Giudice E, Furegato S, Passarotto M, Leon A (2003). Cannabinoids ablate release of TNFalpha in rat microglial cells stimulated with lypopolysaccharide. *Glia* **41**(2): 161-168.

Fakhfouri G, Ahmadiani A, Rahimian R, Grolla AA, Moradi F, Haeri A (2012). WIN55212-2 attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR-gamma pathway. *Neuropharmacology* **63**(4): 653-666.

Fernandez-Solari J, Prestifilippo JP, Bornstein SR, McCann SM, Rettori V (2006). Participation of the endocannabinoid system in the effect of TNF-alpha on hypothalamic release of gonadotropin-releasing hormone. *Ann N Y Acad Sci* **1088**: 238-250.

Fitzgibbon M, Finn DP, Roche M (2015). High Times for Painful Blues: The Endocannabinoid System in Pain-Depression Comorbidity. *Int J Neuropsychopharmacol* **19**(3).

Ford GK, Kieran S, Dolan K, Harhen B, Finn DP (2011). A role for the ventral hippocampal endocannabinoid system in fear-conditioned analgesia and fear responding in the presence of nociceptive tone in rats. *Pain* **152**(11): 2495-2504.

Fraga D, Zanoni CI, Rae GA, Parada CA, Souza GE (2009). Endogenous cannabinoids induce fever through the activation of CB1 receptors. *Br J Pharmacol* **157**(8): 1494-1501.

Gonzalez-Aparicio R, Moratalla R (2014). Oleoylethanolamide reduces L-DOPA-induced dyskinesia via TRPV1 receptor in a mouse model of Parkinson s disease. *Neurobiol Dis* **62**: 416-425.

Henry RJ, Kerr DM, Finn DP, Roche M (2014). FAAH-mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus. *J Neuroimmunol* **276**(1-2): 126-134.

Henry RJ, Kerr DM, Finn DP, Roche M (2016). For whom the endocannabinoid tolls: Modulation of innate immune function and implications for psychiatric disorders. *Prog Neuropsychopharmacol Biol Psychiatry* **64**: 167-180.

Hernangomez M, Mestre L, Correa FG, Loria F, Mecha M, Inigo PM*, et al* (2012). CD200-CD200R1 interaction contributes to neuroprotective effects of anandamide on experimentally induced inflammation. *Glia* **60**(9): 1437-1450.

Kargl J, Brown AJ, Andersen L, Dorn G, Schicho R, Waldhoer M*, et al* (2013). A selective antagonist reveals a potential role of G protein-coupled receptor 55 in platelet and endothelial cell function. *J Pharmacol Exp Ther* **346**(1): 54-66.

Kerr DM, Burke NN, Ford GK, Connor TJ, Harhen B, Egan LJ*, et al* (2012). Pharmacological inhibition of endocannabinoid degradation modulates the expression of inflammatory mediators in the hypothalamus following an immunological stressor. *Neuroscience* **1**(204): 53-63.

Kerr DM, Downey L, Conboy M, Finn DP, Roche M (2013a). Alterations in the endocannabinoid system in the rat valproic acid model of autism. *Behav Brain Res* **249**: 124-132.

Kerr DM, Harhen B, Okine BN, Egan LJ, Finn DP, Roche M (2013b). The monoacylglycerol lipase inhibitor JZL184 attenuates LPS-induced increases in cytokine expression in the rat frontal cortex and plasma: differential mechanisms of action. *Br J Pharmacol* **169**(4): 808-819.

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* **8**(6): e1000412.

Kissin EY, Freitas CF, Kissin I (2005). The effects of intraarticular resiniferatoxin in experimental knee-joint arthritis. *Anesth Analg* **101**(5): 1433-1439.

Krishnan G, Chatterjee N (2012). Endocannabinoids alleviate proinflammatory conditions by modulating innate immune response in muller glia during inflammation. *Glia* **60**(11): 1629-1645.

Madasu MK, Roche M, Finn DP (2015). Supraspinal Transient Receptor Potential Subfamily V Member 1 (TRPV1) in Pain and Psychiatric Disorders. *Mod Trends Pharmacopsychiatri* **30**: 80-93.

