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Endocannabinoid regulation of neuroinflammatory responses following acute systemic viral (TLR3) and bacterial (TLR4) infection

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Abstract

Toll like receptors (TLRs) are key players in host defence, homeostasis and response to injury. However, uncontrolled and aberrant TLR activation has been proposed to trigger the onset of certain neurodegenerative disorders and elicit detrimental effects on the progression and outcome of established disease. There is now accumulating evidence demonstrating potent immunoregulatory effects of the endogenous cannabinoid (endocannabinoid) system on neuroinflammatory processes and suggesting that this system may represent an important therapeutic target in the treatment of neuroinflammatory and neurodegenerative disorders. The main objective of the work presented in this thesis was to investigate the role of the brain's endocannabinoid system in the modulation of acute neuroinflammatory responses, induced following systemic viral (TLR3) and bacterial (TLR4) infection. The enhancement of endocannabinoid tone was achieved by inhibiting the enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), that preferentially catabolise the primary endocannabinoids in the brain, namely anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The data presented herein demonstrate through a series of experiments that the inhibition of FAAH and enhancement of FAAH substrate levels directly within the brain, and not via peripheral modulatory mechanisms, robustly attenuates TLR3-induced neuroinflammatory processes. These effects were shown to be mediated by FAAH substrate-mediated activation of cannabinoid₁ (CB₁) receptors and the peroxisome proliferator-activated receptor (PPAR)- α and - γ within the brain. In comparison, enhancing endogenous 2-AG tone, through MAGL inhibition, was associated with an augmentation of TLR3-induced expression of both interferon (IFN) - and NF- κ B-inducible inflammatory genes within the hippocampus. Thus, these findings indicate differential effects of FAAH substrates (anti-inflammatory) vs. 2-AG (pro-inflammatory) on TLR3-induced neuroinflammatory responses in an *in vivo* model system. In addition, work in this thesis also demonstrated for the first time that enhancement of FAAH substrates directly within the brain, is associated with potent anti-inflammatory effects on TLR4-induced neuroinflammation. Such anti-inflammatory effects were independent of brain cannabinoid (CB₁, CB₂ and G-protein coupled receptor (GPR) 55)) receptors or PPARs (PPAR- α / γ), but rather were shown to be mediated, at least in part, via activation of TRPV1 directly within the brain.

In conclusion, the data presented in this thesis extend the body of knowledge regarding endocannabinoid modulation of neuroinflammatory responses to acute bacterial (TLR4) and viral (TLR3) infection, and may inform the development of novel therapeutics for acute and chronic neuroinflammatory disorders.

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Authors Declaration

I hereby declare that the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland, Galway. The research is original and entirely my own work. The thesis or any part thereof has not be submitted to the National University of Ireland, Galway, or any other institution in connection with any other academic award. Any views expressed herein are those of the author.

Signed:

Date:

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List of Abbreviations

2-AG: 2-arachidonyl glycerol
AA: arachidonic acid
THC: tetrahydrocannabinol
AC: adenylyl cyclase
AD: Alzheimer's disease
AEA: anandamide
AM404: arachidonylethanolamide
ANOVA: Analysis of variance
AP-1: activating protein-1
BDNF: brain derived neurotrophic factor
CB₁: cannabinoid receptor 1
CB₂: cannabinoid receptor 2
CD11b: Cluster of Differentiation molecule 11B
CD68: Cluster of differentiation 68
cDNA: complementary DNA
CNS: central nervous system
COX-2: cyclooxygenase 2
DAMPs: damage-associated molecular pathogens
dsRNA: double-stranded RNA
ELISA: enzyme-linked immunosorbent assays
ERK: extracellular signal-regulated kinases
ERK1/2: extracellular signal-regulated kinase 1/2
FAAH: fatty acid amide hydrolase
GFAP: glial derived neurotrophic factor
GPR55: G-protein-coupled receptor
i.c.v. : intracerebroventricular
i.p. : intraperitoneal
I-CAM: Intracellular cell adhesion protein 1
IFNR1: type I IFN receptor
IFN- α : interferon-alpha
IFN- β : interferon-beta

IFN- γ : interferon-gamma
I κ B α : inhibitor of NF- κ B, inhibitor of κ light chain gene enhancer in B cells (I κ B)
IL-10: interleukin-10
IL-1-R: interleukin-1-receptors
IL-1ra: interleukin-1 receptor antagonist
IL-1 β : interleukin-1beta
IL-6: interleukin-6
iNOS: inducible nitric oxide synthase
IP10: Interferon gamma-induced protein 10
IRF: interferon regulatory transcription factor
JAK/STAT: Janus kinase and Signal transducer and Activator of Transcription
JNK: c-JUN N-terminal kinase
LC-MS/MS: liquid chromatography – tandem mass spectrometry
LOX: lipoxygenases
LPS: lipopolysaccharide
MAGL: monoacylglycerol lipase
MAPK: mitogen-activated protein kinase
MRC: mannose receptor
MS: multiple sclerosis
MyD88: myeloid differentiation primary response gene 88
NF- κ B: Nuclear factor kappa B
NO: nitric oxide
OEA: *N*-oleoylethanolamide
PAMPs: pathogen-associated molecular patterns
PBMCs: peripheral blood mononuclear cells
PD: Parkinson's disease
PEA: *N*-palmitoylethanolamide
PGE₂: Prostaglandin E2
PKA: protein kinase A
PKR: dsRNA-dependent protein kinase R
Poly I:C: Polyinosinic: polycytidylic acid

PPAR- α : peroxisome proliferator-activated receptor-alpha
PPAR- β : peroxisome proliferator-activated receptor
PPAR- γ : peroxisome proliferator-activated receptor-gamma
PRRs: Pathogen recognition receptors
qRT-PCR: Real-Time Polymerase Chain Reaction
SOCS: suppressor of cytokine signalling
TBI: Traumatic brain injury
TLR: Toll-like receptors
TMEV: Theiler's immune encephalomyelitis
TNF: tumour necrosis factor
TRIF: TIR domain-containing adaptor inducing IFN- β
TRIF: Toll-interleukin 1 receptor (TIR)-domain-containing adaptor-inducing interferon- β
TrkB: Tropomyosin receptor kinase B
RXR: Retinoid X receptor
TRPV1: Transient receptor potential cation channel subfamily V member 1
V-CAM: vascular cell adhesion protein 1

Most abbreviations, other than commonly used expressions, are also defined at the first point of occurrence in the text.

List of Publications

Peer-reviewed original research articles

Henry RJ, Kerr DM, Finn DP, Roche M. FAAH-mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus. *J Neuroimmunol.* 2014 Nov 15;276(1-2):126-34.

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Peer-reviewed review and editorial articles

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Kerr DM, **Henry R**, Roche M. Anandamide Modulation of Endotoxin-Induced Inflammation. *Anat Physiol* 2013, 4:1 <http://dx.doi.org/10.4172/2161-0940.1000e130> (invited editorial).

Manuscripts in preparation

Henry RJ, Kerr DM, Finn DP, Roche M. Pharmacological inhibition of FAAH attenuates TLR4-induced increases in NF- κ B-inducible inflammatory genes in the frontal cortex; effects partially mediated by brain TRPV1

Henry RJ, Kerr DM, Finn DP, Roche M. FAAH substrate-mediated modulation of TLR3-induced increases in interferon- and NF- κ B-inducible inflammatory genes in the hippocampus; effects partially mediated by cannabinoid₁ (CB₁) receptor and PPARs (PPAR- α/γ) within the brain

Published abstracts

Henry R, Kerr DM, Finn DP, Roche M (2014). FAAH inhibition modulates TLR3-induced inflammatory responses in the periphery and brain: possible role for CB₁ receptors. *Journal of Neuroimmunology* Volume 276, Issue 1-2, pages 126-134 (15 November 2014) *Proceedings of the XII International Congress of Neuroimmunology (ISNI)*

Henry R, Kerr DM, Finn DP, Roche M (2014). FAAH mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus. *Irish Journal of Medical Sciences* (*in press*)

Flannery L, **Henry R**, Kerr DM, Finn DP, Roche M (2014). Sex-dependent alterations in locomotor activity and interferon expression following TLR3 activation. *Irish Journal of Medical Sciences* (*in press*)

Henry R, Kerr DM, Finn DP, Roche M (2014). FAAH-mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus. *24th Annual Symposium on the Cannabinoids, International Cannabinoid Research Society*, P3-10.

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Neuropsychopharmacology Volume 24, Supplement 1, Pages S11 (March 2014), P.1.010. 10.1016/S0924-977X(14)70012-4

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Conference proceedings

Lydon, S., Healy, O., Roche, M., **Henry, R.**, Mulhern, T., & Hughes, B. M. (2015). *Salivary cortisol levels and challenging behaviour in children with autism spectrum disorder*. Paper presented at the 9th Annual Conference of the Division of Behaviour Analysis, Galway, Ireland.

Hughes, B. M., Lydon, S., Healy, O., Roche, M., **Henry, R.**, Mulhern, T., Reed, P., & Goodwin, M. S. (2015). *Psychophysiological stress responses and challenging behaviour in children with autism spectrum disorder*. Paper presented at the 36th Annual Conference of the Stress and Anxiety Research Society, Tel Aviv, Israel.

Henry R, Kerr D, Finn DP, Roche M (2015). Increasing Fatty acid amide hydrolase substrates attenuates TLR4-induced neuroinflammation independent of central cannabinoid receptor activation. British Neuroscience Association (Poster).

Lydon, S., Healy, O., Roche, M., **Henry, R.**, Mulhern, T., & Hughes, B. M. (2015). Salivary cortisol levels and challenging behavior in children with autism spectrum disorder. 9th Annual Autism Conference of the Association for Behavior Analysis International (ABAI). Las Vegas, NV, USA (Poster).

Fitzgibbon M, **Henry RJ**, Kerr DM, Finn DP, Roche M. Characterisation of the endocannabinoid system in the descending pain pathway in a mouse model of IFN- α induced hyperalgesia. *College of Medicine Research Day, NUIG, May 2015 (Poster)*.

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Henry R, Kerr D, Finn DP, Roche M (2014). FAAH inhibition modulates TLR3-induced inflammatory responses in the periphery and brain: possible role for CB₁ receptors. Careers in Neuroscience Symposium, NUIG (poster).

Henry R, Kerr D, Finn DP, Roche M (2014). FAAH-mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus. *College of Medicine Research Day May 2014 (Talk)*.

Flannery L, **Henry R**, Kerr DM, Finn DP, Roche M. Enhancing endogenous anandamide tone attenuates the expression of TLR3-induced inflammatory mediators in the rat spleen, effects partially mediated by CB₁ receptors. Presented at NUI Galway College of Medicine, Nursing & Health Sciences Postgraduate Research Day, May 2014. Poster

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Henry R, Kerr D, Finn DP, Roche M (2014) *N-acyl ethanolamines modulate early but not late phase neuroinflammatory responses to TLR-3 activation. Presented at Young Life Scientists, Ireland, TCD, Dublin (Poster)*.

Henry R, Kerr DM, Finn DP, Roche M. Central inhibition of fatty acid amide hydrolase attenuates TLR-3 induced expression of interferon-gamma and related genes in the rat (2013). Galway Neuroscience Research Day, NUI Galway (Poster).

Henry R, Kerr D, Finn DP, Roche M (2013). Inhibition of Fatty acid amide hydrolase does not modulate TLR3-induced neuroinflammation or reductions in hippocampal BDNF expression (2013). Young Life Scientists' Symposium 2013 – Cell signalling (Poster).

Henry R, Kerr D, Finn DP, Roche M (2013). Central inhibition of fatty acid amide hydrolase modulates expression of inflammatory mediators in discrete brain regions following a systemic bacterial infection. *College of Medicine Research Day, NUIG, May 2013 (Poster).*

Henry R, Kerr D, Finn DP, Roche M (2013). Central inhibition of fatty acid amide hydrolase modulates expression of inflammatory mediators in discrete brain regions following a systemic bacterial infection. *Presented at College of Science Research Day, NUIG, April (Poster).*

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Chapter 1

General Introduction

1.1 The innate immune system

The innate immune system is an evolutionary ancient part of the host defence mechanisms which provides the first line of defence against a wide array of invading pathogens [For reviews of innate immunity and pathogen host interaction see (Akira *et al.*, 2006; Basset *et al.*, 2003). The innate immune system is composed of anatomical barriers including skin and mucosal membranes; cellular components including monocytes/macrophages, fibroblasts, mast cells, neutrophils, natural killer (NK) cells and dendritic cells (DCs), as well as other circulating leukocytes and also soluble proteins. The ability of the innate immune system to recognise such a wide array of pathogens is due to the presence of a set of germline-encoded pathogen recognition receptors (PRRs) which are located on the cell surface or in intracellular compartments of inflammatory cells. These receptors detect specific microbial sequence patterns termed pattern associated molecular pathogens (PAMPs) that are associated with microbial metabolism, or damage-associated molecular pathogens (DAMPs), released from injured cells. Activation of PRRs by a specific ligand results in activation of signaling cascades which induces production of pro-inflammatory cytokines, type I interferons (IFNs) and other inflammatory mediators which co-ordinate a set of responses to eliminate the invading pathogen from the infected cell. To date, the most widely studied of the PRRs is a class of type-I transmembrane glycoprotein's known as Toll-like receptors (TLRs).

1.2 Toll-like receptors (TLRs)

The Toll protein was first identified in *Drosophila Melanogaster* as a gene involved in controlling embryonic dorsoventral axis formation (Lohs-Schardin *et al.*, 1979). Toll was subsequently reported to be a key player in the *Drosophila* immunity where it was shown to play a critical role in the antifungal immune response in flies (Lemaitre *et al.*, 1996). The first human homolog of the *Drosophila* Toll was named human toll or TLR4 (Medzhitov *et al.*, 1997) and to date 13 mammalian TLRs have been identified; 13 of which are expressed in mice and 10 in humans, all of which play a vital role in mediating host innate immune defences against invading pathogens, maintaining homeostasis and also in controlling adaptive immune responses (see Figure 1.1). TLRs are type 1 integral membrane glycoproteins and are considered members of the interleukin-1-receptors (IL-1-R) super-family, due to

considerable homology in their cytoplasmic regions (Slack *et al.*, 2000). However, despite their similarities in the intracellular domains, TLRs and IL-1-R differ greatly in their extracellular domains with TLR containing 19-25 tandem copies of the leucine-rich repeat (LRR) motifs which form a horseshoe structure. This is in contrast to the extracellular domain of IL-1-R which contains three immunoglobulin-like domains (Akira *et al.*, 2004). TLRs can be further divided into subgroups based on their ability to recognise particular PAMPs; TLR1, TLR2, TLR4, TLR5 and TLR6 sense microbial membrane components while TLR3, TLR7, TLR8 and TLR9 sense microbial and viral nucleic acids. TLRs are expressed on various cell types including epithelial and endothelial cells, immune cells such as neutrophils, macrophages and DCs, and also on cells within the central nervous system (CNS), including microglia, neurons and astrocytes. To date, the most extensively studied TLR is TLR4 which recognises lipopolysaccharide (LPS), a component of the cell wall of Gram negative bacteria, activation of which induces robust inflammatory responses, both in the periphery and within the CNS. However, there is now an increasing amount of interest in examining the inflammatory response induced following activation of additional TLRs, including TLR3 which recognises double-stranded RNA (dsRNA), a component produced by most viruses during their replication, activation of which induces production of type I IFNs and additional inflammatory cytokines. Overall, TLR-induced signalling plays an important role in mediating the host defence mechanisms against both bacterial and viral infections, however uncontrolled or aberrant TLR-induced inflammatory responses have been implicated in the development or exacerbation of various diseases, both in the periphery and within the CNS (Kawai *et al.*, 2010; Lehnardt, 2010; O'Neill *et al.*, 2009).

Figure 1.1 TLR Signalling. There are two types of Toll-like receptors (TLRs), those located at the plasma membrane that sense microbial membrane components and the intracellular ones that sense microbial or viral nucleic acids. All TLRs signal via the adaptor myeloid differentiation primary response gene 88 (MyD88), except TLR3 that can function only via the MyD88-independent/ Toll-interleukin 1 receptor (TIR)-domain-containing adaptor-inducing interferon- β (TRIF). Membrane signalling triggers an inflammatory response via NF- κ B inducible genes whereas intracellular TLR signalling cause the production type I interferons (IFNs) and IFN-inducible genes. Diagram adapted from (Akira *et al.*, 2004).

1.2.1 Ligand recognition by TLR4 and TLR3

TLR4 is the primary PRR for the recognition of Gram negative bacteria due to its activation by LPS, although TLR4 is also known to recognise additional ligands including Lipid A analogs (Lien *et al.*, 2000), mycobacterial components (Means *et al.*, 1999) and viral components including respiratory syncytial virus (Rsv) F protein (Kurt-Jones *et al.*, 2000). LPS associates with mammalian TLR4 in a series of interactions with several proteins which ultimately results in activation of signalling cascades and the production of inflammatory mediators involved in mediating innate immune responses. LPS is a complex glycolipid composed of the hydrophilic polysaccharide “lipid A” which is responsible for the biological activity of LPS (Akira *et al.*, 2001). Prior to binding to TLR4, LPS leaves the Gram negative bacteria and associates with the soluble LPS binding protein (LBP) (Schumann *et al.*, 1994). The LPS/LBP complex is then delivered to membrane bound glycosylphosphatidylinositol (GPI)-linked protein CD14 (Akira *et al.*, 2001). CD14 facilitates the transfer of LPS to myeloid-differentiation protein (MD-2), a small accessory protein associated with the extracellular domain of TLR4. Recognition of LPS by the MD-2/TLR4 complex results in oligomerization of TLR4 and subsequent activation of downstream signalling cascade (Poltorak *et al.*, 1998; Shimazu *et al.*, 1999).

In comparison to TLR4 which is primarily expressed on the cell surface, TLR3 is located on the endosomal compartment of immune and non immune cells such as fibroblasts and epithelial cells (Matsumoto *et al.*, 2008), where it serves to recognise dsRNA (Jacobs *et al.*, 1996). During viral reproduction, dsRNA can arise as a replicative intermediate (Sen *et al.*, 2005) of viruses that have been internalised for delivery to the endosome (Barton *et al.*, 2009). Within the endosome, the N-terminal domain of the TLR3 receptor is primarily responsible for the recognition of dsRNA, while the C-terminal domain induces signalling (Sen *et al.*, 2005). Within the cytoplasm, dsRNA is recognised independent of TLR3, by additional PPRs including RIG-1 (retinoic acid-inducible gene 1) and MDA5 (melanoma-differentiation associated gene 5) (Andrejeva *et al.*, 2004; Yoneyama *et al.*, 2004).

Activation of TLR3 by dsRNA induces a signalling cascade that primarily results in production of type I IFNs, which are pivotal in mediating the hosts anti-viral

responses (Majde, 2000). Poly inosinic:poly cytidylic acid (poly I:C) is a synthetic dsRNA and ligand for TLR3 (Alexopoulou *et al.*, 2001) that is capable of inducing type I IFNs (Cunningham *et al.*, 2007; Fortier *et al.*, 2004). Thus experimentally, many researchers utilize poly I:C to investigate the acute stages of viral infection (Cunningham *et al.*, 2007; Guha-Thakurta *et al.*, 1997; Traynor *et al.*, 2004). In addition to TLR3, other endosomal TLRs are capable of recognising viral components including TLR7/8-induced recognition of single stranded (ss) RNA and TLR9-induced recognition of viral CpG DNA, which also results in production of type I IFNs (West *et al.*, 2006).

1.3 TLR transduction

Following recognition and binding of a specific ligand, TLRs oligomerise and signal via various adaptor molecules to induce an appropriate immune response to protect the host against invading pathogens. Such adaptor molecules include myeloid differentiation primary response gene 88 (MyD88), Toll-interleukin 1 receptor (TIR)-domain-containing adaptor-inducing interferon- β (TRIF), TIR-domain containing adaptor protein (TIRAP)/MyD88-adaptor-like (MAL) and TRIF-related adaptor molecule (TRAM). However, TLR-mediated transduction primarily signals via two main pathways, namely the MyD88-dependent and MyD88-independent/TRIF-dependent signalling pathways, both of which are described in detail below and depicted diagrammatically in Figure 1.2.

1.3.1 MyD88-dependent pathway

MyD88 was the first TIR-domain containing adaptor protein to be identified and is now known to be essential for all TLR-induced signalling cascades upon recognition of their respective ligands, except TLR3 [for review see (Takeda *et al.*, 2004; Takeda *et al.*, 2007)]. To date, the most extensively studied of the TLR family is TLR4, as such this section will specifically describe TLR4-induced MyD88 dependant signalling. Upon LPS binding, MyD88 recruits members of the IL-1 receptor-associated kinase (IRAK) family, namely IRAK1/4 (Akira *et al.*, 2004; Akira *et al.*, 2006). This association results in phosphorylation and subsequent dissociation of IRAK1/4 from MyD88 which in turn leads to activation of tumor necrosis factor receptor-activated factor 6 (TRAF6), which functions as a E3 ubiquitin ligase (Deng

et al., 2000). TRAF6 contains a N-terminal ring RING domain that allows it to associate with ubiquitin-conjugating enzyme 13 (UBC13), which in turn activates the transforming growth factor- β -activated kinase 1 (TAK1) through the assembly of a lysine 63-linked polyubiquitin chain (Wang *et al.*, 2001). TAK-1 is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family and is essential for LPS-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation (Takaesu *et al.*, 2003). TAK-1 forms a complex with the adaptor protein TGF-beta activated kinase-1 (TAB-1) which acts to enhance the kinase activity of TAK-1 (Shibuya *et al.*, 1996). This interaction ultimately leads to nuclear translocation and activation of NF- κ B and increased production of several NF- κ B-responsive inflammatory genes including pro inflammatory cytokines, chemokines and adhesion molecules (Akira *et al.*, 2004; Akira *et al.*, 2006; Ghosh *et al.*, 2002). In addition to NF- κ B transcriptional activation, TAK-1 also phosphorylates the mitogen activated protein kinases (MAPK), namely c-JUN N-terminal kinase (JNK) and p-38 which are also involved in the inflammatory response via activation of the transcription factor activating protein-1 (AP-1).

1.3.2 MyD88-independent pathway

While TLR4 can elicit inflammatory responses via both MyD88-dependent and -independent signalling pathways, TLR3 activation results in activation of the MyD88-independent/TRIF-dependent signalling pathway only, which primarily results in the production of type I IFNs (Akira *et al.*, 2004; Yamamoto *et al.*, 2004). The main adaptor protein identified in the MyD88-independent/TRIF dependent signalling pathway is TRIF (Oshiumi *et al.*, 2003). Upon ligand (dsRNA) recognition by TLR3, TRIF recruits a signalling complex composed of TRAF3 and the non-canonical IkappaB kinases (IKKs) which include TANK-binding kinase-1 (TBK-1) and IKKepsilon, resulting in phosphorylation and activation of the transcription factor interferon regulated factor 3 (IRF3) (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). Activated IRF3 translocates into the nucleus and interacts with the transcriptional co-activator CREB-binding protein (CBP)/p300 where it specifically binds to the positive regulatory domain-1 (PRD-1) of IFN- β , thus inducing increased production of the type I IFN, IFN- β and several IFN-inducible genes including the transcription factor IRF7 (Lin *et al.*, 1998; Lin *et al.*, 1999; Sato

et al., 1998; Yoneyama *et al.*, 1998). Activation and subsequent nuclear translocation of IRF7 leads to enhanced production of the type I IFNs and late phase anti-viral responses via amplified production of type I IFNs and IFN-inducible genes (Taniguchi *et al.*, 2001; Taniguchi *et al.*, 2002). In addition to inducing type I IFNs, signalling through the TRIF-dependent signalling pathway leads to activation of the NF- κ B transcription factor and inducible genes (Akira *et al.*, 2004), albeit with delayed kinetics (Kawai *et al.*, 1999). In this pathway, NF- κ B activation has been reported to be dependent on the physical interaction of the N-terminal region of TRIF with TRAF6 and subsequent recruitment and activation of TAK-1 (Sato *et al.*, 2003). Furthermore, in order to facilitate NF- κ B activation, TRIF interacts via its C-terminal region with the downstream kinase, the receptor-interacting protein-1 (RIP)-1 (Meylan *et al.*, 2004). As stated above, TLR4 is also capable of signalling via the MyD88-independent pathway which results in activation of NF- κ B, MAPK and type I IFN production (Kawai *et al.*, 1999). Notably, while TRIF is utilized by TLR4 they do not directly interact, rather the adaptor molecule TRAM acts co-operatively with TRIF to induce activation of IRF3 and late-phase NF- κ B, following TLR4 activation (Akira *et al.*, 2004; Akira *et al.*, 2006).

Figure 1.2 MyD88-dependent and -independent signalling pathways. MyD88-dependent signalling pathway is used by all TLRs except TLR3. Signalling through the MyD88-dependent pathway leads to the activation of MAPKK and IKK complex resulting in activation and nuclear translocation of AP-1 and NF- κ B, respectively; this results in increased gene transcription and production of pro-inflammatory cytokines. TLR4 is also capable of signalling through the MyD88-independent pathway; however this is the sole signalling mechanism for TLR3. TRIF is the main adaptor protein in the MyD88-independent pathway. Activation of the MyD88-independent pathway leads to the activation of the non-canonical IkappaB kinases (IKKs) which include TBK-1 and IKKepsilon resulting in activation and nuclear translocation of IRF3 and ultimately gene transcription and production of the type I IFN, IFN- β . TLR3 can associate with TRAF6 to activate AP-1 and NF- κ B. In order to facilitate NF- κ B activation, TRIF also interacts with RIP-1. Diagram adapted from (Patel *et al.*, 2012).

1.3.3 NF- κ B signalling

NF- κ B is a key transcription factor of both the MyD88-dependent (TLR4) and the MyD88-independent (TLR3) signalling pathways (Figure 1.2). NF- κ B activation regulates a vast array of inflammatory genes involved in mediating the innate immune system defence mechanisms including: inflammatory cytokines (tumor necrosis factor alpha; TNF α , interleukin 1-beta; IL-1 β , interleukin-6; IL-6 and interleukin 10; IL-10), inducible effector enzymes (inducible nitric oxide synthase; iNOS and cyclooxygenase-2; COX2), chemokines, adhesion molecules, and acute phase proteins (Ghosh *et al.*, 2002; Ghosh *et al.*, 1998). NF- κ B is composed of a group of structurally related and evolutionarily conserved proteins, five of which are found in mammals: Rel (c-Rel), Rel A (p65), Rel B, NF κ B1 (p50 and its precursor p105) and NF κ B2 (p52 and its precursor p100) (Ghosh *et al.*, 1998). These proteins can exist as either homo- or heterodimers and primarily act as activators of transcription of specific target genes (Ghosh *et al.*, 1998; Hayden *et al.*, 2004). The NF- κ B/rel homo-heterodimers all share a conserved 300 amino acid N-terminal Rel homology domain (RHD) which facilitates DNA binding, dimerization and association with the family of cytoplasmic inhibitors of NF- κ B known as the I κ B family (Ghosh *et al.*, 2002; Ghosh *et al.*, 1998). The best characterised member of this inhibitory family is I κ B α which acts to retain NF- κ B in the cytoplasm by blocking the nuclear localisation sequence (NLS) of NF- κ B, via ankyrin-repeat motifs and RHD interactions (Baldwin, 1996; Ghosh *et al.*, 1998). Cell stimulation, including that of TLR-induced activation, results in activation of the I κ B kinase (IKK) signalosome, which is composed of two catalytic subunits: IKK α and IKK β , and one regulatory subunit known as NF- κ B essential modulator (NEMO) or IKK γ (Ghosh *et al.*, 2002). Activation of the IKK signalosome results in phosphorylation and subsequent polyubiquitination of I κ Bs, which ultimately liberates the NF- κ B complex from the cytoplasm and enables it to translocate into the nucleus where it induces increased transcription of target genes involved in mediating the inflammatory response, including pro-inflammatory cytokines (TNF α , IL-1 β and IL-6), chemokines and prostaglandins. The production of these inflammatory mediators encompasses an essential component of the innate immune response to fight infection and restore homeostasis.

Following activation of NF- κ B, cells respond by increasing phagocytic activity, increasing transcription of pro- or anti-inflammatory cytokines, up-regulation of co-stimulatory molecules or maturation of antigen presenting cells (Caamano *et al.*, 2002; Tato *et al.*, 2002). Initially, pro-inflammatory cytokines released by activated macrophages act on their respective receptors to induce transcription of a large number of genes involved in modulating the inflammatory response to infection. Specifically, the immediate actions of TNF α and IL-1 β in the inflammatory response are to induce production of adhesion molecules (E-selectin, intracellular adhesion molecule-1; ICAM-1 and vascular adhesion molecule-1; VCAM-1) and chemokines (IL-8, monocyte chemoattractant protein-1; MCP-1, etc) on the walls of vascular endothelial cells to which neutrophils, monocytes and lymphocytes adhere before migrating across into the tissue to the site of infection. In addition, these cytokines induce COX2 and consequent production of prostaglandin E₂ which causes vasodilatation and enhances perception of pain at the site of infection. Both TNF α and IL-1 β also act in conjunction with additional pro-inflammatory cytokines such as IFN- γ to induce increases in chemotaxis for leukocytes and increased phagocytosis. Furthermore, IL-6 acts as a potent inducer of acute phase proteins (Heinrich *et al.*, 1998). Overall, all of these inflammatory mediators act to induce immune responses to fight infection and protect the host, for a comprehensive review on innate immunity and pathogen-host interaction see (Basset *et al.*, 2003). Under normal physiological conditions this inflammatory response is tightly regulated by several highly regulated mechanisms which modulate cytokine bioavailability, including the synthesis of anti-inflammatory cytokines (interleukin-10; IL-10, interleukin-1 receptor antagonist; IL-1ra and transforming growth factor beta; TGF β) and the production of suppressor of cytokine signalling (SOCS)1 and SOCS3 proteins which primarily act as negative regulators of IFN-(type I and II) and IL-6-induced signalling, respectively (Baker *et al.*, 2009). In addition, excessive NF- κ B activation is limited by I κ B α , which once produced following NF- κ B activation, translocates back into the cytoplasm and acts to inhibit further NF- κ B activation, via an auto regulatory feedback loop (Hoffmann *et al.*, 2006) (See Figure 1.3 for diagrammatical representation of NF- κ B signalling).

Figure 1.3 NF- κ B mechanism of action. Inactive NF- κ B complexed to its inhibitor I κ B resides in the cell cytoplasm. Following activation by extracellular signals, including TLR-induced stimulation, I κ B kinase (IKK) phosphorylates I κ B. Phosphorylated I κ B is then ubiquitinated and marked for degradation by proteasomes thereby releasing NF- κ B dimers from the cytoplasmic NF- κ B-inhibitor complex and allowing its translocation to the nucleus and facilitating the subsequent transcription of various genes including pro-inflammatory cytokines (TNF α , IL-1 β , IL-6) which play a pivotal role in mediating host defences against invading pathogens. Under normal physiological conditions, pro-inflammatory responses are tightly controlled by several mechanisms including production of I κ B α which once produced is transported back into the cytoplasm and inhibits further NF- κ B activation. In addition, anti-inflammatory cytokines including IL-10, IL-1ra and SOCS proteins are also produced following NF- κ B activation where they function to prevent excessive pro-inflammatory cytokine signalling. Diagram adapted from (Ghosh *et al.*, 2002)

1.3.4 Interferon regulatory factors (IRF) and IFN signalling

As previously mentioned, TLR3 recognises dsRNA, a component produced by the majority of viruses during replication (Schulz *et al.*, 2005). Thus, many researchers utilize the synthetic dsRNA poly I:C to mimic the acute stage of viral infection (Guha-Thakurta *et al.*, 1997; Traynor *et al.*, 2004). Experimentally, poly I:C-induced activation of TLR3 induces production of type I IFNs and the NF- κ B-inducible cytokines TNF α , IL-1 β and IL-6 (Cunningham *et al.*, 2007; Fortier *et al.*, 2004). As the immediate role of NF- κ B-inducible cytokines in response to infection has been described above, this section will focus on type I IFN induction and signalling in response to viral infection.

Type I IFNs are a large subgroup of the IFN proteins which include IFN α , IFN β , IFN δ , IFN ϵ , IFN κ , IFN τ and IFN ω . To date, the most extensively studied members of this family are IFN- α and IFN- β (Platanias, 2005), both of which are known to play a key role in mediating anti-viral responses. Interferon regulatory factors (IRFs) are a family of transcription factors which have a key role in the production of type I IFNs in response to viral infection (Honda *et al.*, 2006). To date, the mammalian IRF family comprises of nine members: IRF1-9, each of which contains a well-conserved DNA-binding domain of approximately 120 amino acids and a C-terminal IRF association domain (IAD) that is responsible for signal transduction (Mamane *et al.*, 1999; Taniguchi *et al.*, 2001). The well-conserved DNA-binding domain forms a helix-turn-helix motif which recognises a consensus DNA sequence termed IFN-stimulated response element (ISRE) that is found in the promoter region of all genes activated by IRFs (Taniguchi *et al.*, 2001). Both IRF3 and IRF7 have been identified as key regulators of type I IFN production in response to viral infection, whereby phosphorylation of IRF3 predominantly induces production of IFN- β , whereas IRF7 has the ability to preferentially activate IFN- α (Noppert *et al.*, 2007). IRF3 is constitutively expressed in its latent form in the cytoplasm of all tissues (Au *et al.*, 1995) while in comparison, IRF7 is expressed in much smaller amounts in most cells and is only strongly induced in the presence of type I IFN-induced signalling. In the presence of viral infection, IRF3 undergoes phosphorylation primarily in its C-terminal and forms a dimer with IRF7 which enables the IRF to interact with the co-activators CBP (cyclic-AMP-responsive-element-binding-

protein (CREB)-binding protein) or p300 in the nucleus (Honda *et al.*, 2006; Weaver *et al.*, 1998; Yoneyama *et al.*, 1998), which binds to the DNA response element of target genes (type I IFNs), leading to the subsequent production of type I IFNs. Once produced, the type I IFNs bind to a common cell-surface receptor complex termed the IFN- α receptor (IFNAR) (Pestka *et al.*, 2004; Pestka *et al.*, 1987), where upon activation mediates effects via activation of a janus activated kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signalling pathway (Platanias, 2005). Activation of the type I IFN receptor results in autophosphorylation and subsequent activation of the receptor associated JAKs (Silvennoinen *et al.*, 1993). The activated JAKs then act to regulate the phosphorylation of members of the STAT family of transcription factors including STAT1 and STAT2 via specific binding between STAT Src-homology 2 (Src2) domains and receptor phosphotyrosine residues (Aaronson *et al.*, 2002; Darnell, 1997; Darnell *et al.*, 1994; Stark *et al.*, 1998). Phosphorylation of STAT1 and STAT2 on tyrosine residues results in the formation of a STAT1-STAT2 heterodimer which in turn interacts with IRF9 to form a STAT1-STAT2-IRF9 heterotrimer known as IFN-stimulated gene factor 3 (ISGF3) (Aaronson *et al.*, 2002; Platanias *et al.*, 1999; Stark *et al.*, 1998). The ISGF3 complex translocates into the nucleus where it recognises and binds to a specific DNA response element termed the ISRE which induces transcriptional activation of several IFN stimulated genes (ISGs) including the transcription factor IRF7, which in turn translocates into the nucleus and binds to the positive regulatory domain (PRD) on the IFN- α promoter (Ivashkiv *et al.*, 2014; MacMicking, 2012; Schoggins *et al.*, 2011). Thus, the induction of type I IFNs initiates a positive feedback loop, which enables the infected cells to produce substantial amounts of type I IFNs capable of combating viral infection via several well defined mechanisms including the synthesis of dsRNA-dependent protein kinase R (PKR) (Stark *et al.*, 1998), the IFN-inducible 2-5A synthetases (OAS) (Floyd-Smith *et al.*, 1981; Wreschner *et al.*, 1981) and the IFN-GTP-binding protein Mx (Horisberger, 1995; Stark *et al.*, 1998), all of which act to inhibit viral replication (Stark *et al.*, 1998). In addition, activation of the type I IFNAR induces additional STAT complexes including the formation of STAT1-STAT1 homodimers, which act to recognise and bind to an additional DNA response element known as the IFN γ -activated site (GAS) element (Platanias, 2005; Platanias

et al., 1999), which ultimately leads to increased transcription of genes involved in the inflammatory response (Ivashkiv *et al.*, 2014). Similar to the regulatory effects on pro-inflammatory cytokines, type I IFN signalling is tightly controlled by several mechanisms (Ivashkiv *et al.*, 2014), including IFN-induced production of SOCS1 (Yoshimura *et al.*, 2007). Once produced, SOCS1 acts to negatively regulate type I IFN signalling via direct interaction and inhibition of JAK activation (Kubo *et al.*, 2003) or via direct binding to the type I IFN receptor (IFNAR) (Fenner *et al.*, 2006; Qing *et al.*, 2005). See Figure 1.4 for diagrammatical representation of the canonical type I IFN signalling pathway.

Figure 1.4 The canonical type I IFN signalling pathway. Type I IFN signalling at the type I IFN receptor induces activation of the Jak-Stat signalling pathway. Phosphorylated JAK1 and Tyk2 phosphorylate STAT1 and STAT2, which heterodimerize via SH2 domain, which then associate with IRF9 to form a unique complex known as the interferon-stimulated gene factor-3 (ISGF3). The ISGF3 translocates into the nucleus and binds to the interferon-stimulated response element (ISRE) consensus sequence that induces transcription and production of several interferon-stimulated genes (ISGs) including those involved in mediating anti-viral responses (IRF7, PKR, OAS and Mx). In addition, activation of the type I IFNs results in STAT1-STAT1-homodimers which act to recognise and bind to an additional DNA response element known as the IFN γ -activated site (GAS) element which leads to gene transcription of inflammatory mediators including IFN- γ -induced protein 10 (IP-10). Diagram adapted from (Ivashkiv *et al.*, 2014).

1.4 TLR-induced neuroinflammation and physiological consequences

Circulating inflammatory cytokines including TNF α , IL-1 β and IL-6 induced following systemic TLR3 and TLR4 activation, can communicate with the CNS via several routes, including: (1) entering the brain via the circumventricular organs, (2) transport across the blood brain barrier via transport molecules expressed on brain endothelial cells or (3) cytokine-induced activation of the vagal communication pathway [for review of brain-immune communication pathways see (Dantzer, 2004; Quan *et al.*, 2007)]. This periphery to brain cytokine signalling ultimately results in activation of microglia and astrocytes (the immune cells of the CNS), which in turn play a key role in generating an inflammatory response within the CNS (neuroinflammation). Under normal physiological conditions, this neuroinflammatory response is essential in order for the host to fight infection and allow the CNS microenvironment to return to a state of homeostasis. This is achieved via several highly organised strategies including induction of a fever response (via the production of prostaglandins), activation of the stress-hypothalamic-pituitary adrenal (HPA) axis (Rivest *et al.*, 2000; Romanovsky, 2000) and behavioural alterations such as hypolocomotion, hyperalgesia, anorexia, anhedonia and depressed mood; collectively referred to as “sickness behaviour”. This response to infection acts to conserve energy, reduce pathogen replication, stimulate proliferation of immune cells and minimize thermal loss in the host (Dantzer, 2001; Dantzer, 2004), and usually subsides once the pathogen is removed.

Microglia are the resident immune cells of the CNS originating from myeloid tissue (Rezaie *et al.*, 1999) which play a pivotal role in the modulation of the CNS inflammatory network. Under normal physiological conditions microglia are spread throughout the brain parenchyma where they are responsible for surveying the CNS microenvironment for noxious agents and injurious processes (Nimmerjahn *et al.*, 2005) and maintaining normal cellular homeostasis (Hanisch *et al.*, 2007). Similar to peripheral immune cells, microglia express functional TLRs including TLR3 and TLR4 (Bsibsi *et al.*, 2002; Olson *et al.*, 2004; Town *et al.*, 2006). In addition, they also express receptors for a number of other factors released by damaged neurons including cytokines, glutamate, ATP and growth factors (Hanisch *et al.*, 2007). Microglia have multiple activation phenotypes, dependant on their local microenvironment (Colton, 2009; Kumar *et al.*, 2012). Specifically, in the presence

of infection or injury, microglia are known to promote a “classically activated” M1 phenotype which produces high levels of pro-inflammatory cytokines (TNF α , IL-1 β and IL-6), which in a similar manner to that of peripheral immune responses, are essential for host defence and phagocytic activity against invading pathogens. In comparison, in the presence of anti-inflammatory cytokines such as IL-4 or IL-10, microglia adapt an “alternative activated” M2 phenotype (Ponomarev *et al.*, 2007), which is thought to ultimately promote wound healing, tissue repair and induce suppression of destructive immune responses (Boche *et al.*, 2013; Colton, 2009). Astrocytes are the most abundant cells within the CNS (Kriegstein *et al.*, 2009; Marshall *et al.*, 2003) and under normal conditions play an essential supportive, protective and maintenance role within the CNS (Carson *et al.*, 2006; Farina *et al.*, 2007) where they secrete important neurotrophic factors such as TGF β , brain derived neurotrophic factor (BDNF) and glial fibrillary acidic protein (GFAP) (Farina *et al.*, 2007; Phillips *et al.*, 2014). In addition, astrocytes also express functional TLR receptors (Chung *et al.*, 1990; Farina *et al.*, 2007; Farina *et al.*, 2005), as well as several other PRRs (Farina *et al.*, 2007) and as such are emerging as key players in mediating immune responses within the CNS. In addition to glial cells, LPS-induced activation of TLR4 on neuronal cultures has been shown to result in release of pro-inflammatory cytokines (Leow-Dyke *et al.*, 2012), thus suggesting that neuronal cells express functional TLRs capable of initiating neuroinflammatory responses.

Overall, activation of immune responses by microglia and astrocytes and other cells of the CNS, following systemic infection, are beneficial in response to infection. However if not regulated by an appropriate response (modulation of cytokine bioavailability via production of anti-inflammatory cytokines), may result in detrimental effects on CNS function. Accordingly, there is now a substantial amount of evidence that uncontrolled and aberrant neuroinflammatory responses can elicit potent effects on neurotransmission and neurodegenerative cascades [for reviews see (Arroyo *et al.*, 2011; Lehnardt, 2010; Owens, 2009; van Noort *et al.*, 2009)], which may underlie the pathophysiology associated with various CNS pathologies. Furthermore, viral and bacterial induced activation of TLRs and associated neuroinflammation is proposed to trigger the onset of some neurodegenerative disorders (Deleidi *et al.*, 2012) and elicit detrimental effects on the progression and outcome of established disease (Holmes *et al.*, 2009; Perry, 2004; Teeling *et al.*,

2009). Thus, gaining a greater understanding of TLR-induced neuroinflammatory responses is critical at a fundamental physiological level and for the development of novel, more efficacious treatments for both neurological and psychiatric disorders with an underlying inflammatory component.

Accordingly, there is now accumulating evidence demonstrating potent immunomodulatory effects of cannabis-like compounds (cannabinoids) and the body's own cannabinoids (endocannabinoids) on neuroinflammatory responses, including that following TLR activation [For recent reviews see (Downer, 2011; Jean-Gilles *et al.*, 2010; Rom *et al.*, 2013; Stella, 2010)]. Thus may offer a novel therapeutic target for neuroinflammatory disorders.

1.5 The endocannabinoid system

“By using a plant that has been around for thousands of years, we discovered a new physiological system of immense importance” – *Raphael Mechoulam*

Cannabis Sativa (cannabis) is one of the earliest plants cultivated by man, with its seeds initially used as food *circa* 6000 B.C (Touw, 1981). The plant was first used in China as early as 4000 B.C., where cannabis stems (hemp) were used to manufacture ropes, textiles and paper, some of which were found in the tomb of Emperor Wu of the Han dynasty (104-87 B.C.). It is mentioned in the Hindu sacred text *Atharva Veda* (Science of Charms) as ‘Sacred Grass’, ‘donator of joy’, one of the five sacred plants of India. The medicinal use of cannabis by the Chinese for the treatment of disorders including malaria, constipation and rheumatic pain was first reported in the world’s oldest pharmacopoeia, the *pen-ts’ao ching* (2700 B.C.). Similarly, in India, the medical properties of cannabis have been known for millennia where it was recommended as an analgesic, anticonvulsant, hypnotic, anxiolytic and anti-inflammatory agent. Cannabis was introduced to modern Western medicine in 1839 by Irishman W.B. O’Shaughnessy, a surgeon with the British East India Company and professor at the Calcutta University. He documented its properties as an analgesic and its use for the treatment of rheumatism, muscular spasms, tetanus and rabies, and in 1839 published his findings in “*On the preparations of the Indian hemp, or gunjah*”, thereby laying the foundation stone that has driven cannabinoid research for over a century [for a more detailed review of the history of cannabis see (Zuardi, 2006)]. Even though the medicinal properties of cannabis were evident for millennia, it was not until the isolation and identification of the main psychoactive constituent of cannabis, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), in the 1960s (Mechoulam *et al.*, 1967) that extensive studies revealed the mechanisms underlying the physiological and pharmacological effects of cannabinoids. This discovery of (Δ^9 -THC) led to increased research interest in the field, and approximately 100 other cannabinoids have been identified in the cannabis plant to date. Further research led to the characterisation of a complete signalling system termed the endocannabinoid system that comprises cannabinoid receptors, endogenous ligands and enzymes for biosynthesis and inactivation, and this system is implicated in a broad range of

physiological processes including appetite, gastrointestinal tract function, respiration, cardiovascular, liver and neural function to name but a few.

The endocannabinoid system is widely expressed in all tissues of the body and comprises the cannabinoid (CB)₁ (Devane *et al.*, 1988; Matsuda *et al.*, 1990) and cannabinoid₂ (CB)₂ (Munro *et al.*, 1993) receptors, the naturally occurring endogenous receptor agonists or so-called endocannabinoids, the best characterised of which are arachidonyl ethanolamide (anandamide; AEA) and 2-arachidonoyl glycerol (2-AG) (Devane *et al.*, 1992; Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995), and the enzymes involved in their synthesis and degradation. It should be noted that other endocannabinoid ligands including oleamide (Leggett *et al.*, 2004), O-arachidonoyl ethanolamine (virodamine) (Porter *et al.*, 2002), 2-arachidonoyl glycerol ether (noladin ether) (Hanus *et al.*, 2001) and N-arachidonoyl-dopamine (NADA) (Bisogno *et al.*, 2005; Huang *et al.*, 2001b) have been identified, however, the role of these ligands in physiological processes has not been examined in detail. Once produced and released, endocannabinoids elicit their effect primarily via CB₁ and/or CB₂ receptors. However, in addition to CB₁ and CB₂ receptors, endocannabinoids are now known to also elicit activity at other receptors, namely the transient receptor potential vanilloid 1 (TRPV1), peroxisome proliferator-activated receptors (PPARs), G-protein coupled receptor (GPR) 55 and GPR19 (Huang *et al.*, 2002; Overton *et al.*, 2006b; Ryberg *et al.*, 2007a; Sun *et al.*, 2006b). Activity at these receptors has been proposed to account, at least partially, for some of the differential effects observed with potent selective cannabinoid agonists and modulation of endocannabinoid tone. The following sections of this thesis will focus on describing all components of the endocannabinoid signalling system including biosynthesis, signalling and degradation in greater detail.

1.5.1 Endocannabinoid Biosynthesis

Endocannabinoids are not stored in vesicles but rather their biosynthesis occurs on demand via hydrolysis of cell membrane phospholipid precursors in the post-synaptic nerve terminal (Cadas *et al.*, 1996; Di Marzo *et al.*, 1994; Giuffrida *et al.*, 1999). In addition to being present on neurons, the enzymes responsible for AEA and 2-AG biosynthesis are present in all cells and tissues of the body including

oligodendrocytes (Gomez *et al.*, 2010) and glial (microglia and astrocytes) cells (Hegyí *et al.*, 2012).

1.5.1.1 AEA Biosynthesis

AEA is generated via hydrolysis of a small family of cell membrane phospholipids precursors, the *N*-arachidonoyl-phosphatidylethanolamines (NAPEs) (Cadas *et al.*, 1996; Di Marzo *et al.*, 1994). The canonical route of AEA biosynthesis is referred to as the “transacylation-phosphodiesterase pathway” and is considered the major route for AEA biosynthesis. This pathway is composed of two enzymatic reactions: the stimulus-dependent hydrolytic cleavage of the phospholipid precursor NAPE which is produced by *N*-acyltransferase (NAT) when it catalyses the transfer of arachidonic acid from phosphatidylcholine (PC) to the primary amino group of phosphatidylethanolamine (PE) (Di Marzo *et al.*, 1994), and a second reaction which involves the generation of AEA via the hydrolysis of NAPE by the Ca²⁺ sensitive NAPE-selective phospholipase D (NAPE-PLD) (Cadas *et al.*, 1997; Cadas *et al.*, 1996; Di Marzo *et al.*, 1994). Although this canonical pathway is considered the main pathway for the biosynthesis of AEA, there is now evidence for involvement of detour routes in AEA biosynthesis including: (1) a phospholipase C-dependant pathway which converts *N*-arachidonoyl-PE into phospho-anandamide (Liu *et al.*, 2006a) and (2) hydrolysis of *N*-arachidonoyl-PE by a/b hydrolase domain-containing protein 4 (ABHD-4) (Simon *et al.*, 2008; Simon *et al.*, 2006). [For detailed review see (Tsuboi *et al.*, 2013)].

