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Title	Pharmacological inhibition of FAAH modulates TLR-induced neuroinflammation, but not sickness behaviour: An effect partially mediated by central TRPV1
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Publication Date	2017-02-22
Publication Information	Henry, Rebecca J., Kerr, Daniel M., Flannery, Lisa E., Killilea, Marykate, Hughes, Edel M., Corcoran, Louise, Finn, David P., Roche, Michelle. (2017). Pharmacological inhibition of FAAH modulates TLR-induced neuroinflammation, but not sickness behaviour: An effect partially mediated by central TRPV1. <i>Brain, Behavior, and Immunity</i> , 62, 318-331. doi: <a href="https://doi.org/10.1016/j.bbi.2017.02.016">https://doi.org/10.1016/j.bbi.2017.02.016</a>
Publisher	Elsevier
Link to publisher's version	<a href="https://doi.org/10.1016/j.bbi.2017.02.016">https://doi.org/10.1016/j.bbi.2017.02.016</a>
Item record	<a href="http://hdl.handle.net/10379/15695">http://hdl.handle.net/10379/15695</a>
DOI	<a href="http://dx.doi.org/10.1016/j.bbi.2017.02.016">http://dx.doi.org/10.1016/j.bbi.2017.02.016</a>

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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, lipopolysaccharide (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<sub>1</sub>, CB<sub>2</sub>, PPAR $\alpha$ , PPAR $\gamma$  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; NF $\kappa$ B, 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 TNF $\alpha$ , IL-1 $\beta$ , 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<sub>1</sub> and/or CB<sub>2</sub> 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<sub>1</sub>/CB<sub>2</sub> 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<sub>1</sub> and CB<sub>2</sub> 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<sub>1</sub> 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<sub>1</sub> receptors has been shown to facilitate (De Laurentiis *et al*, 2010), while antagonism of the central CB<sub>1</sub> 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 concomitant 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 ( $21 \pm 2^\circ\text{C}$ ) 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 (100 $\mu\text{g}/\text{kg}$ , 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<sub>1</sub> and CB<sub>2</sub> 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<sub>1</sub>/CB<sub>2</sub> 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 PPAR $\alpha$  antagonist MK886 (300ng), the PPAR $\gamma$  antagonist GW9662 (50 $\mu$ g), the TRPV1 antagonist IRTX (1nmol), the GPR55 receptor antagonist CID (10 $\mu$ M) or vehicle (100% DMSO) in an injection volume of 5 $\mu$ l 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 (100 $\mu$ g/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; Fakhfoury *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<sub>2</sub>; 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<sup>-1</sup> of tissue. The limit of quantification was 1.3pmol g<sup>-1</sup>, 12.1pmol g<sup>-1</sup>, 1.5pmol g<sup>-1</sup>, 1.4pmol g<sup>-1</sup> 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: TNF $\alpha$  (Rn99999017\_m1), IL-1 $\beta$  (Rn00580432\_m1), IL-6 (Rn00561420\_m1), IL-10 (Rn00563409\_m1), IL-1ra (Rn00573488\_m1), I $\kappa$ B $\alpha$  (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°C for 10 min and 40 cycles of 90°C for 15 min followed by 60°C for 1 min.  $\beta$ -actin was used as an endogenous control to normalise gene expression data. Relative gene expression was calculated using the  $\Delta\Delta$ CT 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°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 reprobbed 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- $\kappa$ B-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-1 $\beta$*  [ $t_{(15)} = 5.203$ ,  $p < 0.01$ ], *IL-6* [ $t_{(15)} = 2.23$ ,  $p = 0.04$ ], *TNF $\alpha$*  [ $t_{(15)} = 5.93$ ,  $p < 0.01$ ], *IL-10* [ $t_{(13)} = 2.15$ ,  $p = 0.05$ ], *I $\kappa$ B $\alpha$*  [ $t_{(14)} = 2.85$ ,  $p = 0.01$ ] and *SOCS3* [ $t_{(16)} = 2.88$ ,  $p = 0.01$ ] but not *IL-1 $\alpha$* , 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-1 $\beta$*  [ $F(1,26) = 26.92$ ,  $p < 0.01$ ], *IL-6* [ $F(1,27) = 8.23$ ,  $p < 0.01$ ] and a LPS x PF3845 interaction effect for *TNF $\alpha$*  [ $F(1,27) = 4.53$ ,  $p = 0.04$ ] 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-1 $\beta$*  (Fig 2I) in the frontal cortex 24hrs post administration. Prior systemic administration of PF3845 augmented the LPS-induced increase in *IL-1 $\beta$*  and increased the expression of *TNF $\alpha$*  (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- $\kappa$ B-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-1 $\beta$*  ( $t(15) = 2.38, p = 0.03$ ), *TNF $\alpha$*  ( $t(15) = 3.68, p < 0.01$ ), *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- $\kappa$ B-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- $\kappa$ B-responsive genes *IL-1 $\beta$* , *IL-6* and *TNF $\alpha$*  within the frontal cortex (Fig 4D).