Maione S, De Petrocellis L, de Novellis V, Moriello AS, Petrosino S, Palazzo E*, et al* (2007). Analgesic actions of N-arachidonoyl-serotonin, a fatty acid amide hydrolase inhibitor with antagonistic activity at vanilloid TRPV1 receptors. *Br J Pharmacol* **150**(6): 766-781.

Martins D, Tavares I, Morgado C (2014). "Hotheaded": the role OF TRPV1 in brain functions. *Neuropharmacology* **85**: 151-157.

Mattace Raso G, Russo R, Calignano A, Meli R (2014). Palmitoylethanolamide in CNS health and disease. *Pharmacol Res* **86**: 32-41.

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

Molina-Holgado F, Molina-Holgado E, Guaza C (1998). The endogenous cannabinoid anandamide potentiates interleukin-6 production by astrocytes infected with Theiler's murine encephalomyelitis virus by a receptor-mediated pathway. *FEBS Lett* **433**(1-2): 139-142.

Morgenweck J, Abdel-Aleem OS, McNamara KC, Donahue RR, Badr MZ, Taylor BK (2010). Activation of peroxisome proliferator-activated receptor gamma in brain inhibits inflammatory pain, dorsal horn expression of Fos, and local edema. *Neuropharmacology* **58**(2): 337-345.

Movahed P, Jonsson BA, Birnir B, Wingstrand JA, Jorgensen TD, Ermund A*, et al* (2005). Endogenous unsaturated C18 N-acylethanolamines are vanilloid receptor (TRPV1) agonists. *J Biol Chem* **280**(46): 38496-38504.

Murphy N, Cowley TR, Blau CW, Dempsey CN, Noonan J, Gowran A*, et al* (2012). The fatty acid amide hydrolase inhibitor URB597 exerts anti-inflammatory effects in hippocampus of aged rats and restores an age-related deficit in long-term potentiation. *J Neuroinflammation* **9**: 79.

Nasirinezhad F, Jergova S, Pearson JP, Sagen J (2015). Attenuation of persistent pain-related behavior by fatty acid amide hydrolase (FAAH) inhibitors in a rat model of HIV sensory neuropathy. *Neuropharmacology* **95**: 100-109.

Nass SR, Long JZ, Schlosburg JE, Cravatt BF, Lichtman AH, Kinsey SG (2015). Endocannabinoid Catabolic Enzymes Play Differential Roles in Thermal Homeostasis in Response to Environmental or Immune Challenge. *J Neuroimmune Pharmacol* **10**(2): 364-370.

O'Sullivan JB, Ryan KM, Curtin NM, Harkin A, Connor TJ (2009). Noradrenaline reuptake inhibitors limit neuroinflammation in rat cortex following a systemic inflammatory challenge: implications for depression and neurodegeneration. *Int J Neuropsychopharmacol* **12**(5): 687-699.

O'Sullivan SE, Kendall DA (2010). Cannabinoid activation of peroxisome proliferator-activated receptors: potential for modulation of inflammatory disease. *Immunobiology* **215**(8): 611-616.

Ortega-Gutierrez 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**(2): 163-168.

Paxinos GW, C (2006). *The rat Brain in Stereotaxic Coordinates*

Puffenbarger RA, Boothe AC, Cabral GA (2000). Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells. *Glia* **29**(1): 58-69.

Rea K, Olango WM, Harhen B, Kerr DM, Galligan R, Fitzgerald S*, et al* (2013). Evidence for a role of GABAergic and glutamatergic signalling in the basolateral amygdala in endocannabinoid-mediated fear-conditioned analgesia in rats. *Pain* **154**(4): 576-585.

Read MA, Whitley MZ, Williams AJ, Collins T (1994). NF-kappa B and I kappa B alpha: an inducible regulatory system in endothelial activation. *J Exp Med* **179**(2): 503-512.