Figure 1.5 Biosynthetic pathway(s) involved in the production of anandamide (AEA) (Fezza *et al.*, 2014).

1.5.1.2 2-AG Biosynthesis

2-AG is implicated in multiple routes of lipid metabolism in which it can act interchangeably as an end product for one pathway and a precursor for another. It has been suggested that these diverse metabolic routes of 2-AG may account for its high concentration in the brain tissue which is approximately 200-fold higher when compared to AEA (Stella *et al.*, 1997; Sugiura *et al.*, 1995). The primary biosynthetic pathway involved in the generation of 2-AG is the Ca^{2+} dependent phosphatidylinositol (4,5) biophosphate cascade. This involves the conversion of phosphatidylinositol (PI) into phosphatidylinositol (4,5) bisphosphate ($\text{PI}(4,5)\text{P}_2$), which is then converted into inositol polyphosphate 3 (IP_3) and 1,2-diacylglycerol (1,2-DAG) by activated phospholipase C ($\text{PLC-}\beta$). 1,2-DAG serves as substrate for diacylglycerol kinase which generates phosphatidic acid and DAG lipase (DGL) which in turn hydrolyses DAG into 2-AG (Prescott *et al.*, 1983; Sugiura *et al.*, 1995). In addition, an alternative pathway for 2-AG biosynthesis has been proposed which involves the hydrolysis of phosphatidylinositol by phospholipase A1 (PLA1) which generates intermediary 2-arachidonyl-lysophospholipid which may be hydrolysed by lysophospholipase C (Lyso-PLC) to generate 2-AG (Kobayashi *et al.*, 1996; Sugiura *et al.*, 1995).

Figure 1.6 Biosynthetic pathway(s) involved in the production of 2-arachidonoylglycerol (2-AG)
(Fezza *et al.*, 2014).

1.5.2 Endocannabinoid signalling

CB₁ receptors are G-protein coupled receptors that are highly expressed throughout the human and rodent brain, with particularly high density on the pre-synaptic terminals of GABA and glutamate neurons (Glass *et al.*, 1993; Herkenham *et al.*, 1991; Mackie, 2008; Tsou *et al.*, 1998). Thus, at a neuronal level, newly synthesised endocannabinoids leave the post-synaptic nerve terminal to exert their effects on CB₁ receptor targets on the pre-synaptic nerve terminal, describing a signalling process known as retrograde neurotransmission (see Figure 1.7 for detailed diagrammatical description of endocannabinoid retrograde signalling at a neuronal level). Activation of the CB₁ receptors on the pre-synaptic nerve terminal mediates inhibition of the N- and P/Q-type voltage-activated Ca²⁺ channels and subsequent decreases in intracellular Ca²⁺ levels (Caulfield *et al.*, 1992; Mackie *et al.*, 1993; Mackie *et al.*, 1992), while concurrently activating the inwardly rectifying K⁺ currents (Henry *et al.*, 1995; Mackie *et al.*, 1995; McAllister *et al.*, 1999). As such, activation of CB₁ receptors on neurons directly inhibits intracellular Ca²⁺ levels while concurrently activating inwardly rectifying K⁺ channels. It is this mechanism that is suggested to underlie CB₁-mediated depression of neurotransmitter release.

Figure 1.7 Retrograde signalling of endocannabinoids at neuronal synapses: AEA and 2-AG are synthesised as described previously following an increase in cytosolic calcium (Ca^{++}) due to activation of post-synaptic ion channel or G-protein coupled receptors. Calcium release from intracellular stores triggers the formation of 2-AG from DAG by the enzyme DGL. Activation of ion channel receptors allows the influx of Ca^{++} , which leads to the formation of NAPE from PE and PC via NAT. NAPE is then hydrolysed to AEA by PLD. The cannabinoids are then released from the post-synaptic neuron and travel retrogradely to the pre-synaptic membrane to activate cannabinoid receptors e.g., CB_1 . The activation of the CB_1 receptor results in inhibition of Ca^{++} channels in the presynaptic membrane and a number of other signal transduction-mediated events, which generally result in suppression of neuronal activity and neurotransmitter release. AEA and 2AG are then uptaken into the post and presynaptic neurons respectively where they are catabolised by the cytosolic enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH). 2-AG is catabolised to arachidonyl acid and glycerol by MAGL, while FAAH breaks down AEA to arachidonyl acid and ethanolamine (Rea *et al.*, 2007).

In addition to CB₁ receptor expression on pre-synaptic nerve terminals, there are now several lines of evidence that CB₁ receptors are expressed on glial cells including oligodendrocytes (Molina-Holgado *et al.*, 2002a), astrocytes (Bouaboula *et al.*, 1995a; Molina-Holgado *et al.*, 2002a; Sanchez *et al.*, 1998) and microglia (Cabral *et al.*, 2001; Carlisle *et al.*, 2002; Facchinetti *et al.*, 2003b; Molina-Holgado *et al.*, 2002a). In addition, the CB₂ receptor, also a G-protein coupled receptor, is widely distributed in peripheral tissues, particularly in immune tissues including the spleen, tonsils, thymus, mast cells and blood cells (Berdyshev, 2000; Munro *et al.*, 1993; Sugiura *et al.*, 2000) and on activated glia within the brain (Carlisle *et al.*, 2002; Nunez *et al.*, 2004; Rock *et al.*, 2007). However, there is accumulating evidence that CB₂ receptor protein and mRNA is also expressed on subsets of neurons within the brain (Baek *et al.*, 2008; Gong *et al.*, 2006; Onaivi *et al.*, 2006; Van Sickle *et al.*, 2005; Zhang *et al.*, 2014) and thus this receptor may also directly modulate neurotransmission.

In glia and other immune cells, the production and release of endocannabinoids are proposed to act primarily on CB₂ receptors, and to a lesser extent CB₁ receptor targets, in an autocrine or paracrine manner (Stella, 2009). Similar to all standard G_{i/o}-coupled receptors, both CB₁ and CB₂ receptors are negatively linked to adenylyl cyclase (AC), thus endocannabinoid-induced activation results in a decrease in cytosolic cyclic AMP production and protein kinase A (PKA) phosphorylation (Howlett *et al.*, 1999; Pertwee, 1997; Pertwee, 1999). In addition, CB₁ and CB₂ receptors are positively coupled to several MAPKs including extracellular signal regulated kinase (ERK), JNK and p38 (Howlett *et al.*, 2002), thus activation of CB₁/CB₂ receptors can lead to increased gene expression of proteins involved in cellular function, cell differentiation and survival and also cytokine synthesis (Bouaboula *et al.*, 1995b; Bouaboula *et al.*, 1996; Derkinderen *et al.*, 2003; Derocq *et al.*, 2000; Khaspekov *et al.*, 2004). In this regard, Cabral and Griffin-Thomas proposed a mechanism by which cannabinoids may modulate inflammatory responses via activation of cannabinoid receptors on immune cells including microglia (Cabral *et al.*, 2009); see Figure 1.8 for a detailed description.

Figure 1.8 Cannabinoid Receptor mediated modulation of inflammatory responses on immune cells. G_i-alpha signalling down-regulates the pro-inflammatory immune response via adenylate cyclase. Upon cannabinoid receptor activation, the α subunit of the G_i protein interacts with adenylate cyclase to inhibit its activity. This results in a decrease of cAMP production, which leads to inactivation of protein kinase A (PKA). I κ B- α remains unphosphorylated due to PKA inactivation, thus preventing activation, nuclear translocation and DNA binding of NF κ B and other transcription factors. These events ultimately lead to the down-regulation of pro-inflammatory mediator gene expression. Adapted from (Cabral et al. 2009).

1.5.3 Non-CB₁/CB₂ receptor targets of the endocannabinoids

In addition to mediating effects via activation of CB₁/CB₂ receptor targets there is now evidence that both AEA and 2-AG are capable of interacting with alternative receptor targets including the PPARs, TRPV1, GPR55 and GPR19, all of which are described in greater detail below.

1.5.3.1 Peroxisome proliferator-activated receptors (PPARs)

PPARs belong to a family of nuclear receptors that are activated by fatty acids and their derivatives. Upon activation, PPARs heterodimerize with the retinoid X receptor (RXR), bind to specific PPAR response elements (PPRE) and subsequently induce transcriptional activation of target genes. The PPAR family of receptors consists of three isoforms: PPAR- α , PPAR- β and PPAR- γ , all of which modulate a wide range of biological functions including metabolism, energy homeostasis, inflammation and cell differentiation (O'Sullivan, 2007). The biological functions of individual PPARs largely depends on their tissue distribution (Kota *et al.*, 2005), however all three isoforms are expressed throughout the CNS in both neuronal and glial cells (Cimini *et al.*, 2005; Heneka *et al.*, 2007; Moreno *et al.*, 2004). In addition to their endogenous ligands, there is now an increasing body of evidence suggesting that endocannabinoids mediate some of their biological effects via activation of PPARs (O'Sullivan *et al.*, 2010). Specifically, AEA has been reported to activate PPAR- α (Sun *et al.*, 2006a) and induce transcriptional activation of PPAR- γ (Bouaboula *et al.*, 2005). Furthermore, 2-AG has been reported to induce transcriptional activation of PPAR- γ (Bouaboula *et al.*, 2005) and stimulate the differentiation of fibroblasts to adipocytes and inhibit IL-2 secretion via interaction with NF- κ B and NFAT, in a PPAR- γ dependent manner (Rockwell *et al.*, 2006). See Figure 1.9 for mechanisms of endocannabinoid interaction with PPARs (O'Sullivan, 2007).

Figure 1.9 Possible mechanisms mediating endocannabinoid/PPAR interactions: (1) Endocannabinoids directly bind to PPAR- α/γ to induce targeted gene transcription, (2) metabolic products of endocannabinoid metabolism activate PPARs or (3) endocannabinoids act on surface CB_{1/2} receptors with in turn induce MAPK activation (intracellular signalling cascade) that indirectly induces PPAR activation. (O'Sullivan, 2007).

1.5.3.2 Transient receptor potential cation channel subfamily V member 1 (TRPV1)

The vanilloid VR1 or TRPV1 is a ligand-gated ion channel that is a member of the transient receptor potential (TRP) channels. It is a six-transmembrane spanning protein, composed of intracellular N- and C-terminal domains (Caterina *et al.*, 1997). In addition to its well-described role in the modulation of pain processing (Conway, 2008; Okuse, 2007), there are now several lines of evidence implicating supraspinal TRPV1 in the modulation of brain neurobiology and behaviours related to psychiatric disorders including depression, anxiety and schizophrenia; for detailed overview see (Madasu, 2015).

TRPV1 is highly expressed in the dorsal root ganglia of C and A δ fibres (Caterina *et al.*, 1997; Szallasi *et al.*, 1999), activation of which leads to an influx of ions, predominately Ca²⁺, into the cell which results in neuronal excitation and release of inflammatory mediators including calcitonin gene related peptide (CGRP) and substance P, which in turn, initiate an inflammatory response (Szallasi *et al.*, 2007).

In addition, there are now several lines of evidence of TRPV1 expression throughout the rodent and primate brain including the cortex, hippocampus, basal ganglia, cerebellum and olfactory bulb (Mezey *et al.*, 2000; Szabo *et al.*, 2002) and also on non-neuronal cells including microglia, astrocytes and oligodendrocytes (Zhang *et al.*, 2015). In addition to TRPV1 ligands including capsaicin (the naturally occurring pungent constituent of capsicum peppers), endogenous factors including high temperatures (approx greater than 45°C), low pH (less than 5.4) and lipoxygenase products, there are now several lines of evidence that the endocannabinoid AEA acts as a full agonist at TRPV1 and thus may act as an “endovanilloid” as well as an “endocannabinoid” (Di Marzo *et al.*, 2001; Di Marzo *et al.*, 2002a; Van Der Stelt *et al.*, 2004). It has been proposed that under certain conditions such as inflammation or cell damage, AEA may induce more effects via activation of TRPV1 rather than cannabinoid receptors, thus resulting in contrasting effects on pain perception, inflammation and cell survival in comparison to direct CB₁ activation (Di Marzo *et al.*, 2002b).

1.5.3.3 G-protein-coupled receptor (GPR) 55 & 19

Over the last number of years there has been increased interest in GPR55 as a potential third cannabinoid receptor (Brown, 2007; Pertwee, 2007). Ryberg and colleagues previously described the cloning and protein sequence of human, mouse and rat GPR55 and reported that this orphan G-protein coupled receptor was activated by several cannabinoid receptor agonists, including the endocannabinoids AEA and 2-AG (Ryberg *et al.*, 2007b). Localization of GPR55 to human neutrophils (PMNs) has recently been reported whereby activation was associated with an augmented migratory response of PMNs towards 2-AG, while inhibiting neutrophil degranulation and reactive oxygen species (ROS) production (Balenga *et al.*, 2011). Furthermore, GPR55 expression has been reported to be expressed on the BV-2 microglial cell line where a functional role was demonstrated via increased ERK1/2 activation upon receptor activation with ligand LPI (Pietr *et al.*, 2009). With regards to endocannabinoid-induced activation of GPR55 there have been contradictory reports with some studies demonstrating that AEA acts as a agonist (Ryberg *et al.*, 2007b; Sharir *et al.*, 2012), while others report no agonist activity of either AEA or 2-AG at this receptor (Henstridge *et al.*, 2009), thus indicating that further studies are

required in order to determine the functional significance of endocannabinoid system modulation of this receptor. In addition to GPR55, endocannabinoid-related compounds have also been considered as potential GPR19 ligands, including the AEA related *N*-acylethanolamine OEA, which has been shown to act as an endogenous ligand for this receptor (Lauffer *et al.*, 2009; Overton *et al.*, 2006a).

1.5.4 Endocannabinoid uptake and metabolism

The actions of endocannabinoids are terminated following hydrolysis by intracellular catabolic enzymes which are present in several cell types including neurons and microglia (Stella, 2009; Witting *et al.*, 2004). In order for endocannabinoid signalling to cease they must be translocated across the cell membrane. However, the exact mechanism responsible for endocannabinoid transport across the membrane remains controversial. Accordingly, it has been suggested that endocannabinoids are capable of passing through the lipid bilayer via simple diffusion (Glaser *et al.*, 2003; Kaczocha *et al.*, 2006), however there is also evidence that the endocannabinoids, specifically AEA, are transported across the cell membrane via a selective carrier system in both neuronal and glial cells (Hillard *et al.*, 1997). The nature and existence of such a transporter remained undetermined until Fu and colleagues described a cytosolic variant of the AEA-degrading enzyme FAAH, termed FAAH-like AEA transporter (FLAT-1) which bound AEA with low affinity and facilitated its transport into cells (Fu *et al.*, 2012). Further support for a carrier-mediated process is demonstrated by the reports that inhibitors of AEA transport such as *N*-(4-hydroxyphenyl)-arachidonyl ethanolamide (AM404), OMDM-1 and the FLAT inhibitor ARN272 are capable of inhibiting this transporter and increase extracellular AEA levels (Fu *et al.*, 2012). In comparison, there is a paucity of data with regards to the potential mechanism mediating 2-AG cellular uptake. However, it has been shown that FLAT is not involved in the transport of 2-AG across the pre-synaptic membrane, thus suggesting an independent transporter for 2-AG re-uptake. Nevertheless, it has also been shown that 2-AG is competitively blocked by AEA re-uptake (Beltramo *et al.*, 2000), thus suggesting that both 2-AG and AEA compete for the same transporter and this possibility is further supported by the observations that the transport of both endocannabinoids are blocked by the endocannabinoid transport

inhibitor AM404 (Beltramo *et al.*, 2000). Overall, further studies are required in order to elucidate the possible transporter involved in 2-AG re-uptake.

Regardless of the exact transport mechanism via which endocannabinoids reach cellular targets for degradation, the enzymes involved in endocannabinoid metabolism are well defined. Specifically, fatty acid amide hydrolase (FAAH)-1 is an intracellular serine-hydrolase that has been identified as the primary enzyme involved in the hydrolysis of AEA into ethanolamine and arachidonic acid (AA) (Cravatt *et al.*, 1996b). In addition to AEA, FAAH-1 is also capable of metabolising the related *N*-acylethanolamines OEA and PEA into ethanolamine and oleic and palmitic acid, respectively (Cravatt *et al.*, 1996b). Both OEA and PEA are synthesised via the same pathways as AEA (Ueda *et al.*, 2013). However, despite this, neither OEA nor PEA are considered to be true endocannabinoids due to their lack of affinity for the CB₁/CB₂ receptors (Fu *et al.*, 2003). Instead both OEA and PEA are often referred to as “entourage compounds” due to their ability to enhance the effects of AEA at CB₁/CB₂ receptors by competitively inhibiting hydrolysis by FAAH (Ben-Shabat *et al.*, 1998; Lambert *et al.*, 1999; Mechoulam *et al.*, 1998). A second isoform of FAAH-1 termed FAAH-2 has been identified in humans, elephants and rabbits, but not in rats, mice or dogs (Wei *et al.*, 2006). FAAH-2 shows a distinct tissue distribution from FAAH-1 and decreased activity for AEA, OEA and PEA (Wei *et al.*, 2006). Furthermore, a novel enzyme capable of hydrolysing AEA, namely *N*-acylethanolamine-hydrolysing acid amidase (NAAA) has been identified, however the expression of NAAA is quite low in the healthy brain (Tsuboi *et al.*, 2005). In addition to FAAH, AEA is metabolised by additional enzymes including: (1) COX2 which generates prostamides (Kozak *et al.*, 2002; Yu *et al.*, 1997); (2) lipoxygenases (LOX) which generates hydroperoxy-eicosaetraenoylethanolamides (Hampson *et al.*, 1995; Ueda *et al.*, 1995) and (3) cytochrome P-450 (Bornheim *et al.*, 1993; Urquhart *et al.*, 2014).

In addition, although FAAH-1 is reported to be capable of hydrolysing the endocannabinoid 2-AG (Di Marzo *et al.*, 1998), the main catabolic enzyme implicated in 2-AG metabolism is monoacylglycerol (MAGL) which converts 2-AG into glycerol and AA (Dinh *et al.*, 2002b). Blackman and colleagues demonstrated that MAGL is responsible for approximately 85% of 2-AG hydrolysis in the brain,

while two additional serine hydrolases, namely abhydrolase domain-containing protein (ABDH)-6 and (ABDH)-12 are involved in the hydrolysis of approximately the remaining 15% of 2-AG (Blankman *et al.*, 2007). However, similar to AEA, 2-AG is also subject to oxidative metabolism by COX2 and LOX which generates endocannabinoid metabolic products including prostaglandins (Ueda *et al.*, 2011).

Figure 1.10 Metabolic pathways involved in anandamide (AEA) and 2-arachidonoylglycerol (2-AG) metabolism (Alhouayek *et al.*, 2014b)

1.6 The endocannabinoid system and inflammation

Due to the topological location of all components of this lipid signalling system, all components of the endocannabinoid system have emerged as important players in the modulation of a host of physiological responses ranging from appetite, respiration, metabolism, inflammation, pain and neurotransmission to name but a few. Of particular interest over the past decade has been the discovery that cannabinoids (plant-derived, synthetic and endogenous) elicit potent modulatory effects on inflammatory processes, with clinical and preclinical evidence demonstrating beneficial effects on disease severity and symptoms in several inflammatory conditions (Yoshihara et al., 2005, Storr et al., 2009, Tschop et al., 2009, Yu et al., 2010). However, the precise mechanisms by which cannabinoids modulate immune function depend on the conditions under investigation, and in many cases remain to be determined. Although it is not known if cannabinoids/endocannabinoids have direct activity at TLRs, there has been increasing data to suggest that one mechanism by which endocannabinoids influence innate immune function may be by via modulation of TLR-induced responses. As already mentioned, activation of TLRs participates in host defences, homeostasis and response to injury however, uncontrolled and aberrant TLR activation can elicit potent effects on neurotransmission and neurodegenerative cascades [for reviews see (Owens, 2009, van Noort and Bsibsi, 2009, Lehnardt, 2010, Arroyo et al., 2011)]. Thus, modulation of TLR-associated innate inflammatory responses by cannabinoids may provide a novel therapeutic target for central inflammatory disorders.

1.6.1 Endocannabinoid modulation of TLR4-induced inflammatory responses – *in vitro* studies

Some of the first evidence demonstrating a possible immunomodulatory role for the endocannabinoid system emerged from research investigating the effects of cannabinoids on TLR4-induced inflammatory responses *in vitro*. For example, potent non-selective cannabinoid receptor agonists such as Δ^9 -THC, HU210, CP55940 and WIN55,212-2 have been shown to inhibit TLR4-induced pro-inflammatory cytokine and nitric oxide release, induce apoptosis and inhibit migration of macrophages (Chang *et al.*, 2001; Jeon *et al.*, 1996; Klegeris *et al.*, 2003). Furthermore, these compounds have also been demonstrated to inhibit TLR4-induced inflammatory responses in microglial and astrocyte cultures (Facchinetti *et al.*, 2003a; Puffenbarger *et al.*, 2000), highlighting an important role in modulation of neuroinflammatory responses. Due to the high expression of CB₂ receptors on immune cells and activated glia it was not surprising that many researchers attributed the anti-inflammatory effects of cannabinoids to activity at this receptor. However, while some of these studies demonstrated that modulation of TLR4-induced inflammation was mediated by activation of CB₂ receptors (Correa *et al.*, 2005; Germain *et al.*, 2002; Gui *et al.*, 2013; Merighi *et al.*, 2012; Zhao *et al.*, 2010), a role for CB₁ receptors in mediating effects of some cannabinoids was also noted (Cabral *et al.*, 2001; Esposito *et al.*, 2001; Germain *et al.*, 2002) and a significant proportion of studies indicated non-CB_{1/2} receptor mediated anti-inflammatory effects (Chiba *et al.*, 2011; Chiurchiu *et al.*, 2014; Facchinetti *et al.*, 2003a; Puffenbarger *et al.*, 2000; Ribeiro *et al.*, 2013; Verhoeckx *et al.*, 2006). As discussed above, it is now recognised that cannabinoids also exhibit activity at other receptor targets such as PPARs, TRPV1 and GPR55, effects at which may underlie, at least in part, the anti-inflammatory activity of these compounds in certain cell types.

Enhancing endocannabinoid tone has been proposed as an alternative means of activating cannabinoid receptors without concomitant overt psychotropic effects associated with potent synthetic CB₁ receptor agonists. *In vitro* studies suggest that endocannabinoids elicit anti-inflammatory effects comparable to those of synthetic cannabinoids. Increasing AEA tone, either directly, via inhibition of its primary

catabolic enzyme, FAAH, or by inhibiting its uptake, has been demonstrated to reduce TLR4-induced increases in the levels of pro-inflammatory cytokines and inflammatory mediators such as TNF α , IL-1 β and nitric oxide, and enhance the release of the anti-inflammatory cytokine IL-10 *in vitro* [see Table 1.1] (Chang *et al.*, 2001; Correa *et al.*, 2009a; Correa *et al.*, 2010; Facchinetti *et al.*, 2003a; Molina-Holgado *et al.*, 1997; Ortega-Gutierrez *et al.*, 2005; Puffenbarger *et al.*, 2000; Tham *et al.*, 2007). However, it should be noted that enhancing AEA tone has also been shown to enhance LPS-induced IL-6 levels in astrocytes (Ortega-Gutierrez *et al.*, 2005), thus effects of AEA may depend on the inflammatory mediators and cell type under investigation. Similarly, enhancing 2-AG tone has also been found to induce suppressive effects on TLR4-induced immune activation, namely by reducing proinflammatory cytokines such as IL-6, TNF- α , and expression of COX2 in macrophages and glia [Table 1.1] (Chang *et al.*, 2001; Facchinetti *et al.*, 2003b; Gallily *et al.*, 2000; Zhang *et al.*, 2008). Similar to the effects observed with synthetic cannabinoids, the effects of enhancing endocannabinoid tone have been attributable to CB_{1/2} and non-CB_{1/2} receptor activation (Correa *et al.*, 2008; Correa *et al.*, 2009a; Correa *et al.*, 2010; Facchinetti *et al.*, 2003a; Lu *et al.*, 2014a; Puffenbarger *et al.*, 2000) [Table 1.1]. However, regardless of the receptor mechanism, endocannabinoids have been shown, for the most part, to inhibit TLR4-induced NF- κ B activation (Correa *et al.*, 2010; Du *et al.*, 2011; Lu *et al.*, 2014a; Zhang *et al.*, 2008).

TLR4 and CB_{1/2} receptors share common molecular targets such as MAPK and several studies have demonstrated that this is a key pathway for endocannabinoid modulation of TLR4-induced inflammatory responses. For example, AEA has been shown to augment and attenuate LPS-induced IL-10 and IL-12p70 expression respectively, in mixed glial cultures, effects mediated by CB₂ receptor activation of ERK1/2 and JNK pathways (Correa *et al.*, 2009a; Correa *et al.*, 2010). Similarly, AEA and 2-AG have been shown to up-regulate CB_{1/2} receptors and enhance IL-10 and TGF β expression while concurrently reducing pro-inflammatory cytokine expression in primary muller glial cultures (Krishnan *et al.*, 2012). Thus, the anti-inflammatory effects of endocannabinoids following TLR4 activation have been proposed to be due to enhanced production of the anti-inflammatory cytokine IL-10

(Correa et al., 2010). Furthermore, it has recently been shown that AEA activation of CB₂ receptors blocks the LPS-induced reduction in CD200R1 on microglia (Hernangomez et al., 2012). Activation of CD200R1 was shown to attenuate LPS-induced pro-inflammatory and enhance IL-10 production, and IL-10 increases neuronal expression of CD200, an effect which consequently reduced neuronal cell death (Hernangomez et al., 2012). Thus, AEA-induced up regulation of CD200R1 and IL-10 expression acts to attenuate TLR4-induced microglial activation, limiting the neuroinflammatory response and inducing neuroprotection.

Table 1.1 Endocannabinoid modulation of TLR4-induced immune responses – *in vitro* studies

Modulator	Cell type	Immune/inflammatory response following TLR4 activation	Receptor Mechanism	Reference
Direct administration of Endocannabinoids				
AEA	Human monocytic THP-1 cell line	↓ IL-1β secretion	-	(Klegeris <i>et al.</i> , 2003)
	Human Primary muller glial cultures	↑ IL-10 & TGFβ mRNA production ↓ IL-6, IL-1β, TNFα, IL-2, IFNγ, IL-15, IL-12 & IL-8 mRNA production. ↓ NFκβ, MAPK activation	-	(Krishnan <i>et al.</i> , 2012)
	Mouse J774 macrophages	↓ NO, IL-6, PGE2 release	-	(Chang <i>et al.</i> , 2001)
	Murine RAW 264.7 macrophages	↓ IL-12p40 promoter activity	Non CB ₁ /CB ₂ or TRPV1	(Correa <i>et al.</i> , 2008)
	Mouse primary mixed glial cells	↓ IL-12p35/p40 & IL-23p19 mRNA expression	-	
	Rat primary cortical microglial cells	↓ TNF-α release	Non CB ₁ /CB ₂	(Facchinetti <i>et al.</i> , 2003a)
	Rat primary cortical microglial cells	↓ IL-1α, IL-1β, IL-6, TNFα mRNA expression	Non CB ₁ /CB ₂	(Puffenbarger <i>et al.</i> , 2000)
	Rat primary cortical microglial and astrocyte cell cultures	↑ PGE ₂ , 8- <i>iso</i> -PGF _{2α} production	-	(Navarrete <i>et al.</i> , 2009)
	Mouse primary mixed glial cultures	↓ IL-12 and IL-23 production	CB ₂ mediated	(Correa <i>et al.</i> , 2009a)
	Mouse primary mixed glial cultures	↑ induced IL-10 production, ↓ IκBα phosphorylation & p37 nuclear translocation	CB ₂ mediated	(Correa <i>et al.</i> , 2010)

	Mouse primary mixed glial cultures	↑ CD200R1 expression	CB ₂ mediated	(Hernangomez <i>et al.</i> , 2012)
	Mouse primary cortical mixed neuronal & glial cultures	↓ LPS/IFN-γ induced neuronal death	-	
	Mouse primary cortical astrocytes	↓ NO ₂ , TNF-α release	-	(Molina-Holgado <i>et al.</i> , 1997)
2-AG	Human primary muller glial cultures	↑ IL-10 & TGFβ mRNA production ↓ IL-6, IL-1β, TNFα, IL-2, IFNγ, IL-15, IL-12. ↑ IL-8 mRNA production. ↓ NFκβ activation		(Krishnan <i>et al.</i> , 2012)
	Mouse J774 macrophages	↓ IL-6 and ↑ induced NO release	-	(Chang <i>et al.</i> , 2001)
	Mouse peritoneal macrophages	↓ TNFα levels	-	(Gallily <i>et al.</i> , 2000)
	Rat primary cortical microglial cells	↓ TNFα release	Non CB ₁ /CB ₂ mediated	(Facchinetti <i>et al.</i> , 2003a)
	Rat primary hippocampal neurons	↓ IL-1β-induced COX2 expression	CB ₁ mediated	(Zhang <i>et al.</i> , 2008)
	Rat primary astroglial cultures	↓ COX2 expression		
	Mixed hippocampal neuronal & astroglial cultures			
	Rat primary caudate nucleus neurons	↓ COX2 levels, ↓ pNFκB, pERK1/2 & p-P38 MAPK	CB ₁ mediated	(Lu <i>et al.</i> , 2014b)
	Mouse primary hippocampal neurons	↓ COX2 expression & NFκB p65 phosphorylation	CB ₁ and PPARγ mediated	(Du <i>et al.</i> , 2011)

	Mouse J774 macrophages	↓IL-1β mRNA, NO production	Non CB ₁ /CB ₂	(Alhouayek <i>et al.</i> , 2013)
Endocannabinoid modulators (metabolic enzyme inhibitors)				
URB597 (FAAH inhibitor)	Rat primary microglial cultures	↓ COX2 expression , iNOS, PGE ₂ , NO & TNFα release	Non CB ₁ /CB ₂ mediated	(Tham <i>et al.</i> , 2007)
UCM707 (FAAH inhibitor)	Rat primary astrocyte cultures	↓ iNOS expression, NO levels ↓TNFα, IL-1β & ↑ IL-6 production	CB ₁ /CB ₂ mediated	(Ortega-Gutierrez <i>et al.</i> , 2005)
JZL184 URB602 (MAGL inhibitors)	Mouse primary hippocampal neurons	↓ COX2 expression & NFκB p65 phosphorylation	CB1 and PPARγ. mediated	(Du <i>et al.</i> , 2011)
WWL70 (ABHD6 inhibitor)	Mouse J774 macrophages Thioglycolate-elicited peritoneal macrophages (TGEM) BV2 microglial-like cells	↓IL-1β, PGD ₂ , PGJ ₂ , PGE ₂ in J774 cells ↓IL-1β in TGEM and BV2 cells	Non CB ₁ /CB ₂	(Alhouayek <i>et al.</i> , 2013)

1.6.2 Endocannabinoid modulation of TLR4-induced inflammatory responses – *in vivo* studies

In vivo studies support *in vitro* data demonstrating the immunomodulatory effects of enhanced endocannabinoid tone on TLR4-mediated effects [see Table 1.2]. Some of the first *in vivo* data demonstrating a modulatory role for the endocannabinoid system in TLR4-induced inflammatory responses arose from our data demonstrating that systemic administration of the AEA reuptake inhibitor AM404 attenuates LPS-induced increases in plasma IL-1 β and IL-6 levels (Roche *et al.*, 2008). However, it was also noted that LPS-induced plasma TNF α levels were augmented by systemic administration of either AM404, or the FAAH inhibitor URB597 (Roche *et al.*, 2008). Similar augmentations in LPS-induced plasma TNF α levels were observed following FAAH inhibition directly within the brain, and activation of hypothalamic CB₁ receptors was found to be critical in mediating this response (De Laurentiis *et al.*, 2010). Thus, AEA activation of hypothalamic CB₁ receptors appears to facilitate the production and release of TNF α in the plasma in response to LPS. Hypothalamic IL-1 β has been shown to mediate fever (Murakami *et al.*, 1990) and hypophagia (Kent *et al.*, 1994) in response to LPS, effects which can be attenuated by AEA (Hollis *et al.*, 2011). Furthermore, recent studies from our group have demonstrated that systemic administration of the FAAH inhibitor URB597 increased AEA levels, an effect associated with the attenuation of LPS-induced IL-1 β expression in the hypothalamus (Kerr *et al.*, 2012) and CB₁ receptors have been shown to be critical in mediating the temperature response to LPS (Steiner *et al.*, 2011; Duncan *et al.*, 2013). Thus, taken together, enhancing AEA tone, possibly via CB₁ receptor activation, attenuates TLR4-induced IL-1 β expression in the hypothalamus which may in turn inhibit associated sickness behaviour.

The relatively recent development of potent and selective MAGL inhibitors such as JZL184, KLM129 and MJN110 (Chang *et al.*, 2012; Ignatowska-Jankowska *et al.*, 2014; Long *et al.*, 2009a; Long *et al.*, 2009b; Niphakis *et al.*, 2013) has facilitated more detailed investigation of the role of 2-AG in a number of physiological and pathophysiological processes. Consistent with the *in vitro* data, enhancing 2-AG levels following MAGL inhibition also modulates peripheral and neuroinflammatory

responses following TLR4 activation [Table 1.2], however the exact mechanisms underlying these effects remain unclear. MAGL inhibition has been shown to result in an attenuation of LPS-induced TNF α , IL-6 and MCP-1 levels in bronchoalveolar fluid (BALF) from a mouse model of acute lung injury, effects shown to be mediated by CB₁ and CB₂ receptors (Costola-de-Souza et al., 2013). Furthermore, Alhouayek and colleagues demonstrated that MAGL inhibition was associated with a significant attenuation of colitis-induced increases in endotoxemia as measured by serum LPS levels, circulating inflammatory cytokines and the expression of TNF α and IL-1 β in the liver and brain. The anti-inflammatory effects of MAGL inhibition on mucosal and peripheral inflammation was shown to be partially mediated via CB₁ and CB₂ receptors (Alhouayek et al., 2011). In a subsequent study from this group, the authors demonstrated that inhibition of 2-AG metabolising enzyme ABHD6 attenuated LPS-induced increases in IL-1 β , IL-6 and MCP-1 expression in the cerebellum, lungs and liver of mice (Alhouayek *et al.*, 2013). However, 2-AG levels were increased in the peripheral tissues, but not in the cerebellum, and only in the liver were the anti-inflammatory effects partially attenuated by CB₁ receptor antagonism. The authors went on to conduct further studies, demonstrating that increases in 2-AG within the brain are not responsible for the anti-inflammatory effects of ABHD6 inhibition in the brain and that these are most likely attributed to PGD₂-G (prostaglandin D₂-glycerol ester), a COX2 metabolite of 2-AG (Alhouayek *et al.*, 2013). Thus, enhancing 2-AG tone may modulate TLR4-induced inflammation via differential mechanisms depending on the tissue in question. Similarly, Nomura and colleagues demonstrated that systemic administration of the MAGL inhibitor JZL184, enhanced 2-AG levels both centrally and peripherally and attenuated LPS-induced IL-1 β , IL-1 α , IL-6, TNF α , prostaglandin PGE₂ and arachidonic acid levels in the brain of mice. The central anti-inflammatory effects of MAGL inactivation were shown not to be mediated by CB₁ or CB₂ receptors, but rather attributed to a reduction in arachidonic acid and downstream prostaglandins (Nomura et al., 2011). Recent work from our laboratory further indicate that the mechanisms by which MAGL inhibition results in modulation of TLR4-induced inflammation may be different in the periphery and CNS. Data from our group has demonstrated that systemic administration of JZL184 attenuated LPS-induced increases in cytokine expression in the rat frontal cortex and plasma, effects partially

attenuated by pharmacological blockade of the CB₁ receptor (Kerr *et al.*, 2013b). However, 2-AG levels were only enhanced peripherally and central effects were not accompanied by reduced arachidonic acid and prostaglandin synthesis. Thus, the attenuation of TLR4-induced inflammatory responses in the brain following MAGL inhibition may be mediated by modulation of peripheral systemic innate immune responses that then communicate with the CNS to induce a state of neuroinflammation. Taken together the data to date indicate that while effects of enhancing 2-AG tone on TLR4-induced inflammatory responses in the periphery may be CB_{1/2} mediated, this does not appear to be the mechanism of action in the CNS. Such an effect may be significant, as this would allow for modulation of neuroinflammatory processes without the potential for adverse psychotropic effects that would be associated with central CB₁ receptor activation by 2-AG.

Overall, there is now a substantial amount of evidence, both from *in vitro* and *in vivo* studies that modulation of endocannabinoids exerts potent immunoregulatory effects on TLR4-induced inflammatory processes, both in the periphery and CNS [Table 1.1 & 1.2]. However, further studies are required to elucidate the potential role of the brain's endocannabinoid system on acute neuroinflammatory responses induced following activation of other TLRs.

Table 1.2 Endocannabinoid modulation of TLR4-induced immune responses – *in vivo* studies

<i>Modulator</i>	<i>Response following TLR4 activation</i>	<i>Receptor Mechanism</i>	<i>Reference</i>
Direct administration of Endocannabinoids			
AEA (1mg/kg s.c. rat)	↓ LPS induced fever, and hypophagia ↓ LPS-induced Fos expression in the hypothalamus.	-	(Hollis <i>et al.</i> , 2011)
AEA (50ug/5µl icv rat)	↑ LPS- induced hypothermic response	Possible CB ₁ mediated	(Steiner <i>et al.</i> , 2011)
2-AG (3mg/kg i.p. mice)	↓LPS induced COX2 levels in hippocampus	CB ₁ mediated	(Zhang <i>et al.</i> , 2008)
FAAH inhibitors			
URB597 (50ng/5ul i.c.v. rat)	↑ LPS-induced plasma TNFα and Oxytocin	CB ₁ mediated	(De Laurentiis <i>et al.</i> , 2010)
URB597 (0.3-0.6mg/kg i.p. rat)	↓LPS-induced increase in leukocyte adhesion in intestinal venules ↑ functional capillary density	Leukocyte adhesion CB ₂ mediated	(Kianian <i>et al.</i> , 2013)
URB597 (1mg/kg i.p. rat)	↓LPS-induced IL-1β, SOCS3 expression in hypothalamus	-	(Kerr <i>et al.</i> , 2012)
URB597 (0.6mg/kg i.p. rat)	↑ LPS induced plasma TNFα	-	(Roche <i>et al.</i> , 2008)
URB597 (0.6mg/kg i.v. mouse)	↓ LPS-induced leukocyte adhesion in intestinal V1 & V3 venules	-	(Sardinha <i>et al.</i> , 2014)
MAGL/ABHD6 inhibitors			
JZL184 (16mg/kg i.v. mice)	↓ LPS-induced leukocyte adhesion in intestinal V1 & V3 venules	-	(Sardinha <i>et al.</i> , 2014)

JZL184 (10mg/kg i.p. rat)	↓LPS-induced IL-1 β , IL-6, TNF- α , IL-10 expression in FC . ↓LPS-induced TNF- α , IL-10 levels in plasma	↓in IL-1 β in cortex CB ₁ mediated. ↓in TNF- α , IL-10 in plasma CB ₁ mediated.	(Kerr <i>et al.</i> , 2013b)
JZL184 (40mg/kg i.p. mouse)	↓LPS-induced IL-1 β , IL-1 α , IL-6, TNF α , PGE2 levels in brain	Non CB ₁ /CB ₂	(Nomura <i>et al.</i> , 2011)
JZL184 (16mg/kg i.p. mouse)	↓ LPS induced leukocyte count, TNF α , IL-6, MCP-1 levels in Bronchoalveolar lavage fluid (BALF). ↓LPS-induced lung damage.	CB ₁ and CB ₂ mediated	(Costola-de-Souza <i>et al.</i> , 2013)
WWL70 (20mg/kg i.p. mice)	↓IL-1, IL-6 expression in cerebellum, lung and liver	Effects in liver CB ₁ mediated	(Alhouayek <i>et al.</i> , 2013)
Endocannabinoid re-uptake inhibitor			
AM404 (20mg/kg i.p. rat)	↑ plasma TNF α levels ↓ plasma IL-1 β , IL-6 levels	↓IL-1 β is CB ₁ mediated	(Roche <i>et al.</i> , 2008)

1.6.3 Endocannabinoid modulation of TLR3 and TMEV-induced inflammatory responses – *in vitro* and *in vivo* studies

Although a wealth of evidence has demonstrated a role for endocannabinoid modulation of TLR4-induced inflammation, less is known about the role of this system in the modulation of inflammatory responding to other TLRs, including TLR3. As previously stated, TLR3 activation following systemic administration of the viral antigen acid poly I:C results in enhanced production of the type I IFNs and NF- κ B-inducible inflammatory genes in the CNS (Cunningham *et al.*, 2007; Gibney *et al.*, 2013). This enhanced neuroinflammatory profile is associated with sickness (Cunningham *et al.*, 2007; Gibney *et al.*, 2013; McLinden *et al.*, 2012; Murray *et al.*, 2015b), anxiety- and depressive-like behaviour (Gibney *et al.*, 2013) and has been shown to exacerbate chronic neurodegenerative processes in a model of prion disease (Field *et al.*, 2010). Thus, modulation of TLR3-induced inflammatory responses may provide novel therapeutic approaches for viral-induced neuroinflammation and associated neuronal alterations. Some of the first data demonstrating a direct role of cannabinoids in modulating TLR3-induced inflammatory responses were reported by Downer and colleagues [Table 1.3]. This group demonstrated that the synthetic cannabinoid receptor agonist WIN55,212-2 enhances TLR3-induced IRF3 nuclear translocation and subsequent IFN- β expression, while concurrently attenuating TLR3-induced NF- κ B activation and TNF α expression in astrocytes cultures. These effects are in contrast to the WIN55,212-2 induced attenuation of both IFN- β and TNF α following TLR4 activation (Downer *et al.*, 2011). Furthermore, enhanced IFN- β was necessary for the protective effects of WIN55212-2 in a mouse model of MS (Downer *et al.*, 2011). Examination of the receptor mechanisms underpinning the augmentation of IFN- β by WIN55,212-2 revealed that the effects were independent of CB₁/CB₂ receptor activation, but rather mediated by PPAR- α -induced activation of JNK, AP-1 and positive regulatory domain (PRD) IV and subsequent IFN- β transcriptional activation (Downer *et al.*, 2012; Downer *et al.*, 2011). Thus, cannabinoids appear to induce differential effects on the expression of type I IFNs and NF- κ B-inducible genes following TLR3 activation. Further indirect evidence supporting an immunoregulatory role of the endocannabinoid system on TLR3-induced inflammation is evident from studies examining the effect of Theiler's murine

encephalomyelitis virus (TMEV) which has been shown to induce an inflammatory response primarily via activation of TLR3 (So *et al.*, 2006). To date, several studies have demonstrated that the endocannabinoid system modulates such immune responses to TMEV *in vitro* [Table 1.3]. Administration of AEA or the endocannabinoid reuptake inhibitor OMDM1 attenuates TMEV-induced IL-1 β and IL-12p40 production in macrophages via CB₁/CB₂ receptor activation (Mestre *et al.*, 2005), decreases NOS⁻ and TNF α release in astrocytes (Molina-Holgado *et al.*, 1997) and VCAM-1 production in brain endothelial cells via CB₁ receptor activation (Mestre *et al.*, 2011). Furthermore, a *in vivo* data has demonstrated that administration of the endocannabinoid transport inhibitor UCM707 reduces TMEV-induced VCAM-1 expression and microglial activation in the brain, an effect partially mediated by CB₁ receptors (Mestre *et al.*, 2011) [Table 1.4]. Although Mestre and colleagues did not directly investigate or discuss the role of TLR3, given the early timepoint of pharmacological intervention it is likely that enhancing AEA tone may modulate TMEV-induced inflammatory responses via TLR3. Several other studies have revealed beneficial effects of endocannabinoid modulation on inflammatory and behavioural responses in the chronic phases of TMEV-induced demyelating disease, however the role of TLR3 in mediating effects at this stage is unknown (Correa *et al.*, 2011; Hernangomez *et al.*, 2012; Mestre *et al.*, 2005).

Table 1.3 Endocannabinoid modulation of TLR3/TMEV-induced immune responses – *in vitro* studies

Modulator	Cell type	Response following polyi:c-induced TLR3 activation	Receptor	Reference
WIN55-212,2	TLR3 expressing Human Embryonic Kidney (HEK) 293 cells	↓ NFκB, TNFα ↑ IRF3 translocation and activation	Non CB ₁ /CB ₂ PPARα mediated	(Downer et al., 2011) (Downer et al., 2012)
	Mouse bone marrow-derived macrophages	↑ IFNβ expression		
	Human U373 astrocytoma cells	↓ NFκB, TNFα, ↑ IFNβ expression		
	Mouse primary astrocytes	↓ NFκB, TNFα, ↑ IFNβ expression, ↑ nuclear translocation of IRF3		
		Response following TMEV exposure		
AEA	Mouse macrophage cultures	↓ IL-1β & IL-12p40 production	-	(Mestre et al., 2005)
	Mouse primary cortical astrocytes	↓ NO & TNFα release	-	(Molina-Holgado et al., 1997)
	Mouse primary astrocytes	↑ IL-6 release	CB ₁ mediated	(Molina-Holgado et al., 1998)
	Mouse primary mixed glial cultures	↓ IL-12p70, IL-23 & ↑ IL-10 production	CB ₂ mediated	(Correa et al., 2011)
	Mouse endothelial & astrocyte co-cultures	↓ VCAM-1 production & leukocyte adhesion	CB ₁ mediated	(Mestre et al., 2011)
OMDM1 (EC reuptake inhibitor)	Mouse Macrophage cultures	↓ IL-1β & IL-12p40 production	-	(Mestre et al., 2005)

WIN55-212,2	Mouse endothelial & astrocyte co-cultures	↑ COX2 expression & PGE2 release	Non CB ₁ /CB ₂ or TRPV1	(Mestre <i>et al.</i> , 2006)
		↓ VCAM-1	PPAR γ mediated	(Mestre <i>et al.</i> , 2009)

Table 1.4 Endocannabinoid modulation of TMEV-induced immune responses - *in vivo studies*

Modulator	Immune response in TMEV-infected mice	Receptor	Reference
AEA (3.5mg/kg 7 days)	↓ spinal cord expression of IL12p35 and p19 ↓ serum levels of IL-12p70, IL-23 and IL-17A, ↑ IL-10	-	(Correa <i>et al.</i> , 2011)
Met-AEA (2.5/3.75 mg/kg i.p. 12 days)	↓ spinal cord expression of IL12p35 and IL-23	-	(Correa <i>et al.</i> , 2011)
AA-5HT (5mg/kg., i.p. 12 days)	↓ spinal cord expression of IL12p35,p40 and p19 ↓ serum levels of IL-12p70, IL-23 and IL-17A, ↑ IL-10	-	(Correa <i>et al.</i> , 2011)
AA-5HT (5mg/kg i.p.)	↑spinal cord mRNA expression of CD200 and CD200R1 ↓ IL-1β and IL-6 & ↑ IL-10 mRNA expression in spinal cord	-	(Hernangomez <i>et al.</i> , 2012)
AEA (3.5ug/ul; 1ul/h 7 days)	↑spinal cord mRNA expression of CD200 and CD200R1 ↓ IL-1β and IL-6 & ↑ IL-10 mRNA expression in spinal cord	-	(Hernangomez <i>et al.</i> , 2012)
UCM707 (3mg/kg., i.p. b.i.d. 3 days)	↓ VCAM-1 expression in cortex ↓ microglial activation	Partial CB ₁ receptor	(Mestre <i>et al.</i> , 2011)
UCM707 (5mg/kg., i.p. 10 days)	↑ axonal density ↑ glutamate transporter (GLT-1 and GLAST) expression in spinal cord	-	(Loria <i>et al.</i> , 2010)
OMDM1/2 (2/7 mg/kg i.p. 10 days)	↓ number of reactive microglial cells in spinal cord	-	(Mestre <i>et al.</i> , 2005)

Taken together, the data suggest that cannabinoids (exogenous and endogenous - AEA) modulate TLR3-induced inflammatory responses both peripherally and within the CNS. This may have important implications for neurodegenerative disorders such as MS where enhancing IFN- β with concurrent attenuation of pro-inflammatory cytokines has been shown to be therapeutically beneficial (Javed *et al.*, 2006; Severa *et al.*, 2014). However, to date no studies have examined the effect of endocannabinoid modulation of TLR3-induced inflammation *in vivo*. In addition, the potential role of the brain's endocannabinoid system on TLR-induced neuroinflammation also remains to be determined. This is an important question to address as several neuropathologies including MS (Baker *et al.*, 2001; Loria *et al.*, 2008), PD (Ferrer *et al.*, 2003), ischemic stroke (Berger *et al.*, 2004; Franklin *et al.*, 2003; Schabitz *et al.*, 2002) and brain injury (Hansen *et al.*, 2001; Panikashvili *et al.*, 2001) are associated with increases in endocannabinoid levels within the CNS. Increased endocannabinoids under such conditions are proposed to mediate endogenous protective effects (Zogopoulos *et al.*, 2013), at least in part, via their potent anti-inflammatory properties (Eljaschewitsch *et al.*, 2006; Mechoulam *et al.*, 2002; Panikashvili *et al.*, 2005; Walter *et al.*, 2004). As such, elucidating the potential immunomodulatory role of the brain's endocannabinoid system on acute neuroinflammatory responses may offer a novel therapeutic approach in several neurological disorders with an underlying neuroinflammatory component.