## **FAAH substrate modulation of TLR4-induced neuroinflammation is not mediated via central CB<sub>1</sub> or CB<sub>2</sub> 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<sub>1</sub> and CB<sub>2</sub> receptors in eliciting this response. Statistical analysis (one-way ANOVA) revealed a significant treatment effect on the expression of the NF- $\kappa$ B-inducible genes *IL-1 $\beta$*

[ $F_{3,56}=4.97$ ,  $p < 0.01$ ], *IL-6* [ $F_{3,56}=5.49$ ,  $p < 0.01$ ] and *TNF $\alpha$*  [ $F_{3,56}=4.48$ ,  $p < 0.01$ ], 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-1 $\beta$*  and *IL-6*, but not *TNF $\alpha$* , when compared to vehicle-vehicle-LPS-treated counterparts (Fig 5). Central (i.c.v.) administration of the selective CB<sub>1</sub> or CB<sub>2</sub> antagonists AM251 or AM630 respectively, did not alter the decrease in frontal cortical expression of *IL-1 $\beta$*  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 *TNF $\alpha$*  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<sub>1</sub> and CB<sub>2</sub> receptors is positively coupled to MAPK activation which in turn is known to be important in modulating NF- $\kappa$ B-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<sub>1/2</sub> 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<sub>1</sub> and CB<sub>2</sub> 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 PPAR $\alpha$ , PPAR $\gamma$ , 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-1 $\beta$*  [F<sub>5,74</sub>=4.02, *p* < 0.01], *IL-6* [F<sub>5,73</sub>=2.27, *p* < 0.05] and *TNF $\alpha$*  [F<sub>5,73</sub>=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-1 $\beta$*  and *IL-6*, but not *TNF $\alpha$*  [Vehicle-PF3845-LPS vs. Vehicle-vehicle-LPS] (Fig 6A-C). Central (i.c.v.) administration of IRTX (TRPV1 antagonist), but not MK886 (PPAR $\alpha$  antagonist), GW9662 (PPAR $\gamma$  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  $\pm$  34% vs. IRTX-Vehicle-LPS 67  $\pm$  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<sub>4,41</sub>=2.46, *p* = 0.05], and reduced body weight [F<sub>4,41</sub>=6.05, *p* < 0.01] and food intake [F<sub>4,41</sub>=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<sub>1</sub> or CB<sub>2</sub> 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 hippocampus. 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-1 $\beta$  and TNF $\alpha$ ) 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-induced 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 associated 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 TLR4-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-1 $\beta$  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- $\kappa$ B, 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 *I $\kappa$ B $\alpha$* , often used as an indirect measure of NF- $\kappa$ B 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<sub>1</sub>/CB<sub>2</sub> 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<sub>1</sub> receptors in LPS-induced increases in plasma TNF $\alpha$  (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-1 $\beta$  and IL-6, is likely not mediated by central CB<sub>1</sub> or CB<sub>2</sub> 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<sub>1</sub> or CB<sub>2</sub> antagonism did PF3845 attenuate LPS-induced TNF $\alpha$  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<sub>1</sub> or CB<sub>2</sub> 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 TNF $\alpha$ . 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<sub>1</sub> or CB<sub>2</sub> 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<sub>1</sub>/CB<sub>2</sub> 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- $\gamma$  has been shown to inhibit IL-2 release (Rockwell and Kaminski, 2004) and PPAR- $\gamma$  was shown to mediate, at least in part, the effect of the putative AEA reuptake inhibitor AM404, on increases in plasma TNF $\alpha$ , induced following systemic TLR4 activation (Roche *et*

*al*, 2008). However, the present findings demonstrated that blockade of either PPAR- $\alpha$  or PPAR- $\gamma$ , or the newly classified cannabinoid receptor GPR55, directly within the brain, does not alter FAAH substrate-mediated attenuation of *IL-1 $\beta$*  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 NF $\kappa$ B activation. Evaluating the possible receptor mechanisms revealed that the effects are independent of central cannabinoid receptors (CB<sub>1</sub>, CB<sub>2</sub>, and GPR55) or PPARs (PPAR- $\alpha/\gamma$ ) 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

### **Acknowledgements**

The authors would like to gratefully acknowledge funding received from the Science Foundation Ireland Research Frontiers Programme (*Grant no. 11/RFP/NES/3175*), The Research Office and College of medicine, Nursing and Health Sciences National University of Ireland Galway. The authors would also like to thank Patricia Calcagno for technical assistance during this study.

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## 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). <sup>a</sup>  $p < 0.05$  LPS effect in ANOVA. <sup>b</sup>  $p < 0.05$  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<sub>1</sub> or CB<sub>2</sub> antagonism on the expression of (A) IL-1 $\beta$ , (B) IL-6 and (C) TNF $\alpha$  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 phosphorylation 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-1 $\beta$ , (B) IL-6 and (C) TNF $\alpha$  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).