Reus GZ, Fries GR, Stertz L, Badawy M, Passos IC, Barichello T*, et al* (2015). The role of inflammation and microglial activation in the pathophysiology of psychiatric disorders. *Neuroscience* **300**: 141-154.

Roche M, Diamond M, Kelly JP, Finn DP (2006). In vivo modulation of LPS-induced alterations in brain and peripheral cytokines and HPA axis activity by cannabinoids. *J Neuroimmunol* **181**(1-2): 57-67.

Roche M, Kelly JP, O'Driscoll M, Finn DP (2008). Augmentation of endogenous cannabinoid tone modulates lipopolysaccharide-induced alterations in circulating cytokine levels in rats. *Immunology* **125**(2): 263-271.

Roche M, Kerr DM, Hunt SP, Kelly JP (2012). Neurokinin-1 receptor deletion modulates behavioural and neurochemical alterations in an animal model of depression. *Behav Brain Res* **228**(1): 91-98.

Rock EM, Limebeer CL, Ward JM, Cohen A, Grove K, Niphakis MJ*, et al* (2015). Interference with acute nausea and anticipatory nausea in rats by fatty acid amide hydrolase (FAAH) inhibition through a PPARalpha and CB1 receptor mechanism, respectively: a double dissociation. *Psychopharmacology (Berl)* **232**(20): 3841-3848.

Rockwell CE, Kaminski NE (2004). A cyclooxygenase metabolite of anandamide causes inhibition of interleukin-2 secretion in murine splenocytes. *J Pharmacol Exp Ther* **311**(2): 683-690.

Rubino T, Realini N, Castiglioni C, Guidali C, Vigano D, Marras E*, et al* (2008). Role in anxiety behavior of the endocannabinoid system in the prefrontal cortex. *Cereb Cortex* **18**(6): 1292-1301.

Sayd A, Anton M, Alen F, Caso JR, Pavon J, Leza JC*, et al* (2015). Systemic administration of oleoylethanolamide protects from neuroinflammation and anhedonia induced by LPS in rats. *Int J Neuropsychopharmacol* **18**(6).

Skaper SD, Facci L, Barbierato M, Zusso M, Bruschetta G, Impellizzeri D*, et al* (2015). N-Palmitoylethanolamine and Neuroinflammation: a Novel Therapeutic Strategy of Resolution. *Mol Neurobiol* **52**(2): 1034-1042.

Starowicz K, Makuch W, Korostynski M, Malek N, Slezak M, Zychowska M*, et al* (2013). Full inhibition of spinal FAAH leads to TRPV1-mediated analgesic effects in neuropathic rats and possible lipoxygenase-mediated remodeling of anandamide metabolism. *PLoS One* **8**(4): e60040.

Steiner AA, Molchanova AY, Dogan MD, Patel S, Petervari E, Balasko M*, et al* (2011). The hypothermic response to bacterial lipopolysaccharide critically depends on brain CB1, but not CB2 or TRPV1, receptors. *J Physiol* **589**(Pt 9): 2415-2431.

Suardiaz M, Estivill-Torrus G, Goicoechea C, Bilbao A, Rodriguez de Fonseca F (2007). Analgesic properties of oleoylethanolamide (OEA) in visceral and inflammatory pain. *Pain* **133**(1-3): 99-110.

Tchantchou F, Tucker LB, Fu AH, Bluett RJ, McCabe JT, Patel S*, et al* (2014). The fatty acid amide hydrolase inhibitor PF-3845 promotes neuronal survival, attenuates inflammation and improves functional recovery in mice with traumatic brain injury. *Neuropharmacology* **85**: 427-439.

Thabuis C, Tissot-Favre D, Bezelgues JB, Martin JC, Cruz-Hernandez C, Dionisi F*, et al* (2008). Biological functions and metabolism of oleoylethanolamide. *Lipids* **43**(10): 887-894.

Tham CS, Whitaker J, Luo L, Webb M (2007). Inhibition of microglial fatty acid amide hydrolase modulates LPS stimulated release of inflammatory mediators. *FEBS Lett* **581**(16): 2899-2904.