1.7 Overall Research objectives

Activation of TLRs participate in host defence, homeostasis and response to injury, however, uncontrolled and aberrant TLR activation can elicit potent effects on neurotransmission and neurodegenerative cascades [for reviews see (Arroyo *et al.*, 2011; Lehnardt, 2010; Owens, 2009; van Noort *et al.*, 2009)]. Furthermore, TLR expression has been reported to be increased in the post-mortem brain of patients with neurodegenerative and psychiatric disorders (Brudek *et al.*, 2013; Salaria *et al.*, 2007). Thus, the need to develop a greater understanding of the neurobiological mechanisms mediating TLR-induced neuroinflammation is critical at a fundamental physiological level and for the development of novel, more efficacious treatments. Accordingly, over the past number of decades, a substantial body of evidence has demonstrated that the endocannabinoid system exerts potent anti-inflammatory effects [For recent reviews see (Downer, 2011; Jean-Gilles *et al.*, 2010; Rom *et al.*, 2013; Stella, 2010)] and thus may offer a novel therapeutic target for neuroinflammatory disorders.

Furthermore, there are now several reports of increased endocannabinoid levels within the CNS in conditions such as PD, MS and ischemic stroke. However, the extent to which enhancing endocannabinoid levels directly within the brain modulates acute neuroinflammatory responses is not known. Thus, the overall question addressed in this thesis was to investigate if pharmacological modulation of the brain's endocannabinoid system altered neuroinflammatory responses in discrete brain regions often affected in neurological and psychiatric disorders, induced following systemic viral (TLR3) or bacterial (TLR4) infection. Furthermore, the studies carried out in this thesis aimed to investigate the neuro-immuno-modulatory effects mediating endocannabinoid-induced modulation of TLR-induced neuroinflammation.

The specific aims of the studies described in this thesis were:

1. Investigation of the effect of enhancing FAAH substrate (AEA, OEA and PEA) tone on TLR3-induced inflammation in the periphery (serum) and brain (hippocampus and frontal cortex) over time.
2. Investigation of the effect of enhancing FAAH substrate (AEA, OEA and PEA) tone directly within the brain on TLR3-induced neuroinflammation in the hippocampus and frontal cortex.
3. Investigation of the potential receptor mechanisms within the brain underlying FAAH substrate-mediated modulation of TLR3-induced neuroinflammation in the hippocampus.
4. Investigation of the effect of enhancing endogenous 2-AG tone, both systemically and directly within the brain, on TLR-induced neuroinflammation in the hippocampus.
5. Investigation of the effect of enhancing endogenous FAAH substrate tone directly within the brain on TLR4-induced neuroinflammation in the frontal cortex and hippocampus, and the potential receptor mechanisms within the brain involved in mediating such responses. A further aim was to investigate the effect of enhanced FAAH substrate tone on long-term neuro-immune and behavioural outcomes, following systemic TLR4 activation.

Chapter 2

General Methods

2.1 Animal husbandry

Experiments were carried out on male Sprague Dawley rats (weight 220-260g; Charles River, UK), housed singly in plastic bottomed cages (45 X 25 X 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 0800 to 2000 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 an intraperitoneal (i.p.) injection of sterile saline (0.89% NaCl) for 3-4 days prior to experimentation in order 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 licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

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

A guide cannula (5mm, Plastics One Inc., Roanoke, Virginia, USA) was stereotaxically implanted into the right lateral ventricle, see Fig 2.1 for i.c.v. implantation (anterior posterior (AP): -0.07mm; medial lateral (ML): -0.15mm relative to bregma, dorsal ventral (DV): -0.30mm from skull surface (The Rat Brain in Stereotaxic Coordinates, 6th Edition; (Paxinos, 2006)), of rats under isoflurane anaesthesia (1-3% in O₂; 0.5L/min) (Fig 2.1 for implantation of guide cannula into right lateral cerebral ventricle of the rat brain). In addition, the local anaesthetic Norocaine (Norbrook Laboratories, Ireland) was applied topically to the skull surface prior to incision. The cannula was permanently fixed to the skull using stainless steel screws and dental acrylic cement. A stylet made from stainless steel tubing (Plastics One Inc., USA) was inserted into the guide cannula to prevent blockage by debris. The broad spectrum antibiotic, enrofloxacin (2.5mg/kg s.c.) (Baytril, Bayer Ltd., Ireland), was administered during surgery to prevent post-operative infection. Following cannulae implantation, the rats were housed singly and administered enrofloxacin (2.5mg/kg s.c.) for a further 3 days. Rats were allowed to recover for at least 6 days prior to experimentation. During this period, the rats were handled and their body weight and general health monitored on a daily basis. Correct cannula placement was verified by the Angiotensin (Ang) II drinking test 3 days prior to

the experiment as previously described (De Fanti *et al.*, 2002). Briefly, Ang II (Sigma, USA) was dissolved in sterile phosphate buffered saline + calcium (PBS⁺) at a concentration of 20ng/μl. Using a 50μl Hamilton 1700 Series Syringe (Hamilton, Switzerland), connected to a Harvard Appartus PHD 200 Infusion pump (Harvard Appartus, USA), set at a diameter of 1.031mm, rats received a i.c.v. microinjection of Ang II in an injection volume of 5μl infused over 1 min. Following microinjection, animals were placed back into their homecage and their drinking response was immediately assessed. An increased drinking response of at least 3ml of water within 20 min following i.c.v. injection of 100ng ANG II confirmed correct cannula placement. Animals were considered non-responders if drinking reponse was less than 3mls and as a result not included in the experiment. In all experiments carried out, the average number of non-responders was less than 5%.

Figure 2.1 Implantation of guide cannula into right lateral cerebral ventricle of the rat brain. Figure obtained from *The Rat Brain in Stereotaxic Coordinates*, 6th Edition (Paxinos, 2006).

2.3 Animal sacrifice and tissue collection

Rapid decapitation was used as the humane experimental endpoint for all studies.

2.3.1 Blood collection and spleen removal

Following decapitation, trunk blood was immediately collected in sterile 15ml red-cap tubes, allowed to clot and stored at 4°C for approximately 4 hours until centrifugation at 14,000g for 15min at 4°C. The serum was then removed and stored in aliquots at -80°C. In addition, an incision was made with a scissors along the midline of the rat's abdomen and the skin was pulled back so as to expose the abdominal cavity. The spleen was located along the left side of the upper abdomen and excised out, placed in a 24-well plate and snap-frozen on dry ice and stored at -80°C for further analysis.

2.3.2 Removal of brains

Following decapitation, an incision was made using a scissors along the top of the head and the skin pulled back to expose the skull. At this point the guide cannula was removed from the rat brain by pulling the cannula straight up out of the brain. Next, the optic ridge between the eyes and the back of the skull was broken with a rongeur. Using a scissors, a cut was made carefully along the midline of the skull from the back, maintaining pressure away from the brain surface, and the parietal and frontal skull was removed. The remaining bone along the sinus between the olfactory bulbs and frontal cortex was carefully removed, as was the bone over the nasal cavity and eye socket. The dura mater was removed, the trigeminal nerve was cut and the brain removed from the skull using a curved forceps.

2.3.3 Brain dissection

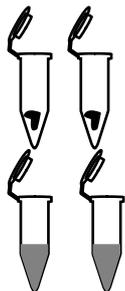
Immediately after removal of the brain from the skull, discrete brain regions (hippocampus and frontal cortex) were dissected on an ice-cold plate which was sprayed with RNaseZap® (Invitrogen, Ireland) to inhibit RNases. Each region was divided into two pieces; one piece was placed into a 1.5ml eppendorf (Starstedt, Ireland) and was used as spare tissue, while the other half of tissue was subdivided into two pieces of tissue (approx 30mg), one of which was placed in RNase free 2ml tubes (Starstedt, Ireland) for PCR analysis, while the other piece of tissue was weighed and placed in 1.5ml eppendorfs for mass-spectrometry analysis. All samples were snap-frozen on dry ice and stored at -80°C for further analysis.

2.4 Analysis of gene expression using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.4.1 RNA isolation

Total RNA was extracted from homogenised discrete brain regions and spleen samples using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Fisher Scientific, Ireland). In brief, 354µl of RA1 lysis buffer containing 1% β-mercaptoethanol (M6250: Sigma-Aldrich, Ireland) was added to approx 30mg tissue prior to homogenisation with an Ultra-Turrax Polytron tissue disrupter (Fisher Scientific, Ireland) for 20 seconds. Homogenates were then transferred to a Nucleospin filter column (purple) and centrifuged at 14,000g for 1 min. 350µl of 70% molecular grade ethanol (E7023: Sigma-Aldrich, Ireland) was added to the lysates and mixed by pipetting up and down 10 times. The samples were then transferred to another set of Nucleospin RNA II columns (blue) and centrifuged at 14,000g for 30 seconds to allow RNA bind to the column. Following centrifugation, the columns were placed in a new collection tubes and 350µl of membrane desalting buffer (MDB, supplied with kit) was added prior to centrifugation at 14,000g for 1 minute. Genomic DNA was digested using a 10% v/v rDNase solution prepared in DNase reaction buffer (supplied). 95µl of the rDNase solution was pipetted directly onto the centre of each column and allowed to stand for 15 minutes at room temperature, following which 200µl RA2 buffer was added to each column prior to centrifugation at 14,000g for 30 seconds. The columns were then placed in new

collection tubes and 600µl of RA3 wash buffer was added followed by centrifugation at 14,000g for 30 seconds. The eluent was discarded and 250µl of RA3 wash buffer was added followed by centrifugation at 14,000g for 2 minutes. The columns were then placed in RNase-free collection tubes and the RNA was eluted by the addition of 60µl of RNase-free water (W4503: Sigma-Aldrich, Ireland) followed by centrifugation at 14,000g for 1 minute. The eluted RNA was then stored at -80°C until quantification and reverse transcription (See flow diagram for RNA isolation protocol using NucleoSpin, Macherey-Nagel)



RNA Isolation using NucleoSpin, Macherey-Nagel

30mg tissue(max) keep on dry ice. Do not let thaw.

Prepare RA1+10% B-mercapto(350 μ l RA1+3.5 μ l B-mercaptoethanol)

For 8 samples 3ml+30 μ l beta mercaptoethanol

Add 354 ul RA1 mix to each tube then add tissue and homogenise



Place violet column in 2ml collection tube.

Add homogenate. Spin @ 11000g for 1 min.



Discard filter.

Add 350 ul 70% ethanol to sample lysate.

(Make up using molecular grade ethanol and RNase free water.)



Place blue column in new 2ml collection tube.
Mix by pipetting up+down 10 times

Add lysate. Spin @ 11000g for 30 seconds. (Can combine above 2 steps)



Place blue column in new 2ml collection tube.



Add 350 μ l MDB (membrane desalting buff)

Spin 11000g for 1 min.

Make up working Dnase mixture.

(for each sample add 10 μ l reconstituted DNase to 90 μ l reaction buffer for rDNase)



Add 95 μ l of DNase solution to centre of the column.mLeave at RT for 15 mins.



Add 200 μ l of RA2 solution to centre of the column.

Spin at 11000g for 30 secs.

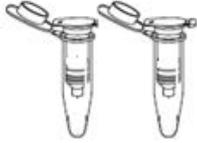


Place column into new 2ml collection tube.



Add 600 μ l of RA3 solution to centre of the column.

Spin at 11000g for 30 secs.



Discard contents of tube and place column back into collection tube.

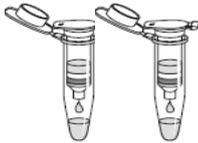


Add 250 μ l of RA3 solution to centre of the column.

Spin at 11000g for 2 mins.

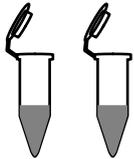


Place column into 1.5 ml collection tube (provided with kit, RNase free)



Add 60 μ l of RNase free water to centre of the column.

Spin at 11000g for 1 min.



Cap tubes and store eluted RNA at -80.

2.4.2 RNA quantification and equalization

The quantity, purity and quality of RNA was assessed using an Eppendorf Biophotometer plus (Unitech Ltd, Ireland) or a ND-2000 Nanodrop spectrophotometer (Mason Technology, Ireland). RNA quantity was determined by measuring optical density (OD) at 260nm (1 OD unit at 260nm corresponds to 40µg/ml RNA). RNA quality was determined by measuring the OD_{260}/OD_{280} ratio where a value of approximately 1.6-2.1 was deemed indicative of pure RNA. All RNA samples with a ratio >1.6 were accepted. Prior to cDNA synthesis, all samples were equalised to the same concentration of RNA (2-4µg/20µl) by addition of RNase free water. Equalised samples were then stored at -80°C until reverse transcribed.

2.4.3 Reverse Transcription of mRNA to cDNA

A high capacity complementary DNA (cDNA) kit (Cat # 4368814: Applied Biosystems, UK) was used to reverse transcribe RNA samples. 10µl of equalised RNA was added to an equal volume of 2X master mix in a PCR mini-tube. The 2X master mix was prepared as follows: 2.0µl 10X RT buffer, 0.8µl 25X dNTP mix, 2.0µl 10X RT random primers, 1.0µl Multiscribe Reverse Transcriptase and 4.2µl RNase free water. A negative control replacing mRNA with RNase-free water was included. Samples were then placed in an 'MJ research' thermal cycler (Bio-Rad, Ireland) and incubated at 25°C for 10 minutes, 37°C for 2 hours and 85°C for 5 minutes. The resultant cDNA was stored at -80°C until quantification by qRT-PCR.

2.4.4 Quantitative Real-time PCR (qRT-PCR) analysis of gene expression

Gene expression of target proteins were determined using commercially available TaqMan gene expression assays (Applied Biosystems, UK) containing specific forward and reverse target primers and FAM-labelled MGB probes. β -actin was used as an endogenous control to normalise gene expression between samples and was quantified using a β -actin endogenous control assay (Product code: 4352340E) containing specific primers and a VIC-labelled MGB probe. Assay IDs for the genes examined are given in Table 2.1.

A reaction master mixture was first prepared and stored on ice for each target gene. This consisted of 0.625 μ l target primers, 0.625 μ l β -Actin (multiplex version) and 6.25 μ l of Bioline one master mix (Cat no BIO-91005, Bioline, Ireland) per sample. cDNA samples were diluted 1:4 and 5 μ l of each diluted sample was pipetted in duplicate onto a MicroAmp® optical 96-well plate (Starstedt, Ireland). 7.5 μ l of the relevant reaction mixture was then added to each well giving a total reaction volume of 12.5 μ l. Non template controls (NTC) containing the master mix without cDNA for each target gene were also included as was the non-RNA control sample from the reverse transcription step. Plates were then covered with optical adhesive covers and spun at 1000g for 1 minute to ensure complete mixing and elimination of bubbles. The plate was then placed in the real time PCR thermocycler (ABI prism 7500, Applied Biosystems, UK) pre-set to run the following Relative Quantification protocol: step 1: 95°C for 10 minutes, step 2: 95°C for 15 seconds followed by one minute at 60°C. Step 2 was repeated 40 times and the fluorescence read during the annealing and extension phase (60°C) for the duration of the programme.

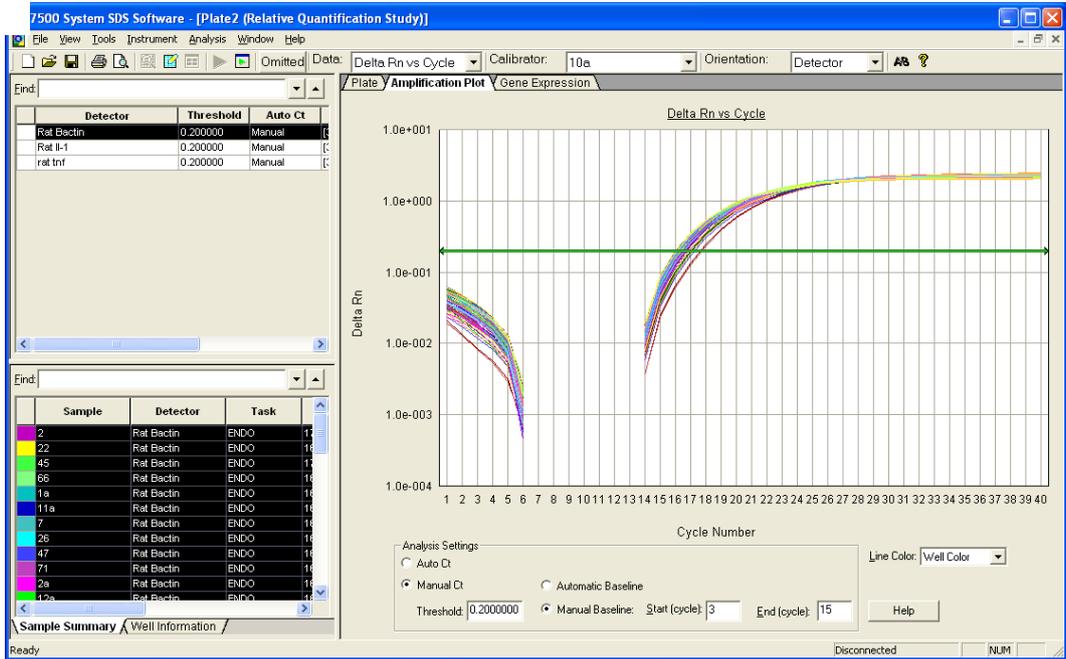
Target Gene	Assay number
Inflammatory cytokines/mediators	
<i>IL-1β</i>	Rn00580432_m1
<i>TNFα</i>	Rn99999017_m1
<i>IL-6</i>	Rn00561420_m1
<i>IL-10</i>	Rn00563409_m1
<i>SOCS3</i>	Rn00585674_s1
<i>IL-1ra</i>	Rn00573488_m1
<i>IL-4</i>	Rn01456866_m1
<i>IκBα</i>	Rn00585674_m1
<i>NOS2 (iNOS)</i>	Rn00561646_m1
<i>COX-2</i>	Rn01483828_m1
<i>m-PGE-s</i>	Rn00572047_m1
<i>CGRP</i>	Rn01511353_g1
IFN-related genes	
<i>IFN-α</i>	Rn02395770_g1
<i>IFN-β</i>	Rn00569434_g1
<i>IFN-γ</i>	Rn00594078_m1
<i>CXCL-10 (IP-10)</i>	Rn00566603_m1
<i>SOCS1</i>	Rn00595838_s1
<i>IRF7</i>	Rn01450778_g1
<i>PKR</i>	Rn01515440_m1
<i>Fas</i>	Rn00685720_m1
Markers of glial activation, neurotrophins and enzymes	
<i>CD11b</i>	Rn00709342_m1
<i>GFAP</i>	Rn00566603_m1
<i>MRC2</i>	Rn01456616_m1
<i>CD68</i>	Rn01495634_g1
<i>BDNF</i>	Rn 01441749_m1
<i>TrkB</i>	Rn 02531967_s1
Housekeeping gene	
B-actin	4352340E

Table 2.1 List of Taqman gene expression assays used

2.4.5 Analysis of qRT-PCR

Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems 7500 System SDS Software 1.3.1. Ct values for each sample were analysed after setting the threshold to the linear exponential phase of the amplification plots and exporting to Microsoft Excel for final analysis (Fig. 2.1). The $2^{-\Delta\Delta Ct}$ method was used to determine gene expression (Livak *et al.*, 2001). This method is used to assess relative gene expression by comparing gene expression of experimental samples to control samples, allowing determination of the fold change in mRNA expression between experimental groups. This method involves 3 steps: (1) Normalisation to endogenous control (β -actin) where ΔCt is determined: $\Delta Ct = Ct \text{ Target gene} - Ct \text{ Endogenous control}$; (2) Normalisation to control sample where $\Delta\Delta Ct$ is determined: $\Delta\Delta Ct = \Delta Ct \text{ Sample} - \text{average } \Delta Ct \text{ of Control group}$; and (3) where the fold difference is given by $2^{-\Delta\Delta Ct}$. The $2^{-\Delta\Delta Ct}$ values for each sample were then expressed as a percentage of the average of the $2^{-\Delta\Delta Ct}$ values for the control group which in Chapters 3-6 was vehicle-saline-treated animals, while in Chapter 7 it was the vehicle-LPS-treated animals. In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.

(a)



(b)

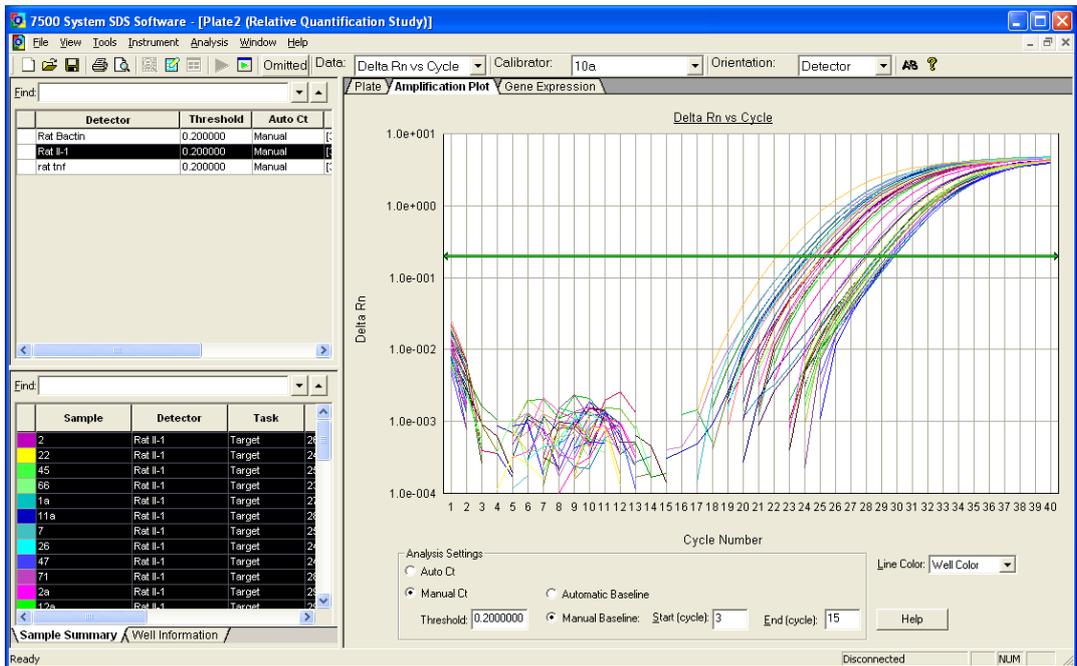


Figure 2.2 Sample Amplification Plots for (a) the endogenous control β -actin and (b) IL-1 β

2.5 Determination of cytokine protein levels using enzyme-linked immunosorbent assay (ELISA)

Serum IP-10 (catalog no: 900-K449), IFN- γ (catalog no: 900-K109), TNF α (catalog no: 900-K73), IL-1 β (catalog no: 900-K91) and IL-6 (catalog no. 900-K86) protein concentrations were determined using specific rat enzyme-linked immunosorbent assays (ELISAs) performed using antibodies and standards obtained from Peprotech, USA.

2.5.1 ELISA Assay protocol

- Maxisorb Nunc microtitre plates (Biosciences Ltd. Ireland) were coated with 100 μ l of goat or rabbit anti-rat cytokine antibodies (1-2 μ g/ml in PBS) covered with an adhesive plate cover and left at room temperature overnight.
- Plates were then aspirated washed four times with 300 μ l wash buffer (0.05% Tween 20 in PBS, pH 7.4) on a ELx50 BIO-TEK plate washer (Mason Technology, Ireland) and blocked for at least an hour at room temperature with 300 μ l reagent diluent (0.05% Tween 20, 1% BSA in PBS, pH 7.4).
- Standards for cytokines were prepared (5000-0 pg/ml) in reagent diluent (0.05% Tween 20, 1% BSA in PBS, pH 7.4).
- Following blocking, the plates were washed a further four times, following which 100 μ l aliquots of samples or standards were added to the wells in duplicate, and plates were incubated at room temperature for 2 hours.
- After four washes, 100 μ l of specific biotinylated goat or rabbit anti-rat cytokine detection antibody (0.25-0.5 μ g/ml) was added to each well and plates incubated for 2 hours at room temperature.
- After four washes, 100 μ l of Avidin-HRP Conjugate (1:2000) was added to each well, and plates were incubated at room temperature for 30 minutes, covered from light.
- Following four washes, 100 μ l of 2,2'-azino-bis 3-ethylbenzothiazoline-6sulphonic acid (ABTS) substrate solution (A3219: Sigma-Aldrich, Ireland) was added to each well.
- Absorbance was read upon colour development at 405nm on a μ Quant Bio-Tek plate reader (Mason Technology, Ireland) and at 5 minute intervals for approximately 35 minutes.

2.6 Quantification of endocannabinoid and *N*-acylethanolamine concentrations using liquid chromatography – tandem mass spectrometry (LC-MS/MS)

2.6.1 Preparation of Standards

Non-deuterated stock solutions were prepared in 100% acetonitrile for AEA, 2-AG, OEA and PEA (Cayman Chemicals, Cambridge Biosciences, UK) at concentrations of 2.5mg/ml for AEA, PEA, OEA and 0.5mg/ml for 2-AG. A single stock solution of all the above standards was then prepared containing 2-AG at a concentration of 5µg/ml (250ng/50µL) and AEA, PEA and OEA at 0.5µg/ml (25ng/50µL) in 100% acetonitrile. This was then used to construct a standard curve for use on the mass spectrometry system for the determination of EC levels.

Deuterated stock solutions of the deuterated form of AEA(d8), 2-AG(d8), OEA(d2) and PEA(d4) (Cayman chemicals, Cambridge Biosciences, UK) were prepared in acetonitrile, each at a concentration of 100µg/ml. From these stocks, a deuterated homogenising buffer was prepared containing 2-AG(d8) at 100ng/400µl and AEA(d8), OEA(d2) and PEA(d4) at 5ng/400µl. The deuterated homogenizing buffer was then dispensed into 50ml aliquots and stored with non-deuterated standards at -80°C.

2.6.2 Preparation of standard curve and samples

A 10 point standard curve was prepared in acetonitrile by carrying out a 4-fold serial dilution of the undueterated standard giving a concentration range of 18.75ng to 71.5pg for AEA, PEA and OEA and 187.5 to 715fg for 2AG. 400µl of the deuterated homogenising buffer was then added to each point of the standard curve.

Quantitation of the concentration of endocannabinoids and N-acylethanolamines was carried out essentially as previously described (Kerr *et al.*, 2012; Kerr *et al.*, 2013b). Pre-weighed frozen tissue (~30mg) was first homogenised using a Branson sonicator in 400µl of the deuterated homogenising buffer. Homogenates were centrifuged at 14,000g for 15 minutes at 4°C and the supernatant was collected and evaporated to dryness along with the standard curve in a centrifugal evaporator (Thermo SPD131DDA-230, Fischer Scientific, Ireland). Lyophilised samples and standards were resuspended in 40µl 65% acetonitrile and 2µl were injected onto a Zorbax® C18 column (150 × 0.5 mm internal diameter) from a cooled autosampler maintained at 4°C. Mobile phases consisted of A (HPLC grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), maintained at a flow rate of 12µL/min. Analytes were eluted under gradient elution (Table 2.3) and the total run time was 30 min.

Time(min)	%B(CH ₃ CN, 0.1% formic acid)	%A (H ₂ O, 0.1% formic acid)
0	65	35
10	100	0
20	100	0
20.1	65	35
30	STOP	

Table 2.2 Gradient used to elute analytes

Under these conditions, AEA, 2AG, PEA and OEA eluted at the following retention times: 11.36 min, 12.8 min, 14.48 min and 15.21 min, respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies, UK). Instrument conditions and source parameters including fragmentor voltage and collision energy were optimised for each analyte of interest prior to assay of samples. Quantitation of target endocannabinoids was achieved by positive ion electrospray ionization and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [M + H⁺] of the analytes of interest and the deuterated forms of the internal standards.

In MRM mode the first quadrupole mass filter was set to allow only ions of the target mass (parent ions) to pass through into the second quadrupole collision cell where they collide with gas molecules producing product ions and neutral fragments. The third quadrupole was set to mass filter product ions produced in the collision cell, namely daughter ions. Thus retention times in combination with parent---daughter transition allowed the unique identification of each analyte and its corresponding deuterated internal standard

Analyte	Parent----Daughter transition
AEA	348.3-----62.1
2AG	379.3-----287.2
PEA	300.3-----62.1
OEA	326.0-----62.1
AEA(D8)	356.3-----63.1
2AG(D8)	387.3-----294.2
PEA(D4)	304.3-----62.1
OEA(D2)	328.3-----62.1

Table 2.3 Parent-Daughter transitions (mass charge ratios (m/z)) for the deuterated (internal standard) and non deuterated forms of each analytes .

Quantification of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was performed using Masshunter Quantitative Analysis Software (Agilent Technologies, UK). The amount of analyte in unknown samples was calculated from a standard curve of Relative response vs. Relative concentration for each analyte i.e. $(\text{Peak Area analyte}_{(\text{undeuterated})} / \text{Peak area analyte}_{(\text{deuterated})})$ vs $(\text{Conc analyte}_{(\text{undeuterated})} / \text{Conc analyte}_{(\text{deuterated})})$ (Fig 2.2). As 1-AG and 2-AG fail to completely separate from one another, the combined intergration of both was used to represent total 2-AG levels. The limit of quantification was 1.32 pmol/g, 12.1 pmol/g, 1.5 pmol/g, 1.41 pmol/g for AEA, 2AG, PEA and OEA respectively. Sample chromatograms are shown below (Fig 2.2). Final concentrations for each analyte was divided by the tissue weight in order to get ng/g of tissue. Values for AEA, OEA and PEA, but not 2-AG, were multiplied by 1000 in order to express as pg/g of tissue. In order to express as nmol/g (2-AG) or pmol/g (AEA, OEA and PEA) of tissue, each analyte was divided by its molecular weight (MW), for example the X concentration of 2-AG (ng/gram of tissue) was divided by 379.3 (2-AG MW) to get X nmol/gram of tissue.

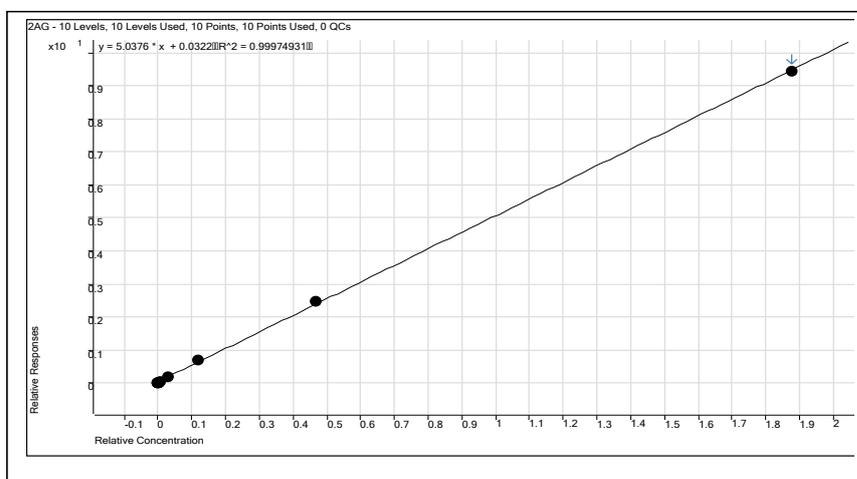


Figure 2.3 Plot of Relative Response vs. Relative Concentration for 2-AG.

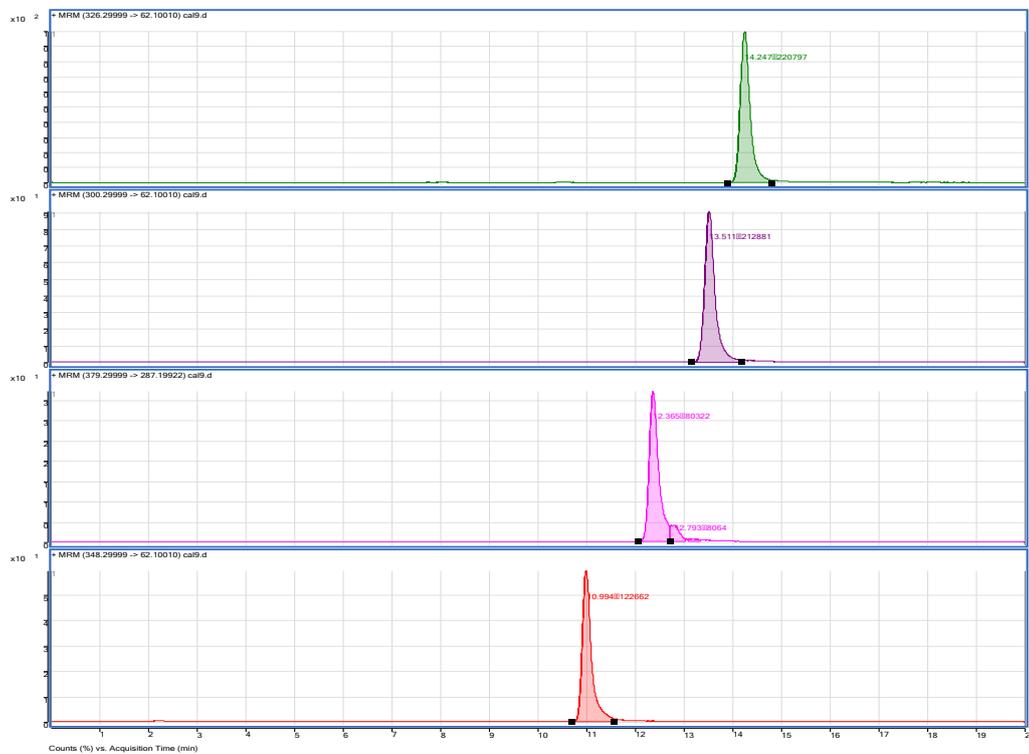


Figure 2.4 Chromatograms of 2-AG, AEA, OEA and PEA

2.7 Qualitative detection of JZL184 in hippocampal tissue following systemic administration using LC-MS/MS

The system protocol employed was similar to that described for detection of endocannabinoids with the following modifications (Kerr *et al.*, 2013b). A standard picomolar solution of JZL184 was initially injected onto an Agilent 6510 QTOF LC/MS system (Agilent Technologies, UK) to obtain accurate m/z for parent and daughter ions generated using standard collision energy of 20eV. Separate injections were carried out with the system operating in both positive and negative ion electrospray mode, respectively. Samples were then run under optimised conditions on an Agilent 1100 HPLC system coupled to a triple quadrupole 6410 mass spectrometer. The protocol employed was similar to that described for the detection of endocannabinoid and *N*-acylethanolamine levels with the following modifications: Hippocampal samples previously prepared and analysed for endocannabinoid determination were resuspended in approximately 50µl of 100% acetonitrile and 4µl injected onto a Zorbax® SB C18 column (150 × 0.5 mm internal diameter). Mobile phases consisted of solvent A (0.1% formic acid (v/v) in water) and solvent B (0.1% (v/v) formic acid in acetonitrile) maintained at a flow rate of 12µl per minute. JZL184 was eluted under gradient elution. Under these conditions, JZL184 eluted at 14 minutes. JZL184 detection was carried out using electrospray-positive ionisation and multiple reaction monitoring (MRM) mode where the parent-daughter transition of 503.1>199.1 was monitored with a collision energy of 25V. Chromatograms were examined qualitatively for the presence of a JZL184 peak. A sample chromatogram is shown below (Fig 2.5).

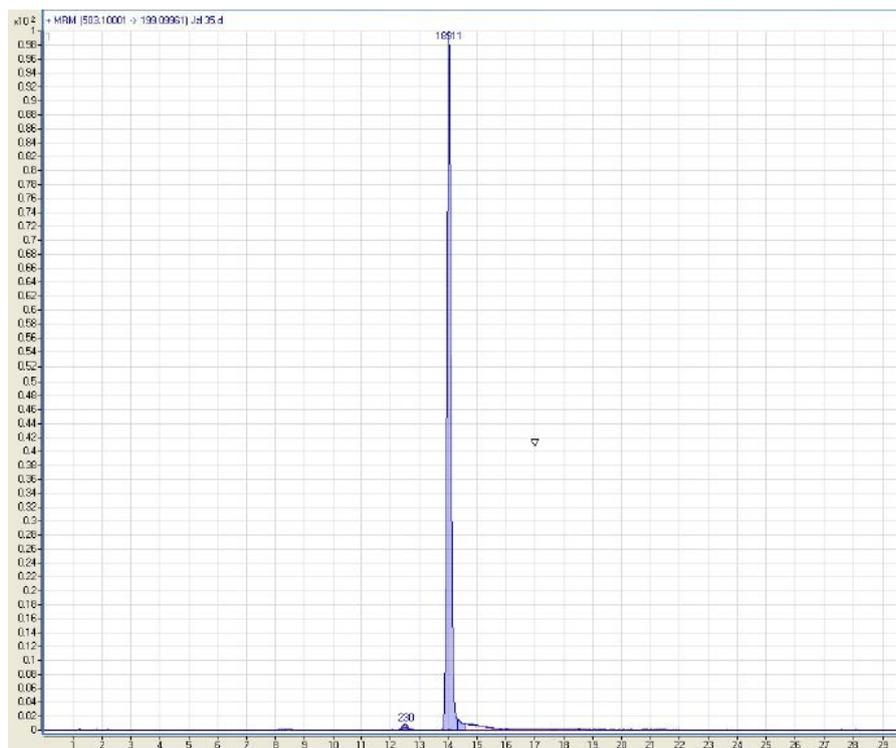


Figure 2.5 Chromatogram of JZL184 in the rat hippocampus

2.8 Western immunoblotting

Approximately 30mg of cortical tissue was lysed in 400 μ l of RIPA lysis buffer containing protease and phosphatase inhibitors (Table 2.4) and homogenised using an ultrasonic sonicator (Mason Technology, Ireland) for approximately 20 seconds followed by centrifugation at 13,000g at 4°C for 20 min. The supernatant was collected and protein content was determined by the Bradford assay (Bradford, 1976) as described below (Appendix 1). Samples were then diluted in ice-cold lysis buffer to give equal protein concentrations (30 μ g in 15 μ l of each sample) followed by the addition of (5 μ l) of sample buffer containing 20% 2-mercaptoethanol (Table 2.4). Lysates were heated at 95°C for 5 minutes and proteins were then separated by SDS-PAGE electrophoresis using 12% polyacrylamide gels (Appendix 2) and semi dry electroblotted onto an Immoblian P PVDF membrane (0.45 μ m; Millipore, Cat# IPVH00010, Sigma Aldrich, Ireland) as described below (Appendix 3). Membranes were then rocked in blocking solution (5% milk, 0.1% Tween 20 in TBS) for 1hr at room temperature (RT). Individual membranes were then incubated in primary antibody diluent (1:1000 dilution in 5% milk, 0.1% Tween 20 in TBS) overnight at 4°C containing the relevant primary diluted antibody (Cell Signaling Technologies (CST), USA) (Table 2.5). The following day, after three 5 minute washes in wash buffer, (0.25% Tween 20 in TBS), membranes were then rocked at RT for one hour in secondary antibody solution (1:10,000 dilution: LI-COR Biosciences UK) diluted in 1% milk, 0.1% Tween 20 in TBS (Table 2.5). After washing in wash solution membranes were placed in distilled water following which they were analysed using the fluorescence ODYSSEY CLx scanner (LI-COR Biosciences UK). Membranes were subsequently stripped with stripping buffer (Table 2.4) and reprobed for other target protein as well as β -actin. Densitometry analysis of integrated band density was carried out using Image Studio Lite software V5.0 (LI-COR Biosciences UK). In the Image Studio software, a rectangle was drawn around each individual visible band on the gel of interest and using the Image Studio software, the optical density for each band was calculated and the integrated optical density values for each protein of interest was exported to an excel file and normalised to their respective integrated optical density for β -actin (endogenous control). Following which, the normalised protein of interest was divided by the average of the control group (vehicle-LPS-treated) to obtain the relative concentrations of both the total and phosphorylated protein. In addition, the relative phosphorylated (p)-protein was divided by the total (t)-

protein p-protein to express as p-protein:t-protein of interest, see below for example calculation for JNK:

	Optical density	Adjusted density	Average density control group	Relative density	pJNK:tJNK
β-action	3050000				1.66
tJNK	4210	0.001	0.002	0.5	
pJNK	3920	0.001	0.0012	0.83	

Table 2.4 Western Blotting Solutions

Solution	Ingredients
4X Sample buffer _ store at -20°C	SDS (1g) 1M Tris-base pH 6.8 (5mL) Glycerol (4mL) 1% Bromophenol blue (500µL in PBS) Make up 20mL with dH ₂ O and dispense into 800µL aliquots (Sigma Aldrich Ireland Ltd).
RIPA lysis buffer _ store at 4°C	250µL 1M Tris-HCL stock 1000µL 10% Triton X-100 1000µL 10% sodium deoxycholate 300µL of 5M NaCl 57.1µL of 175mM sodium orthovanadate 200µL of 500mM NaF 100µL 10% SDS stock Dissolve to a final amount of 6mL of dH ₂ O. Dispense into 990µL aliquots and immediately prior to use, add 10µL/mL of protease inhibitor cocktail (Sigma Aldrich Ireland Ltd).
1.5M Tris, pH 8.8 _ stored at 4°C	Trizma-base (90.82g) added to a final volume of 500mL of dH ₂ O. Adjust pH to 8.8 with 50%(v/v) HCl

0.5M Tris, pH 6.8 _ stored at 4°C	Trizma-base (12.11g) added to a final volume of 200mL of dH ₂ O. Adjust pH to 6.8 with 50%(v/v) HCl (Sigma Aldrich Ireland Ltd).
5X Running buffer _ stored at 4°C	Trizma-base (15g) Glycine (28.8g) 20% SDS (25mL) Make up to a final volume of 1L in distilled H ₂ O (Sigma Aldrich Ireland Ltd).
10X Transfer buffer _ stored at 4°C	Trizma-base (30g) Glycine (144g) Make up to a final volume of 1L in dH ₂ O (Sigma Aldrich Ireland Ltd).
1X Transfer buffer _ stored at 4°C	Transfer buffer (10X) (150ml) 100% Methanol (300mL) Make up to a final volume of 1.5L in dH ₂ O (Sigma Aldrich Ireland Ltd).
10X Tris-Buffered Saline (TBS) _ stored at 4°C	Trizma-base (30g) NaCl (80g) KCl (2g) pH to 7.4 and bring to final volume of 1L with dH ₂ O. Dilute to 1X TBS prior to use (Sigma Aldrich Ireland Ltd).
Washing solution (0.1% TBS-T) _ stored at 4°C	Tween 20 (200µL) in a final volume of 100mL of 1X TBS (Sigma Aldrich Ireland Ltd).
Blocking solution (5% milk) _ stored at 4°C	Dawn condensed milk (2.5g) Make up to a final volume of 50mL with washing solution
Blocking solution (1% milk) _ stored at	Dawn condensed milk (0.5g)

4°C	Make up to a final volume of 50mL with washing solution
0.1% Ponceau stain in 5% Acetic acid _ stored at 4°C	Ponceau stain (100mg) Acetic acid (1mL) Make up to a final volume with 95mL of dH ₂ O (Sigma Aldrich Ireland Ltd).
Stripping buffer _ stored at 4°C 25mM Glycine-Hcl pH 2, 1%SDS	Add 1.876g of Glycine-HCL to 950mL of dH ₂ O, pH to 2.0 with 50% Hcl. Add 50 ml of 20% SDS (Sigma Aldrich Ireland Ltd).

Frontal cortical protein levels of pERK (CST; cat no. 9101), ERK (CST; cat no. 9102), p-p38 (CST; cat no. 9211), p38 (CST; cat no. 9212), p-JNK (CST; cat no. 9251) and JNK (CST; cat no. 9252) were determined using western blot analysis using antibodies listed below.

Table 2.5 List of primary and secondary Antibodies

Primary Antibody	Dilution Required	Secondary Antibody	Dilution Required
Phospho-p44/42 MAPK (pERK1/2) antibody_ #9101	1:1000	Goat Anti-Rabbit IRDye Cat# 926-3211 (LI-COR Biosciences UK)	1:10,000
p44/42 MAPK (ERK1/2) antibody_ #9102	1:1000	Goat Anti-Rabbit IRDye Cat# 926-3211 (LI-COR Biosciences UK)	1:10,000
Phospho-p38 MAPK (Thr180/Tyr182) antibody_ #9211	1:1000	Goat Anti-Rabbit IRDye Cat# 926-3211 (LI-COR Biosciences UK)	1:10,000
p38 MAPK antibody_ #9212	1:1000	Goat Anti-Rabbit IRDye Cat# 926-3211 (LI-COR Biosciences UK)	1:10,000
Phospho-SAPK/JNK (Thr183/Tyr185) antibody_ #9251	1:1000	Goat Anti-Rabbit IRDye Cat# 926-3211 (LI-COR Biosciences UK)	1:10,000
SAPK/JNK antibody_ #9252	1:1000	Goat Anti-Rabbit IRDye Cat# 926-3211 (LI-COR Biosciences UK)	1:10,000
Mouse α β -Actin (Sigma; cat no. A5441)	1:1000	Donkey anti-mouse IRDye Cat#92668022 (LI-COR Biosciences UK)	1:10,000

2.9 Statistical Analysis

SPSS (IBM, New York, USA) statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro–Wilks and Levene test $p > 0.05$, respectively. When comparing the means of two unrelated groups, parametric data were analysed using unpaired t -test. One-way ANOVA was used to compare the mean of more than two groups on one factor. *Post-hoc* analysis was performed using Fisher's LSD test. Data were considered significant when $p < 0.05$. All graphs representing data were constructed using GraphPad Prism 5.0 and results expressed as group means + standard error of the mean (SEM).

Chapter 3

Systemic administration of the selective FAAH inhibitor URB597 modulates TLR3-induced inflammation, both in the periphery and within the brain, at discrete time points post systemic TLR3 activation

3.1 Introduction

Neuroinflammation is a key component underlying several neurological diseases including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS) and psychiatric disorders including depression. Viral and bacterial infections induce systemic and central inflammation, an effect proposed to trigger the onset of some neurodegenerative disorders including multiple sclerosis (Deleidi *et al.*, 2012) and elicit detrimental effects on the progression and outcome of established disease (Holmes *et al.*, 2009; Perry, 2004; Teeling *et al.*, 2009). Toll-like receptors (TLRs), which recognize and induce an immune response to bacterial (TLR4) and viral (TLR3) infection, are expressed on neurons, astrocytes and microglia (Bsibsi *et al.*, 2002). While activation of TLRs participates in host defences, homeostasis and response to injury, uncontrolled and aberrant TLR activation can elicit potent neurodegenerative cascades [for reviews see (Arroyo *et al.*, 2011; Lehnardt, 2010; Owens, 2009; van Noort *et al.*, 2009)] and TLR expression has been reported to be increased in the post-mortem brain of patients exhibiting neurodegenerative disorders (Brudek *et al.*, 2013; Salaria *et al.*, 2007). Thus, understanding the neurobiological mechanisms mediating TLR-induced neuroinflammation is critical at a fundamental physiological level and for the development of novel, more efficacious treatments.

The endogenous cannabinoid (endocannabinoid) system has been shown to exhibit a potent modulatory role on neuroinflammatory responses, including those induced by TLR activation (Downer, 2011; Jean-Gilles *et al.*, 2010; Nagarkatti *et al.*, 2009). The endocannabinoid system comprises the cannabinoid₁ (CB₁) and cannabinoid₂ (CB₂) receptors, the naturally occurring endogenous ligands for these receptors, the best characterised of which include AEA and 2-AG, and enzymes involved in their synthesis and degradation. AEA, and related fatty acid amides, *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA), are primarily catabolised by the enzyme fatty acid amide hydrolase (FAAH), although it should be noted that *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) represents an important metabolic enzyme for PEA in macrophages and peripheral tissues (Solorzano *et al.*, 2010; Tsuboi *et al.*, 2007). Anti-inflammatory and neuroprotective effects of AEA and PEA, have been documented (Bisogno *et al.*, 2010; Correa *et al.*, 2009b; Esposito *et al.*, 2013; Murphy *et al.*, 2012; Scuderi *et al.*, 2013). *In vitro* studies have shown that increasing AEA tone directly, or inhibiting FAAH activity and thereby elevating substrate levels, in microglia or astrocyte

cultures, attenuates TLR4-induced increases in pro-inflammatory cytokines and inflammatory mediators such as TNF α , IL-1 β and nitric oxide, and enhances the release of the anti-inflammatory cytokine IL-10 (Correa *et al.*, 2009a; Correa *et al.*, 2010; Facchinetti *et al.*, 2003b; Molina-Holgado *et al.*, 1997; Puffenbarger *et al.*, 2000; Tham *et al.*, 2007). *In vivo* studies have demonstrated that AEA activation of hypothalamic CB₁ receptors facilitates (De Laurentiis *et al.*, 2010), while antagonism of the central CB₁ receptors attenuates (Steiner *et al.*, 2011), TLR4-induced increases in plasma TNF α levels. In addition, recent data have shown that enhancing endogenous FAAH substrate levels attenuates the TLR4-induced increase in *IL-1 β* expression while concurrently augmenting the expression of *SOCS3* in the hypothalamus (Kerr *et al.*, 2012). However, there is a paucity of data regarding the effect of FAAH substrates on neuroinflammatory responses elicited by other TLRs. The potent cannabinoid receptor agonist WIN55,212-2 has been shown to attenuate TLR3-induced increases in TNF α while concurrently augmenting IRF3 and the expression of the type I IFN IFN- β , an effect necessary for the protective effects of WIN55212-2 in a rat model of multiple sclerosis (Downer *et al.*, 2012; Downer *et al.*, 2011). TMEV induction of inflammatory processes is thought to be primarily mediated via TLR3 activation (So *et al.*, 2006). *In vitro* data have demonstrated that AEA downregulates pro-inflammatory cytokines IL-12, IL-17, TNF α and enhances IL-10 and IL-6 levels in TMEV-infected astrocytes (Correa *et al.*, 2011; Hernangomez *et al.*, 2012; Molina-Holgado *et al.*, 1997; Molina-Holgado *et al.*, 1998). Furthermore, PEA or FAAH inhibition attenuates microglial activation, the expression of pro-inflammatory cytokines and ameliorates motor symptoms in the TMEV-model of multiple sclerosis (Correa *et al.*, 2011; Hernangomez *et al.*, 2012; Loria *et al.*, 2010; Loria *et al.*, 2008; Mestre *et al.*, 2005; Ortega-Gutierrez *et al.*, 2005). However, to date there have been no studies examining the effect of enhancing FAAH substrate levels specifically on TLR3-induced neuroinflammatory responses *in vivo*.

Systemic administration of the TLR3 agonist poly I:C to rodents has been shown to enhance the expression of type I IFNs and pro-inflammatory cytokines in several brain regions including the hippocampus and frontal cortex (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013). Poly I:C-induced increases in expression of neuroinflammatory cytokines including TNF α , IL-1 β and IL-6 have been reported to be associated with decreases in both hippocampal and frontal cortical expression of *BDNF* and its receptor tropomyosin receptor kinase (*TrkB*), while concurrently increasing

expression of *CD11b* and *GFAP*, markers of microglia and astrocyte activation, respectively (Gibney *et al.*, 2013). BDNF is known to play a vital role in several processes including neurogenesis, neuronal growth and synaptic plasticity and there is now evidence that the pro-inflammatory cytokine IL-1 β impairs hippocampal-dependent memory processes via decreases in hippocampal BDNF (Barrientos *et al.*, 2004; Lapchak *et al.*, 1993). Thus, modulation of TLR3-induced signalling within the brain may alter long term alterations in neuro-immune processes and thus subsequent functional outcomes.

Hypothesis: Based on these data we hypothesised that enhancing endocannabinoid tone would modulate neuroinflammatory responses following TLR3 activation. Therefore the aims of the studies described in this chapter were to:

1. Determine if systemic administration of the FAAH inhibitor URB597 modulates levels of peripheral inflammatory cytokines produced following systemic administration of the TLR3 agonist poly I:C.
2. Examine the effect of systemic administration of the FAAH inhibitor URB597 on TLR3-induced alterations in IFN- and NF- κ B-inducible genes in two discrete rat brain regions (the hippocampus and frontal cortex) at discrete time points post systemic poly I:C administration. In addition, the ability of URB597 to enhance AEA and *N*-acylethanolamine levels in these brain regions will be confirmed.
3. Examine the effects of systemic administration of URB597 on markers of microglial and astrocyte activation, *BDNF* and its receptor *TrkB* (the neurotrophin system) in both in the hippocampus and frontal cortex at 24 hours post systemic poly I:C administration.

3.2 Methodology and Experimental design

Experiments were carried out on male Sprague Dawley rats (weight 220-260g; Charles River, UK), housed singly in plastic bottomed cages (45 X 25 X 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 0800 to 2000 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 an intraperitoneal (i.p.) injection of sterile saline (0.89% NaCl) for 3-4 days prior to experimentation in order 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 licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

Rats were randomly assigned into one of nine treatment groups, see table below

Group	Time of kill (hour post poly I:C)	Number of animals per group
1. Veh-veh-saline	2,4, 8 & 24	12
2. Veh-veh-poly I:C	2	8
3. Veh-URB-poly I:C	2	8
4. Veh-veh-poly I:C	4	8
5. Veh-URB-poly I:C	4	8
6. Veh-veh-poly I:C	8	8
7. Veh-URB-poly I:C	8	8
8. Veh-veh-poly I:C	24	8
9. Veh-URB-poly I:C	24	8

The vehicle-saline-treated control group comprised of 3 rats at each time point examined, which were subsequently pooled because ANOVA revealed no significant effect of time on cytokine expression in vehicle-saline-treated animals. The FAAH inhibitor URB597 (1mg/kg, Cayman Chemicals, Estonia) or vehicle (ethanol:cremaphor:saline; 1:1:18) were administered i.p. in a single, acute systemic injection in a volume of 2ml/kg followed 30 min later by a single, acute i.p. injection of poly I:C (3mg/kg, GE Healthcare, Ireland) or saline vehicle (sterile 0.89% NaCl) administered in an injection volume of 1.5ml/kg. The dose of URB597 was determined on the basis of previous published work demonstrating that systemic administration of URB597 at this dose enhanced levels of AEA and the related *N*-acylethanolamines in the brain (Fegley *et al.*, 2005; Kathuria *et al.*, 2003; Kerr *et al.*, 2012). Furthermore, work from our group has previously demonstrated that the dose of URB597 utilized in this study does not alter peripheral or brain cytokine expression in the absence of an immune stimulus (Kerr *et al.*, 2012), thus the effect of URB597 was only evaluated in the presence of poly I:C administration in this and subsequent chapters. The dose and time of poly I:C administration were chosen on the basis of previous published work demonstrating enhanced cytokine expression in the brain (Gibney *et al.*, 2013; Katafuchi *et al.*, 2005; Katafuchi *et al.*, 2003). Animals were sacrificed by decapitation at 2, 4, 8 or 24 hours post poly I:C/saline administration and blood samples were obtained from trunk blood. Blood samples were stored at 4°C for approximately 4 hours and were centrifuged at 14,000g for 15 min at 4°C to obtain serum. In addition, the hippocampus and frontal cortex were excised, snap-frozen on dry ice and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression. (The methodology was essentially as described in Chapter 2).

3.2.1 Statistical Analysis

SPSS (IBM, New York, USA) statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro–Wilks and Levene test $p > 0.05$, respectively. A one-way ANOVA was used to compare the mean of more than two groups on one factor. *Post-hoc* analysis was performed using Fisher's LSD test. Data were considered significant when $p < 0.05$. All graphs representing data were constructed using GraphPad Prism 5.0 and results expressed as group means + standard error of the mean (SEM).

3.3 Results

3.3.1 Systemic administration of URB597 attenuates the poly I:C-induced increases in peripheral inflammatory mediators, in a time dependent manner

Cytokine levels were examined using ELISA analysis from serum samples collected at 2, 4, 8 and 24 hours post systemic poly I:C administration. Analysis revealed a significant effect of treatment on peripheral levels of IL-6 [$F_{8, 63} = 3.921, p < 0.01$] and IFN- γ [$F_{8, 64} = 2.189, p < 0.05$] (Fig 3.1). However, no significant effect of treatment was reported on levels of either IL-1 β or TNF α at any of the time points examined in the current study. Poly I:C treatment significantly increased the serum levels of IL-6 and IFN- γ at 4 and 8 hours when compared to vehicle-saline-treated counterparts, with levels decreasing thereafter and returned to baseline levels at 24 hours post poly I:C administration. Prior administration of URB597 significantly attenuated the poly I:C-induced increases in the serum levels of IL-6 and at 4 and 8 hours, and IFN- γ at 4 hours when compared to vehicle-poly I: C-treated counterparts (Fig 3.1a,b).

--- Vehicle-saline □ Vehicle-poly I: C ■ URB597-poly I: C

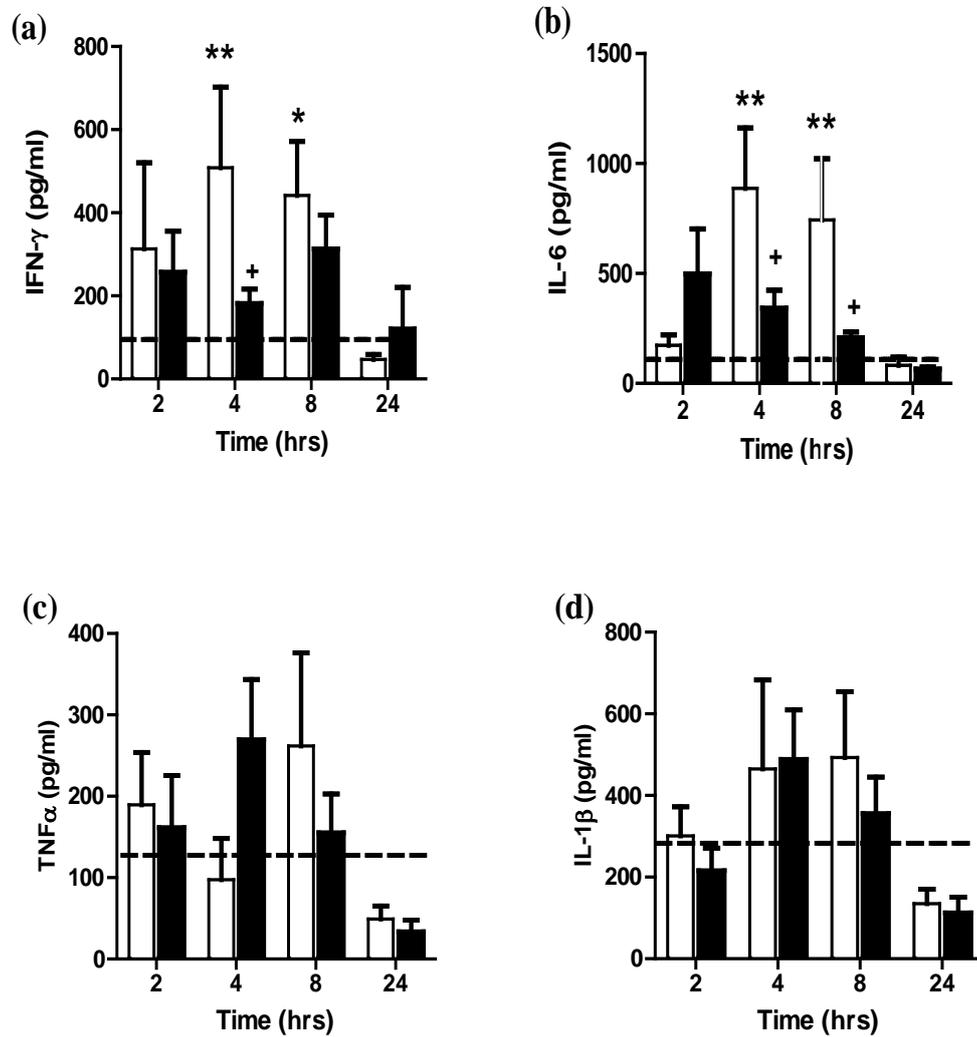


Figure 3.1 The effect of systemic administration of URB597 on serum levels of IFN- γ , IL-6, TNF α and IL-1 β , at discrete time points post poly I:C administration. URB597 attenuates the poly I:C-induced increases in (a) IFN- γ at 4 hours, and (b) IL-6 at 4 and 8 hours post poly I:C administration. In comparison, there was no significant effect of the current treatment regime on serum levels of (c) TNF α or (d) IL-1 β . Data expressed as mean + SEM (n = 8-12 per group). * $p < 0.05$, ** $p < 0.01$ vs. vehicle-saline-treated counterparts. + $p < 0.05$ vs. vehicle-poly I:C-treated counterparts.

3.3.2 Systemic administration of poly I:C induces time dependent increases in TLR3-dependent inflammatory genes in the rat hippocampus; effects of which are partially modulated by prior systemic administration of URB597

Statistical analysis revealed a significant effect of poly I:C treatment on hippocampal mRNA expression of the type I IFN *IFN- α* [$F_{8,65} = 2.844$, $p < 0.01$], the IFN-inducible chemokine *IP-10* [$F_{8,61} = 10.076$, $p < 0.01$] and the NF- κ B-inducible cytokines *IL-6* [$F_{8,60} = 4.228$, $p < 0.01$], *TNF α* [$F_{8,57} = 2.249$, $p < 0.05$] and *IL-1 β* [$F_{8,69} = 3.805$, $p < 0.01$], when compared to vehicle-saline-treated counterparts. However, this treatment regime failed to significantly alter hippocampal mRNA expression of *IFN- β* . Systemic administration of the TLR3 agonist poly I:C significantly increased inflammatory gene expression at discrete time points post challenge: *IFN- α* at 24 hours, *IP-10* at 4 and 8 hours, *TNF α* at 4 and 8 hours and *IL-1 β* expression at 4 hours, when compared to vehicle-saline-treated counterparts (Fig 3.2). At 24 hours post poly I:C, hippocampal expression of *IP-10*, *TNF α* , *IL-1 β* and *IL-6* had all returned to baseline levels of expression. Prior systemic administration of URB597 significantly decreased the mRNA expression of both *TNF α* and *IL-1 β* , while concurrently increasing hippocampal expression of *IL-6* at 4 hours post poly I:C challenge when compared to vehicle-poly I:C-treated counterparts (Fig 3.2d,e,f). Furthermore, URB597-poly I:C-treated animals displayed significant increases in hippocampal expression of *IFN- α* , when compared to their vehicle-poly I:C-treated counterparts, at 4 hours post poly I:C challenge (Fig 3.2 a).

--- Vehicle-saline □ Vehicle-poly I:C ■ URB597-poly I:C

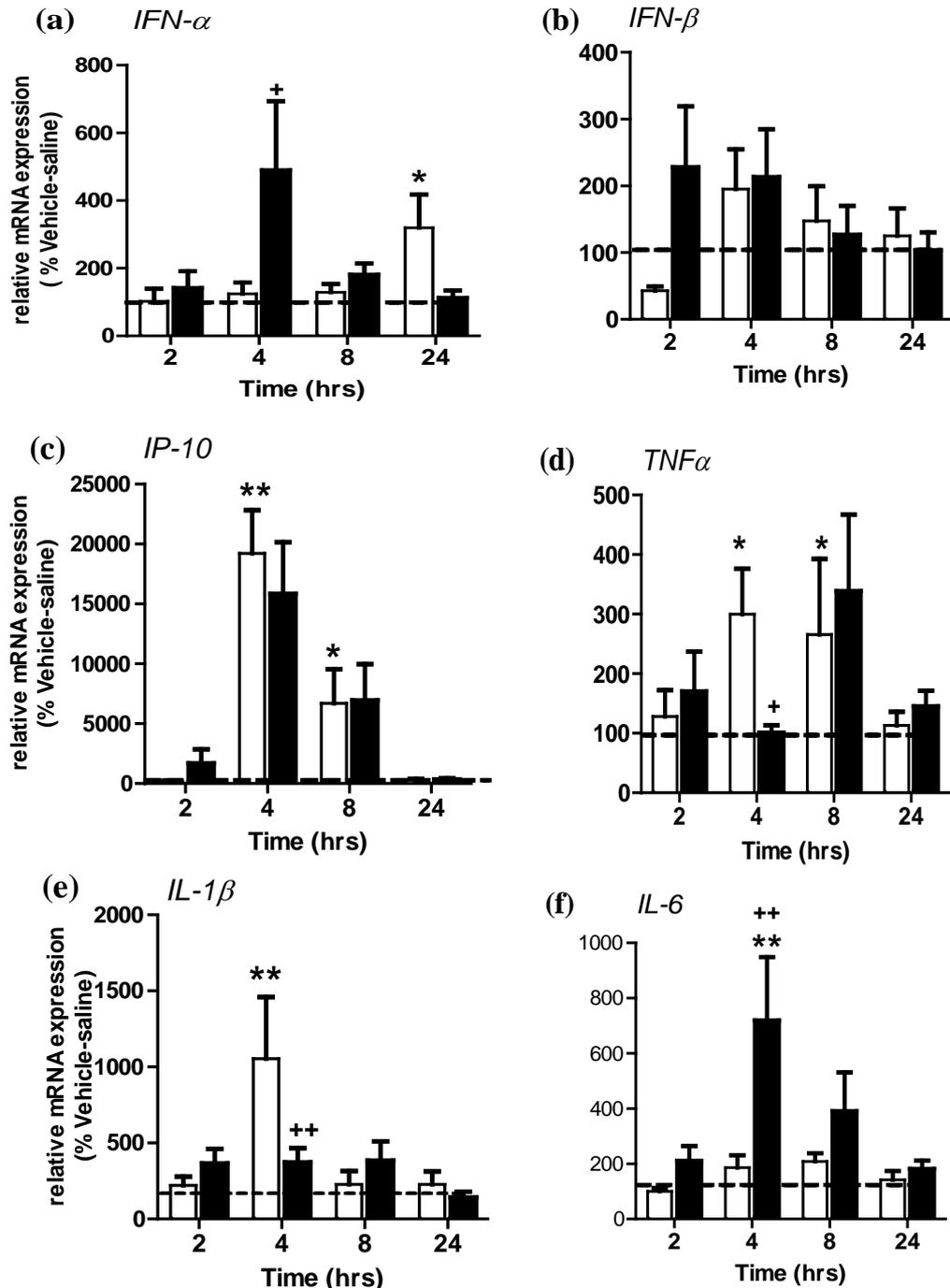


Figure 3.2 Systemic administration of URB597 modulates hippocampal expression of inflammatory genes, in a time-dependent manner, following systemic administration of poly I:C. URB597 enhances hippocampal expression of (a) *IFN-α* and (f) *IL-6*, while concurrently attenuating poly I:C-induced increases in (d) *TNFα* and (e) *IL-1β*, when compared to vehicle-poly I:C-treated counterparts, 4 hours post poly I:C, with no reported effect of treatment on (b) *IFN-β*. Data expressed as mean + SEM (n = 8-12 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated counterparts + $p < 0.05$ ++ $p < 0.01$ vs. vehicle-poly I:C-treated counterparts.

3.3.3 Systemic administration of poly I:C induces time dependent increases in TLR3-dependent inflammatory genes in the rat frontal cortex, in the absence or presence of systemic administration of URB597

Similar to the hippocampus, analysis revealed a significant effect of poly I:C treatment on *IL-1 β* [$F_{8,66} = 2.858$, $p < 0.01$], *IL-6* [$F_{8,65} = 5.32$, $p < 0.01$], *TNF α* [$F_{8,65} = 5.106$, $p < 0.001$] and *IP-10* [$F_{8,66} = 8.371$, $p < 0.01$] mRNA expression in the frontal cortex (Fig 3.3). However, there was no significant effect of treatment on frontal cortical mRNA expression of either of the type I IFNs, *IFN- α* and *IFN- β* . Poly I:C treatment significantly increased expression of *IL-1 β* at 2 and 4 hours, *IL-6* expression at 4 and 8 hours, *TNF α* at 8 hours and *IP-10* at 4 and 8 hours, when compared to vehicle-saline-treated counterparts (Fig 3.3). As reported in the hippocampus, frontal cortical expression of *IP-10*, *TNF α* , *IL-1 β* and *IL-6* all returned to baseline levels of expression at 24 hours post poly I:C administration. In addition, prior systemic administration of URB597 enhanced the poly I:C-induced increases in frontal cortical expression of *IL-6*, at 8 hours post poly I:C administration (Fig 3.3f).

--- Vehicle-saline □ Vehicle-poly I:C ■ URB597-poly I:C

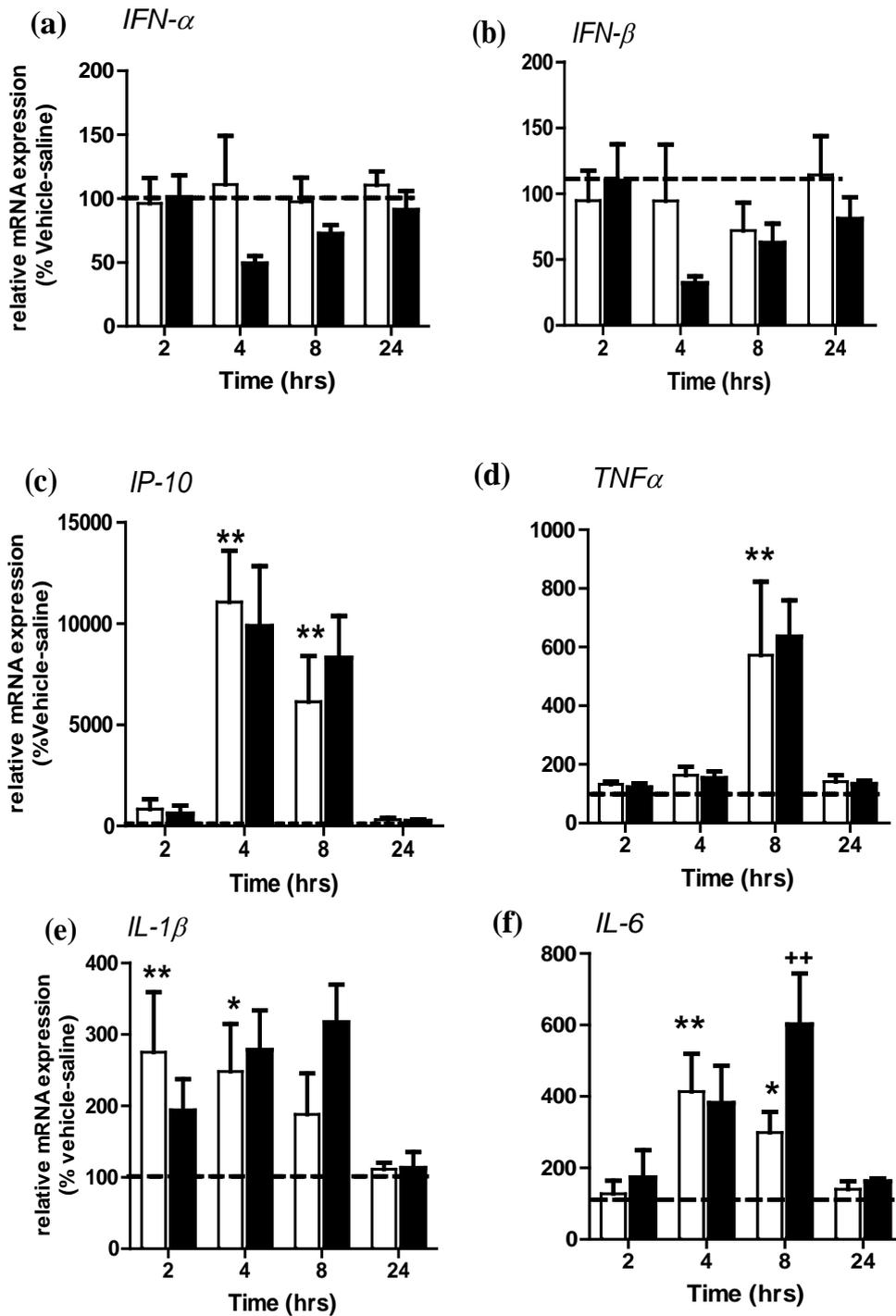


Figure 3.3 Systemic administration of URB597 modulates frontal cortical expression of inflammatory genes in a time-dependent manner, following systemic poly I:C administration. URB597 does not alter expression of (a) *IFN-α*, (b) *IFN-β*, (c) *IP-10*, (d) *TNFα* or (e) *IL-1β* in the frontal cortex at 2, 4, 8 and 24 h post poly I:C. However, URB597 increases poly I:C-induced increase in (e) *IL-6* expression, 8 hours post poly I:C administration. Data expressed as mean + SEM (n = 8-12 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated counterparts ++ $p < 0.01$ vs. vehicle-poly I:C-treated counterparts.

3.3.4 Poly I:C induced an increase in the expression of *CD11b* and reduced *BDNF* expression in the hippocampus, effects not altered by systemic URB597 administration

As already mentioned, hippocampal mRNA expression of acute inflammatory mediators returned to baseline levels of expression at 24 hours post poly I:C administration. However, previous studies have demonstrated that systemic administration of poly I:C-induced increases in mRNA expression of markers of glial activation while concurrently inducing a decrease in *BDNF* and *TrkB* expression in both the hippocampus and frontal cortex, at 24 hours post administration (Gibney *et al.*, 2013). Thus, we aimed to examine if similar effects occurred in our study and if this was modulated by systemic URB597 administration. Analysis reported a significant effect of poly I:C treatment on hippocampal mRNA expression of *CD11b* [$F_{2,19}=4.054$, $p <0.05$] and *BDNF* [$F_{2,18}=6.946$, $p <0.01$], while treatment just failed to alter hippocampal expression of *GFAP* [$F_{2,18}=3.346$, $p =0.058$], when compared to vehicle-saline-treated counterparts. However, this treatment regime failed to affect hippocampal expression of the *BDNF* receptor *TrkB* (Fig 3.4). Systemic administration of poly I:C significantly increased hippocampal expression of *CD11b* (marker of microglial activation) while concurrently inducing decreases in hippocampal expression of *BDNF*, when compared to vehicle-saline-treated counterparts (Fig 3.4). Such poly I:C-induced effects were not altered by prior systemic administration of URB597.

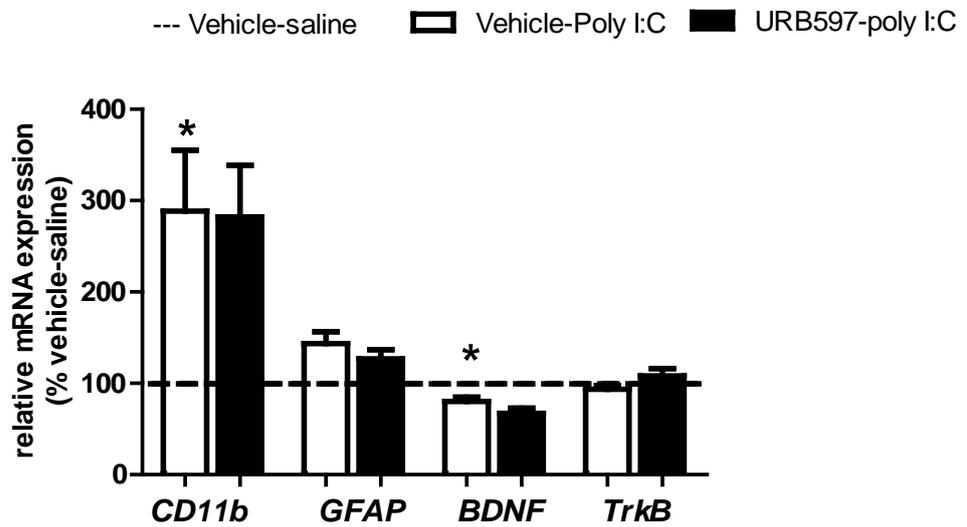


Figure 3.4 The effect of systemic administration of URB597 on hippocampal expression of *CD11b*, *GFAP*, *BDNF* and *TrkB*, 24 hours post systemic poly I:C administration. URB597 does not alter the poly I:C-induced increases in hippocampal expression of *CD11b* or *GFAP*, nor the poly I:C-induced decrease in expression of *BDNF*, 24 h post poly I:C. In addition, there was no effect of treatment on hippocampal expression of *TrkB*. Data expressed as mean + SEM (n = 8-12 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated counterparts.

3.3.5 URB597 attenuates the poly I:C-induced increases in the expression of *CD11b* in the frontal cortex

Similar to results reported in the hippocampus, the poly I:C-induced increases in frontal cortical mRNA expression of acute inflammatory mediators returned to baseline levels of expression at 24 hours post poly I:C administration. However at this timepoint, poly I:C-induced a significant increase in *CD11b* [$F_{2,15}=6.424$, $p < 0.01$] mRNA expression in the frontal cortex, when compared to vehicle-saline-treated counterparts. Prior administration of URB597 attenuated this poly I:C-induced increase in frontal cortical expression of *CD11b* (Fig 3.5). In addition, there was no significant effect of poly I:C on frontal cortical mRNA expression of *GFAP*, *BDNF* or its receptor *TrkB*, when compared to vehicle-saline-treated counterparts.

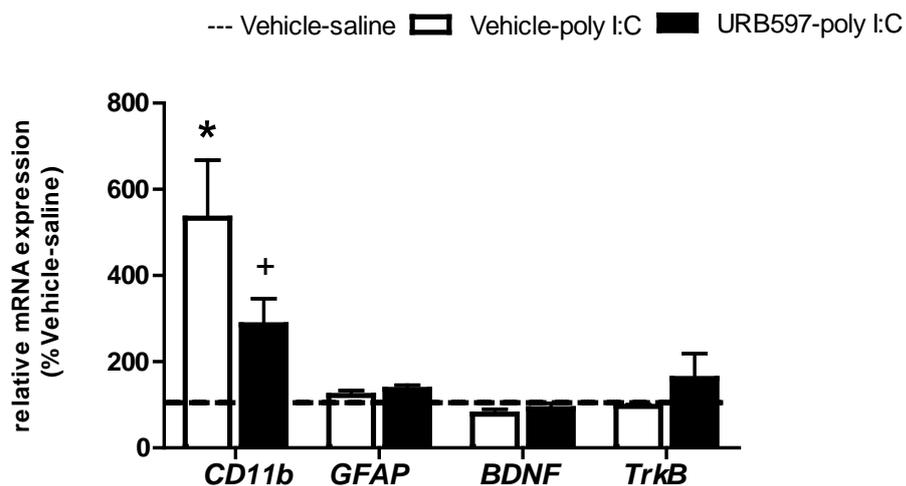


Figure 3.5 The effect of systemic administration of URB597 on frontal cortical expression of *CD11b*, *GFAP*, *BDNF* and *TrkB*, 24 hours post poly I:C. URB597 decreases the poly I:C-induced increases in frontal cortical expression of *CD11b*. In addition, there was no effect of treatment on *GFAP*, *BDNF* or *TrkB* expression in the frontal cortex. Data expressed as mean + SEM (n = 8-12 per group). * $p < 0.05$ vs. vehicle-saline-treated counterparts + $p < 0.01$ vs. vehicle-poly I:C-treated counterparts.

3.3.6 Systemic administration of URB597 increases hippocampal levels of the *N*-acylethanolamines OEA and PEA, but not AEA or 2-AG, at discrete time points following systemic administration of poly I:C

A one way ANOVA revealed a significant effect of URB597 on both OEA [$F_{8, 65} = 11.417$ $p < 0.01$] and PEA [$F_{8, 65} = 11.664$ $p < 0.01$] levels in the hippocampus (Fig 3.6b,c). *Post-hoc* analysis revealed that URB597 significantly increased the levels of OEA and PEA in the hippocampus at 2, 4 and 8 hours post poly I:C treatment when compared to both vehicle-saline- and vehicle-poly I:C-treated counterparts. Although there is a trend for URB597 to induce increases in hippocampal levels of OEA and PEA, when compared to their vehicle-saline-treated counterparts at 24 hours post poly I:C administration, this effect failed to reach statistical significance. In addition, there was no significant effect of URB597 on AEA or 2-AG in the hippocampus, at any of the time points examined in the present study (Fig 3.6a,d).

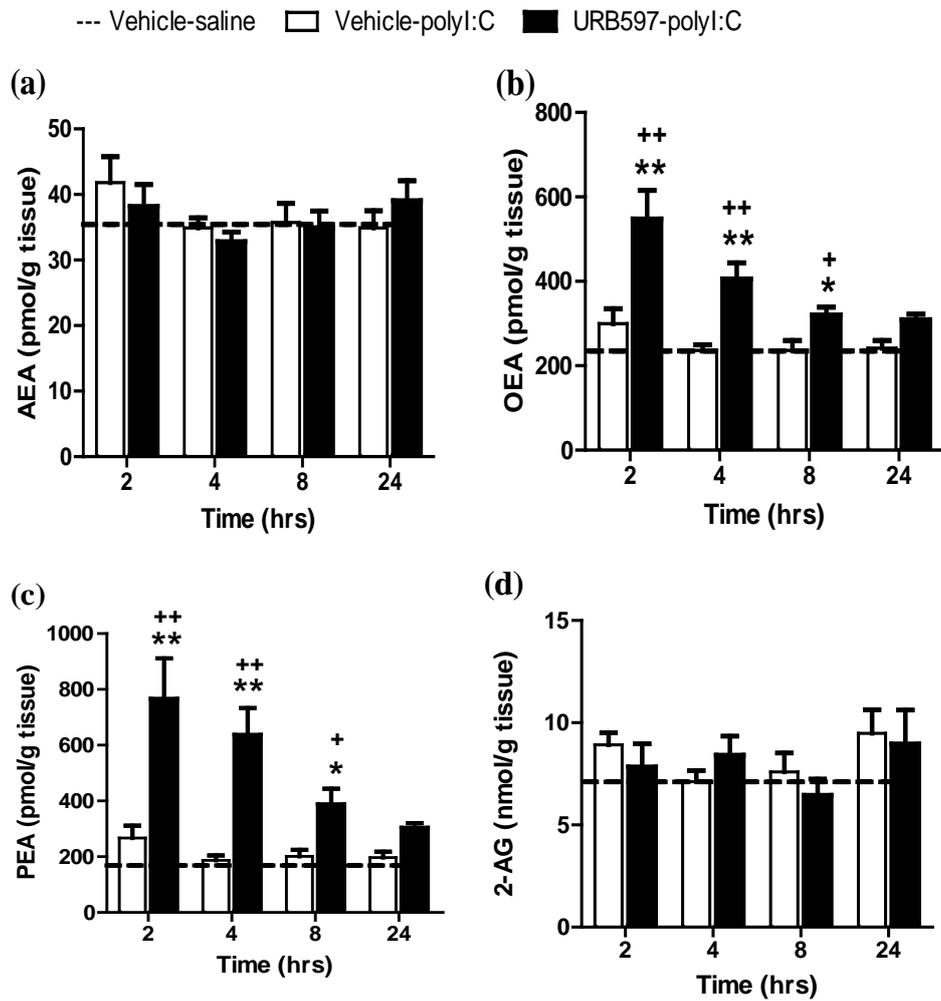


Figure 3.6 The effect of systemic administration of URB597 on hippocampal levels of AEA, OEA, PEA and 2-AG. URB597 increases hippocampal levels of (b) OEA and (c) PEA, but not (a) AEA or (d) 2-AG, when compared to vehicle-saline-treated counterparts, at 2, 4 and 8 hours post systemic poly I:C administration. URB597 does not significantly alter hippocampal levels of any of the metabolites examined, 24 hours post poly I:C administration. Data expressed as mean + SEM (n = 8-12 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated counterparts + $p < 0.05$ ++ $p < 0.01$ vs. vehicle-poly I:C-treated counterparts.

3.3.7 Systemic administration of URB597 increases levels of the *N*-acylethanolamines OEA and PEA, but not AEA or 2-AG in the frontal cortex, at discrete time points following systemic administration of poly I:C

A one way ANOVA revealed a significant effect of URB597 on both OEA [$F_{8, 67} = 14.031$ $p < 0.01$] and PEA [$F_{8, 67} = 13.719$ $p < 0.01$] levels in the frontal cortex (Fig 3.7b,c). *Post-hoc* analysis revealed that URB597 significantly increased the levels of OEA and PEA in the frontal cortex at 2, 4 and 8 hours post poly I:C treatment, when compared to saline-vehicle-treated counterparts. Like that reported in the hippocampus, there was a trend for URB597 to increase frontal cortical levels of OEA and PEA at 24 hours post poly I:C, however this effect failed to reach statistical significance. Furthermore, there was no significant effect of systemic administration of URB597 on AEA or 2-AG concentration in the frontal cortex at any of the time points examined (Fig 3.7a,d).

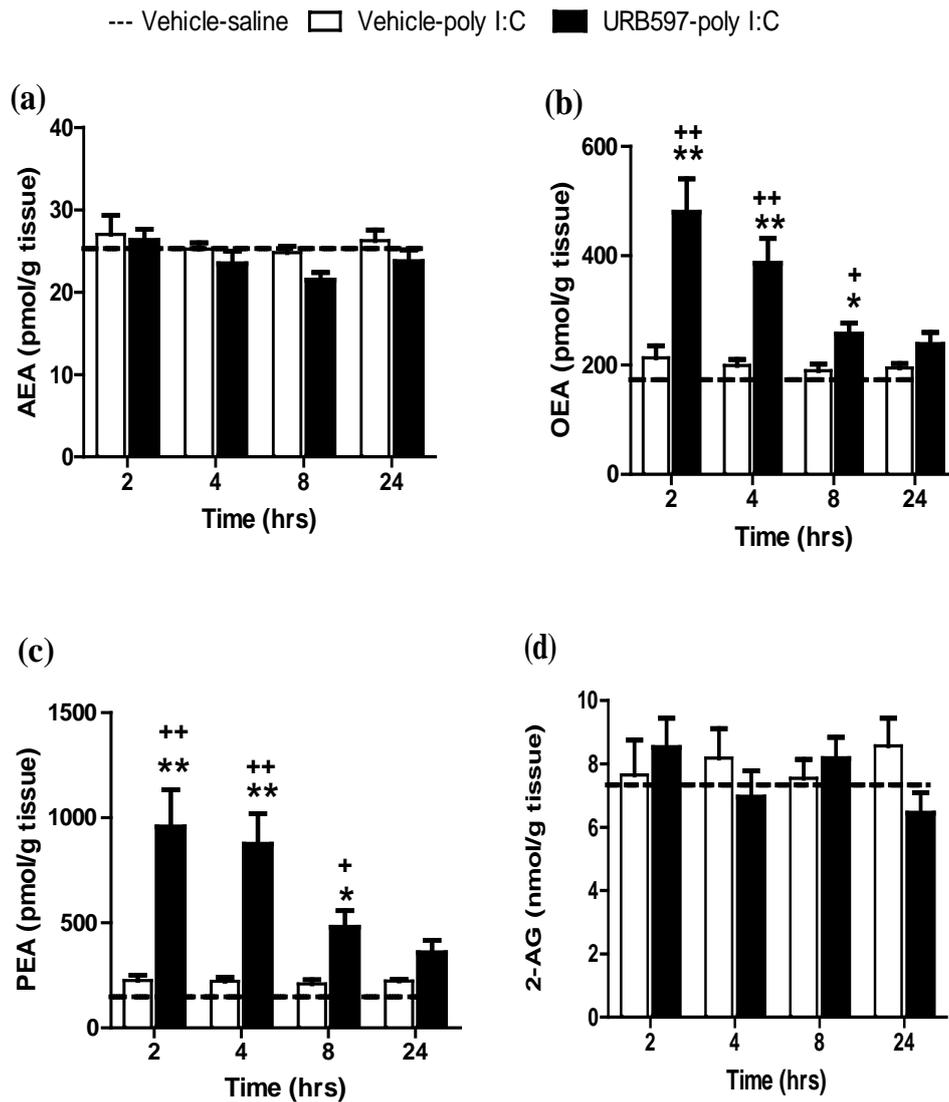


Figure 3.7 The effect of systemic administration of URB597 on AEA, OEA, PEA and 2-AG levels in the frontal cortex. URB597 increases frontal cortical levels of (b) OEA and (c) PEA, but not (a) AEA or (d) 2-AG, when compared to vehicle-saline-treated counterparts, at discrete time points following systemic poly I:C challenge. Data expressed as mean + SEM (n = 8-12 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated counterparts + $p < 0.05$ ++ $p < 0.01$ vs. vehicle-poly I:C-treated counterparts.

3.4 Discussion

The present study demonstrated that systemic administration of the FAAH inhibitor URB597 induced increases in brain (hippocampal and frontal cortical) levels of OEA and PEA, but not AEA, at discrete time points. Such increases in FAAH substrates were associated with a modulation of TLR3-induced increases in inflammatory mediators in both of these brain regions and also in the periphery. Specifically, prior systemic administration of URB597 attenuated the poly I:C-induced increases in serum levels of both IFN- γ and IL-6, at 4 and 8 hours post poly I:C administration. In addition, prior administration of URB597 was associated with an attenuation of hippocampal expression of the pro-inflammatory cytokines *IL-1 β* and *TNF α* , while concurrently inducing increases in both the type I IFN, *IFN- α* and the *NF- κ B*-inducible cytokine *IL-6*, at 4 hours post poly I:C administration. Similarly, URB597 enhanced the TLR3-induced increases in frontal cortical expression of *IL-6*, at 8 hours post poly I:C. Prior administration of URB597 attenuated frontal cortical, but not hippocampal expression of the microglial activation marker *CD11b*, at 24 hours post poly I:C administration, but failed to alter the poly I:C-induced decreases in hippocampal expression of the neurotrophic factor *BDNF*. Overall, these findings suggest a key immunomodulatory role for increased endogenous FAAH substrates, following systemic administration of URB597, on TLR3-induced increases in IFN- and *NF- κ B*-inducible inflammatory mediators, both in the periphery and within the CNS.

As previously described in Chapter 1, activation of TLR3 primarily results in increased production of type I IFNs which play a pivotal role in mediating the host's anti-viral responses and also late phase *NF- κ B*-inducible inflammatory mediators. It has previously been reported that systemic poly I:C administration induces robust increases in plasma levels of the type I IFN IFN- β and the *NF- κ B*-inducible genes *IL-1 β* , *IL-6* and *TNF α* , at discrete time points post systemic administration (Cunningham *et al.*, 2007; Fortier *et al.*, 2004). Accordingly, the current study demonstrated that systemic administration of the TLR3 agonist poly I:C increased serum levels of both IFN- and *NF- κ B*-inducible inflammatory mediators at discrete time points post administration. Specifically, we reported no effect of poly I:C on serum levels on any of the cytokines examined at 2 hours post poly I:C administration, however poly I:C increased serum levels of both IFN- γ and IL-6, at 4 and 8 hours post challenge, with levels returning to

baseline levels by 24 hours post poly I:C administration. However, this treatment regime failed to increase serum levels of either IL-1 β or TNF α at any of the time points examined in the current study. Other cytokines, chemokines and IFNs may also have been induced following TLR3 activation, however due to the limited amount of serum obtained in this study it was unfortunately not possible for us to measure additional inflammatory mediators in the current study. Examining the effect of enhancing FAAH substrate levels on TLR3-induced cytokine levels in serum revealed that prior systemic administration of URB597 partially attenuated the TLR3-induced increases in both IL-6 and IFN- γ at 4 and 8 hours. It has previously been demonstrated that both systemic or i.c.v. administration of URB597 potentiated the TLR4-induced increases in plasma levels of TNF α (De Laurentiis *et al.*, 2010; Roche *et al.*, 2008), while systemic administration of the endocannabinoid reuptake inhibitor AM404 attenuated TLR4-induced increases in plasma levels of both IL-1 β and IL-6 (Roche *et al.*, 2008). Such findings indicate that modulation of FAAH substrates, following either URB597 or AM404 administration, is associated with immunoregulatory effects on peripheral immune mediators induced following TLR4 activation. However, to our knowledge this is the first study to report that systemic administration of URB597 and subsequent increases in FAAH substrates (OEA, PEA and possibly AEA) is associated with immunoregulatory effects on TLR3-induced increases in serum cytokine levels. As stated above due to the limited amount of serum obtained in this study it was unfortunately not possible for us to measure these effects of URB597 on type I IFNs or other cytokines. However, although not presented in this thesis, recent work from within our group has demonstrated that systemic administration of URB597 and subsequent increases in splenic FAAH substrates failed to alter TLR3-induced increases in gene expression of *IFN- α* or several other NF- κ B-inducible genes at 4 hours post poly I:C administration (Flannery *et al.*, unpublished data), indicating that URB597 may not modulate TLR3-induced type I IFN induction. Peripherally produced cytokines are capable of signalling to the brain via several different routes including entry into the brain via the circumventricular organs, transported across the blood brain barrier via transport molecules expressed on brain endothelial cells, or cytokine-induced activation of the vagal communication pathway, for review see (Dantzer, 2004). This in turn results in microglial activation, the production of cytokines, modulation of neuronal function and the induction of a sickness response. Thus modulation of peripheral inflammatory

responses may in turn alter CNS function. Alternatively, certain pharmacological agents may mediate their effects on neuroinflammatory responses to acute infection directly within the brain, without significant effect on peripheral inflammatory responses. In this regard, it has previously been demonstrated that URB597 failed to alter TLR4-induced increases in plasma levels of TNF α , IL-1 β and IL-6, while concurrently attenuating and enhancing TLR4-induced increases in hypothalamic expression of *IL-1 β* and *SOCS3*, respectively (Kerr *et al.*, 2012). These data suggest that the reported immunoregulatory effects of increased FAAH substrates on TLR4-induced neuroinflammatory responses are mediated directly at the level of the brain, and not via indirect modulation of peripheral immune responses. In this respect, although the present study reported that systemic administration of URB597 partially modulated TLR3-induced increases in serum levels of IFN- γ and IL-6, whether this is responsible for subsequent TLR3-induced neuroinflammatory responses remains to be fully elucidated.

In addition to examining the effects on peripheral cytokines a primary focus of this study was to examine the effect of URB597 on hippocampal and frontal cortical expression of neuroinflammatory mediators, following TLR3 activation. Systemic administration of poly I:C robustly increased hippocampal expression of *IFN- α* and the NF- κ B-responsive genes, *TNF α* , *IL-1 β* and *IL-6* and the IFN-inducible chemokine *IP-10* and increased frontal cortical expression of *IP-10*, *TNF α* , *IL-1 β* and *IL-6*, effects which correlate with previous reports in both rats and mice (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013). The pattern of inflammatory gene expression, both in the hippocampus and frontal cortex, mirrored that of serum cytokine levels induced following systemic TLR3 activation, namely systemic administration of poly I:C did not alter either hippocampal or frontal cortical levels of inflammatory gene expression at 2 hours, while inflammatory gene (*TNF α* , *IL-1 β* , *IL-6* and *IP-10*) expression peaked at 4 and 8 hours and returned to baseline levels at 24 hours post poly I:C administration. This was the case for all genes examined except for hippocampal expression of *IFN- α* which peaked at 24 hours post poly I:C administration. In addition to inducing increases in both *IFN-* and *NF- κ B*-inducible genes within the brain this study also demonstrated that systemic administration of poly I:C was associated with an increase in expression of *CD11b* (marker of microglial activation) in both the hippocampus and frontal cortex and a concurrent decrease in hippocampal expression of *BDNF*, at 24 hours post poly I:C administration, which is in accordance with previously published data (Gibney *et al.*,

2013). Overall, these data and others (Gibney *et al.*, 2013) indicate lasting alterations in neuro-immune processing following acute TLR3 activation.