Toth A, Blumberg PM, Boczan J (2009). Anandamide and the vanilloid receptor (TRPV1). *Vitam Horm* **81**: 389-419.

Tsuji F, Murai M, Oki K, Seki I, Ueda K, Inoue H*, et al* (2010). Transient receptor potential vanilloid 1 agonists as candidates for anti-inflammatory and immunomodulatory agents. *Eur J Pharmacol* **627**(1-3): 332-339.

Ueda K, Tsuji F, Hirata T, Takaoka M, Matsumura Y (2008). Preventive effect of TRPV1 agonists capsaicin and resiniferatoxin on ischemia/reperfusion-induced renal injury in rats. *J Cardiovasc Pharmacol* **51**(5): 513-520.

Wang X, Miyares RL, Ahern GP (2005). Oleoylethanolamide excites vagal sensory neurones, induces visceral pain and reduces short-term food intake in mice via capsaicin receptor TRPV1. *J Physiol* **564**(Pt 2): 541-547.

Figure Legends

Fig 1 The effect of systemic administration of PF3845 on levels of (A) AEA, (B) OEA and (C) PEA and the expression of (D-E) inflammatory mediators in the frontal cortex, 2 hours post LPS administration. Data expressed as mean + SEM (n = 8-10 per group). ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

Fig 2 The effect of systemic administration of LPS and/or PF3845 on (A) nocturnal homecage activity, (B) fluid intake, (C) sucrose preference, the expression of (D-E) markers of glial activity, (F) BDNF and (E-K) pro-inflammatory cytokines, in the frontal cortex 24 post LPS administration. Data expressed as mean + SEM (n=7-9 per group). ^{a} $p < 0.05$ LPS effect in ANOVA. ^{b} $p < 0.05$ PF3845 effect in ANOVA; * $p < 0.05$, ** $p < 0.01$ vs. vehicle-saline-treated counterparts, + $p < 0.05$ vs. vehicle-LPS-treated counterparts (SNK post hoc).

Fig 3 The effect of i.c.v. administration of PF3845 on levels of (A) AEA, (B) OEA and (C) PEA and (D) the expression of inflammatory mediators in the frontal cortex, 2 hours post LPS administration. Data expressed as mean + SEM (n = 8-10 per group). ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

Fig 4 The effect of systemic administration of URB937 on levels of (A) AEA, (B) OEA and (C) PEA and expression of (D) pro-inflammatory cytokines in the frontal cortex, 2 hours post LPS administration. Data expressed as mean + SEM (n = 8 per group). * $p < 0.05$, ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

Fig 5 The effect of central CB_1 or CB_2 antagonism on the expression of (A) IL-1β, (B) IL-6 and (C) TNFα within the frontal cortex of PF3845-LPS treated animals. Data expressed as mean + SEM (n = 10-13 per group) *$p < 0.05$ vs. vehicle-vehicle-LPS-treated animals (SNK post hoc).

The effect of systemic administration of PF3845 on (D) ERK and (E) JNK phosphorlyation in the frontal cortex, 2 hours post LPS administration. Data expressed as mean + SEM (n = 5-6 per group).

Fig 6 The effect of i.c.v. administration of selective PPAR, TRPV1 and GPR55 antagonists on the expression of (A) IL-1β, (B) IL-6 and (C) TNFα within the frontal cortex of PF3845-LPS treated animals. Data expressed as mean + SEM (n = 9-10 per group) * $p < 0.05$, vs. vehicle-vehicle-LPS-treated counterparts, + $p < 0.05$ vs. vehicle-PF3845-LPS-treated counterparts (SNK post hoc).

Fig 7 The effect of systemic administration of PF3845, in the presence or absence of central TRPV1 antagonism, on LPS-induced sickness behaviour. (A) temperature 2hrs post LPS, (B) body weight gain, (C) Food intake (D) Fluid intake, (E) water intake and (F) sucrose preference over 24 hours post LPS. Data expressed as mean +SEM (n = 8-9 per group). *P<0.05 vs Vehicle-Vehicle-Saline (SNK post hoc).