Although the effects of cannabinoids and endocannabinoid modulators, including URB597, on the expression of inflammatory cytokines in the brain following systemic administration of the TLR4 agonist LPS, has been demonstrated (Duncan *et al.*, 2013; Kerr *et al.*, 2012; Kerr *et al.*, 2013b; Roche *et al.*, 2008), there has been a paucity of studies examining if similar responses occur following activation of other TLRs. The synthetic cannabinoid WIN55,212-2 inhibits TLR3-induced NF- κ B activation and the expression of TNF α , while concurrently enhancing IRF3 signalling and IFN- β expression in a human astrocyte cell line (Downer *et al.*, 2011). TMEV induction of cytokines such as IL-6, IL-10, IL-12 and IL-23 is primarily mediated by TLR3 (So *et al.*, 2006) and modulated by enhancing AEA tone (Correa *et al.*, 2011; Molina-Holgado *et al.*, 1998). Furthermore, the inhibition of FAAH or direct administration of PEA has been shown to limit inflammation and ameliorate motor deficits in TMEV-infected rodents (Correa *et al.*, 2011; Hernangomez *et al.*, 2012; Loria *et al.*, 2010; Loria *et al.*, 2008; Mestre *et al.*, 2005; Ortega-Gutierrez *et al.*, 2005). Thus, modulation of type I IFNs and inflammatory cytokines may provide a novel therapeutic strategy for viral-induced neuroinflammation and associated neurodegeneration. The current data are the first to demonstrate that enhancing FAAH substrate levels *in vivo* can modulate the expression of TLR3-induced inflammatory genes in the brain. Specifically, the current data demonstrated that enhancing FAAH substrate levels following systemic administration of URB597 was associated with an increase in the hippocampal expression of the type I IFN, IFN- α , and IL-6, while concurrently attenuating the hippocampal expression of TNF α and IL-1 β , at 4 hours following TLR3 activation. In addition, increased FAAH substrates was associated with an increase in frontal cortical expression of IL-6, at 8 hours following systemic TLR3 activation; however frontal cortical expression of any of the other type I IFN- or NF- κ B inflammatory genes examined were not altered in the presence of increased FAAH substrates. The relative increases in FAAH substrates (OEA and PEA) in both the hippocampus and frontal cortex are almost identical, indicating that FAAH substrates at receptor targets would be capable of modulating TLR3-induced responses to a similar extent in both regions. However, it may be possible that although FAAH substrates are increased to the same extent, the density of receptor targets within each region may be different, which may in

turn account for the differential effects reported between the hippocampus and frontal cortex.

Although IFNs have been shown to elicit pro-inflammatory effects and deleterious effects on neuronal function, several lines of evidence also indicate anti-inflammatory effects associated with these immune modulators. For example, type I IFNs have been regarded as anti-inflammatory within the brain due to their ability to limit leukocyte infiltration (Prinz *et al.*, 2008) and reduce expression of pro-inflammatory cytokines including IL-1 β and TNF α (Teige *et al.*, 2006). Furthermore, enhancement of both type I IFNs limits inflammation and disease progression in models of multiple sclerosis (Bowen *et al.*, 2013; Lin *et al.*, 2007; Naves *et al.*, 2013). Thus, increasing type I IFN expression in combination with a reduction in pro-inflammatory cytokines may limit the neuroinflammatory and possibly neurodegenerative cascades in the hippocampus following TLR3 activation. Although an increase in *IL-6* expression in both the hippocampus and frontal cortex may seem at odds with this hypothesis, IL-6 activates STAT3 signalling cascades. Recent evidence has demonstrated that endocannabinoids released in response to electroacupuncture mediate neuroprotective effects in a model of ischemic reperfusion, via CB₁ receptor activation of STAT3 (Wang *et al.*, 2009; Zhou *et al.*, 2013). Interestingly, the inhibition of FAAH or direct administration of AEA has been shown to increase TLR4- and TMEV-induced production of the anti-inflammatory cytokine IL-10 in microglial cells (Correa *et al.*, 2011; Correa *et al.*, 2010). Thus, it may be likely that the reported immunoregulatory effects of increased FAAH substrates in the current study are being mediated via increases in IL-10, however further in depth studies are required to determine the precise mechanism by which FAAH substrates modulate TLR3-induced inflammatory responses in the brain.

In addition to modulating acute neuroinflammatory responses (4 hours post poly I:C challenge) this study also reported that systemic administration of URB597 was associated with an attenuation of TLR3-induced increase in frontal cortical expression of the microglial marker *CD11b*, at 24 hours post poly I:C administration. This TLR3-induced increase in expression of *CD11b* is likely mediated in a similar manner to that previously suggested (Gibney *et al.*, 2013) whereby the TLR3-induced increases in early expression of neuroinflammatory cytokines acted on corresponding receptors on microglia, inducing their activation and further cytokine production, which in turn led to

a feed-forward neuroinflammatory response and increases in hippocampal and frontal cortical expression of *CD11b* by 24 hours post poly I:C administration. The data presented here demonstrated that prior systemic administration of URB597 attenuated this TLR3-induced increase in the expression of *CD11b* in the frontal cortex, but not hippocampus. It has previously been reported that chronic administration of URB597 attenuated microglia activation, exemplified by a decrease in age-related increases in hippocampal expression of *CD11b*, major histocompatibility complex (*MHC*) class II and cluster of differentiation 68 (*CD68*) and cortical expression of *MHC II* cortex, which in turn was associated with beneficial effects on synaptic plasticity (Murphy *et al.*, 2012). It is possible that effects may have been observed in the hippocampus at a time point not examined in the present study. Alternatively, despite the immunomodulatory effects of URB597 on TLR3-induced cytokine expression, the magnitude of the inflammatory response in the hippocampus may have been too great for the increase in FAAH substrates to fully attenuate. Furthermore, given that all microglia, regardless of their activation state express *CD11b*, it is possible that URB597 may have induced a switch in the microglial phenotype to an M2 vs. M1 profile, thus generating a more anti-inflammatory effect. However, further studies are required in order to determine the long-term significance of modulating neuroinflammatory responses to TLR3 activation *in vivo*. In addition to examining the effects of systemic administration of URB597 on neuroinflammatory responses induced following poly I:C administration, we also examined the effect of such a treatment regime on hippocampal and frontal cortical expression of *BDNF* and its receptor *TrkB*. *BDNF* is a neurotrophic factor that plays a vital role in neurogenesis, neuronal growth and synaptic plasticity (Tapia-Arancibia *et al.*, 2004). This study reported that systemic administration of poly I:C induced decreases in hippocampal expression of *BDNF*, which is consistent with previous findings demonstrating that poly I:C induces decreases in expression of *BDNF* and *TrkB* in both the hippocampus and frontal cortex, at 24 hours post poly I:C administration (Gibney *et al.*, 2013). *IL-1 β* has been implicated as one of the primary cytokines involved in compromising the *BDNF* pathway where it has been reported to decrease hippocampal expression of *BDNF* (Barrientos *et al.*, 2004; Lapchak *et al.*, 1993). Although our data demonstrated that increased levels of FAAH substrates were associated with a robust reduction in the TLR3-induced increase in hippocampal expression of *IL-1 β* , this was not associated with an alteration in hippocampal

expression of *BDNF*. However, it is important to note that we have only examined mRNA expression and we cannot rule out the possibility that BDNF protein levels are not reduced/altered. Additionally, we cannot exclude the possibility that URB597-induced changes in BDNF are not occurring at earlier time points as it has been showed that poly I:C also induces decreases in expression at 6 hours post poly I:C administration (Gibney *et al.*, 2013).

It is important to note that the effects of URB597 on neuroinflammatory responses induced by/following TLR3 activation are accompanied by time-dependant increases in both hippocampal and frontal cortical levels of OEA and PEA up to 8 hours post administration. Although the endocannabinoid AEA is a FAAH substrate and reported to be increased in the rodent brain following systemic administration of URB597 (Fegley *et al.*, 2005; Kathuria *et al.*, 2003; Kerr *et al.*, 2012; Murphy *et al.*, 2012), we did not report increases in either hippocampal or frontal cortical levels of AEA at any of the time points examined in the current study. However, there are a number of differences between studies which may account for the reported discrepancies including the specific brain regions examined and the presence of an immune stimulus, where in accordance with the present findings, systemic administration of URB597 increased OEA and PEA, but not AEA levels in the dorsal hippocampus (Butler *et al.*, 2012). In addition, it is also possible that the increase in AEA occurred at an earlier time point (1hr) or was below the limit of detection given that whole tissue levels of the *N*-acylethanolamines, rather than synaptic levels, were evaluated. Furthermore, although URB597 is known to inhibit FAAH activity, several off-targets have been identified including serine hydrolases in peripheral tissues (Alexander *et al.*, 2005; Lichtman *et al.*, 2004; Zhang *et al.*, 2007) and also exhibits agonistic activity at the transient receptor potential (TRP) ion channel TRPA1 (Niforatos *et al.*, 2007), which may account for the lack of increases in AEA in the current study. In any case, the present data confirm that URB597 does inhibit FAAH in the CNS resulting in enhanced endogenous OEA and PEA (and possibly AEA) levels.

3.4.1 Conclusion

The data presented herein demonstrate that systemic administration of the FAAH inhibitor URB597 increases FAAH substrate levels and potently modulates TLR3-induced increases in acute inflammatory mediators both in the periphery and within the CNS. Overall, such findings provide further evidence that FAAH substrates exert a modulatory effect on TLR3-induced neuroinflammatory responses *in vivo*.

Chapter 4

FAAH substrate-mediated modulation of TLR3-induced neuroinflammation in the rat hippocampus and frontal cortex

4.1 Introduction

The hippocampus is a key brain structure within the cortico-limbic system involved in memory and learning processes and is a region affected in several neurological disorders (Hu *et al.*, 2014; Nikonenko *et al.*, 2009; Shankar, 2010; Sierra *et al.*, 2015) many of which have a key underlying inflammatory component. TLR3 activation following systemic administration of poly I:C results in robust increases in hippocampal expression of type I IFNs and NF- κ B-inducible pro-inflammatory cytokines (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013; Katafuchi *et al.*, 2003) which are reported to be associated with lasting alterations in neuro-immune function (Gibney *et al.*, 2013) and exacerbation of existing neurodegenerative processes within the hippocampus (Field *et al.*, 2010). Furthermore, TLR3-induced signalling within the hippocampus induces alterations in hippocampal glutamatergic transmission and spontaneous interictal-like activity (Costello *et al.*, 2013), both of which are known features of epileptogenesis (Frasca *et al.*, 2011), while activation of brain TLR3 has been reported to be associated with increases in long-term seizure susceptibility (Galic *et al.*, 2009). Further evidence implicating a detrimental role for TLR3 signalling within the hippocampus comes from a report demonstrating that TLR3 deficient mice exhibit enhanced hippocampal-dependent working memory, increased hippocampal cellular plasticity, increased hippocampal neurogenesis, and increased levels of the AMPA receptor subunit Glu1 in the CA1 region of the hippocampus (Okun *et al.*, 2010), while i.c.v. administration of the TLR3 agonist poly I:C induces impairments in working and contextual memory processes (Galic *et al.*, 2009; Okun *et al.*, 2010). In addition to the hippocampus, systemic TLR3 activation is reported to be associated with robust increases in expression of neuroinflammatory mediators, reactive oxygen species and neuronal death (Qin *et al.*, 2012), and also long-term alterations in neuro-immune function (Gibney *et al.*, 2013) within the frontal cortex. Thus, modulation of TLR3-induced inflammatory responses may provide novel therapeutic approaches for viral-induced neuroinflammation and associated neuronal alterations.

Accordingly, our results from Chapter 3 demonstrated key immunoregulatory effects of FAAH substrates on TLR3-induced neuroinflammatory responses within the hippocampus, with robust effects observed 4 hours post systemic poly I:C administration. Specifically, systemic administration of the FAAH inhibitor URB597

increased brain levels of the FAAH substrates OEA and PEA which in turn were associated with an increase in hippocampal expression of *IFN- α* and *IL-6* and a concurrent decrease in hippocampal expression of the pro-inflammatory cytokines *TNF α* and *IL-1 β* . These findings were the first to report that pharmacological modulation of FAAH substrates exerted robust immunoregulatory effects on TLR3-induced increases in hippocampal expression of neuroinflammatory mediators. Downer and colleagues previously demonstrated that the synthetic CB₁/CB₂ receptor agonist WIN,55,212-2 differentially modulated TLR-induced signalling, exemplified by an increase in IRF3 signalling and IFN- β expression, while concurrently attenuating TNF α production in astrocyte cultures (Downer *et al.*, 2011). In addition to modulating TLR3-induced increases in hippocampal expression of IFN- and NF- κ B-inducible inflammatory mediators, we also reported that systemic administration of URB597 attenuated serum levels of the type II IFN, IFN- γ and the NF- κ B-inducible cytokine IL-6 (Chapter 3). The question therefore arises as to whether the reported immunoregulatory effects of increased FAAH substrates on hippocampal expression of inflammatory mediators are due to indirect modulation of TLR3-induced increases in peripheral immune responses, or mediated directly at the level of the brain. This is an important question to address as several neuropathologies are reported to be associated with increases in FAAH substrate levels within the CNS; specifically, focal cerebral ischemia is associated with increases in OEA and PEA within the brain (Degn *et al.*, 2007; Franklin *et al.*, 2003) whereby systemic administration of the FAAH inhibitor URB597 has been found to decrease infarct volume (Degn *et al.*, 2007). In addition, increased cerebral spinal fluid (CSF) levels of AEA have been reported in epilepsy (Gesell *et al.*, 2013) and PD (Pisani *et al.*, 2010), increased spinal cord levels of PEA have been reported in the TMEV model of MS (Loria *et al.*, 2008) and increases in both AEA and PEA are observed in the chronic relapsing experimental allergic encephalomyelitis (CREAE) model of MS (Baker *et al.*, 2001). It has been proposed that the increases in these substrates may act as an endogenous compensatory mechanism to limit neuroinflammatory processes associated with CNS diseases and thus protect neuronal function. Thus, modulation of FAAH substrates directly within the brain may have important implications for targeting FAAH in the treatment of neuroinflammatory and neurodegenerative disorders.

Hypothesis: Based on these data we hypothesised that increasing FAAH substrates directly within the brain would reduce neuroinflammatory processes associated with TLR3 activation. Therefore, the aims of the studies described in this chapter were to:

1. Investigate the effect of intracerebroventricular (i.c.v.) administration of URB597, a selective FAAH inhibitor, on endocannabinoid and *N*-acylethanolamine levels in the hippocampus and frontal cortex, at 4 hours following systemic administration of the TLR3 agonist poly I:C.
2. Investigate the effect of systemic and i.c.v. administration of URB597 on TLR3-induced increases in expression of both IFN- and NF- κ B-inducible neuroinflammatory genes in the hippocampus and frontal cortex, at 4 hours post poly I:C administration.
3. Investigate the effect of systemic administration of the peripherally restricted FAAH inhibitor URB937 on FAAH substrate levels and expression of TLR3-induced increases in neuroinflammatory genes in the hippocampus, at 4 hours post systemic poly I:C administration.

4.2 Methodology and Experimental design

Experiments were carried out on male Sprague Dawley rats (weight 220-260g; Charles River, UK), housed singly in plastic bottomed cages (45 X 25 X 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 0800 to 2000 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 an i.p. injection of sterile saline (0.89% NaCl) for 3-4 days prior to experimentation in order 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 licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

4.2.1 *Experiment 1: Investigation of the effect of FAAH inhibition following systemic administration of URB597 on poly I:C-induced increases in inflammatory gene expression in the hippocampus and frontal cortex*

Rats were randomly assigned to one of three groups: Vehicle-Saline, Vehicle-poly I:C and URB597-poly I:C (n=6-8 per group). The FAAH inhibitor URB597 (1mg/kg i.p.) or vehicle (ethanol:cremaphor:saline; 1:1:18) were administered in a single, acute i.p. injection, in an injection volume of 2ml/kg followed 30 min later by a single, acute i.p. injection of poly I:C (3mg/kg i.p.) or saline vehicle (sterile 0.89% NaCl) administered in an injection volume of 1.5ml/kg. The dose of URB597 was determined on the basis of previous published work demonstrating that systemic administration of URB597 at this dose enhanced levels of AEA and the related *N*-acylethanolamines in the brain (Fegley *et al.*, 2005; Kathuria *et al.*, 2003; Kerr *et al.*, 2012) and also our previous findings in Chapter 3, sections 3.3.6 & 3.3.7. The dose and time of poly I:C administration were chosen on the basis of previous published work (Gibney *et al.*, 2013; Katafuchi *et al.*, 2005; Katafuchi *et al.*, 2003) and studies in Chapter 3 demonstrating enhanced cytokine expression in the brain; sections 3.3.2 & 3.3.3. Animals were sacrificed by decapitation at 4 hours post poly I:C/saline administration, the brain rapidly removed, hippocampus

and frontal cortex excised, snap-frozen on dry ice and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression. (The methodology was essentially as described in Chapter 2).

4.2.2 *Experiment 2: Investigation of the effect of FAAH inhibition within the brain following i.c.v. administration of URB597 on poly I:C-induced increases in inflammatory gene expression in the hippocampus and frontal cortex*

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol in Chapter 2), rats were randomly assigned into one of three treatment groups: Vehicle-Saline, Vehicle-poly I:C and URB597-poly I:C (n= 6-10 per group). Rats received a single, acute i.c.v. injection of URB597 (50µg) or vehicle (100% DMSO) in an injection volume of 5µl infused over 1 min, followed 30 min later by a single, acute i.p. injection of poly I:C (3mg/kg, i.p.) or sterile saline (0.89%) in an injection volume of 1.5ml/kg. The dose and time of URB597 was chosen on the basis of previous findings demonstrating blockade of receptor agonist effects *in vivo* (De Laurentiis *et al.*, 2010) and pilot work within our laboratory demonstrating increases in FAAH substrate levels within the brain. Animals were returned to their home cages and sacrificed by decapitation at 4 hours following poly I:C administration, brain removed, hippocampus and frontal cortex excised, snap-frozen and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression.

4.2.3 Experiment 3: Investigation of the effect of systemic administration of the peripherally restricted FAAH inhibitor URB937 on poly I:C-induced increases in inflammatory gene expression in the hippocampus

Rats were randomly assigned to one of two treatment groups: Vehicle-poly I:C and URB937-poly I:C (n = 8 per group). The peripherally restricted FAAH inhibitor URB937 (1mg/kg, Cayman Chemical) or vehicle (ethanol: cremophor: saline; 1:1:18) were administered in a single, acute i.p. injection, in an injection volume of 1ml/kg, followed 1 hour later by a single, acute i.p. injection of poly I:C (3mg/kg) or sterile saline (0.89% NaCl) in an injection volume of 1.5ml/kg. The dose of URB937 was chosen based on previous published work demonstrating increases in AEA levels and inhibition of FAAH activity outside of the CNS (Clapper *et al.*, 2010). Animals were sacrificed by decapitation at 4 hours post-poly I:C challenge, hippocampal tissue excised, dissected in half, snap-frozen on dry ice and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression.

4.2.4 Experiment 4: Investigation of the effect of i.c.v. administration of URB597 on hippocampal expression of IFN- and NF- κ B-inducible genes, in the absence of systemic administration of poly I:C

Rats were randomly assigned into one of two treatment groups: Vehicle-saline and URB597-saline (n = 4 per group). Rats received a single, acute i.c.v. injection of URB597 (50 μ g) or vehicle (100% DMSO) in an injection volume of 5 μ l infused over 1 min, followed 30 min later by a single, acute i.p. injection of sterile saline (0.89%; 3mg/kg) in an injection volume of 1.5ml/kg. Animals were returned to their home cages and sacrificed by decapitation at 4 hours following saline administration, brain removed, hippocampus excised, snap-frozen and stored at -80°C until assayed for cytokine expression.

4.2.5 Statistical Analysis

SPSS (IBM, New York, USA) statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro–Wilks and Levene test $p > 0.05$, respectively. When comparing the means of two unrelated groups, parametric data were analysed using unpaired t -test. One-way ANOVA was used to compare the mean of more than two groups on one factor. *Post-hoc* analysis was performed using Fisher's LSD test. Data were considered significant when $p < 0.05$. All graphs representing data were constructed using GraphPad Prism 5.0 and results expressed as group means + standard error of the mean (SEM).

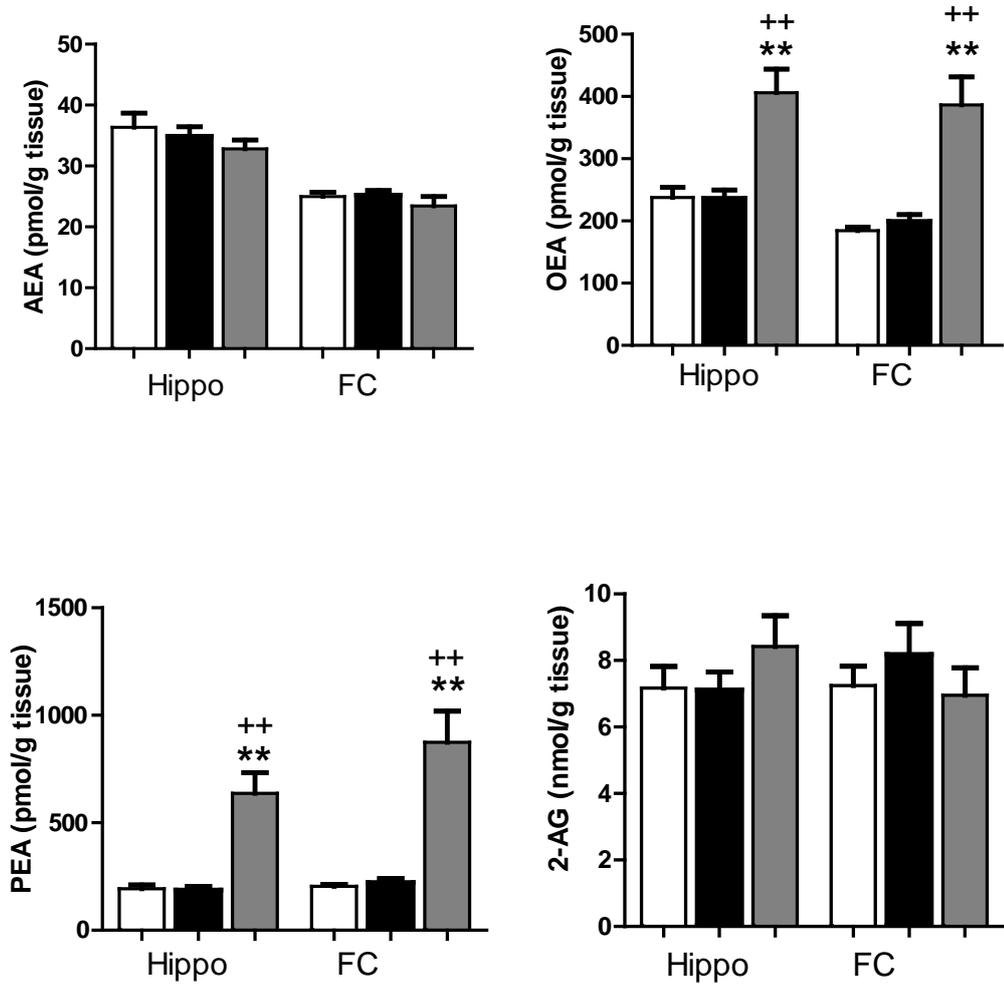
4.3 Results

4.3.1 Experiment 1 & 2: Systemic and i.c.v. administration of URB597 enhances OEA and PEA levels in the hippocampus and frontal cortex

Systemic administration of poly I:C did not alter levels of the endocannabinoids or *N*-acylethanolamines in the hippocampus or frontal cortex at 4 hours following administration. Systemic and i.c.v. administration of URB597 increased OEA [i.p.: $F_{2,25}=16.198$, $p < 0.001$; i.c.v.: $F_{2,19}=15.695$, $p < 0.001$] and PEA [i.p. $F_{2,25}=24.835$, $p < 0.001$; i.c.v. $F_{2,19}=9.036$, $p < 0.01$] levels in the hippocampus, when compared to both vehicle-saline- and vehicle-poly I:C-treated counterparts (Fig 4.1). In addition, both treatment regimes significantly increased levels of OEA [i.p.: $F_{2,25}=16.198$, $p < 0.01$; i.c.v. $F_{3,31}=5.633$, $p < 0.01$] and PEA [i.p.: $F_{2,25}=24.835$, $p < 0.01$; i.c.v. $F_{3,31}=6.270$, $p < 0.01$] in the frontal cortex, when compared to vehicle-saline- and vehicle-poly I:C-treated counterparts (Fig 4.1). URB597 did not alter levels of AEA or 2-AG in the hippocampus or frontal cortex of poly I:C-treated animals following either systemic or central administration (Fig 4.1).

□ vehicle-saline ■ vehicle-poly I:C ▒ URB597-poly I:C

(a) URB597 (i.p.)



(b) URB597 (i.c.v.)

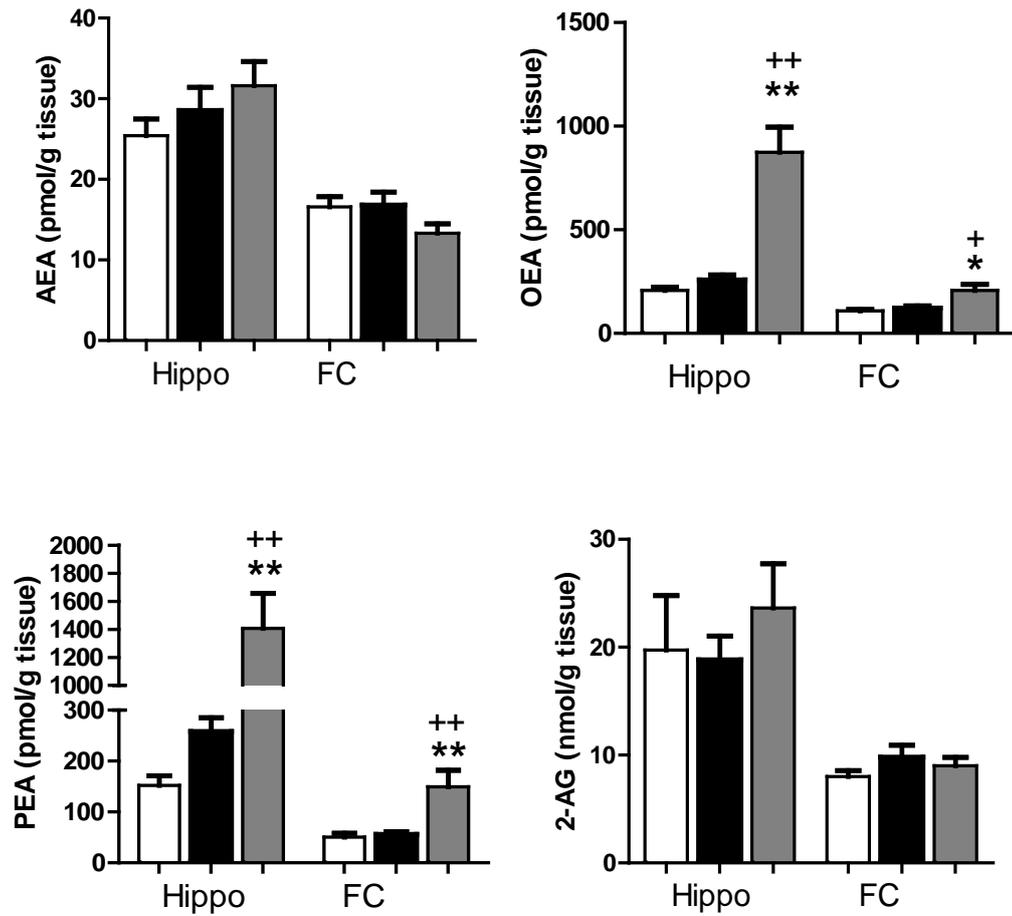


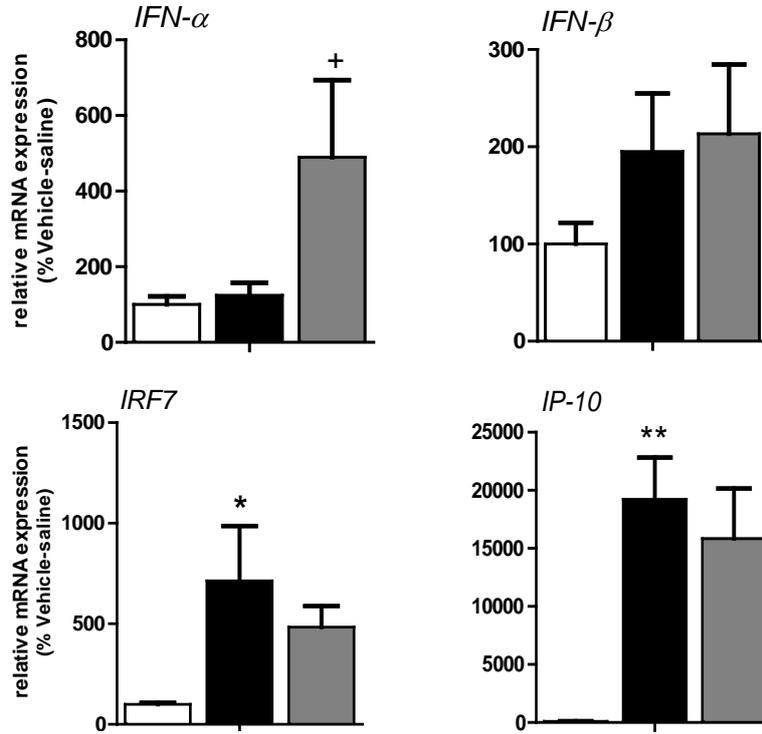
Figure 4.1 The effect of systemic and i.c.v. administration of URB597 on hippocampal and frontal cortical (FC) levels of AEA, OEA, PEA and 2-AG. Both (a) systemic and (b) i.c.v. administration of URB597 increase hippocampal and FC levels of the *N*-acylethanolamines, OEA and PEA, but not AEA or 2-AG, 4 hours post poly I:C administration. Data expressed as mean + SEM (n = 6-10 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated animals + $p < 0.05$ ++ $p < 0.01$ vs. vehicle-poly I:C-treated animals.

4.3.2 Experiment 1 & 2: Differential effects of systemic and i.c.v. administration of URB597 on hippocampal expression of type I IFN-responsive genes following poly I:C administration

Systemic administration of poly I:C did not significantly alter the mRNA expression of the type I IFNs; *IFN- α* and *IFN- β* , however mRNA expression of the IFN-induced transcription factor (*IRF7*) [i.p.: $F_{2,12}=4.137$, $p < 0.05$; i.c.v.: $F_{2,20}=8.866$, $p < 0.01$] and the chemokine *IP-10* [i.p. $F_{2,21}=18.121$, $p < 0.01$; i.c.v.: $F_{2,20}=21.090$, $p < 0.001$] were significantly increased when compared to vehicle-saline-treated counterparts (Fig 4.2). The robust transcription of *IRF7* and *IP-10* suggests that *IFN- α/β* are produced at the mRNA and protein level in the hippocampus following systemic poly I:C administration, possibly at an earlier time point than that examined in the present study, and were active at the type I IFN receptor. Systemic administration of the FAAH inhibitor URB597 robustly increased *IFN- α* expression [$F_{2,24}=3.981$, $p < 0.05$] in the hippocampus of poly I:C-treated animals, an effect not observed following i.c.v. administration (Fig 4.2). In comparison, i.c.v. administration of URB597 partially attenuated the poly I:C-induced increase in *IP-10* expression, with a similar effect also observed on *IRF7* expression, although this just failed to reach statistical significance ($p = 0.059$).

□ Vehicle-saline ■ Vehicle-poly I:C ▒ URB597-poly I:C

(a) URB597 (i.p.)



(b) URB597 (i.c.v.)

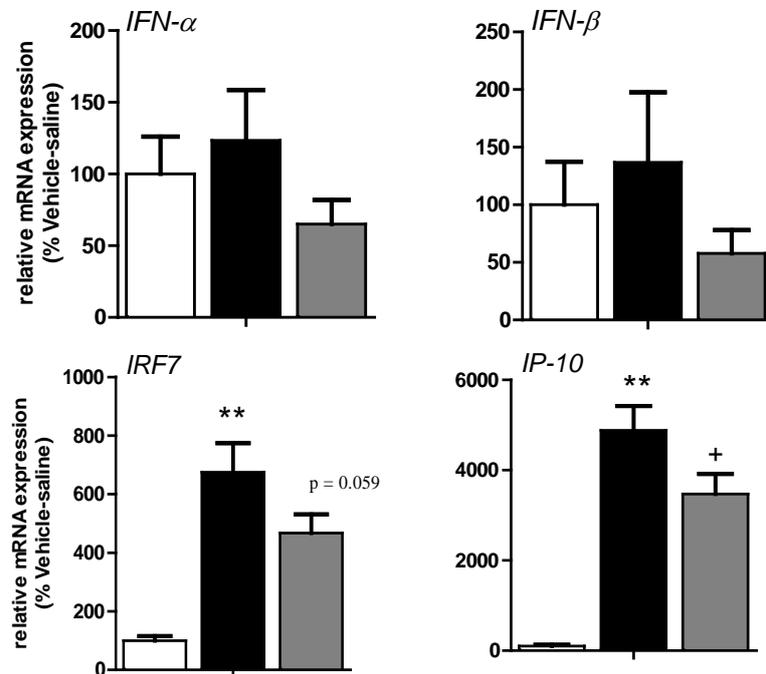


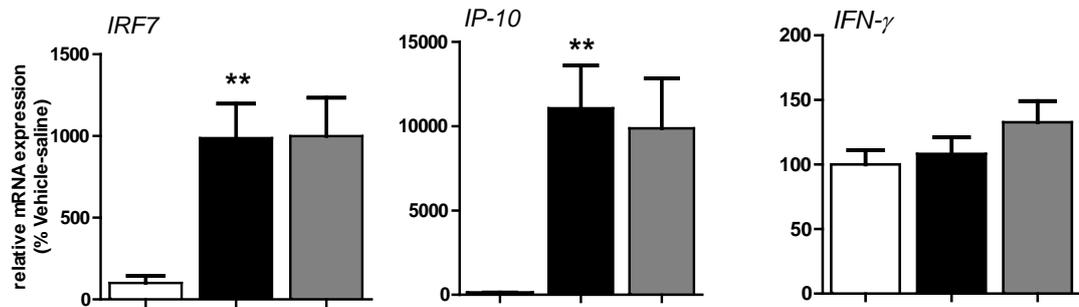
Figure 4.2 Prior (a) systemic and (b) i.c.v. administration of URB597 alters hippocampal expression of the IFN-related genes, 4 hours post poly I:C administration. Data expressed as mean + SEM (n = 6-10 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated animals + $p < 0.05$ vs. vehicle-poly I:C-treated animals.

4.3.3 Experiment 1 & 2: I.c.v., but not systemic, administration of URB597 attenuates TLR3-induced increases in *IRF7* and *IP-10* expression in the frontal cortex

Although there was a trend for systemic administration of URB597 to attenuate frontal cortical mRNA expression of both *IFN- α* and *IFN- β* , at 4 hours post poly I:C administration as observed in Chapter 3, this was not significant. As such, in this study we examined the mRNA expression of downstream genes of the type I IFN signalling pathway including *IRF7* and *IP-10* as indicators of IFN signalling in this brain region and also the type II IFN *IFN- γ* . Analysis revealed a significant effect of treatment on frontal cortical expression of the *IFN*-inducible genes *IRF7* [i.p.: $F_{2,10}=6.680$, $p < 0.05$; i.c.v.: $F_{2,15}=12.198$, $p < 0.01$] and *IP-10* [i.p.: $F_{2,25}=10.861$, $p < 0.001$; i.c.v.: $F_{2,20}=46.785$, $p < 0.001$], but not *IFN- γ* , at 4 hours post poly I:C administration. Prior i.c.v., but not systemic, administration of URB597 attenuated the poly I:C-induced increases in frontal cortical expression of *IRF7* and *IP-10* (Fig 4.3).

□ Vehicle-saline ■ Vehicle-poly I:C ▒ URB597-poly I:C

(a) URB597 (i.p.)



(b) URB597 (i.c.v.)

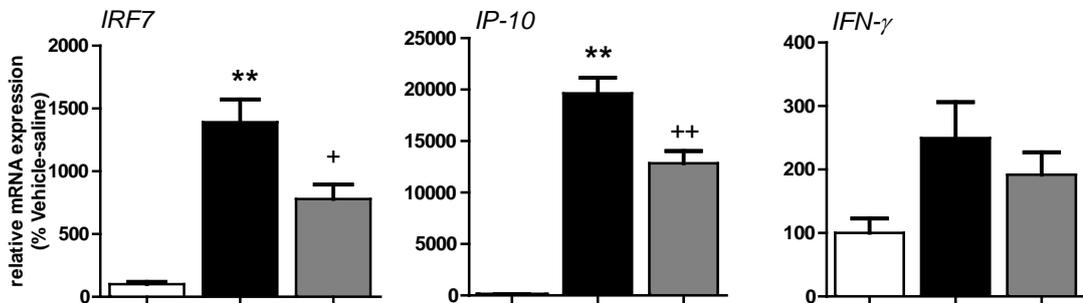


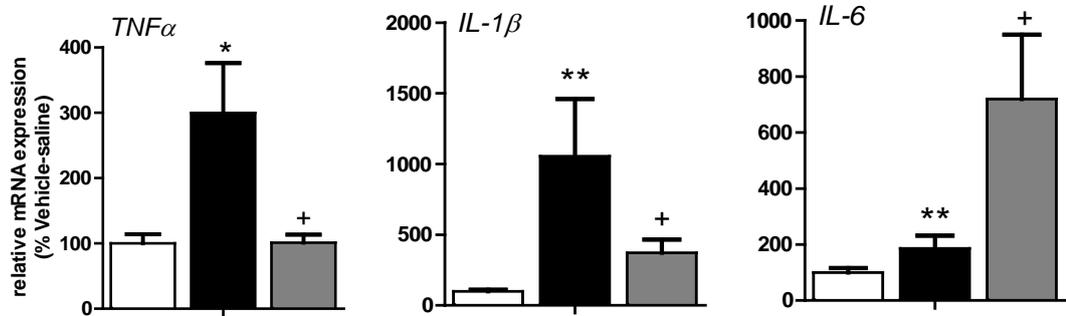
Figure 4.3 The effect of systemic and i.c.v. administration of URB597 on frontal cortical expression of *IRF7*, *IP-10* and *IFN-γ*, 4 hours post poly I:C administration. Prior (b) i.c.v., but not (a) systemic administration of URB597 attenuates the poly I:C-induced increases in expression of the IFN-inducible inflammatory genes *IRF7* and *IP-10*, 4 hours post poly I:C administration. In comparison, there was no effect of either treatment on *IFN-γ* expression. Data expressed as mean + SEM (n = 6-10 per group). ** $p < 0.01$ vs. vehicle-saline-treated animals + $p < 0.05$ ++ $p < 0.01$ vs. vehicle-poly I:C-treated animals.

4.3.4 Experiment 1 & 2: Systemic and i.c.v. administration of URB597 modulates the expression of poly I:C-induced increases in NF-κB-inducible pro-inflammatory cytokines in the rat hippocampus

TLR3 activation stimulates both the IRF3 and NF-κB signal transduction pathways. As shown in Fig 4.4, systemic administration of poly I:C significantly increased hippocampal mRNA expression of the NF-κB-dependant pro-inflammatory cytokines *TNFα*, *IL-1β* and *IL-6*, 4 hours post administration (vehicle-saline vs. vehicle-poly I:C; Fig 4.4). Both systemic [$F_{2,23} = 7.112$, $p < 0.01$] and i.c.v. [$F_{2,19} = 4.550$, $p < 0.05$] administration of URB597 attenuated the poly I:C-induced increase in *TNFα* expression in the hippocampus (Fig 4.4). The poly I:C-induced increase in hippocampal *IL-1β* expression was attenuated by systemic [$F_{2,22} = 5.995$, $p < 0.01$], but not i.c.v. administration of URB597 (Fig 4.4). In comparison, the increase in hippocampal *IL-6* expression following poly I:C administration was augmented in rats receiving systemic URB597 [$F_{2,20} = 6.009$, $p < 0.01$], while i.c.v. administration of URB597 tended to attenuate this response (Fig 4.4).

□ Vehicle-saline ■ Vehicle-poly I:C ▒ URB597-poly I:C

(a) URB597 (i.p.)



(b) URB597 (i.c.v.)

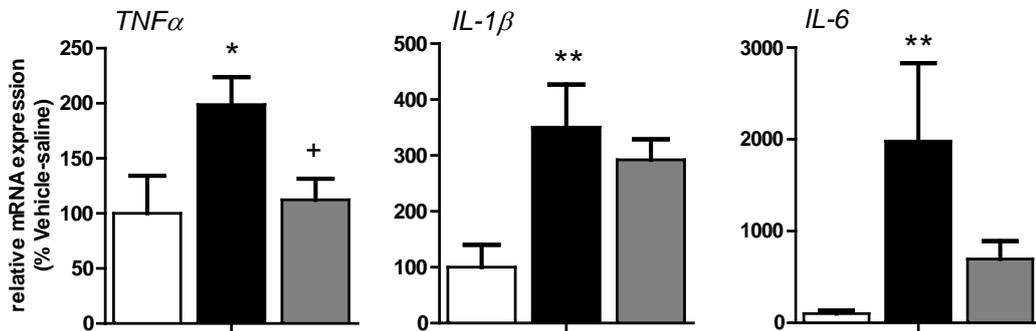


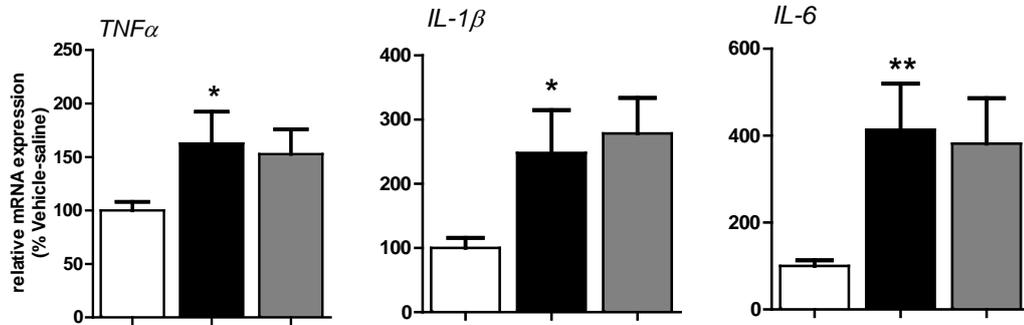
Figure 4.4 The effect of systemic and i.c.v. administration of URB597 on hippocampal expression of *TNFα*, *IL-1β* and *IL-6*, 4 hours post poly I:C administration. Both (a) systemic and (b) i.c.v administration of URB597 attenuates hippocampal expression of *TNFα*, while only (a) systemic URB597 attenuates hippocampal expression of *IL-1β*, while concurrently enhancing expression of *IL-6*, 4 hours following poly I:C administration. Data expressed as mean + SEM (n = 6-10 per group). * p < 0.05 ** p < 0.01 vs. vehicle-saline-treated animals + p < 0.05 vs. vehicle-poly I:C-treated animals.

4.3.5 Experiment 1 & 2: I.c.v., but not systemic, administration of URB597 attenuates poly I:C-induced increases in *TNF α* expression in the frontal cortex

In addition to inducing IFN-responsive genes in the frontal cortex, systemic administration of poly I:C significantly increased frontal cortical mRNA expression of *TNF α* [i.p. : $F_{2,24}=3.558$, $p < 0.05$; i.c.v: $F_{2,20}=4.630$, $p < 0.05$], *IL-1 β* [i.p. : $F_{2,25}=5.122$, $p < 0.05$; i.c.v: $F_{2,20}=6.066$, $p < 0.01$] and *IL-6* [i.p.: $F_{2,25}=6.007$, $p < 0.01$; i.c.v: $F_{2,20}=4.059$, $p < 0.05$], compared to vehicle-saline-treated counterparts, 4 hours post administration (Fig 4.5). Prior i.c.v., but not systemic administration of URB597 significantly attenuated the poly I:C-induced increases in frontal cortical expression of *TNF α* , while neither treatment regimes significantly altered the poly I:C-induced increases in frontal cortical expression of *IL-1 β* or *IL-6* (Fig 4.5).

□ Vehicle-saline ■ Vehicle-poly I:C ▒ URB597-poly I:C

(a) URB597 (i.p.)



(b) URB597 (i.c.v.)

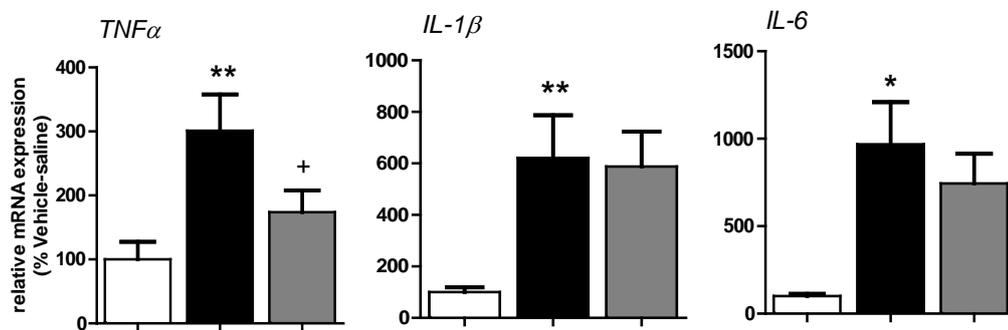


Figure 4.5 The effect of systemic and i.c.v. administration of URB597 on frontal cortical expression of the NF- κ B-inducible genes *TNF α* , *IL-1 β* and *IL-6*, 4 hours post poly I:C administration. Prior (b) i.c.v., but not (a) systemic, administration of URB597 significantly attenuated the poly I:C-induced increase in *TNF α* expression in the frontal cortex. Neither systemic or i.c.v. administration altered the poly I:C-induced increases in *IL-1 β* or *IL-6*. Data expressed as mean + SEM (n = 6-10 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated animals + $p < 0.05$ vs. vehicle-poly I:C-treated animals.

4.3.6 Experiment 1 & 2: Evidence of further anti-inflammatory effects in the hippocampus following i.c.v., but not systemic, administration of URB597

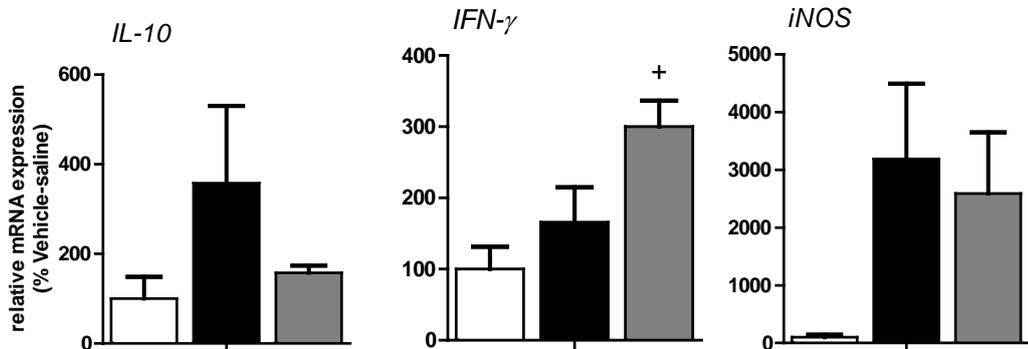
Because the most prominent immunoregulatory effects on TLR3-induced neuroinflammatory responses were observed in the hippocampus, following both systemic and i.c.v. administration of URB597, it was decided to examine downstream molecular targets in the TLR3 signalling cascade in the hippocampus only. Accordingly, the mRNA expression of the anti-inflammatory cytokine *IL-10* in the hippocampus following systemic administration of poly I:C tended to be increased (3-4 fold), although this effect was not statistically significant when compared to saline-treated counterparts (Fig 4.6). However, i.c.v., but not systemic, administration of URB597 significantly increased the expression of *IL-10* in the hippocampus of poly I:C-treated rats, when compared to both vehicle-saline- and vehicle-poly I:C-treated counterparts [$F_{2,17}=8.346$, $p < 0.01$].

Expression of the type II IFN *IFN- γ* , was significantly increased in poly I:C-treated animals following systemic administration of URB597 [URB597-poly I:C vs. Vehicle-poly I:C : $F_{2,10}=5.310$, $p < 0.05$]. In comparison, i.c.v. administration of URB597, attenuated the poly I:C-induced increase in *IFN- γ* expression in the hippocampus [$F_{2,17}=4.491$, $p < 0.05$] (Fig 4.6).

Poly I:C induced an increase in *iNOS* expression [$F_{2,18}=5.579$, $p < 0.05$] in the hippocampus, that was unaltered by prior systemic or i.c.v. administration of URB597 (Fig 4.6).

□ Vehicle-saline ■ Vehicle-poly I:C ▒ URB597-poly I:C

(a) URB597 (i.p.)



(b) URB597 (i.c.v.)

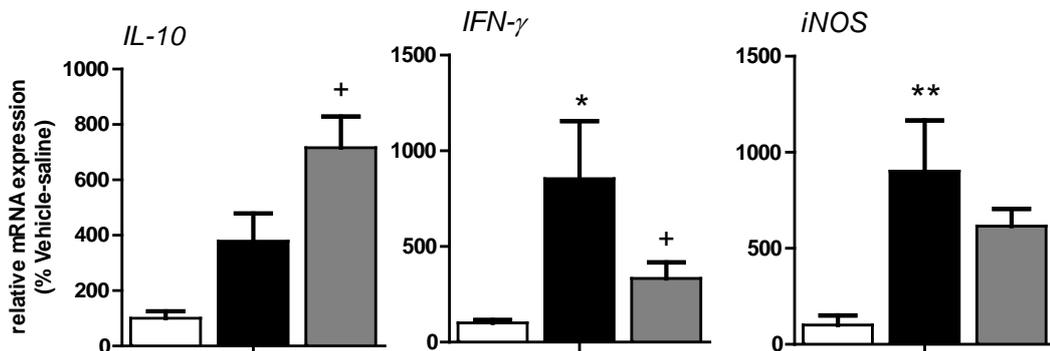


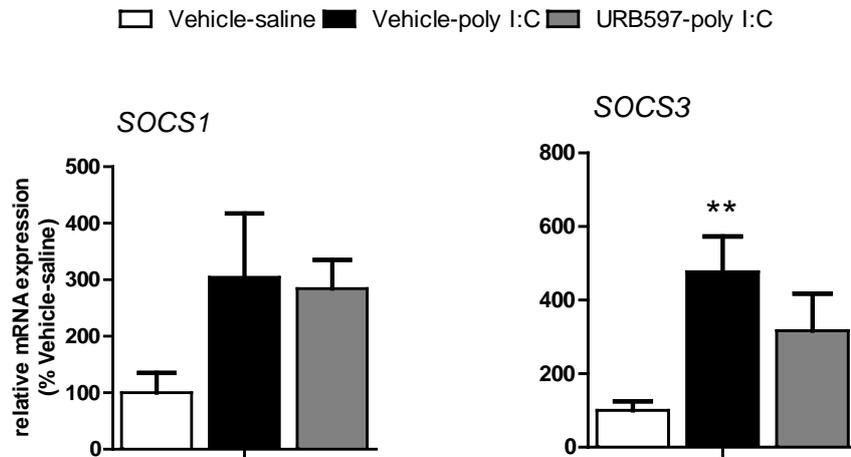
Figure 4.6 The effect of systemic and i.c.v. administration of URB597 on hippocampal expression of *IL-10*, *IFN-γ* and *iNOS*, 4 hours post poly I:C administration. (b) i.c.v. administration of URB597 enhances hippocampal expression of *IL-10*, while concurrently attenuating expression of *IFN-γ*, when compared to vehicle-poly I:C-treated counterparts. In comparison, (a) systemic administration of URB597 increases expression of *IFN-γ*, but not *IL-10*, when compared to vehicle-poly I:C-treated counterparts. Neither (a) systemic or (b) i.c.v. administration of URB597 altered hippocampal expression *iNOS*. Data expressed as mean + SEM (n = 6-10 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated animals + $p < 0.05$ vs. vehicle-poly I:C-treated animals.

4.3.7 Experiment 1 & 2: I.c.v. administration of URB597 attenuates poly I:C-induced increases in *SOCS1*, but not *SOCS3*, expression in the hippocampus

Inflammation associated increases in cytokines and chemokines are self-regulated by concurrent increases in the expression of suppressors of cytokine signalling (SOCS), namely *SOCS1* which primarily acts to inhibit the biological activity of IFN- α and IFN- γ ; and *SOCS3* which primarily acts to inhibit IL-6 signalling. Thus, a further aim of this study was to examine if such regulatory mechanisms were altered by systemic or i.c.v. administration of URB597.

Systemic administration of poly I:C induced a robust increase in mRNA expression of both *SOCS1* [i.p. $F_{2,12}=3.759$, $p = 0.05$; i.c.v.: $F_{2,21}=10.736$, $p < 0.01$] and *SOCS3* [i.p. $F_{2,11}=6.790$, $p < 0.05$; i.c.v.: $F_{2,19}=8.095$, $p < 0.05$] in the hippocampus. Prior systemic administration of URB597 did not alter the poly I:C-induced increase in the expression of either the *SOCS 1* or *3* gene. In comparison, i.c.v. administration of URB597 significantly attenuated the poly I:C-induced increase in the expression of *SOCS1* (Vehicle-poly I:C vs. URB597-poly I:C) (Fig 4.7).

(a) URB597 (i.p.)



(b) URB597 (i.c.v.)

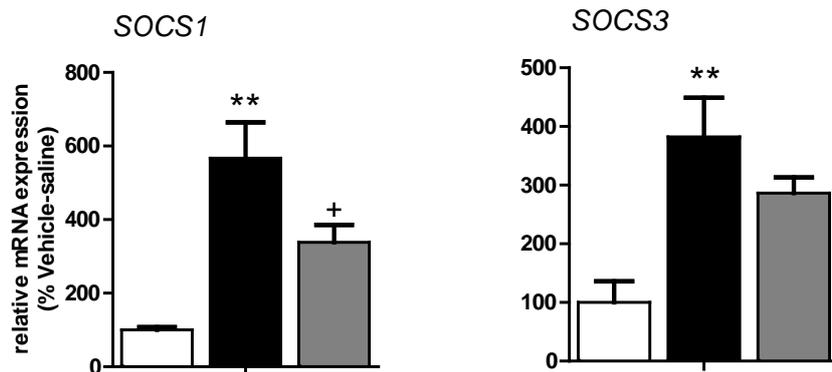
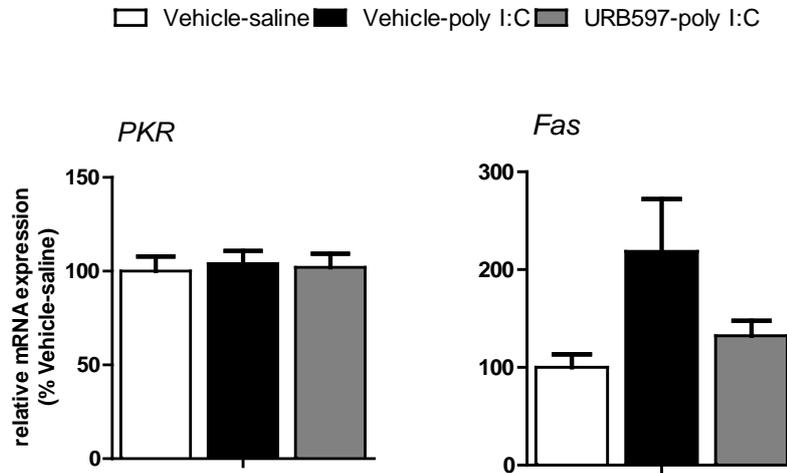


Figure 4.7 The effect of systemic and i.c.v. administration of URB597 on hippocampal expression of *SOCS1* and *SOCS3*, 4 hours post poly I:C administration. Prior (b) i.c.v., but not (a) systemic, administration of URB597 attenuates poly I:C-induced increases in *SOCS1*, but not *SOCS3*, expression in the hippocampus. Data expressed as mean + SEM (n = 6-10 per group). ** $p < 0.01$ vs. vehicle-saline-treated animals + $p < 0.05$ vs. vehicle-poly I:C-treated animals.

4.3.8 Experiment 1 & 2: URB597 does not alter poly I:C-induced increases in the pro-apoptotic gene *Fas* in the hippocampus

Activation of the type I IFN receptor (IFNR1) by IFN- α/β results in increased transcription of anti-viral genes including interferon-induced dsRNA-dependent protein kinase (PKR) which in turn can induce the expression of the pro-apoptotic genes *Fas* and *Bax* and activation of caspase 3 signalling resulting in cell loss. As such, a further aim was to examine if the neuroimmunomodulatory effects of URB597 were associated with changes in mRNA expression of the pro-apoptotic genes *PKR* and *Fas*. Systemic administration of poly I:C increased the expression of *Fas* [$F_{2,20}=4.686$, $p < 0.05$], but not *PKR*, in the hippocampus (Fig 4.8). Although systemic administration of URB597 tended to reduce the poly I:C-induced increase in *Fas*, the effect failed to reach statistical significance [$F_{2,13}=3.339$, $p = 0.068$]. I.c.v. administration of URB597 did not alter poly I:C-induced increases in *Fas* expression (Fig 4.8).

(a) URB597 (i.p.)



(b) URB597 (i.c.v.)

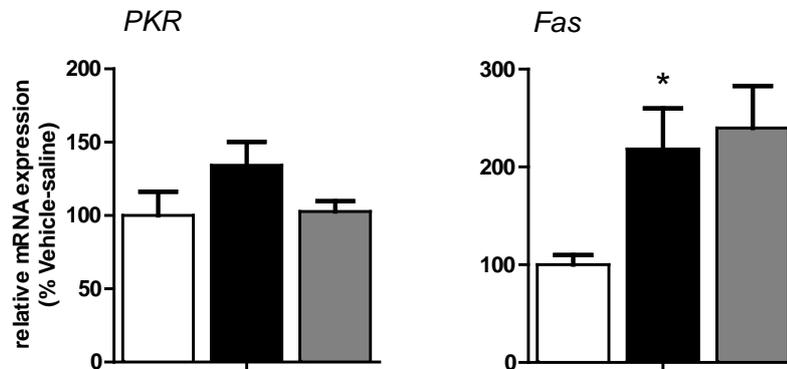


Figure 4.8 The effect of systemic and i.c.v. administration of URB597 on hippocampal expression of the pro-apoptotic genes *PKR* and *Fas*, 4 hours post poly I:C administration. (b) i.c.v. administration of URB597 does not alter the poly I:C-induced increases in hippocampal expression of *Fas*. In addition, there was no effect of either (a) systemic or (b) i.c.v. administration of URB597 on *PKR* expression, compared to vehicle-poly I:C-treated counterparts. Data expressed as mean + SEM (n = 6-10 per group). * $p < 0.05$ vs. vehicle-saline-treated animals.

4.3.9 Experiment 3: Systemic administration of the peripherally restricted FAAH inhibitor URB937 does not alter hippocampal levels of FAAH substrates, nor poly I:C-induced increases in hippocampal expression of IFN- or NF- κ B-inducible genes

In order to further determine if the potent immunoregulatory effects of systemic and i.c.v. administration of URB597 were mediated by enhancement of FAAH substrates directly within the brain, the effect of systemic administration of the peripherally restricted FAAH inhibitor URB937 on TLR3-induced inflammatory responses within the hippocampus was examined. Analysis revealed that systemic administration of URB937 failed to significantly alter hippocampal levels of the FAAH substrates or 2-AG, when compared to their vehicle-poly I:C-treated counterparts, 4 hours post poly I:C administration (Fig 4.9a). Furthermore, systemic administration of URB937 did not significantly alter the poly I:C-induced increase in hippocampal mRNA expression of the *IFN- γ* , *IP-10* or the NF- κ B-inducible cytokines *TNF α* , *IL-1 β* and *IL-6*, 4 hours post systemic poly I:C administration (Fig 4.9b).

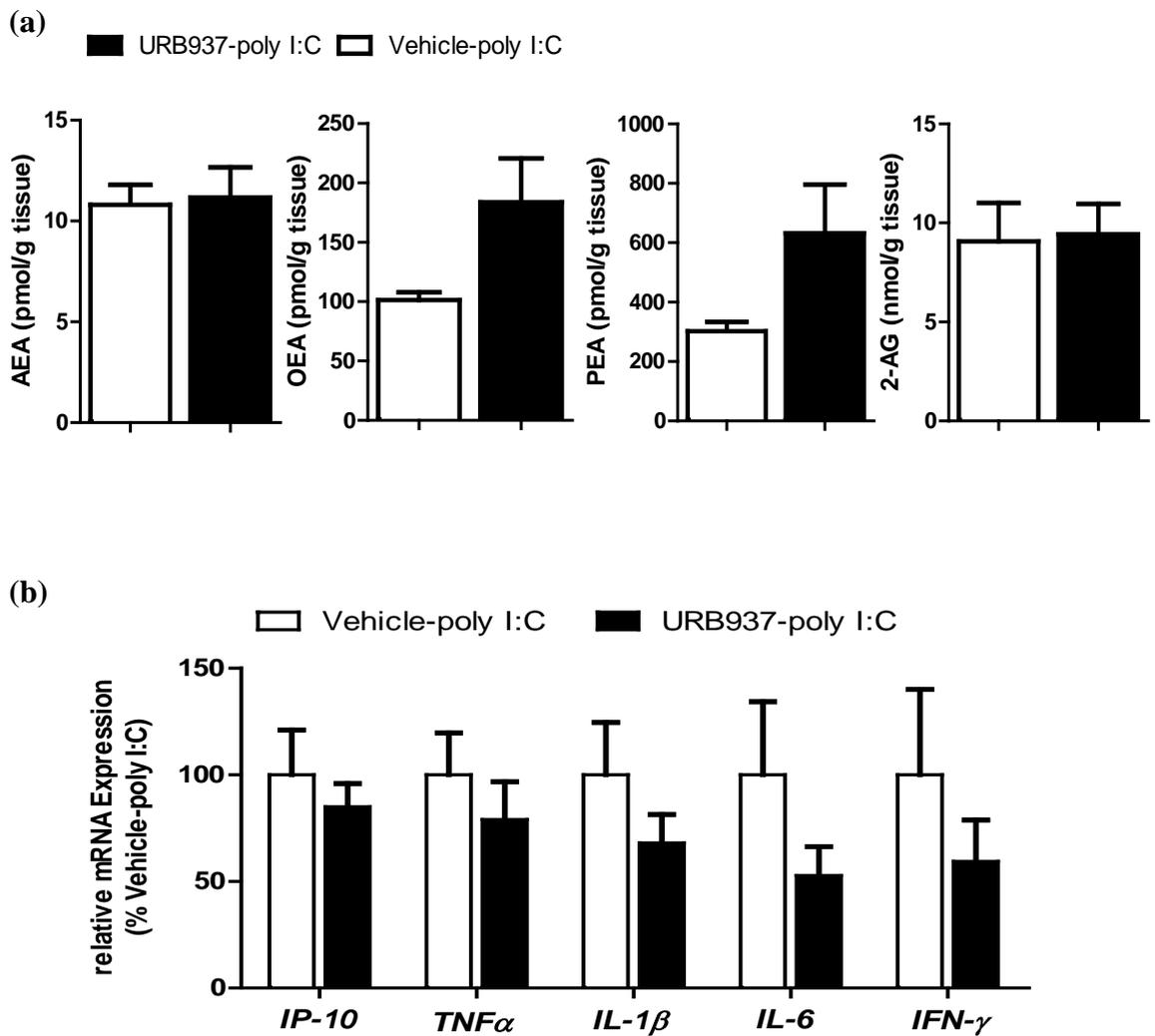


Figure 4.9 The effect of systemic administration of the peripherally restricted FAAH inhibitor URB937 on hippocampal levels of endocannabinoids and related *N*-acylethanolamines, and hippocampal expression of inflammatory mediators, 4 hours post poly I:C administration. (a) Systemic administration of the peripherally restricted FAAH inhibitor URB937 does not significantly increase hippocampal levels of AEA, OEA, PEA or 2-AG levels, nor does it alter (b) poly I:C-induced increases in hippocampal expression of *IP-10*, *TNF α* , *IL-1 β* , *IL-6* or *IFN- γ* , 4 hours post poly I:C administration. Data expressed as mean + SEM (n = 8 per group).

4.3.10 Experiment 4: Elevation of FAAH substrates following i.c.v. administration of URB597 does not alter hippocampal expression of *IP-10* or *TNF α* , in the absence of poly I:C

Previous work from our laboratory demonstrated that systemic administration of URB597 does not alter neuroinflammatory gene expression in the absence of an immune stimulus (Kerr et al., 2012). As such, we next wanted to determine if i.c.v. administration of URB597 in the absence of systemic administration of poly I:C altered hippocampal mRNA expression of IFN-(*IP-10*) and NF- κ B-inducible (*TNF α*) genes in the hippocampus. Similar to that reported following systemic administration of URB597, statistical analysis revealed that i.c.v. administration of URB597 does not alter hippocampal expression of *IP-10* or *TNF α* , in the absence of an immune stimulus (Fig 4.10).

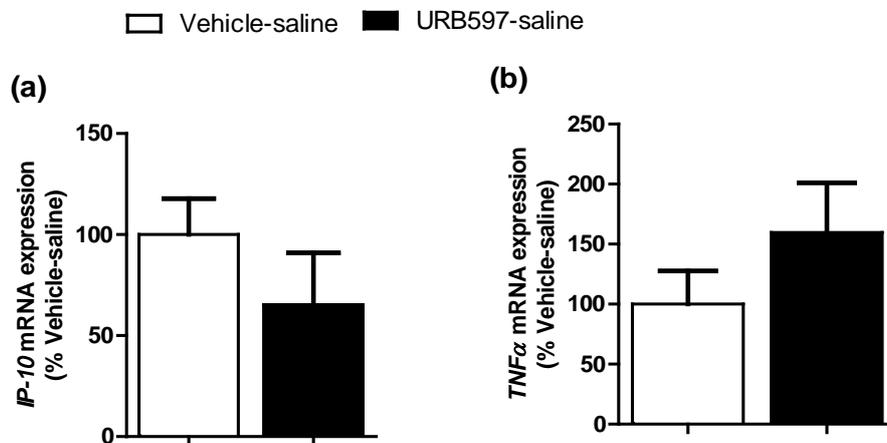


Figure 4.10 The effect of i.c.v. administration on hippocampal expression of *IP-10* and *TNF α* , in the absence of systemic poly I:C administration. I.c.v. administration of URB597 does not alter hippocampal expression of (a) the IFN-inducible chemokine *IP-10* or (b) the NF- κ B-inducible pro-inflammatory cytokine *TNF α* , in the absence of poly I:C. Data expressed as mean + SEM (n = 3-4 per group).

4.4 Discussion

The present study demonstrates that both systemic and i.c.v. administration of the FAAH inhibitor URB597 increases FAAH substrates within the brain and exerts robust immunoregulatory effects on TLR3-induced increases in neuroinflammatory mediators within the hippocampus, while only i.c.v. administration of URB597 modulates TLR3-induced inflammatory mediators within the frontal cortex. Specifically, systemic administration of URB597 increased hippocampal expression of the type I and type II IFNs, *IFN- α* and *IFN- γ* respectively, and attenuated the poly I:C-induced increase in the expression of NF- κ B responsive pro-inflammatory genes, *IL-1 β* and *TNF α* , while concurrently augmenting the expression of *IL-6*. In comparison, i.c.v. administration of URB597, attenuated the poly I:C-induced increase in hippocampal expression of *IFN- γ* , the IFN-inducible chemokine *IP-10* and IFN regulatory gene *SOCS1*. Furthermore, the poly I:C-induced increase in the expression of *TNF α* and *IL-10* were attenuated and augmented respectively, by i.c.v. administration of URB597. Within the frontal cortex, i.c.v., but not systemic, administration of URB597 attenuated the poly I:C-induced increases in *IP-10* and *TNF α* . The data presented also demonstrate that systemic administration of the peripherally restricted FAAH inhibitor URB937 does not alter hippocampal levels of FAAH substrates, nor does this treatment regime alter the TLR3-induced increases in hippocampal expression of either IFN- or NF- κ B-responsive genes. Taken together, these data provide strong evidence for an important immunoregulatory role of increased endogenous FAAH substrates (OEA and PEA) directly within the brain on TLR3-induced neuroinflammatory responses within the hippocampus and to a lesser extent the frontal cortex, both of which are affected in several neurological disorders with an underlying inflammatory component.

TLR3 activation is associated with induction of anti-viral type I IFNs, namely IFN- α/β , activation of IFNR1 and increased transcription of genes including *IRF7* and other anti-viral genes including pro-apoptotic genes. Although hippocampal (current chapter) and frontal cortical (Chapter 3) expression of IFN- α/β were not increased following systemic poly I:C administration, the robust induction of *IRF7*, and the IFN-inducible chemokine *IP-10* suggests strongly that type I IFNs were produced at the protein level and active in both the hippocampus and frontal cortex. In addition to inducing IFN- α/β production and signalling, TLR3 stimulation also induces activation of the NF- κ B signalling pathway

and subsequent production of inflammatory cytokines. Accordingly, the current data demonstrate that systemic poly I:C administration robustly increases hippocampal expression of the NF- κ B-responsive genes, *TNF α* , *IL-1 β* , *IL-6*, *IL-10* and *iNOS* and frontal cortical expression of *TNF α* , *IL-1 β* and *IL-6* which correlates with published reports in both rats and mice (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013). TLR3-induced increases in hippocampal expression of *IL-1 β* and *IFN- β* are reported to be associated with increases in expression of IFN-inducible pro-apoptotic genes and increases in neuronal cell death within the hippocampus; effects which are substantially greater in diseased animals compared to normal animals (Field *et al.*, 2010), while repeated poly I:C challenge induces successive episodes of acute neurological deficits in an already existing diseased state (Field *et al.*, 2010). In addition, TLR3-induced increases in pro-inflammatory cytokines are reported to be associated with increases in oxidative stress and neuronal death within the frontal cortex (Qin *et al.*, 2012) and lasting alterations in neuro-immune responses, exemplified by increases in microglial (increased *CD11b* expression) activation, and a concurrent decrease in expression of *BDNF* within the hippocampus and frontal cortex (Chapter 3) and (Gibney *et al.*, 2013). Thus modulation of TLR3-induced neuroinflammatory responses may afford some protection against neurodegenerative processes.

The data reported in Chapter 3 demonstrated that systemic administration of URB597 exerted potent immunoregulatory effects on hippocampal, but not frontal cortical expression of TLR3-induced neuroinflammatory responses. Further extending these findings, this chapter found that systemic administration of URB597, in addition to attenuating the TLR3-induced increases in hippocampal expression of *TNF α* and *IL-1 β* , and enhancing the TLR3-induced increases in *IFN- α* and *IL-6*, also increased hippocampal expression of the type II IFN *IFN- γ* , but not the IFN-inducible genes including *IRF7* and *iNOS* or the potent anti-inflammatory cytokine *IL-10*. IFN- γ is a multifunctional cytokine that is a known potent activator of microglia (Hausler *et al.*, 2002; Lyons *et al.*, 2011; Mutnal *et al.*, 2011) which promotes neuroinflammation largely through its ability to increase inflammatory mediators including TNF α , IL-1 β and IL-6 [for reviews see (Hanisch, 2002; Lynch, 2014)] and subsequent production of iNOS which untimely leads to neuronal injury and death (Jung *et al.*, 2010; Sohn *et al.*, 2007). As such, the reported URB597-induced increases in hippocampal expression of *IFN- γ* may seem at odds with our hypothesis that increased FAAH substrates mediate

anti-inflammatory effects, however under certain experimental conditions IFN- γ has been shown to elicit anti-inflammatory effects (Muhl *et al.*, 2003), protect neurons from damage initiated by viral infection (Geiger *et al.*, 1997; Rodriguez *et al.*, 2003), protect cultured hippocampal neurons against glutamate-induced excitotoxic death (Lee *et al.*, 2006b) and alleviate status epilepticus-induced neuronal damage in the hippocampus of rats (Ryu *et al.*, 2010). Taken together, the data suggest that enhancing FAAH substrates within the brain following systemic URB597 administration alters the expression of neuroinflammatory genes in the hippocampus, but not the frontal cortex, following TLR3 activation, likely inhibiting the neuroinflammatory process and protecting neuronal function.

A key question of this research was to decipher whether increasing FAAH substrates directly within the brain or FAAH substrate modulation of peripheral inflammatory responses were responsible for the effects of URB597 on TLR3-induced neuroinflammation. Evaluating the potential role of the brain's FAAH substrates on neuroinflammatory processes is of particular importance as several neuropathologies including ischemia, PD and MS are associated with increases in FAAH substrate levels within the brain (Baker *et al.*, 2001; Degen *et al.*, 2007; Franklin *et al.*, 2003; Loria *et al.*, 2008). Accordingly, our findings demonstrate that increasing FAAH substrates (OEA and PEA) directly within the brain (following i.c.v. administration of URB597) is associated with a robust anti-inflammatory effect, exemplified by a decrease in hippocampal expression of the *TNF α* , *IL-1 β* , *IFN- γ* and *IP-10*, while concurrently increasing hippocampal expression of the anti-inflammatory cytokine *IL-10*. In addition, i.c.v. administration of URB597 decreases frontal cortical expression of *TNF α* and the IFN-inducible inflammatory genes *IRF7* and *IP-10*, further suggesting an important role for FAAH substrates directly within the brain in the modulation of TLR3-induced neuroinflammatory responses. Although it has previously been reported that i.c.v. administration of URB597 modulates TLR4-induced increases in plasma *TNF α* (De Laurentiis *et al.*, 2010), to our knowledge this is the first study to demonstrate that enhancing FAAH substrates specifically within the brain modulates TLR-induced neuroinflammatory responses. Furthermore, systemic administration of the peripherally restricted FAAH inhibitor URB937 (Clapper *et al.*, 2010) increased peripheral (data not shown) but not hippocampal FAAH substrate levels or the expression of TLR3 associated increases in inflammatory cytokines, further highlighting that the effects on

neuroinflammation observed are most likely mediated at the level of the brain. Thus, while systemic administration of URB597 was associated with enhanced IFNs and reduced pro-inflammatory gene expression; i.c.v. administration elicits a more profound attenuation of TLR3-induced neuroinflammatory responses, exemplified by a decrease in pro-inflammatory cytokines and a concurrent increase in *IL-10* in the hippocampus. IL-10 is a potent anti-inflammatory cytokine and key negative regulator of microglial activation. The inhibition of FAAH or direct administration of AEA has been shown to increase TLR4- and TMEV-induced IL-10 levels in microglial cells (Correa *et al.*, 2011; Correa *et al.*, 2010). Follow-up studies from the same group have demonstrated that IL-10 plays a pivotal role in AEA-induced neuroprotection both *in vitro* and *in vivo*, at least in part, by increasing expression of CD200 on neurons and amplifying interactions with CD200R1 on microglia, and limiting harmful inflammatory processes (Hernangomez *et al.*, 2012). Thus, it is possible that enhancing FAAH substrate levels directly within the brain increase the expression and levels of IL-10 which in turn stabilise microglial activation and inhibit the expression of TNF α and IFN- γ (and the associated downstream genes including IP-10), thus limiting TLR3-induced neuroinflammatory processes in the hippocampus. Our data indicate that the most robust immunoregulatory effects on TLR3-induced inflammatory responses are within the hippocampus, and thus the potential molecular mechanisms mediating such responses within this region were further examined. This is of particular importance given the reports that TLR3-induced increases in hippocampal cytokine expression are accompanied by enhanced neuronal excitability in the hippocampus (Costello *et al.*, 2013), alterations in memory consolidation (Kranjac *et al.*, 2012) and seizure susceptibility (Galic *et al.*, 2009), while TLR3 deficient mice exhibit enhanced hippocampal-dependent working memory, increased hippocampal volume and neurogenesis (Okun *et al.*, 2010). SOCS1 and SOCS3 represent a rapid self-regulating mechanism to limit IFN (type I and type II) and IL-6 signalling, respectively (Alexander *et al.*, 1999; Fenner *et al.*, 2006; Shuai *et al.*, 2003). The transcription of SOCS1 and SOCS3 are primarily mediated by activation of the JAK/STAT pathway, via cytokines including IL-6, IL-10 and IFN- γ binding to their respective cell-surface receptors which in turn results in a cascade of phosphorylation of STAT transcription factors which untimely leads to increased production of SOCS1 and SOCS3 (Baker *et al.*, 2009). Once produced SOCS1 and SOCS3 feed back to negatively regulate JAK/STAT activation and therefore cytokine signalling. Specifically, over-

expression of SOCS1 protects against deleterious effects of IFN- γ (Balabanov *et al.*, 2006) while SOCS3, in addition to inhibiting IL-6 signalling, has been reported to inhibit IL-1 β -induced transcription and NF- κ B and MAPK activation (Frobose *et al.*, 2006; Karlsen *et al.*, 2004). In addition, SOCS3 has also been suggested to mediate some of the anti-inflammatory effects of IL-10 (Berlato *et al.*, 2002; Qin *et al.*, 2006). Although our current findings demonstrate that i.c.v. administration of URB597 increases hippocampal expression of the anti-inflammatory cytokine *IL-10*, this is not associated with an alteration in hippocampal expression of *SOCS3*, thus suggesting that URB597-induced increases in *IL-10* is mediating anti-inflammatory effects independent of increase *SOCS3* expression, following systemic TLR3 activation. Furthermore, i.c.v., but not systemic, administration of URB597 attenuates rather than increases the poly I:C-induced increase in hippocampal expression of *SOCS1*. It has been previously demonstrated that cannabinoid ligands increase SOCS expression in the periphery (Caraceni *et al.*, 2009; Lavon *et al.*, 2003), however our current study suggests that the reported anti-inflammatory effects on TLR3-induced neuroinflammation, following both systemic and i.c.v. administration of URB597, are occurring independent of increased SOCS signalling.

Activation of the type I IFN receptor results in production of viral transcripts including the IFN-induced dsRNA-dependent protein kinase (PKR) which induces apoptosis via up-regulation of the pro-apoptotic genes *Fas* and *Bax* and activation of caspase 3 signalling resulting in cell loss (Balachandran *et al.*, 2000; Chawla-Sarkar *et al.*, 2003). Although poly I:C did not alter the expression of the anti-viral gene *PKR*, the increased hippocampal expression of the pro-apoptotic gene *Fas* suggests the activation of downstream IFN-mediated apoptotic events. In this regard, it has previously been reported that poly I:C induces increases in hippocampal expression of both *PKR* and *Fas* and caspase-3/TUNEL-positive cell death which in turn were suggested to contribute to acceleration of already existing neurodegenerative processes (Field *et al.*, 2010). Prior i.c.v., but not systemic, administration of URB597 attenuated IFN downstream inflammatory genes including *IRF7*, *IP-10*, *IFN- γ* and *SOCS1*, however, this treatment regime did not modulate the TLR3-induced increases in the hippocampal expression of the pro-apoptotic gene *Fas*, however, it cannot be ruled out that an effect may occur at a later time point or via an alternative mechanism. Overall, the current study demonstrates

some differential effects of systemic FAAH inhibition vs. FAAH inhibition directly within the brain on TLR3-induced neuroinflammatory responses in terms of individual cytokines modulated, with a more robust anti-inflammatory profile observed when FAAH substrates are increased directly within the brain, which may be due to the fact that i.c.v. administration of URB597 caused significantly greater relative increases in both hippocampal levels of OEA and PEA, when compared to those observed following systemic URB597 administration.

4.4.1 Conclusion

The data presented herein demonstrate that both systemic and i.c.v. administration of URB597 and subsequent increases in FAAH substrates within the brain modulates the expression of TLR3-responsive inflammatory genes in two discrete brain regions, and suggest that the effects are most likely mediated at the level of the brain. As TLR3-induced inflammatory mediators in the hippocampus are associated with impairments in learning and memory and also seizure susceptibility, and given that the most robust effects of FAAH inhibition were observed in this brain region, these data may have important implications for targeting FAAH in the treatment of neuroinflammatory and neurodegenerative disorders.

Chapter 5

***Investigation of the potential receptor mechanisms
underlying FAAH substrate-mediated modulation
of TLR3-induced neuroinflammation***

5.1 Introduction

To date the most widely studied endocannabinoid receptors are the CB₁ and CB₂ receptors. These are both G_{i/o}-coupled receptors which are negatively linked to AC and thus upon ligand activation there is a decrease in cAMP production and PKA phosphorylation (Howlett *et al.*, 1999; Pertwee, 1997; Pertwee, 1999). On immune cells, this mechanism has been proposed to inhibit NF-κB activation and subsequent pro-inflammatory cytokine expression (Cabral *et al.*, 2009). Cannabinoid receptors are also positively coupled to MAPK (ERK, JNK) activation, which regulates gene expression of several proteins including inflammatory cytokine synthesis (Bouaboula *et al.*, 1995b; Bouaboula *et al.*, 1996; Derkinderen *et al.*, 2003; Derocq *et al.*, 2000; Khaspekov *et al.*, 2004). CB₁ receptors are densely expressed within the CNS, and in particular on pre-synaptic nerve terminals and dendritic spines (Ong *et al.*, 1999) where they function to modulate neuronal activity via inhibition of N- and P/Q-type voltage-activated Ca²⁺ channels (Caulfield *et al.*, 1992; Huang *et al.*, 2001a; Mackie *et al.*, 1993; Mackie *et al.*, 1992). However, in addition to neuronal expression, there are now several lines of evidence that CB₁ receptors are expressed on glial cells including oligodendrocytes (Molina-Holgado *et al.*, 2002a), astrocytes (Bouaboula *et al.*, 1995a; Molina-Holgado *et al.*, 2002a; Sanchez *et al.*, 1998) and microglia (Cabral *et al.*, 2001; Carlisle *et al.*, 2002; Facchinetti *et al.*, 2003b; Molina-Holgado *et al.*, 2002a). In comparison, CB₂ receptors were initially identified on peripheral immune cells (Munro *et al.*, 1993) and thought not to be expressed within the CNS (Carlisle *et al.*, 2002; Griffin *et al.*, 1999; Munro *et al.*, 1993; Sugiura *et al.*, 2000). However, there is now evidence of CB₂ receptor expression on activated microglia (Benito *et al.*, 2008; Carlisle *et al.*, 2002; Facchinetti *et al.*, 2003b; Klegeris *et al.*, 2003; Walter *et al.*, 2003); activation of which is associated with decreased production of pro-inflammatory cytokines (Ehrhart *et al.*, 2005; Klegeris *et al.*, 2003) and increased release of anti-inflammatory cytokines (Molina-Holgado *et al.*, 2003); resulting in associated decreases in neurotoxicity (Eljaschewitsch *et al.*, 2006; Klegeris *et al.*, 2003; Molina-Holgado *et al.*, 2003).

Direct activation of CB₁ receptors with potent agonists is known to be associated with adverse psychoactive side effects including short term memory loss, catalepsy, increased appetite, impairments in motor co-ordination, depression and anxiety, thus the therapeutic potential of drugs which directly activate cannabinoid receptors are limited

due to these adverse side effects. As such, enhancement of the endogenous cannabinoid tone, via inhibition of the metabolic enzymes, and therefore indirect activation of cannabinoid receptors may offer an alternative approach. In this respect, our previous data (Chapter 3 and 4) have demonstrated that both systemic and i.c.v. administration of the selective FAAH inhibitor URB597, exerted potent immunoregulatory effects on TLR3-induced increases in neuroinflammatory mediators. However, the question remains as to the potential receptor mechanisms underlying the reported immunoregulatory effects of increased FAAH substrates on TLR3-induced neuroinflammatory responses. To date, many of the studies examining cannabinoid modulation of TLR-induced neuroinflammation have been carried out *in vitro*. Perhaps not surprisingly, a key role for microglial CB₂ receptors has been demonstrated in AEA-mediated modulation of TLR4-induced neuroinflammation (Correa *et al.*, 2009a; Correa *et al.*, 2010; Hernangomez *et al.*, 2012; Krishnan *et al.*, 2012; Malek *et al.*, 2015), although a role for CB₁ receptor activation has also been implicated in mediating such responses (Krishnan *et al.*, 2012; Molina-Holgado *et al.*, 2002b). However, a number of studies have also reported non-CB₁/CB₂ mediated effects of AEA-induced modulation of TLR4-induced increases of pro-inflammatory mediators (Facchinetti *et al.*, 2003b; Puffenbarger *et al.*, 2000; Tham *et al.*, 2007), suggesting possible alternative receptor mechanisms. Within the *in vivo* setting, CB₁ receptors have been implicated in AEA-induced modulation on TLR4-induced increases in plasma levels of TNF α (De Laurentiis *et al.*, 2010) and IL-1 β (Roche *et al.*, 2008). Furthermore, CB₂ receptors have been shown to be involved in AEA-mediated modulation of TLR4-induced increases in leukocyte adhesion in intestinal venules (Kianian *et al.*, 2013). Our data (Chapter 3 & 4) and that previously published (Kerr *et al.*, 2012) have demonstrated that URB597, and subsequent increases in FAAH substrates within the brain, exerted robust immunoregulatory effects on both TLR3 and TLR4-induced neuroinflammation, in discrete brain regions. However, to our knowledge there have been no studies to date examining the receptor mechanisms involved in FAAH substrate-mediated modulation of TLR-induced neuroinflammation.

In addition to AEA, there are additional FAAH substrates including the related *N*-acylethanolamines OEA and PEA which are known to exert potent anti-inflammatory and neuroprotective effects (Gonzalez-Aparicio *et al.*, 2014; Lambert *et al.*, 2002; Sayd *et al.*, 2015; Skaper *et al.*, 2015; Skaper *et al.*, 2013). Accordingly, our previous data

reported that both systemic and i.c.v. administration of the FAAH inhibitor URB597 increased OEA and PEA, but not AEA levels within the brain and as such it is likely that the increases in these FAAH substrates are involved in mediating some of the reported immunoregulatory effects of URB597 on TLR3-induced neuroinflammation. However, it is important to note that although we did not report increases in AEA at the particular time point we examined, we cannot rule out that AEA was not increased at an earlier time point as it has previously been shown that URB597, at the same dose used in our work, increased AEA levels in the hypothalamus, at 2.5 hours post administration (Kerr *et al.*, 2012).

Although derived from the same biosynthetic pathway as AEA, it is now generally accepted that neither OEA nor PEA bind directly to CB₁ or CB₂ receptors (Fu *et al.*, 2003; LoVerme *et al.*, 2005; Sugiura *et al.*, 2000). Rather these FAAH substrates, as well as AEA, are capable of binding to and mediating biological effects via activation of a family of non-cannabinoid receptors called the PPARs (O'Sullivan, 2007; O'Sullivan *et al.*, 2010). PPARs belong to a family of ligand-activated nuclear receptors which comprises of three isoforms: α , δ and γ . Ligand binding to each PPAR isoform results in heterodimerization of the individual PPAR isoform with the RXR, this heterodimer then binds to the promoter region of the PPARE, with the recruitment of co-activators (see Fig 1.9 for diagrammatical representation; Chapter 1). This subsequently induces the transcription of genes involved in a plethora of biological functions including metabolism, energy homeostasis, cell differentiation and inflammation (Bishop-Bailey *et al.*, 2009; Ferre, 2004; Kota *et al.*, 2005; Stienstra *et al.*, 2007). Each PPAR isoform exhibits distinct tissue expression patterns (Auboeuf *et al.*, 1997); however, all three isoforms are expressed throughout the CNS in both neuronal and glial cells (Cimini *et al.*, 2005; Heneka *et al.*, 2007; Moreno *et al.*, 2004). Both OEA and PEA increase the transcriptional activation of PPAR- α (Fu *et al.*, 2003) while AEA is capable of directly binding to PPAR- γ (Bouaboula *et al.*, 2005; Gasperi *et al.*, 2007), a mechanism by which it has been shown to inhibit IL-2 release (Rockwell *et al.*, 2004). There are now several reports that both OEA and PEA exert potent anti-inflammatory (Lo Verme *et al.*, 2005; Scuderi *et al.*, 2012; Sun *et al.*, 2007) and neuroprotective effects (D'Agostino *et al.*, 2012; Di Cesare Mannelli *et al.*, 2013; Esposito *et al.*, 2012; Scuderi *et al.*, 2012; Sun *et al.*, 2007; Zhou *et al.*, 2012) in several experimental disease models, via activation of PPAR- α . Mechanistically the anti-inflammatory properties of PPAR- α

agonists have been attributed to be mediated via inhibition of the transcription factor NF- κ B and the subsequent production of inflammatory mediators (Poynter *et al.*, 1998).

To date, the majority of research has focused on endocannabinoid-mediated modulation of TLR4-induced neuroinflammation with just one study examining the potential receptor mechanisms mediating cannabinoid-induced immunoregulation on TLR3-induced responses. This work carried out by Downer and colleagues demonstrated that the synthetic CB₁/CB₂ receptor agonist WIN55,212-2 enhanced TLR3-induced increases in IRF3 activation and subsequent IFN- β expression, independent of either CB₁ or CB₂ receptor activation (Downer *et al.*, 2011). Follow up studies from this same group eloquently described a PPAR- α -dependent mechanism for WIN55,212-2-induced enhancement of IFN- β expression, following TLR3 activation (Downer *et al.*, 2012). Further evidence implicating a role for PPAR activation in modulation of TLR3-induced responses can be found in reports that administration of selective PPAR- γ agonists attenuate the TLR3-induced increases in TNF α and IL-12p40 release from glial cells (Gurley *et al.*, 2008) and attenuate TLR3-induced increases of IFN- β secretion from peritoneal macrophages (Zhao *et al.*, 2011).

Hypothesis: Based on these data we hypothesised a potential role for FAAH substrate receptor targets including cannabinoid (CB₁, CB₂) and non-cannabinoid (PPAR- α , PPAR- γ) in FAAH substrate-mediated modulation of TLR3-induced neuroinflammation within the hippocampus.

Therefore the specific aims of the studies described in this chapter were to:

1. Investigate the effect of CB₁, CB₂, PPAR- α and PPAR- γ antagonism, on FAAH substrate-mediated modulation of TLR3-induced increases in inflammatory mediators in the hippocampus and serum.
2. Investigate if CB₁, CB₂, PPAR- α or PPAR- γ directly within the brain mediate the effects of FAAH substrates on TLR3-induced increases of neuroinflammatory genes in the hippocampus. This was achieved by i.c.v. administration of selective antagonists of CB₁, CB₂, PPAR- α or PPAR- γ prior to the enhancement of FAAH substrate levels using URB597.
3. Determine if CB₁, CB₂, PPAR- α or PPAR- γ antagonists alone elicit effects on TLR3-induced increases in neuroinflammatory gene expression in the hippocampus

5.2 Methodology and Experimental design

Experiments were carried out on male Sprague Dawley rats (weight 220-260g; Charles River, UK), housed singly in plastic bottomed cages (45 X 25 X 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 0800 to 2000 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 an intraperitoneal (i.p.) injection of sterile saline (0.89% NaCl) for 3-4 days prior to experimentation in order 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 licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

5.2.1 Experiment 1: Investigation of the effect of systemic administration of selective CB_1 receptor, CB_2 receptor, PPAR- α or PPAR- γ antagonists on FAAH substrate-mediated modulation of TLR3-induced increases of neuroinflammatory genes in the hippocampus

Rats were randomly assigned into 1 of 7 treatment groups: Vehicle-vehicle-saline, Vehicle-vehicle-poly I:C, Vehicle-URB597-poly I:C, AM251-URB597-poly I:C, AM630-URB597-poly I:C, MK866-URB597-poly I:C and GW9662-URB597-poly I:C (n = 9-16 per group). Rats were co-administered with a single, acute systemic injection combining the FAAH inhibitor URB597 (Cayman Chemicals, Estonia; 1mg/kg i.p.) with either the CB_1 receptor antagonist AM251 (Cayman Chemicals, Estonia; 1mg/kg i.p.), the CB_2 receptor antagonist AM630 (Cayman Chemicals, Estonia; 1mg/kg i.p.), the PPAR- α antagonist MK886 (Cayman Chemicals, Estonia; 1mg/kg i.p.), the PPAR- γ antagonist GW9662 (Cayman Chemicals, Estonia; 2mg/kg i.p.) or Vehicle (ethanol: cremaphor:saline; 1:1:18) at an injection volume of 2ml/kg. Animals received a single, acute i.p. injection of poly I:C (3mg/kg i.p.) or sterile saline (0.89% NaCl) in an injection volume of 1.5 ml/kg, 30 min following URB597/vehicle-antagonist co-administration. Refer to Chapter 3: section 3.2 for rationale for chosen doses of both URB597 and poly I:C. The antagonist doses were chosen based on previous studies demonstrating antagonistic activity *in vivo* (Gonzalez *et al.*, 2011; Jayamanne *et al.*,

2006; Mazzola *et al.*, 2009; Roche *et al.*, 2008). Animals were sacrificed by decapitation 4 hours post poly I:C/saline administration and trunk blood collected and left to clot. Blood samples were stored at 4°C for approximately 4 hours and were centrifuged at 14,000g for 15 min at 4°C to obtain serum. In addition, the brain was rapidly removed and the hippocampus excised, snap-frozen on dry ice. All samples were stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression. (The methodology was essentially as described in Chapter 2).

5.2.2 Experiment 2: Investigation of the effect of i.c.v. administration of selective CB₁ receptor, CB₂ receptor, PPAR- α or PPAR- γ antagonists on FAAH substrate-mediated modulation of TLR3-induced increases of neuroinflammatory genes in the hippocampus

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol in Chapter 2); rats were randomly assigned into one of six treatment groups: Vehicle-vehicle-saline, Vehicle-vehicle-poly I:C, Vehicle-URB597-poly I:C, AM251-URB597-poly I:C, AM630-URB597-poly I:C, MK886-URB597-poly I:C and GW9662-URB597-poly I:C and (n= 6-10 per group). Rats received a single, acute i.c.v. injection of AM251 (2.5 μ g), AM630 (5 μ g), MK886 (300ng), GW9662 (50 μ g), or vehicle (100% DMSO) in an injection volume of 5 μ l infused over 1 min, followed 15 min later by a single, acute i.c.v. injection of URB597 (50 μ g) or vehicle (100% DMSO), in an injection volume of 5 μ l infused over 1 min. Animals received a single, acute i.p. injection of poly I:C (3mg/kg, i.p.) or sterile saline (0.89%) in an injection volume of 1.5ml/kg, 30 min post URB597/vehicle. Antagonist doses were chosen based on previous published work demonstrating antagonistic activity *in vivo* (dos Santos *et al.*, 2012; Morgenweck *et al.*, 2010; Rea *et al.*, 2013). Following poly I:C/saline injections animals were returned to their home cages and sacrificed by decapitation at 4 hours following poly I:C administration. Blood samples were stored at 4°C for approximately 4 hours and were centrifuged at 14,000g for 15 min at 4°C to obtain serum. In addition, the brain was rapidly removed and the hippocampus excised, snap-frozen on dry ice and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression.

5.2.3 Experiment 3: Investigation of the effect of i.c.v. administration of the CB₁ receptor, CB₂ receptor, PPAR- α or PPAR- γ antagonists alone on TLR3-induced increases of neuroinflammatory genes in the hippocampus

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol in Chapter 2); rats were randomly assigned into 1 of 6 treatment groups: Vehicle-vehicle-saline, Vehicle-vehicle-poly I:C, AM251-vehicle-poly I:C, AM630-vehicle-poly I:C, MK886-vehicle-poly I:C and GW9662-vehicle-poly I:C (n=5-10 per group). Rats received a single, acute i.c.v. injection of AM251 (2.5 μ g), AM630 (5 μ g), MK886 (300ng), GW9662 (50 μ g), or vehicle (100% DMSO) in an injection volume of 5 μ l infused over 1 min, followed 15 min later by a single, acute i.c.v. injection of Vehicle (100% DMSO), also in an injection volume of 5 μ l infused over 1 min. Animals received a single, acute i.p. injection of poly I:C (3mg/kg, i.p.) or sterile saline (0.89%) in an injection volume of 1.5ml/kg, 30 min post vehicle administration. Animals were returned to their home cages and sacrificed by decapitation at 4 hours following poly I:C administration, the brain excised, the hippocampus removed, snap-frozen and stored at -80°C until assayed for cytokine expression.

3.2.4 Statistical Analysis

SPSS (IBM, New York, USA) statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro–Wilks and Levene test $p > 0.05$, respectively. When comparing the means of two unrelated groups, parametric data were analysed using unpaired t -test. One-way ANOVA was used to compare the mean of more than two groups on one factor. *Post-hoc* analysis was performed using Fisher's LSD test. Data were considered significant when $p < 0.05$. All graphs representing data were constructed using GraphPad Prism 5.0 and results expressed as group means + standard error of the mean (SEM).

5.3 Results

5.3.1 Experiment 1 & 2: Systemic and i.c.v. administration of URB597 increases levels of the *N*-acylethanolamines in the hippocampus, at 4 hours post poly I:C

Poly I:C was previously reported not to alter hippocampal levels of endocannabinoids or *N*-acylethanolamines, when compared to their vehicle-saline-treated counterparts (Chapters 3 and 4), as such we compared vehicle-vehicle-poly I:C-treated animals vs. vehicle-URB597-poly I:C-treated animals in the current analysis. In accordance with previous findings, both systemic and i.c.v. administration of URB597 increased the levels of the *N*-acylethanolamines OEA [i.p. $t_{(14)}=2.220$, $p < 0.05$; i.c.v. $t_{(12)}=3.687$, $p < 0.01$] and PEA [i.p. $t_{(14)}=2.487$, $p < 0.05$; i.c.v. $t_{(12)}=4.264$, $p < 0.01$] in the hippocampus, when compared to vehicle-vehicle-poly I:C-treated counterparts (Fig 5.1b,c). In addition, i.c.v., but not systemic, administration of URB597 significantly increased hippocampal levels of AEA [$t_{(12)}=4.264$, $p < 0.01$], when compared to their vehicle-vehicle-poly I:C-treated counterparts, at 4 hours post poly I:C challenge (Fig 5.1a). Furthermore, neither systemic or i.c.v. administration of URB597 significantly altered 2-AG in the hippocampus.

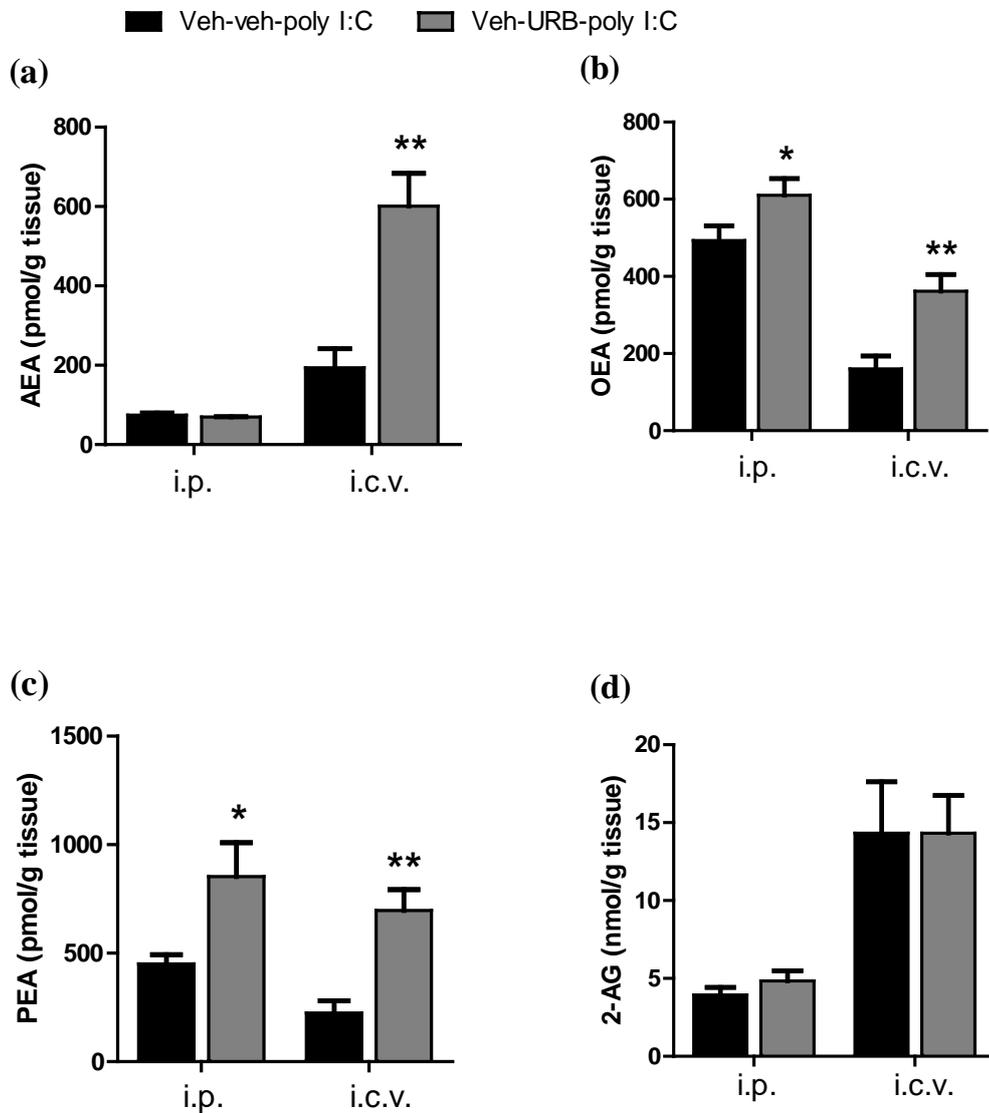


Figure 5.1 The effect of systemic and i.c.v. administration of URB597 on hippocampal levels of endocannabinoids and *N*-acylethanolamines. Systemic and i.c.v. administration of the FAAH inhibitor URB597 increases hippocampal levels of (b) OEA and (c) PEA. In addition, i.c.v., but not systemic, administration of URB597 increases (a) AEA, when compared to vehicle-vehicle-poly I:C-treated counterparts, 4 hours post poly I:C challenge. Neither systemic or i.c.v. administration of URB597 altered hippocampal levels of (d) 2-AG. * $p < 0.05$ ** $p < 0.01$ vs. vehicle-vehicle-poly I:C-treated counterparts. Data expressed as mean + SEM ($n=6-10$ per group).

5.3.2 Experiment 1: The effect of systemic administration of CB₁, CB₂, PPAR- α or PPAR- γ antagonists, in the presence of systemic administration of URB597, on TLR3-induced increases in hippocampal expression of inflammatory genes

Our initial data demonstrated that systemic administration of the FAAH inhibitor URB597 and subsequent increases in hippocampal levels of OEA and PEA was associated with immunoregulatory effects on TLR3-induced increases in inflammatory genes within the hippocampus, but not the frontal cortex, at 4 hours post administration (Chapters 3 and 4). As such we next aimed to elucidate the potential role of CB₁, CB₂, PPAR- α or PPAR- γ in FAAH substrate-mediated modulation of TLR3-induced neuroinflammatory responses. Accordingly, statistical analysis revealed a significant effect of treatment on hippocampal mRNA expression of *IP-10* [$F_{6,62}=5.286$, $p < 0.001$], *TNF α* [$F_{6,62}=2.486$, $p < 0.05$] and *IL-6* [$F_{6,62}=3.122$, $p = 0.01$], 4 hours post poly I:C administration. In comparison to previous findings, the current treatment regime failed to significantly affect hippocampal mRNA expression of *IFN- α* , 4 hours post systemic poly I:C administration. Systemic administration of poly I:C significantly increased hippocampal expression of the IFN-inducible chemokine *IP-10*, when compared to vehicle-vehicle-saline-treated counterparts (Fig 5.2b,d). Although there was a trend for a poly I:C-induced increase in *TNF α* and *IL-6*, these effects failed to reach statistical significance (Fig 5.2c,d). Furthermore, although AM251-URB597-treated animals displayed significantly higher hippocampal expression of *IP-10* when compared to vehicle-URB597-treated counterparts (Fig 5.2b), none of the other antagonists examined exerted any significant effect on hippocampal expression of inflammatory mediators, induced following systemic administration of poly I:C. It is important to note that hippocampal expression of *IFN- β* was not examined in the current study as we have previously demonstrated that neither systemic administration of poly I:C or URB597 affected hippocampal expression of this gene, at 4 hours post poly I:C administration (Chapters 3 and 4).

Veh-veh-saline
 AM251-veh-poly I:C
 MK886-URB-poly I:C
 Veh-veh-poly I:C
 AM630-URB-poly I:C
 GW9662-URB-poly I:C
 Veh-URB-poly I:C
 I:C

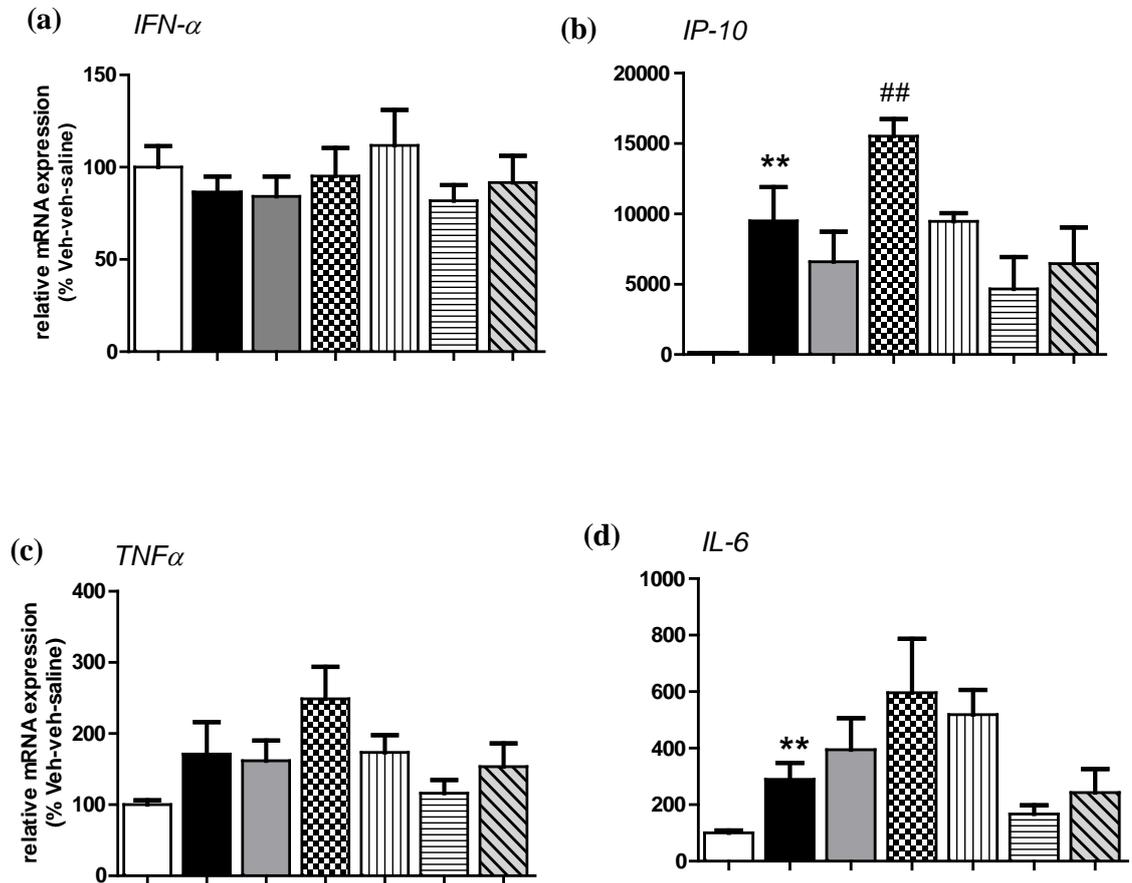


Figure 5.2 The effect of systemic administration of CB₁, CB₂, PPAR- α or PPAR- γ antagonists, in the presence of systemic administration of URB597, on TLR3-induced increases in hippocampal expression of *IFN- α* , *IP-10*, *TNF α* and *IL-6*. Systemic administration of poly I:C significantly increases hippocampal expression (b) *IP-10* and (d) *IL-6*, but not (a) *IFN- α* or (c) *TNF α* , 4 hours post poly I:C challenge. Although systemic administration of URB597 did not alter poly I:C-induced increases in either *IP-10* or *IL-6*, co-administration of the CB₁ receptor antagonist AM251 with URB597 significantly increased hippocampal expression of (b) *IP-10*, when compared to vehicle-URB597-treated counterparts. ** $p < 0.01$ ## $p < 0.01$ vs. Vehicle-URB597-poly I:C-treated counterparts. Data expressed as mean + SEM (n = 9-16 per group).

5.3.3 Experiment 1: The effect of systemic administration of CB₁, PPAR- α or PPAR- γ antagonists, in the presence of systemic administration of URB597, on TLR3-induced increases in serum levels of inflammatory mediators

Our initial data demonstrated that systemic administration of URB597 significantly attenuated TLR3-induced increases in serum levels of the inflammatory cytokines IL-6 and IFN- γ , at 4 hours post poly I:C challenge, when compared to vehicle-saline-treated counterparts (Chapter 3). As such we next aimed to investigate the potential receptor mechanisms responsible for mediating such responses. Specifically the potential involvement of the CB₁ receptor, PPAR- α or PPAR- γ was examined. Serum samples for CB₂ receptor antagonist group were accidentally destroyed and thus could not be analysed. Although poly I:C tended to increase IFN- γ or IL-6, at 4 hours post administration, statistical analysis revealed that there was no significant effect of treatment on serum levels of IFN- γ , IL-6, TNF α or IL-1 β levels (Fig 5.3a-d).

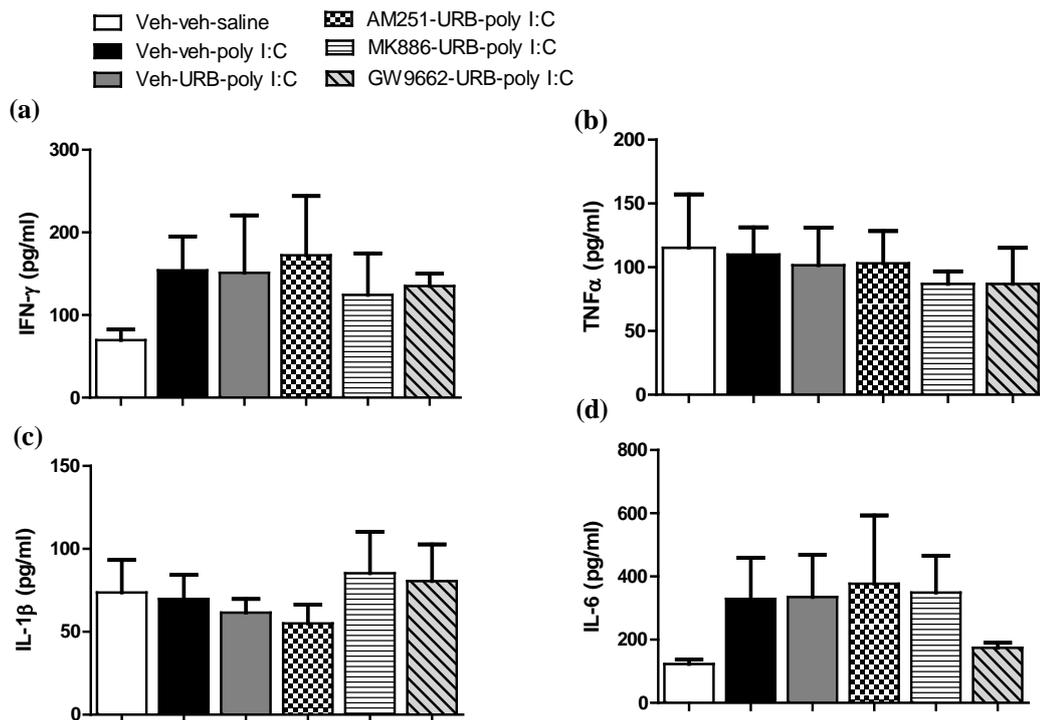
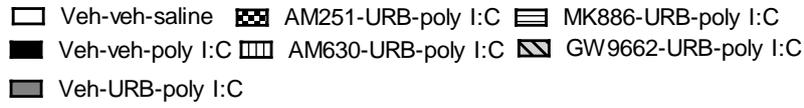


Figure 5.3 The effect of URB597 in the presence or absence of CB₁, PPAR α or PPAR γ antagonists on serum levels of IFN- γ , TNF α , IL-1 β and IL-6, 4 hours post poly I:C administration. Data expressed as mean + SEM (n= 5-8 per group).

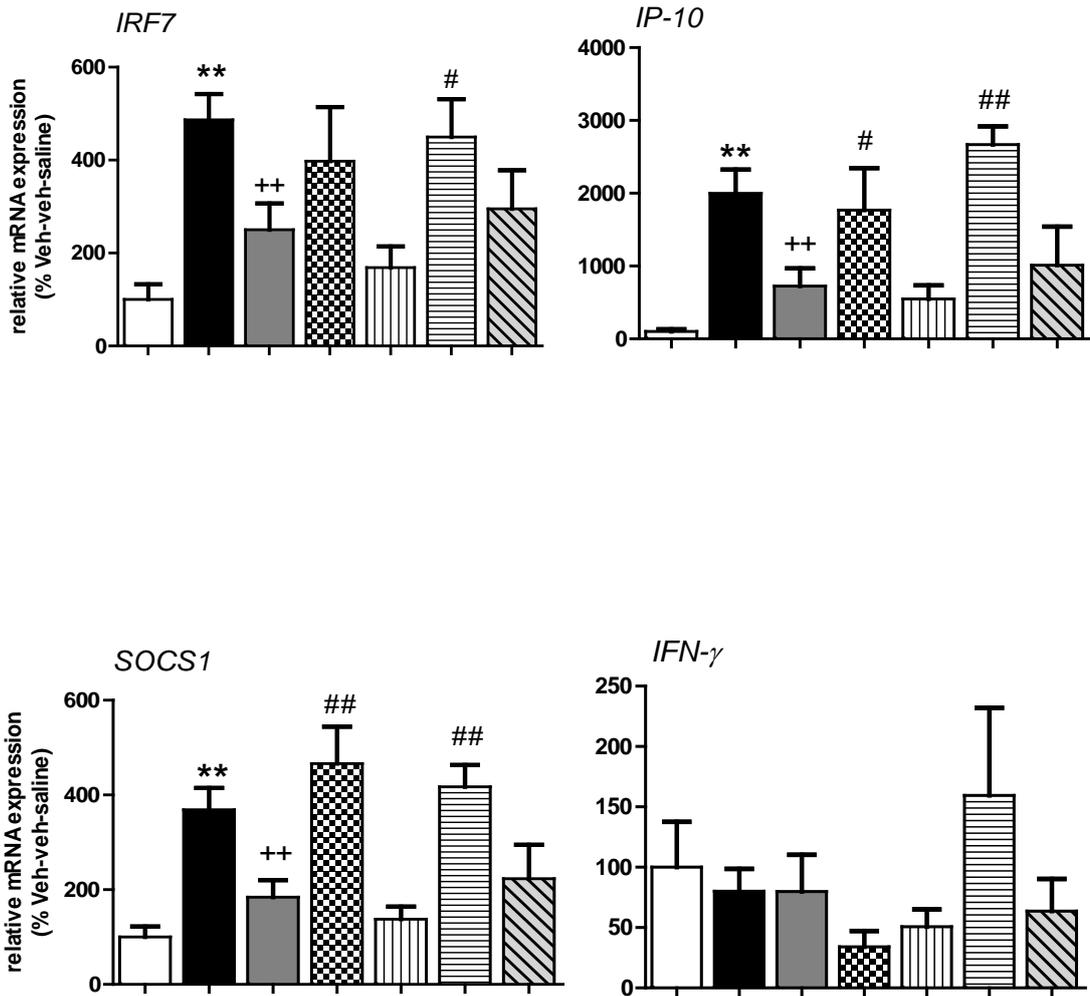
5.3.4 Experiment 2: Antagonism of the CB₁ receptor, PPAR- α or PPAR- γ directly within the brain attenuates FAAH substrate-mediated attenuation of TLR3-induced increases in IFN- and NF- κ B-inducible genes within the hippocampus

Although systemic administration of URB597 and subsequent increases in the FAAH substrates OEA and PEA exerted immunoregulatory effects on TLR3-induced increases in hippocampal expression of inflammatory genes, our previous results demonstrated that increasing FAAH substrates directly within the brain was associated with a more robust anti-inflammatory profile; exemplified by attenuation in TLR3-induced increases in mRNA expression of the pro-inflammatory mediators (*TNF α* , *IFN- γ* and *IP-10*) and a concurrent increase in hippocampal expression of the anti-inflammatory cytokine *IL-10* (Chapter 4). As such, we next wanted to elucidate the potential central receptor mechanisms underlying FAAH substrate-mediated modulation of TLR3-induced neuroinflammatory genes within the hippocampus. Statistical analysis revealed a significant effect of treatment on hippocampal mRNA expression of several IFN-inducible genes including: *IRF7* [$F_{8,51}=3.548$, $p < 0.01$], *IP-10* [$F_{5,43}=5.913$, $p = 0.000$] and *SOCS1* [$F_{8,49}=5.296$, $p = 0.000$]. In comparison, this treatment did not significantly alter hippocampal expression of the type II IFN *IFN- γ* , at 4 hours post poly I:C administration (Fig 5.4a). In addition, statistical analysis revealed a significant effect of treatment on hippocampal mRNA expression of the NF- κ B-inducible cytokines *IL-6* [$F_{6,45}=6.815$, $p = 0.000$], *TNF α* [$F_{6,47}=4.702$, $p < 0.01$] and also *SOCS3* [$F_{8,49}=6.319$, $p = 0.000$], at 4 hours post poly I:C administration. However, no significant effect of treatment on hippocampal expression of the anti-inflammatory cytokine *IL-10* was reported (Fig 5.4b). Prior i.c.v. administration of URB597 significantly attenuated TLR3-induced increases in hippocampal expression of *IRF7*, *IP-10* and *SOCS1*, when compared to vehicle-vehicle-poly I:C-treated counterparts. Furthermore, the URB597-induced decreases in hippocampal expression of *IP-10* and *SOCS1* were significantly reversed in both AM251- and MK886-URB597-poly I:C-treated animals, when compared to their vehicle-URB597 treated counterparts. The URB597-induced decrease in *IRF7* was significantly reversed in MK886-URB597-poly I:C-treated animals (Fig 5.4a). In addition, the URB597-induced decrease in *IL-6* and was significantly reversed in AM251- MK886- and GW9662-URB597-treated animals, while the URB597-induced decrease in *SOCS3* was significantly reversed in MK886- and GW9662-URB597-poly

I:C-treated animals, when compared to vehicle-URB597-poly I:C-treated counterparts (Fig 5.4b).



(a) IFN-inducible inflammatory genes.



(b) NF-κB-inducible inflammatory genes

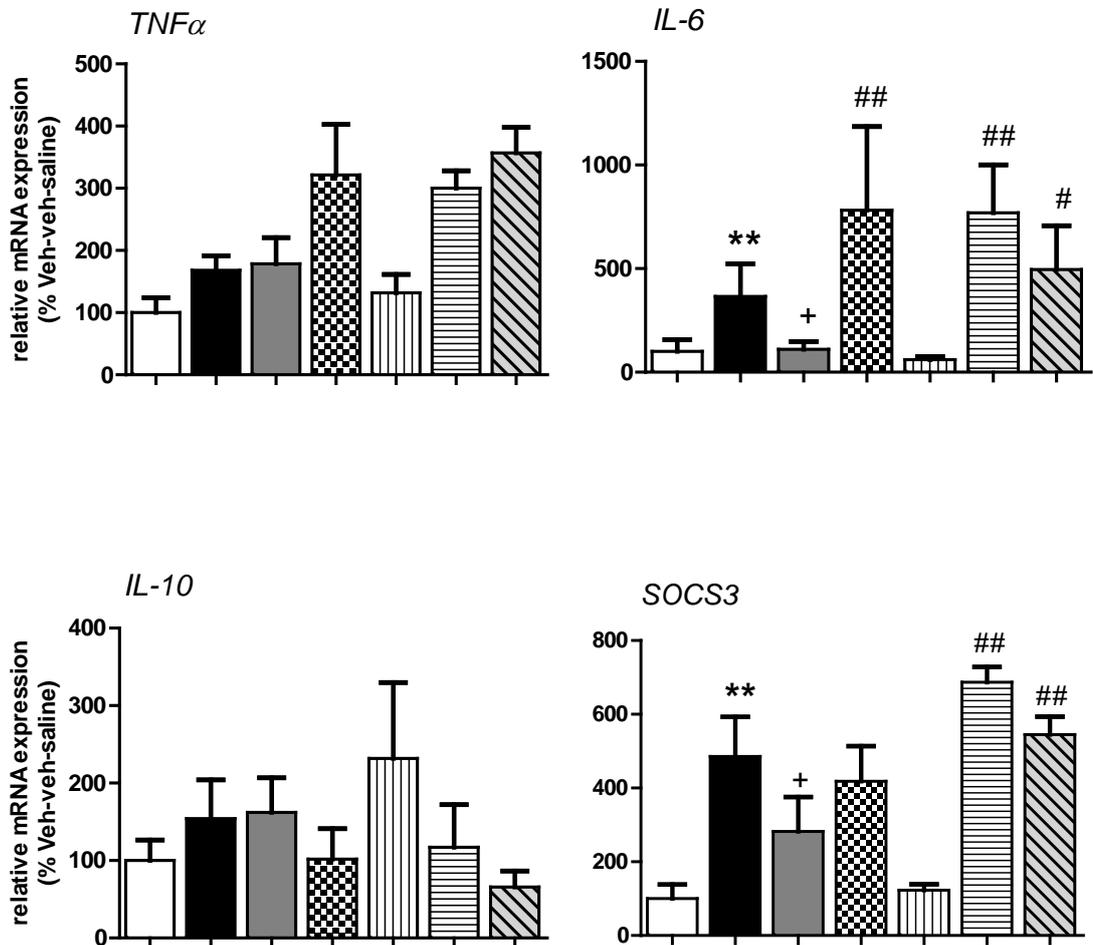


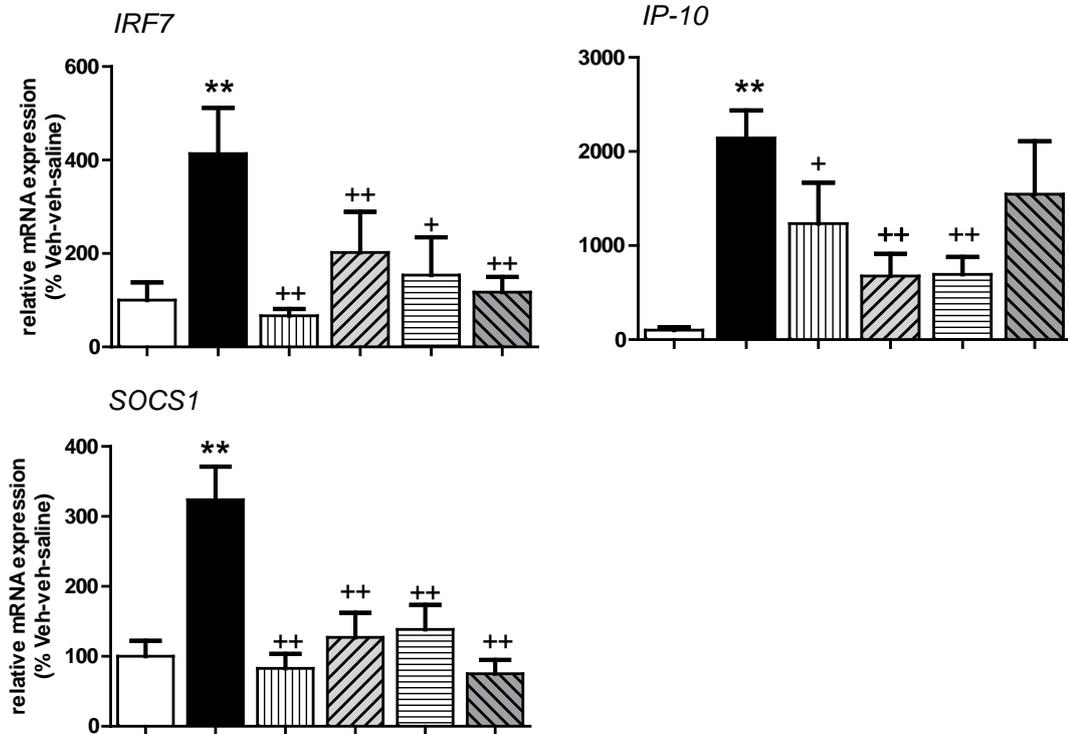
Figure 5.4 The effect of blockade of cannabinoid receptors and PPARs directly within the brain on FAAH substrate-mediated modulation of TLR3-induced increases of hippocampal expression of IFN- and NF-κB-inducible genes. (a) i.c.v. administration of AM251 and MK886 reverses the URB597-induced decrease in hippocampal expression of the IFN-inducible genes *IP-10* and *SOCS1*, while MK886 reverses the URB597-induced decrease in hippocampal expression of the IFN-inducible transcription factor *IRF7*, when compared to vehicle-URB597-poly I:C-treated counterparts, 4 hours following systemic poly I:C challenge. In addition, (b) i.c.v. administration of AM251, MK886 and GW9662 reverses the URB597-induced decrease of the NF-κB-inducible cytokine *IL-6* when compared to vehicle-URB597-poly I:C-treated counterparts and MK886 and GW9662 reverses the URB597-induced decrease of *SOCS3*. ** $p < 0.01$ vs. vehicle-vehicle-saline + $p < 0.05$ vs. vehicle-vehicle-poly I:C ++ $p < 0.01$ vs. vehicle-vehicle-poly I:C # $p < 0.05$ vs. vehicle-URB597-poly I:C ## $p < 0.01$ vs. vehicle-URB597-poly I:C-treated counterparts. Data expressed as mean + SEM (n=6-11 per group).

5.3.5 Experiment 3: CB₁, CB₂, PPAR- α and PPAR- γ antagonism directly within the brain induces immunosuppressive effects on TLR3-induced increases in IFN- and NF- κ B-inducible genes within the hippocampus

Previous findings demonstrated that i.c.v. administration of the cannabinoid (CB₁) receptor and non-cannabinoid (PPAR- α/γ) antagonists reverses FAAH substrate-mediated attenuation of TLR3-induced IFN- and NF- κ B-inducible genes in the hippocampus. However, we next wanted to examine if i.c.v. administration of these antagonists alone, exerted effects on TLR3-induced neuroinflammation. Statistical analysis revealed a significant effect of treatment on hippocampal mRNA expression of the IFN-inducible genes *IRF7* [$F_{5,32}=8.127$, $p =0.000$], *IP-10* [$F_{5,35}=5.899$, $p =0.000$] and *SOCS1* [$F_{5,27}=7.724$, $p =0.000$] (Fig 5.5a). Furthermore, statistical analysis revealed a significant effect of treatment on mRNA expression of the NF- κ B-inducible cytokine *IL-6* [$F_{5,35}=3.617$, $p <0.05$] and also *SOCS3* [$F_{5,30}=3.926$, $p <0.01$], at 4 hours post systemic poly I:C administration (Fig.5b). AM251- and AM630-vehicle-poly I:C-treated animals displayed significantly reduced hippocampal mRNA expression of the IFN-inducible genes *IRF7*, *IP-10* and *SOCS1*, when compared to vehicle-vehicle-poly I:C-treated counterparts. Furthermore, MK886- and GW9662-vehicle-poly I:C-treated animals displayed significantly decreased hippocampal mRNA expression of both *IRF7* and *SOCS1*, while only MK886-vehicle-poly I:C-treated animals showed decreased mRNA expression of *IP-10*, when compared to vehicle-vehicle-treated counterparts, at 4 hours post poly I:C administration (Fig 5.5a). Analysis also revealed that both AM630- and MK886-vehicle-poly I:C-treated animals displayed significantly decreased hippocampal mRNA expression of *IL-6*. In addition, AM251-vehicle-treated animals displayed a trend for decreased *IL-6* expression, however, this effect failed to reach statistical significance. Furthermore, AM251- AM630- MK886 and GW9662-vehicle-poly I:C-treated animals displayed decreased mRNA expression of *SOCS3*, when compared to vehicle-vehicle-poly I:C-treated counterparts (Fig 5.5b).

Veh-veh-saline
 AM251-veh-poly I:C
 MK886-veh-poly I:C
 Veh-veh-poly I:C
 AM630-veh-poly I:C
 GW9662-veh-poly I:C

(a) IFN-inducible inflammatory genes



(b) NF-κB-inducible inflammatory genes

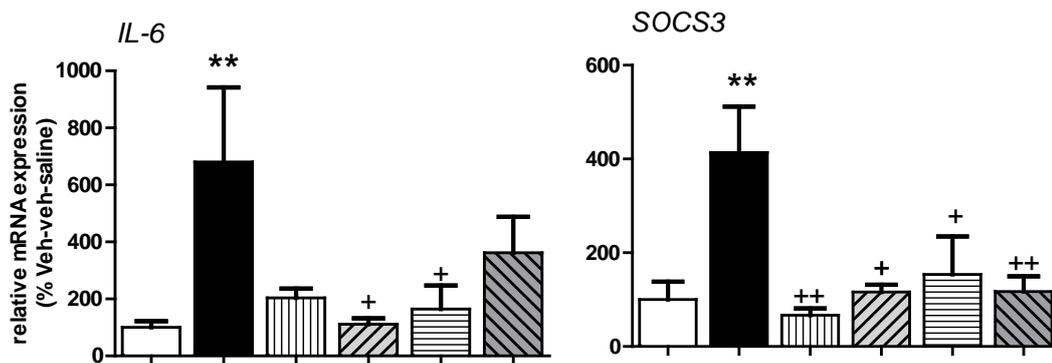


Figure 5.5 The effect of i.c.v. administration of selective receptor antagonists, in the absence of i.c.v. administration of URB597, on TLR3-induced neuroinflammation in the hippocampus. (a) I.c.v. administration of selective receptor antagonists, in the absence of i.c.v. administration of URB597, induces immunosuppressive on hippocampal expression of (a) IFN- inducible and (b) NF-κB-inducible genes, 4 hours post poly I:C. ** $p < 0.01$ vs. vehicle-vehicle-saline + $p < 0.05$ vs. vehicle-vehicle-poly I:C ++ $p < 0.01$ vs. vehicle-vehicle-poly I:C-treated counterparts. Data expressed as mean + SEM (n=5-10 per group).

5.3.6 Experiment 2: I.c.v. administration of URB597, in the presence or absence of i.c.v. administration of selective receptor antagonists, does not modulate serum cytokine levels following TLR3 activation

It has previously been demonstrated that i.c.v. administration of URB597 facilitates TLR4-induced increases of TNF α , while blockade of CB₁ receptors directly within the brain using the selective antagonist AM251 attenuates TLR4-induced increases in plasma TNF α levels (De Laurentiis *et al.*, 2010). As such we examined if i.c.v. administration of URB597, in the absence or presence of i.c.v. administration of CB₁, PPAR- α or PPAR- γ antagonists, modulated peripheral inflammatory mediators, induced following systemic TLR3 activation. Statistical analysis revealed that systemic administration of poly I:C increased serum levels of IP-10 [F_{5,39}=2.800, $p < 0.032$], but not TNF α , IL-1 β or IL-6, when compared to vehicle-vehicle-saline-treated counterparts, at 4 hours post administration. Prior i.c.v. administration of URB597, in the absence or presence of i.c.v. administration of the AM251, MK886 or GW9662, did not significantly alter the TLR3-induced increases in serum levels of IP-10 (Fig 5.6a). In addition, it should also be noted that i.c.v. administration of URB597 does not alter peripheral (splenic) levels of AEA, OEA or PEA, 4 hours post poly I:C administration (data not shown).

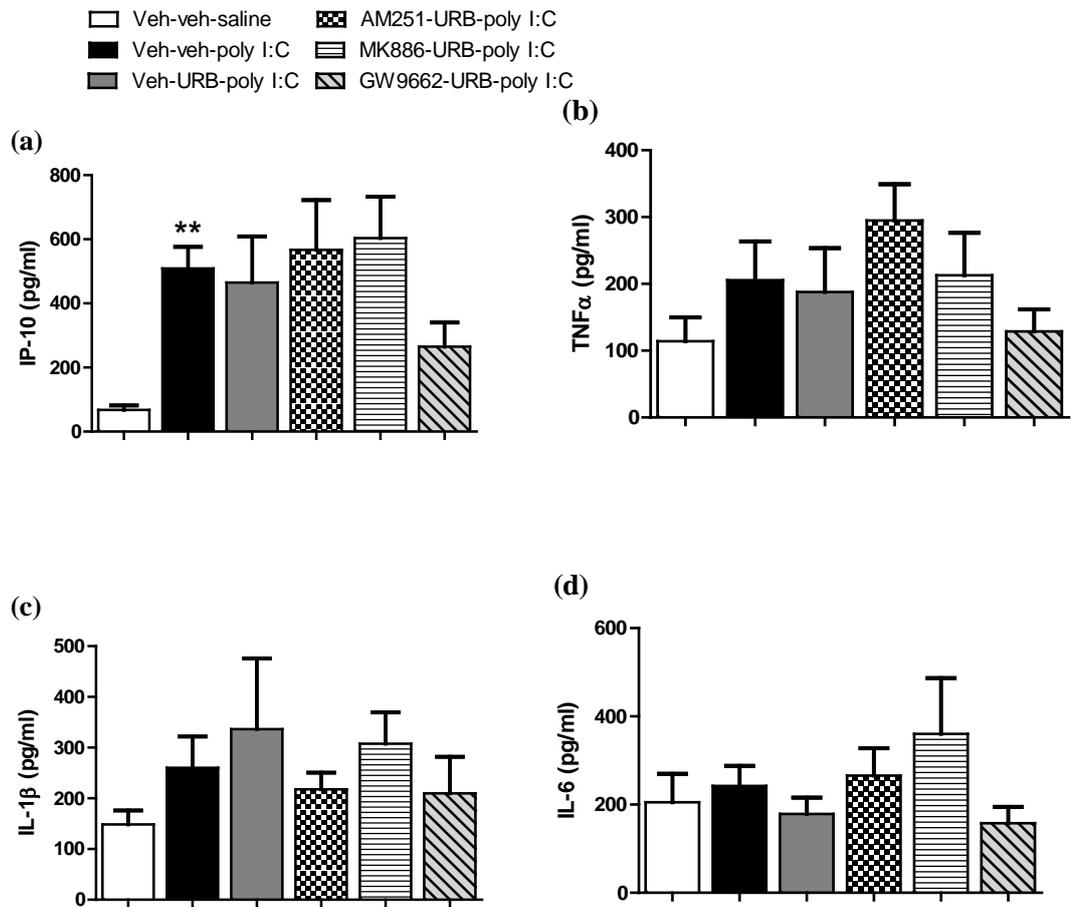


Figure 5.6 The effect of i.c.v. administration of URB597, in the absence or presence of i.c.v. administration of CB₁, PPAR- α or PPAR- γ receptor antagonists, on TLR3-induced inflammation in the periphery. (a) I.c.v. administration of URB597 does not alter serum levels of IP-10, nor was there a significant effect of this treatment regime on serum levels of (b) TNF α , (c) IL-1 β or (d) IL-6, 4 hours post systemic poly I:C administration. Data expressed as mean + SEM (n= 5-8 per group). ** $p < 0.01$ vs. vehicle-vehicle-saline-treated counterparts.

5.4 Discussion

The data presented herein demonstrate that both systemic and i.c.v. administration of the FAAH inhibitor URB597 increase hippocampal levels of the FAAH substrates OEA and PEA, while only i.c.v. administration of URB597 increases hippocampal levels of AEA. Prior i.c.v., but not systemic, administration of URB597 attenuated TLR3-induced increases in hippocampal expression of the IFN-inducible inflammatory mediators *IRF7*, *IP-10* and *SOCS1*. In addition, i.c.v. administration of URB597 attenuated hippocampal expression of the NF- κ B-inducible genes *IL-6* and *SOCS3*. Blockade of CB₁ and PPAR- α directly within the brain following i.c.v. administration of the selective antagonists AM251 and MK886, respectively, completely reversed the FAAH substrate-mediated decrease in hippocampal expression of the IFN-inducible genes *IP-10* and *SOCS1*. Furthermore, antagonism of PPAR- α directly within the brain reversed the FAAH substrate-mediated decrease in hippocampal expression of the IFN-inducible transcription factor *IRF7*. In addition, antagonism of CB₁, PPAR- α and PPAR- γ directly within the brain, following i.c.v. administration of selective antagonists, resulted in a reversal of the FAAH substrate-mediated decrease in hippocampal expression of the NF- κ B-inducible cytokine *IL-6*, while only antagonism of PPAR- α or PPAR- γ directly within the brain reversed the FAAH substrate-mediated decrease in *SOCS3* expression. Somewhat surprisingly, antagonism of CB₁, CB₂ or the PPARs directly within the brain, following i.c.v. of selective antagonists, exerted potent immunosuppressive effects on hippocampal expression of both IFN – and NF- κ B-inducible genes following systemic TLR3 activation. Additionally, increased FAAH substrate levels directly within the brain, either in the absence or presence of specific antagonists, did not alter TLR3-induced increases in serum levels of IP-10, further suggesting that the effects observed are mediated within the CNS rather than modulation of peripheral immune responses. Overall, these findings demonstrate an important role for cannabinoid (CB₁) and non-cannabinoid (PPAR- α/γ) receptors directly within the brain in FAAH substrate-mediated modulation of TLR3-induced neuroinflammation within the hippocampus.

The initial part of this chapter focused on examining the potential receptor mechanisms involved in mediating the effects of increased FAAH substrates following systemic administration of URB597, on TLR3-induced increases in inflammatory genes within the hippocampus and periphery. Systemic administration of URB597 increased

hippocampal levels of both OEA and PEA, however unlike that previously reported in Chapter 3; this treatment regime failed to alter TLR3-induced increases in inflammatory mediators in either the hippocampus or serum. Such discrepancies between studies may be attributed to differential treatment regimes; specifically animals in Chapter 3 received two i.p. injections, whereas initially in the current study animals received three i.p. injections (data not shown). Although this initial treatment regime was based on previously published work (Kerr *et al.*, 2013b; Roche *et al.*, 2008), we found that there was quite a substantial amount of variability within groups and as a result, data were difficult to interpret. Previous work examining the potential receptor mechanisms involved in mediating cannabinoid-induced immunoregulation, following systemic TLR4 activation utilized an approach whereby animals were co-administered (i.p.) with a cannabinoid receptor agonist and cannabinoid receptor antagonist (Roche *et al.*, 2006) which would reduce possible stress effects associated with a 2 injection procedure. As such, we repeated our experiment whereby animals received co-administration (i.p.) of the specific receptor antagonists and URB597, followed by i.p. administration of poly I:C. However, this approach also failed to replicate FAAH substrate-mediated modulation of TLR3-induced inflammatory responses within the hippocampus observed in Chapter 3 (data not shown) which may again have been due to the variability within groups. Furthermore, although the relative increases in hippocampal levels of the FAAH substrates were comparable between both studies (present study and Chapter 3), the TLR3-induced induced increases in both serum levels and hippocampal expression of inflammatory mediators were reduced in the present study when compared to those reported in Chapter 3. Thus, it may be likely that the reduced immune response in the present study may be partially responsible for lack of FAAH substrate-mediated modulation of cytokine expression/levels following systemic TLR3 administration.

In comparison to the data from systemically administered URB597, i.c.v. administration of URB597 and subsequent increases in FAAH substrates directly within the brain, exerted a robust anti-inflammatory profile on TLR3-induced neuroinflammation similar to that observed in Chapter 4. In accordance with data reported in Chapter 4, i.c.v. administration of URB597 increased hippocampal levels of OEA and PEA; however the treatment regime used in the present study also increased hippocampal levels of AEA. This increase in AEA may be due to the additional vehicle i.c.v. injection which may have in itself induced an increase in AEA release, which is then maintained when

URB597 inhibits FAAH activity. In any case, the increase in FAAH substrates directly within the brain were associated with an attenuation of the expression of both the IFN-inducible genes *IRF7*, *IP-10* and *SOCS1* and also the NF- κ B-inducible cytokine *IL-6* and *SOCS3* in the hippocampus, further suggesting that FAAH substrates directly in the brain have potent immunomodulatory effects following TLR3 activation. A key aim of this study was to determine the receptor mechanisms that underlie these immunomodulatory effects. As highlighted, FAAH substrates can activate multiple receptor targets, most notably cannabinoid (CB_{1/2}) receptors and PPARs. The data presented here show that antagonism of the CB₁ receptor, but not the CB₂ receptor, directly within the brain, completely reversed the FAAH substrate-mediated attenuation of hippocampal expression of both the IFN-inducible genes *IP-10* and *SOCS1*, and with a strong trend for a reduction in *IRF7*. In addition to the IFN-inducible genes, blockade of CB₁ directly within the brain also attenuated FAAH substrate-mediated decreases in expression of the NF- κ B-inducible cytokine *IL-6*, but not *SOCS3*. Our data revealed increases in hippocampal levels of the FAAH substrates AEA, OEA and PEA, all of which may be involved in mediating such immunoregulatory effects on TLR3-induced neuroinflammation. However, out of these three FAAH substrates, only AEA is known to display affinity for the cannabinoid receptors. In this regard, there are now several lines of evidence from *in vitro* studies that AEA mediates anti-inflammatory effects on TLR4-induced neuroinflammation, in a CB₁ receptor-dependent manner (Krishnan *et al.*, 2012; Molina-Holgado *et al.*, 2002b; Ortega-Gutierrez *et al.*, 2005). As such, it is likely that AEA-induced activation of CB₁ receptors directly within the brain is responsible, at least in part, for mediating the reported immunoregulatory effects on TLR3-induced neuroinflammation within the hippocampus. Findings in Chapter 4 demonstrated that increased FAAH substrates directly within the brain were associated with a decrease in TLR3-induced increases in pro-inflammatory cytokines, with concurrent increases in expression of the anti-inflammatory cytokine *IL-10*. In this respect, AEA-induced activation of cannabinoid receptors has been proposed to mediate anti-inflammatory effects on TLR4-induced inflammation via enhanced production of *IL-10* (Correa *et al.*, 2010). However, this mechanism is not likely in the present study as we do not report any significant effect of treatment on hippocampal expression of *IL-10*, which therefore suggests that AEA-induced activation of CB₁ receptors within the brain, is mediating immunoregulatory effects on TLR3-induced inflammation

independent of increased hippocampal expression of *IL-10*. In addition to CB₁, there are now several lines of evidence implicating an important role for CB₂ receptors in AEA-induced modulation of TLR4-induced neuroinflammation, in mixed glial cultures (Correa *et al.*, 2009a; Correa *et al.*, 2010; Malek *et al.*, 2015). However, findings in the current study indicate that AEA-induced modulation of TLR3-induced neuroinflammation occurs independent of activation of CB₂ receptors within the brain, exemplified by the findings that antagonism of the CB₂ receptor, via i.c.v. administration of the selective antagonist AM630, does not alter FAAH substrate-mediated modulation of TLR3-induced increases of either IFN- or NF-κB-inducible inflammatory genes in the hippocampus. Overall, our current data indicate an important role for activation of CB₁, but not CB₂ receptors directly within the brain in FAAH substrate-mediated attenuation of TLR3-induced neuroinflammation.

However, it is important to note that i.c.v. administration of the CB₁ and CB₂ receptor antagonists, AM251 and AM630, alone, exerted immunosuppressive effects on TLR3-induced neuroinflammation in the hippocampus. This may seem as odds with the blockade of the URB597 associated effects, however several reports have demonstrated that selective CB₁ and CB₂ receptor antagonists exert immunosuppressive effects on TLR-induced inflammation *in vivo* (De Laurentiis *et al.*, 2010; Kerr *et al.*, 2013b; Roche *et al.*, 2008), although this is the first to examine effects specifically within the brain. The precise mechanisms mediating such responses remains to be determined, however there are suggestions that the reported immunosuppressive effects of cannabinoid receptor antagonists may be associated with their ability to block the effects of endocannabinoids on CB₁ and CB₂ receptors and as a result unmask endocannabinoid action at additional receptor targets including PPARs (Rockwell *et al.*, 2004). In addition, there are also reports that select cannabinoid receptor antagonists may act as partial agonists at CB₁ and CB₂ receptors when administered alone (Crocchi *et al.*, 2003; Roche *et al.*, 2006; Roche *et al.*, 2008; Smith *et al.*, 2000). In any case, regardless of the exact mechanism via which cannabinoid receptor antagonists within the brain may be mediating immunosuppressive effects, our current data has shown that antagonism of the CB₁ receptor directly within the brain is associated with a complete reversal of the FAAH substrate-mediated attenuation of TLR3-induced increases in hippocampal expression of IFN- and NF-κB-inducible genes.

In addition to mediating effects via cannabinoid receptors, there is now evidence that AEA is capable of directly activating alternative receptor targets including PPAR- γ (Bouaboula *et al.*, 2005; Gasperi *et al.*, 2007) where AEA-induced decreases in IL-2 has been proposed to be mediated via a PPAR- γ -dependent activation (Rockwell *et al.*, 2004). Furthermore, as previously mentioned, our findings demonstrate that in addition to increased hippocampal levels of AEA, URB597 also increased hippocampal levels of the FAAH substrates OEA and PEA, which are also agonists at PPAR- α/γ where they have been shown to exert potent anti-inflammatory and neuroprotective effects (Gonzalez-Aparicio *et al.*, 2014; Lambert *et al.*, 2002; Sayd *et al.*, 2015; Skaper *et al.*, 2015; Skaper *et al.*, 2013) in several experimental models. Accordingly, the data generated in this study demonstrated that blockade of PPAR- α directly within the brain attenuated the FAAH substrate-mediated decreases in hippocampal expression of the IFN-inducible genes *IP-10*, *SOCS1* and *IRF7* and the NF- κ B-inducible genes *IL-6* and *SOCS3*, induced following TLR3 activation. Furthermore, blockade of PPAR- γ directly within the brain reversed the FAAH substrate-mediated attenuation of *IL-6* and *SOCS3* expression, while blockade of this receptor has no effect on any of the IFN-inducible genes examined. There is evidence that all PPAR isoforms are expressed within the CNS, in both neuronal and glial cells (Cimini *et al.*, 2005; Heneka *et al.*, 2007; Moreno *et al.*, 2004). To our knowledge this is the first study to demonstrate an involvement of PPAR- α and PPAR- γ within the brain in FAAH substrate-mediated modulation of TLR3-induced increases in neuroinflammatory mediators. There are now several reports that activation of PPAR- α with its agonist fenofibrate is associated with potent anti-inflammatory and neuroprotective effects in animal models of TBI (Besson *et al.*, 2005; Chen *et al.*, 2007), PD (Barbiero *et al.*, 2014), ischemic stroke (Deplanque *et al.*, 2003) and MS (Xu *et al.*, 2005), anti-inflammatory effects of which are reported to be attributed to inhibition of NF- κ B activation and subsequent decreases in pro-inflammatory cytokines (Poynter *et al.*, 1998). In accordance, fenofibrate-induced activation of PPAR- α has been shown to attenuate TLR3 induction of the NF- κ B reporter gene (Downer *et al.*, 2012), while PEA-induced activation of PPAR- α inhibits NF- κ B activation (D'Agostino *et al.*, 2009). Our current findings demonstrate that blockade of PPAR- α within the brain attenuates the FAAH substrate-mediated decrease in hippocampal expression of *IL-6* and *SOCS3*; a key mediator downstream of *IL-6* signalling, which therefore suggests that activation of PPARs directly within the brain

are involved in FAAH substrate-mediated modulation of TLR3-induced neuroinflammation, which may at least in part, be mediated via inhibition of NF- κ B. In addition, this study also demonstrated that increased FAAH substrates directly within the brain attenuated TLR3-induced increases in hippocampal expression of several IFN-inducible inflammatory mediators including *IRF7*, *IP-10* and *SOCS1*; effects of which are all completely reversed in the presence of blockade of PPAR- α directly within the brain. In contrast with our current findings, Downer and colleagues identified that the synthetic cannabinoid WIN55,212-2 acted via PPAR- α to increase TLR3-induced activation of JNK, AP-1, and subsequent increased transcriptional activation of IFN- β in primary astrocyte cultures (Downer *et al.*, 2012). Discrepancies may be due to the model under investigation (*in vitro* vs. *in vivo*; or the cannabinoid in question WIN55212-2 vs. FAAH substrates). It has previously been reported that in addition to inducing decreases in NF- κ B activation following TLR4- or IFN- β -induced stimulation of microglia; administration of PPAR agonists also attenuates IFN- β -induced STAT1 phosphorylation and subsequent production of IP-10 (Si *et al.*, 2004). This may account, at least in part, for some of the effects of FAAH substrate-induced activation of PPAR- α observed in the present study, as *IRF7*, *IP-10* and *SOCS1* are all produced downstream of STAT1 activation. Furthermore, STAT1 is reported to contribute to *IL-6* expression (Chaudhuri *et al.*, 2008; Lee *et al.*, 2006a), where it has recently been demonstrated that basal type I IFN-induced signalling is necessary to facilitate robust expression of *IL-6* within the hippocampus, following systemic TLR3 activation (Murray *et al.*, 2015b). Thus, it seems plausible to suggest that increased FAAH substrates within the CNS may be exerting modulatory effects on type I IFN-inducible genes, via activation of CB₁ and/or PPAR- α directly within the brain, which in turn attenuates hippocampal expression of *IL-6* and downstream genes including *SOCS3*. However, further studies are required in order to fully elucidate the exact molecular mechanisms involved in mediating the reported effects on TLR3-induced neuroinflammatory responses.

In addition to PPAR- α , findings in the present study also demonstrate that antagonism of PPAR- γ directly within the brain was associated with a reversal of the FAAH substrate-mediated attenuation of TLR3-induced increases in hippocampal expression of *IL-6* and *SOCS3*. In this regard, it has previously been reported that activation of PPAR- α and - γ directly within the brain, via i.c.v. administration of receptor agonists, was associated with both anti-inflammatory and anti-hyperalgesic effects in experimental models of

inflammatory pain (D'Agostino *et al.*, 2009; D'Agostino *et al.*, 2007; Morgenweck *et al.*, 2010); effects of which, at least in the case of PPAR- γ , were shown to be mediated via direct actions of PPAR- γ within the brain (Morgenweck *et al.*, 2010). With regards to TLR-induced inflammation, it has previously been reported that blockade of PPAR- γ attenuates AM404-induced increases in plasma TNF α , following TLR4 activation (Roche *et al.*, 2008), however to our knowledge this is the first study to examine and report effects of PPAR- γ directly within the brain on TLR-induced neuroinflammatory responses. Evidence from *in vitro* studies report that stimulation of PPAR- γ with the endogenous ligand prostaglandin J₂ (15d-PGJ₂) is associated with an attenuation the production of neuroinflammatory mediators induced following TLR4-induced stimulation of microglial cells (Bernardo *et al.*, 2000; Gurley *et al.*, 2008; Storer *et al.*, 2005), anti-inflammatory effects of which have recently been attributed to be, at least in part, due to the ability of 15d-PGJ₂ to inhibit TLR4-induced alterations in CD200-CD200R1 function (Dentesano *et al.*, 2014). With regards to TLR3-induced responses, recent reports suggest that the synthetic PPAR- γ agonist troglitazone attenuates TLR3-induced increases of IFN- β secretion from peritoneal macrophages (Zhao *et al.*, 2011), thus suggesting that activation of PPAR- γ may directly modulate type I IFN signalling, following TLR3 activation. However, our current findings demonstrate that unlike PPAR- α , antagonism of PPAR- γ within the brain does not alter FAAH substrate-mediated attenuation of TLR3-induced increases in type I IFN signalling. It has previously been reported that 15d-PGJ₂-induced activation of PPAR- γ is associated with a decrease in NF- κ B activation (Doyle *et al.*, 2013). Furthermore, activation of PPAR- γ has been shown to inhibit TLR4-induced acute lung injury via inhibition of NF- κ B and subsequent cytokine production (Lin *et al.*, 2015). Thus it is likely that FAAH substrate mediated activation of PPAR- γ within the brain may act to inhibit NF- κ B activation and subsequent attenuation in hippocampal expression of *IL-6* and *SOCS3*. However, further studies are required in order to fully elucidate the exact mechanism of action.

Similar to the immunosuppressive effects reported following i.c.v. administration of both AM251 and AM630, i.c.v. administration of the PPAR- α and PPAR- γ antagonists MK886 and GW9662, alone, also exerted immunosuppressive effects on TLR3-induced neuroinflammation in the hippocampus. In this respect, although MK886 is known to act as a non-competitive inhibitor of PPAR- α (Kehrer *et al.*, 2001), it was originally identified as a potent inhibitor of leukotriene biosynthesis (Vickers, 1995) which may

account for the immunosuppressive effects on TLR3-induced neuroinflammation. As such, further studies with more selective antagonists may be required in order to fully delineate effects (PPAR- α antagonist vs. leukotriene inhibitor) from each other. In any case, our current data has shown that blockade of PPARs directly within the brain reverses FAAH substrate-mediated attenuation of TLR3-induced neuroinflammation in the hippocampus.

Finally, it has previously been reported that i.c.v. administration of URB597 and AM251, at the same doses used in the present study, were capable of enhancing and attenuating TLR4-induced increases in plasma levels of TNF α levels, respectively (De Laurentiis *et al.*, 2010). However, our findings report that i.c.v. administration of URB597 and subsequent increases in FAAH substrates directly within the brain, but not the periphery (data not shown), either in the absence or presence of i.c.v. administration of AM251, MK886 or GW9662, does not alter TLR3-induced increases in serum levels of IP-10, nor does this treatment regime alter serum levels of TNF α , IL-6 or IL-1 β . These results further supports the findings that the effects of FAAH substrates on TLR3 associated neuroinflammation are mediated directly within the brain.

5.4.1 Conclusion

Overall, the current data have reaffirmed our initial findings demonstrating that increased levels of FAAH substrates directly within the brain are associated with robust immunoregulatory effects on TLR3-induced neuroinflammatory mediators within the hippocampus. Furthermore, our findings suggest that FAAH substrate-mediated regulation of TLR3-induced neuroinflammation within the hippocampus is likely mediated via activation of both (cannabinoid) CB₁ receptor - and (non-cannabinoid) - PPAR- α/γ - targets directly within the brain.

Chapter 6

Investigation of the effect of systemic and i.c.v. administration of selective MAGL inhibitors on TLR-induced neuroinflammation in the hippocampus

6.1 Introduction

2-AG is the most abundant endocannabinoid within the brain, present at approximately 200-fold higher concentrations than AEA (Bisogno *et al.*, 1999). 2-AG acts as a full agonist for CB₁ and CB₂ receptors and is primarily metabolised (85%) by the enzyme MAGL into AA and glycerol, in the rodent brain (Blankman *et al.*, 2007; Dinh *et al.*, 2002a). However, additional enzymes have also been identified as being involved in 2-AG metabolism including the serine hydrolases ABHD6 and ABHD12 (Blankman *et al.*, 2007), FAAH (Di Marzo *et al.*, 1998), COX-2 (Kozak *et al.*, 2002; Kozak *et al.*, 2000) and carboxylesterases (Xie *et al.*, 2010); all of which together account for approximately 15% of 2-AG metabolism. Increased levels of 2-AG have been reported in animal models of AD, PD, MS and TBI (Baker *et al.*, 2001; Panikashvili *et al.*, 2001; van der Stelt *et al.*, 2005; van der Stelt *et al.*, 2006), where it is suggested to mediate an endogenous protective response (Panikashvili *et al.*, 2001; Shohami *et al.*, 2011; Zogopoulos *et al.*, 2013). 2-AG-induced neuroprotection is suggested to be mediated, at least in part, via its potent anti-inflammatory properties, which have now been extensively reported *in vitro* (Alhouayek *et al.*, 2013; Chang *et al.*, 2001; Facchinetti *et al.*, 2003b; Gallily *et al.*, 2000; Krishnan *et al.*, 2012; Zhang *et al.*, 2008). Accordingly, direct administration of 2-AG or enhancement of endogenous 2-AG levels by pharmacologically inhibiting its hydrolysis is associated with neuroprotective effects following excitotoxic (A β , kainic acid or glutamate)- or inflammatory (LPS)-induced insults via a CB₁ receptor-dependent inhibition of ERK/p38 MAPK, NF- κ B phosphorylation and COX2 expression in hippocampal neurons in culture (Chen *et al.*, 2011; Zhang *et al.*, 2008) and caudate nucleus neurons (Lu *et al.*, 2014b). Furthermore, 2-AG attenuates TLR4/IL-1 β -induced increases in NF- κ B phosphorylation, COX-2 expression and excitatory synaptic transmission via CB₁-dependent increases in PPAR- γ expression (Du *et al.*, 2011). Thus, evidence from *in vitro* studies suggests that 2-AG-induced neuroprotection is at least partially mediated via modulation of inflammatory mediators. In accordance with these findings, administration of 2-AG has been shown to exert neuroprotective effects in the closed head injury (CHI) model of TBI, at least in part, via a CB₁ receptor-mediated decrease in NF- κ B activation (Panikashvili *et al.*, 2005). A subsequent study from the same group reported that 2-AG reduced BBB permeability and expression of the NF- κ B-inducible pro-inflammatory cytokines TNF α ,

IL-1 β and IL-6 while concurrently increasing anti-oxidants in CHI mice (Panikashvili *et al.*, 2006). However, despite these evident immunoregulatory and neuroprotective effects of 2-AG within the CNS; a key limitation in investigating the role of 2-AG on such responses *in vivo* has been the lack of pharmacological tools available to increase 2-AG levels within the CNS and thus avoid the unwanted side effects of direct activation of CB₁ receptors with potent agonists. First generation MAGL inhibitors included the reversible N-biphenyl carbamate URB602 which selectively increased 2-AG concentrations in rat brain cultures when injected locally (Hohmann *et al.*, 2005) and elicited anti-inflammatory effects in animal models of pain (Comelli *et al.*, 2007; Guindon *et al.*, 2011). However, the use of this compound was limited due to various factors including low solubility and potency (Hohmann *et al.*, 2005) and also its lack of selectivity for MAGL over FAAH (Vandevorode *et al.*, 2007). To this end, the development of the novel and potent irreversible MAGL inhibitor JZL184 (Long *et al.*, 2009a; Long *et al.*, 2009b) enabled researchers to examine the effects of increasing endogenous 2-AG in several physiological and pathological processes (Mulvihill *et al.*, 2013). In terms of inflammation, there are now several lines of evidence demonstrating that systemic administration of JZL184 exerts immunoregulatory and anti-inflammatory effects on TLR4-induced inflammation, both peripherally (Costola-de-Souza *et al.*, 2013; Sardinha *et al.*, 2014) and within the CNS (Kerr *et al.*, 2013b; Nomura *et al.*, 2011). Although some of these anti-inflammatory effects have been attributed to 2-AG-induced activation of CB₁/CB₂ receptors (Alhouayek *et al.*, 2011; Costola-de-Souza *et al.*, 2013), cannabinoid receptor-independent effects have also been reported whereby Nomura and colleagues demonstrated that 2-AG-induced anti-inflammatory effects on TLR4-induced neuroinflammation occurred via a reduction in AA levels and a consequent reduction in prostaglandin production within the CNS (Nomura *et al.*, 2011).

To date, the majority of *in vivo* studies examining the effects of JZL184 on TLR4-induced inflammation have been carried out on mice, with only one study to date examining such responses in rats (Kerr *et al.*, 2013b). In this study, it was suggested that the effects of systemic administration of JZL184 on TLR4-induced neuroinflammation were most likely not mediated at the level of the rat brain but rather via modulation of TLR4-induced peripheral inflammation, as JZL184 was not detected in the brain, nor was MAGL activity or 2-AG levels altered within the frontal cortex (Kerr *et al.*, 2013b).

In this respect, although JZL184 acts as a potent irreversible inhibitor of MAGL, it is known to have reduced affinity for rat MAGL compared with the murine enzyme (Long *et al.*, 2009b). As such, over the last number of years novel MAGL inhibitors with greater selectivity for rat MAGL and increased BBB permeability have been developed, including the N-Hydroxysuccinimidyl (NHS) carbamate, MJN110 (Niphakis *et al.*, 2013), which may offer a more suitable tool for examining the physiological effects of enhanced 2-AG tone *in vivo*.

Although increased levels of FAAH substrates following administration of the FAAH inhibitor URB597 are associated with potent immunoregulatory effects on TLR3-induced neuroinflammation within the hippocampus (Chapters 3-5), to date no studies have examined the effect of modulation of endogenous 2-AG levels on TLR3-induced inflammation, either in the periphery or within the CNS. Furthermore, there are now several reports of increased 2-AG levels within the CNS of animal models of AD, PD, MS, ischemic stroke and TBI (Baker *et al.*, 2001; Panikashvili *et al.*, 2001; van der Stelt *et al.*, 2005; van der Stelt *et al.*, 2006) where it is reported to exert neuroprotective effects, at least in part, via its anti-inflammatory effects (Panikashvili *et al.*, 2005; Panikashvili *et al.*, 2006). However, to date no studies have examined the effect of pharmacological inhibition of MAGL directly within the brain on endogenous 2-AG levels and TLR-mediated neuroinflammatory responses. Such studies would greatly enhance our understanding of the role of elevated 2-AG in neurological and neuroinflammatory disorders.

Hypothesis: Based on these data we hypothesised that enhancing 2-AG tone both globally and directly within the CNS would modulate TLR3 and TLR4-induced increases in neuroinflammation. Therefore the aims of the studies described in this chapter were to:

1. Investigate the effect of systemic administration of MJN110, a selective inhibitor of MAGL, the enzyme that is primarily responsible for the metabolism of 2-AG, on TLR3-induced increases in IFN- and NF- κ B-inducible inflammatory genes within the hippocampus.

2. Previous studies have demonstrated that systemic administration of the MAGL inhibitor JZL184 modulates TLR4-induced neuroinflammation. However it is unknown if the effects of this compound are due to elevated levels of 2-AG directly within the brain. Thus the second aim of the studies described in this chapter was to investigate the effect of i.c.v. administration of JZL184, on 2-AG levels and NF- κ B-inducible inflammatory cytokines within the hippocampus, following systemic administration of the TLR4 agonist LPS.

3. Given the lack of effect of JZL184 on 2-AG levels in the brain, this study investigated the effect of direct i.c.v. administration of the alternative MAGL inhibitor MJN110 in different vehicle preparations on levels of 2-AG within the hippocampus.

4. Investigate the effect of i.c.v. administration of MJN110 and subsequent increases in 2-AG levels directly within the brain, on TLR4-induced neuroinflammation within the hippocampus.

6.2 Methodology and Experimental design

Experiments were carried out on male and female Sprague Dawley rats (weight 220-260g; Charles River, UK), housed singly in plastic bottomed cages (45 X 25 X 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 0800 to 2000 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 an intraperitoneal (i.p.) injection of sterile saline (0.89% NaCl) for 3-4 days prior to experimentation in order 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 licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

6.2.1 *Experiment 1: Investigation of the effect of systemic administration of the MAGL inhibitor MJN110 on hippocampal levels of 2-AG, 4 hours post administration*

Rats were randomly assigned into one of 2 treatment groups: Vehicle- and MJN110-treated animals (n = 3-4 per group). Animals received a single, acute systemic injection of MJN110 (Gifted from Ben Cravatt Lab; 5mg/kg, i.p.) or vehicle (1:1:18; ethanol:cremaphor:saline) in an injection volume of 2ml/kg. The dose of MJN110 was based on previous findings demonstrating that this dose increased brain levels of 2-AG (Niphakis et al., 2013). Animals were sacrificed by decapitation at 4 hours post vehicle/MJN110 administration, the brain was rapidly removed and the hippocampus excised, snap-frozen on dry ice and stored at -80°C until assayed for the levels of endocannabinoids and *N*-acylethanolamines. (The methodology was essentially as described in Chapter 2).

6.2.2 *Experiment 2: Investigation of the effect of systemic administration of MJN110 on hippocampal expression of inflammatory mediators induced following acute systemic poly I:C administration*

Rats were randomly assigned into one of two treatment groups: Vehicle-poly I:C and MJN110-poly I:C (n = 4-8 per group). Animals received a single, acute systemic injection of MJN110 (5mg/kg, i.p.) or vehicle (1:1:18; ethanol:cremaphor:saline) in an injection volume of 2ml/kg, followed 1 hour later with a single, acute i.p. injection of poly I:C (3mg/kg, i.p.) or vehicle (0.89% sterile saline), in an injection volume of 1.5ml/kg. We have previously demonstrated that the dose of MJN110 utilized in this study does not alter inflammatory cytokine expression either in the periphery or within the CNS in the absence of a poly I:C challenge; as such we did not include this group in the current analysis (unpublished data). Animals were returned to their home cages and sacrificed by decapitation at 4 hours following poly I:C administration, brain removed, hippocampus excised, snap-frozen and stored at -80°C until assayed for cytokine expression.

6.2.3 *Experiment 3: Investigation of the effect of i.c.v. administration of the MAGL inhibitor JZL184 on 2-AG levels and hippocampal expression of neuroinflammatory mediators following systemic administration of the TLR4 agonist LPS*

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol in Chapter 2), rats were randomly assigned into one of two treatment groups: Vehicle-LPS and JZL184-LPS (n = 8 per group). Rats received a single, acute i.c.v. injection of JZL184 (NIMH drug synthesis programme; 0.7µmoles) or vehicle (100 % DMSO) at a flow rate of 3µl infused over 1 min. Animals received a single, acute i.p. injection of LPS (100ug/kg, B0111:B4; Sigma Aldrich, Dublin, Ireland) or vehicle (0.89% sterile saline) 15 min post JZL184/vehicle administration. The dose and time of JZL184 was chosen on the basis of pilot work within our lab demonstrating increased 2-AG levels within the brain. The dose of LPS administration was chosen on the basis of previous work in our laboratory demonstrating evident increases in cytokine expression and protein levels in discrete brain regions at 2-4 hours post systemic LPS administration (Kerr et al., 2011; Roche et al., 2006). Animals were returned to their home cages and sacrificed by decapitation at 2 hours following LPS administration, brain removed, hippocampus excised, snap-frozen and stored at -80°C until assayed for

levels of endocannabinoids and *N*-acylethanolamines and cytokine expression. The presence of JZL184 in the hippocampal tissue was confirmed using Q-TOFF mass spectrometry analysis (See Chapter 2 for method).

6.2.4 *Experiment 4: Investigation of the effect of i.c.v. administration of MJN110 on hippocampal levels of 2-AG*

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol Chapter 2), rats were randomly assigned into one of eight treatment groups: Vehicle (100% DMSO), MJN110 (100 % DMSO), Vehicle (100 % DMF), MJN110 (100 % DMF), Vehicle (50% DMSO: 50% PEG), MJN110 (50% DMSO: 50% PEG), Vehicle (50% DMF: 50% PEG) and MJN110 (50% DMF: 50% PEG) with n = 3 per group). Animals received a single, acute i.c.v. injection of MJN110 (0.7 μ moles) or appropriate vehicle at a flow rate of 5 μ l infused over 1 min. The dose of MJN110 was based on that chosen for JZL184. Animals were returned their home cages and sacrificed by decapitation at 2 hours post MJN110/vehicle administration, brain removed, hippocampus excised, snap-frozen and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines.

6.2.5 *Experiment 5: Investigation of the effect of i.c.v. administration of MJN110 on inflammatory mediators in the hippocampus, induced following systemic administration of LPS*

Rats were randomly assigned into one of two treatment groups: Vehicle-LPS, MJN110-LPS (n = 7-8 per group). Animals received a single, acute i.c.v. injection of MJN110 (0.7 μ moles) or vehicle (100% DMF) at a flow rate of 5 μ l infused over 1min, followed 15 min later with a single, acute i.p. injection of LPS (100 μ g/kg, i.p.) or vehicle (0.89% sterile saline). Animals were returned their home cages and sacrificed by decapitation at 2 hours post LPS administration, brain removed, hippocampus excised, snap-frozen and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression.

6.2.6 Statistical Analysis

SPSS (IBM, New York, USA) statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro–Wilks and Levene test $p > 0.05$, respectively. When comparing the means of two unrelated groups, parametric data were analysed using unpaired t -test. One-way ANOVA was used to compare the mean of more than two groups on one factor. *Post-hoc* analysis was performed using Fisher's LSD test. Data were considered significant when $p < 0.05$. All graphs representing data were constructed using GraphPad Prism 5.0 and results expressed as group means + standard error of the mean (SEM).

6.3 Results

6.3.1 Experiment 1: Systemic administration of the MAGL inhibitor MJN110 increases hippocampal levels of 2-AG

Our previous findings have demonstrated that increasing FAAH substrates within the brain, following both systemic and i.c.v. administration of the FAAH inhibitor URB597 exerted potent immunoregulatory effects on TLR3-induced neuroinflammatory responses. We next wanted to investigate the potential involvement of 2-AG on such TLR3-induced inflammatory responses. In the first instance we examined the effect of systemic administration of the potent MAGL inhibitor MJN110 on hippocampal levels of 2-AG, 4 hours post administration; the time point at which the most robust immunoregulatory effects of URB597 on poly I:C-induced inflammation were reported in previous chapters (Chapters 3-5). Statistical analysis revealed that systemic administration of the potent MAGL inhibitor MJN110 significantly increased hippocampal levels of 2-AG [$t_{(2)}=57.748$, $p =000$] (Fig 6.1a), but not AEA, OEA or PEA, when compared to vehicle-treated counterparts, at 4 hours post systemic MJN110 administration (Fig 6.1b,c,d).

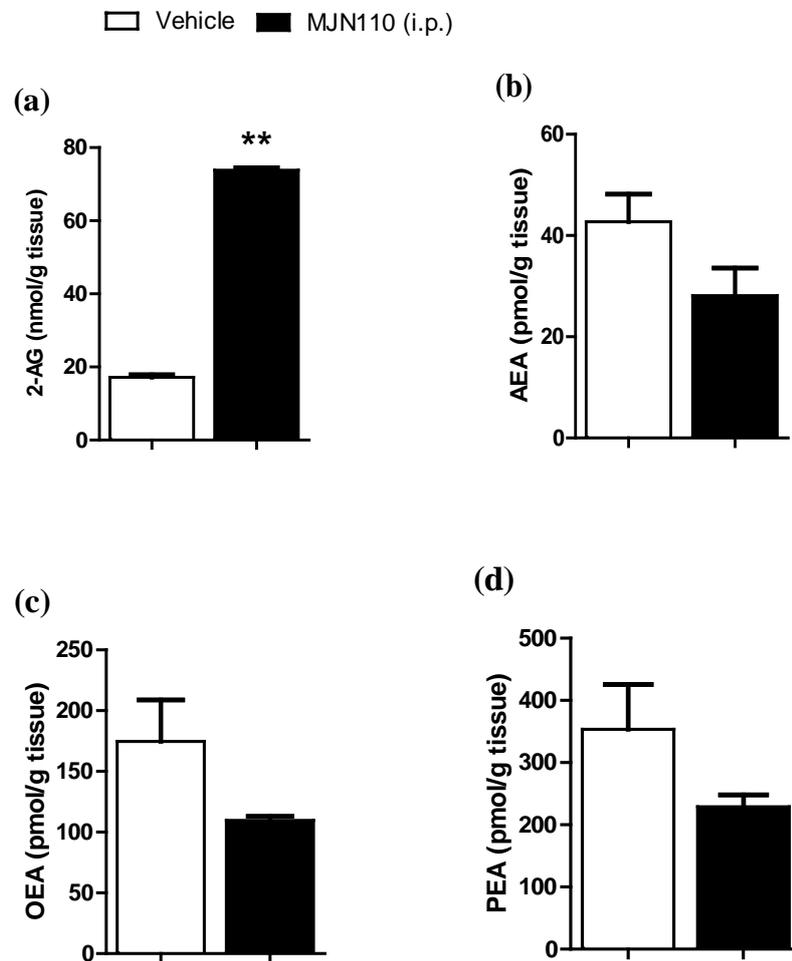


Figure 6.1 The effect of systemic administration of the MAGL inhibitor MJN110 on hippocampal levels of 2-AG. Systemic administration of the potent MAGL inhibitor MJN110 significantly increased hippocampal levels of (a) 2-AG, but not (b) AEA, (c) OEA or (d) PEA, 4 hours post systemic MJN110 administration. Data expressed as mean + SEM (n = 3-4 per group). ** $p < 0.01$ vs. vehicle-treated animals.

6.3.2 Experiment 2: Systemic administration of MJN110 enhances hippocampal expression of IFN-inducible inflammatory genes, 4 hours following systemic TLR3 activation

In accordance with our previous findings, systemic administration of poly I:C did not alter expression of *IFN- α* or *IFN- β* in the hippocampus, when compared to vehicle-saline-treated counterparts (Fig 6.2a,b). However, systemic administration of poly I:C significantly increased hippocampal mRNA expression of type I IFN-inducible inflammatory genes including *STAT1* [$F_{2,14}=13.794$, $p =0.000$], *IRF7* [$F_{2,13}=19.812$, $p =000$] and *IP-10* [$F_{2,13}=19.559$, $p <0.01$], when compared to vehicle-saline-treated counterparts (Fig 6.2c,d,e). The robust increases in hippocampal mRNA expression of these gene transcripts strongly suggests that type I IFN signalling was induced in the hippocampus, following systemic administration of poly I:C, possibly at an earlier time point than examined in the present study. Systemic administration of the MAGL inhibitor MJN110 significantly enhanced the poly I:C-induced increases in hippocampal mRNA expression of *STAT1* and *IRF7*, when compared to vehicle-poly I:C-treated counterparts (Fig 6.2c,d). In addition, although there was a trend for systemic administration of MJN110 to increase hippocampal mRNA expression of *IP-10* in poly I:C-treated animals; this effect failed to reach statistical significance (Fig 6.2e).

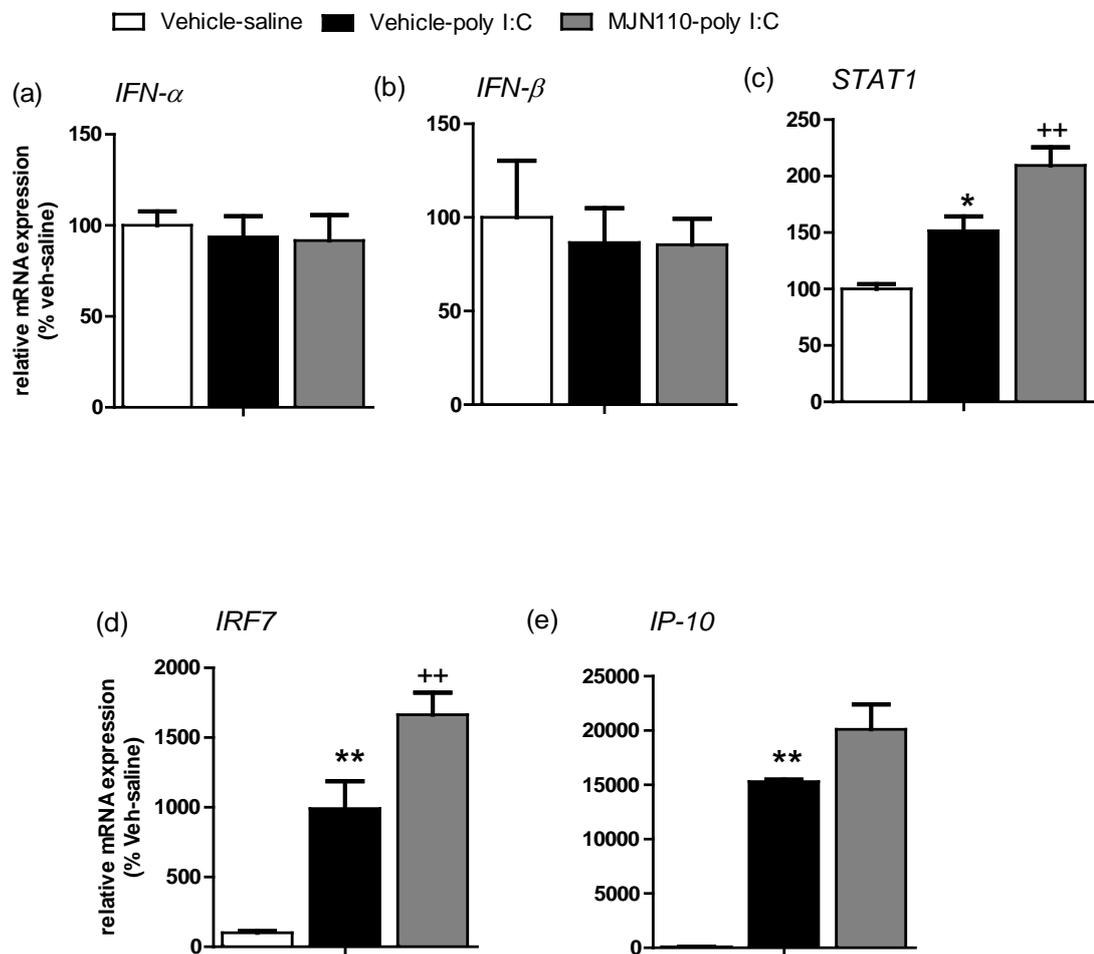


Figure 6.2 The effect of systemic administration of MJN110 on hippocampal expression of type I IFN-inducible genes, 4 hours post poly I:C administration. Although this treatment does not alter either (a) *IFN-α* or (b) *IFN-β*, systemic administration of MJN110 enhances the poly I:C-induced increases in hippocampal expression of (c) *STAT1* and (d) *IRF7*, but not (e) *IP-10*. Data expressed as mean + SEM (n = 4-8 per group). * $p < 0.05$ ** $p < 0.01$ + $p < 0.05$ ++ $p < 0.01$ vs. vehicle-poly I:C-treated animals.

6.3.3 Experiment 2: Systemic administration of MJN110 enhances hippocampal expression of the pro-inflammatory cytokine *IL-1 β* , 4 hours following systemic TLR3 activation

In addition to type I IFN responses, our data (Chapters 3-5) and that previously published (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013) has demonstrated that systemic administration of poly I:C also induces NF- κ B-inducible inflammatory cytokines within the hippocampus (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013). In accordance, statistical analysis revealed that systemic administration of poly I:C significantly increased hippocampal mRNA expression of the NF- κ B-inducible cytokines *IL-1 β* [$F_{2,14}=15.132$, $p =0.000$] and *IL-6* [$F_{2,14}=8.676$, $p <0.01$], but not *TNF α* , when compared to vehicle-saline-treated counterparts, 4 hours post systemic poly I:C administration (Fig 6.3). Prior systemic administration of MJN110 significantly increased the poly I:C-induced increases in mRNA expression of *IL-1 β* and although there was a trend for an increase in *IL-6*, this failed to reach significance (Fig 6.3b,c).

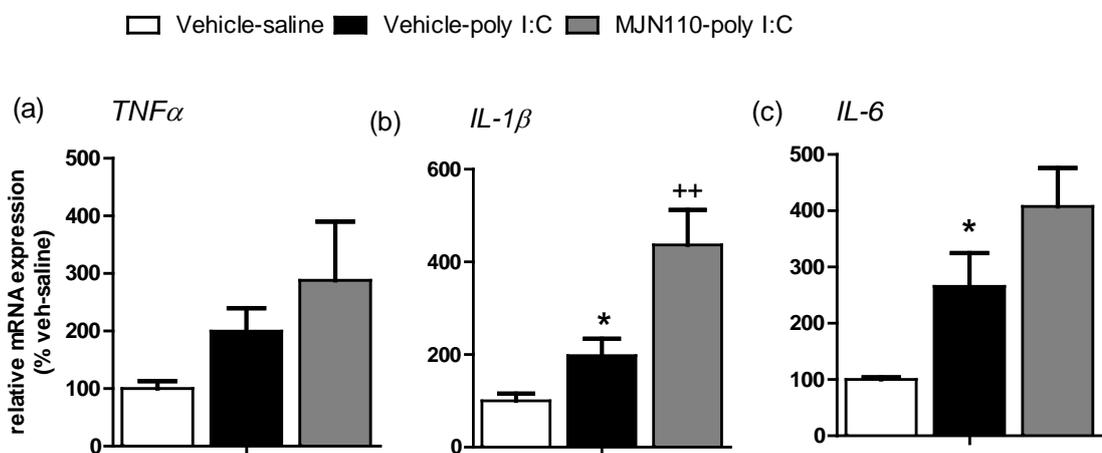


Figure 6.3 The effect of systemic administration of MJN110 on hippocampal expression of the NF- κ B-inducible genes *TNF α* , *IL-1 β* and *IL-6*, 4 hours post poly I:C administration. Systemic administration of MJN110 enhances hippocampal expression of (b) *IL-1 β* , but not (a) *TNF α* or (c) *IL-6*. Data expressed as mean + SEM (n = 4-8 per group). * $p < 0.05$ ** $p < 0.01$ vs. vehicle-saline-treated animals ++ $p < 0.01$ vs. vehicle-poly I:C-treated animals.

6.3.4 Experiment 2: Systemic administration of MJN110 enhances hippocampal expression of the anti-inflammatory cytokines *IL-10* and *SOCS1*, 4 hours following systemic TLR3 activation

In order to assess the molecular mechanism responsible for mediating 2-AG-induced modulation of TLR3 signalling, additional inflammatory mediators implicated in TLR3-induced signalling were examined including both pro- (*IFN- γ* and *iNOS*) and anti- (*IL-10* and *SOCS1*) inflammatory mediators. Statistical analysis revealed a significant effect of treatment on hippocampal mRNA expression of *IL-10* [$F_{2,11}=4.509$, $p <0.05$], *SOCS1* [$F_{2,14}=8.724$, $p <0.01$] and *iNOS* [$F_{2,14}=17.072$, $p =0.000$], 4 hours post poly I:C challenge. (Fig 6.4a,d).

Although there was a trend for poly I:C to induce an increase in hippocampal expression of *IL-10* (2 fold vs. vehicle-saline-treated counterparts), this effect failed to reach statistical significance (Fig 6.4a). In addition, hippocampal expression of the anti-inflammatory cytokine *SOCS1* was also increased in the hippocampus, when compared to vehicle-saline-treated counterparts, however, this effect just failed to reach statistical significance ($p =0.052$) (Fig 6.4b). Furthermore, although poly I:C failed to significantly alter hippocampal expression of the type II IFN, *IFN- γ* (Fig 6.4c), it did significantly increase expression of the downstream pro-inflammatory mediator *iNOS*, when compared to vehicle-saline-treated counterparts (Fig 6.4d). Prior administration of MJN110 significantly increased hippocampal expression of both *IL-10* and *SOCS1*, when compared to both vehicle-saline- and vehicle-poly I:C-treated counterparts, 4 hours post poly I:C challenge (Fig 6.4a,b). However, prior administration of MJN110 did not alter expression of *iNOS* (Fig 6.4d).

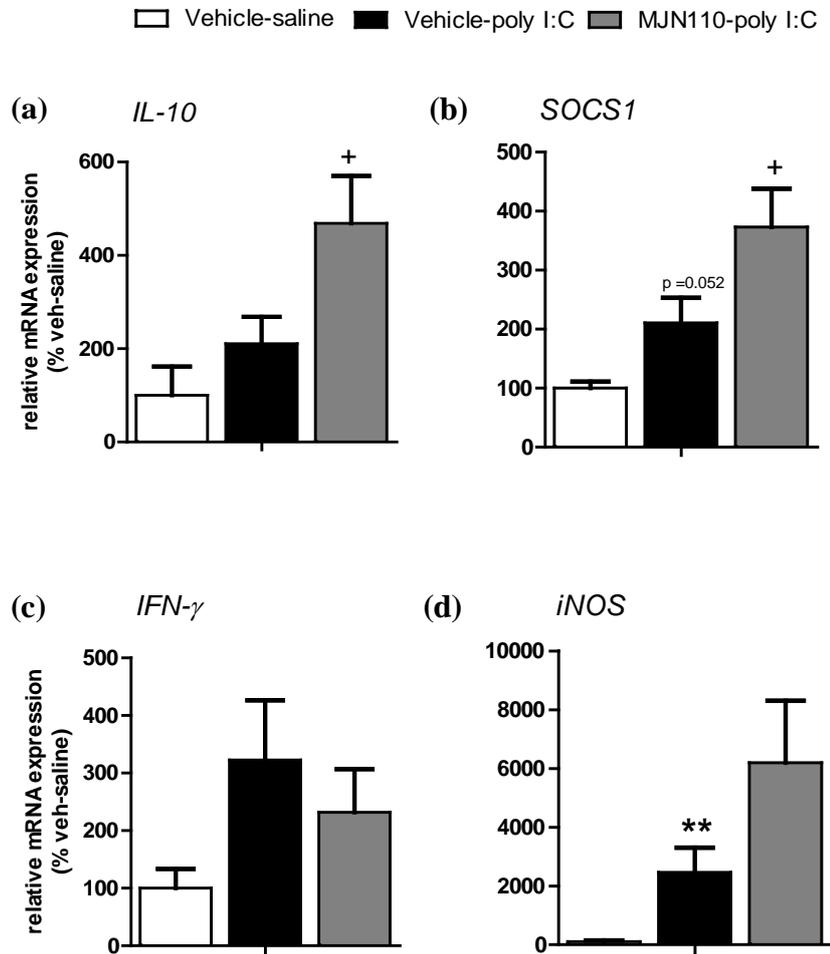


Figure 6.4 The effect of systemic administration of MJN110 on hippocampal expression of both anti- (*IL-10*, *SOCS1*) and pro- (*IFN-γ*, *iNOS*) inflammatory mediators, 4 hours post poly I:C administration. Systemic administration of MJN110 enhances hippocampal expression of (a) *IL-10* and (b) *SOCS1*. In comparison, MJN110 did not significantly alter hippocampal expression of (c) *IFN-γ* or (d) *iNOS*, 4 hours post poly I:C administration. Data expressed as mean + SEM (n = 4-8 per group). ** $p < 0.01$ vs. vehicle-saline-treated animals + $p < 0.05$ vs. vehicle-poly I:C-treated animals.

6.3.5 Experiment 2: The effect of systemic administration of poly I:C on hippocampal expression of the pro-apoptotic marker *Fas*, in either the absence or presence of MJN110

Activation of the type I IFN receptor (IFNR1) by IFN α/β results in increased transcription of anti-viral genes including interferon-induced dsRNA-dependent PKR which in turn can induce the expression of the pro-apoptotic genes *Fas* and *Bax* and activation caspase 3 signalling resulting in cell loss. In this regard, we have demonstrated previously that systemic administration of poly I:C did not alter hippocampal mRNA expression of *PKR*, when compared to vehicle-saline-treated counterparts, 4 hours post systemic poly I:C administration. However, we did report that poly I:C significantly increased hippocampal mRNA expression of the *PKR*-inducible gene *Fas*, an effect which was unaltered by administration of the FAAH inhibitor URB597 (Chapter 4). As such, we examined the hippocampal mRNA expression of *Fas* under the current treatment regime. However, statistical analysis revealed no effect of treatment on *Fas* expression (Fig 6.5).

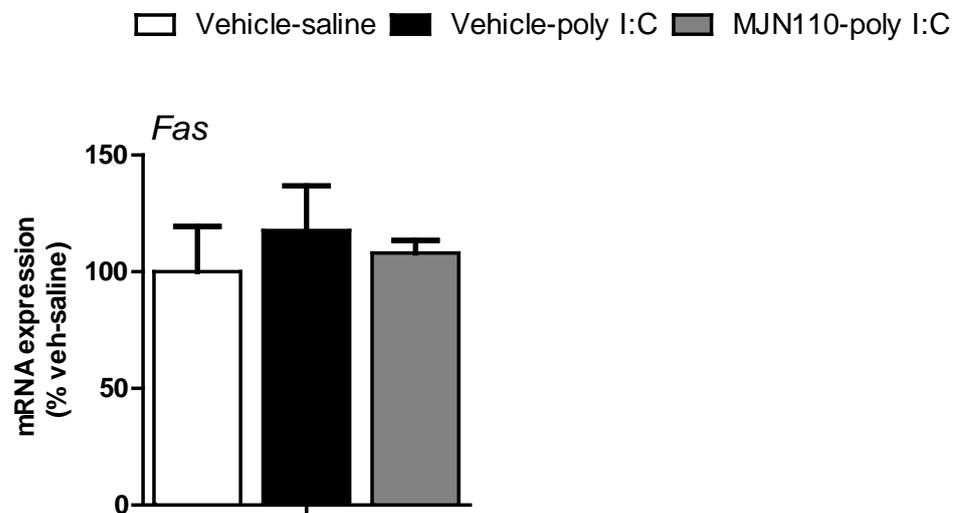


Figure 6.5 The effect of systemic administration of MJN110 on hippocampal expression of the pro-apoptotic gene *Fas*, 4 hours post poly I:C administration. Systemic administration of poly I:C in either the absence or presence of MJN110 does not alter hippocampal expression of *Fas*. Data expressed as mean + SEM (n = 4-8 per group).

6.3.6 Experiment 2: Systemic administration of MJN110 does not alter expression of either IFN- or NF-κB-inducible genes in the spleen, 4 hours post systemic TLR3 activation

In order to examine whether the reported immunoregulatory effects of increased 2-AG levels on TLR3-induced neuroinflammation was occurring directly at the level of the brain or via indirect modulation of peripheral immune responses, we examined the effect of systemic administration of MJN110 on peripheral (splenic) mRNA expression of TLR3-induced inflammatory responses. Statistical analysis revealed that although poly I:C did not significantly alter splenic mRNA expression of *IFN-β*, it did significantly increase mRNA expression of the IFN-inducible genes, *IRF7* [$F_{2,13}=46.739$, $p =0.000$] and *IP-10* [$F_{2,13}=26.410$, $p =0.000$], and the NF-κB-inducible cytokine *TNFα* [$F_{2,13}=8.466$, $p <0.01$], when compared to vehicle-saline-treated counterparts, 4 hours post poly I:C administration (Fig 6.6). Although systemic administration of MJN110 significantly increased levels of 2-AG in the spleen (Vehicle-poly I:C: 7.57nmol/tissue vs. MJN110-poly I:C: 31.76nmol/g tissue; $p =0.000$), this was not associated with alterations in poly I:C-induced increases in splenic mRNA expression of *IRF7*, *IP-10* or *TNFα*, 4 hours post poly I:C challenge (Fig 6.6).

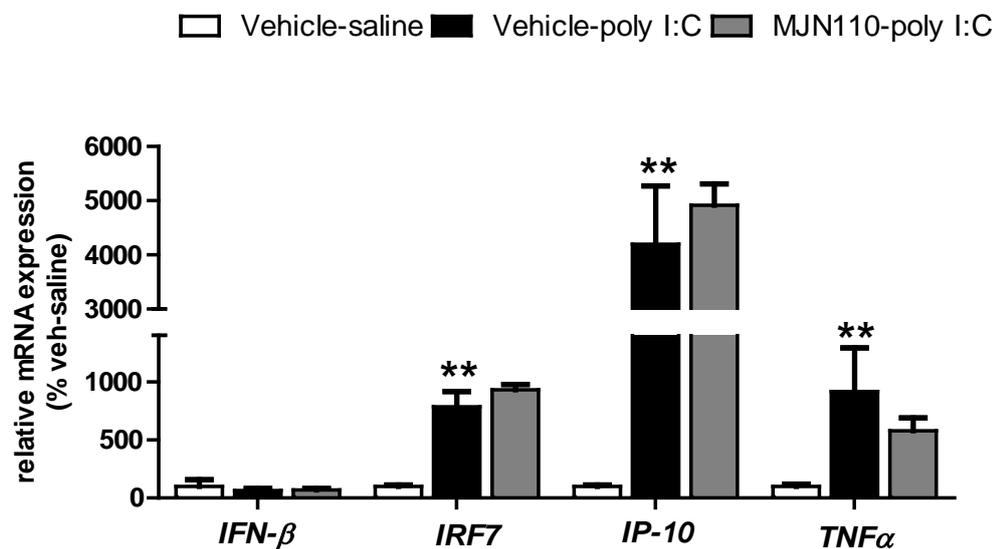


Figure 6.6 The effect of systemic administration of MJN110 on expression of type I IFN- and NF-κB-inducible genes in the spleen, 4 hours post poly I:C administration. Systemic administration of MJN110 does not alter poly I:C-induced increases in splenic expression of *IRF7*, *IP-10* or *TNFα*. There was no effect of treatment on splenic expression of *IFN-β*. Data expressed as mean + SEM (n = 4-8 per group). ** $p < 0.01$ vs. vehicle-poly I:C-treated animals.

6.3.7 Experiment 3: I.c.v. administration of the MAGL inhibitor, JZL184 does not alter 2-AG levels in the rat hippocampus, despite being present in high levels within the hippocampus

An additional aim of this work was to elucidate the potential role of modulation of 2-AG directly within the brain, on TLR-induced neuroinflammation. Previous work within our group has demonstrated that systemic administration of the MAGL inhibitor JZL184 attenuated TLR4-induced increases in several NF- κ B-inducible genes including *TNF α* , *IL-1 β* , *IL-6* and *IL-10* in the rat frontal cortex, 2 hours post systemic LPS administration (Kerr *et al.*, 2013b). However, it is unknown if the effects of this compound are due to elevated levels of 2-AG directly within the brain. As such, we decided to investigate the effect of i.c.v. administration of JZL184 on TLR4-induced increases in NF- κ B-inducible inflammatory cytokines within the hippocampus, 2 hours post systemic administration of LPS. Although JZL184 was detected within the hippocampus (Fig 6.7e), statistical analysis revealed that it did not significantly alter hippocampal levels of 2-AG or the FAAH substrates AEA, OEA or PEA, when compared to vehicle-LPS-treated counterparts, 2 hours post LPS challenge (Fig 6.7a,b,c,d). Furthermore, although i.c.v. administration of JZL184 tended to enhance TLR4-induced increases in mRNA expression of *IL-1 β* and *TNF α* expression, analysis revealed no significant effect of JZL184 on TLR4-induced increases in hippocampal expression of *TNF α* , *IL-1 β* or *IL-6* (Fig 6.7f).

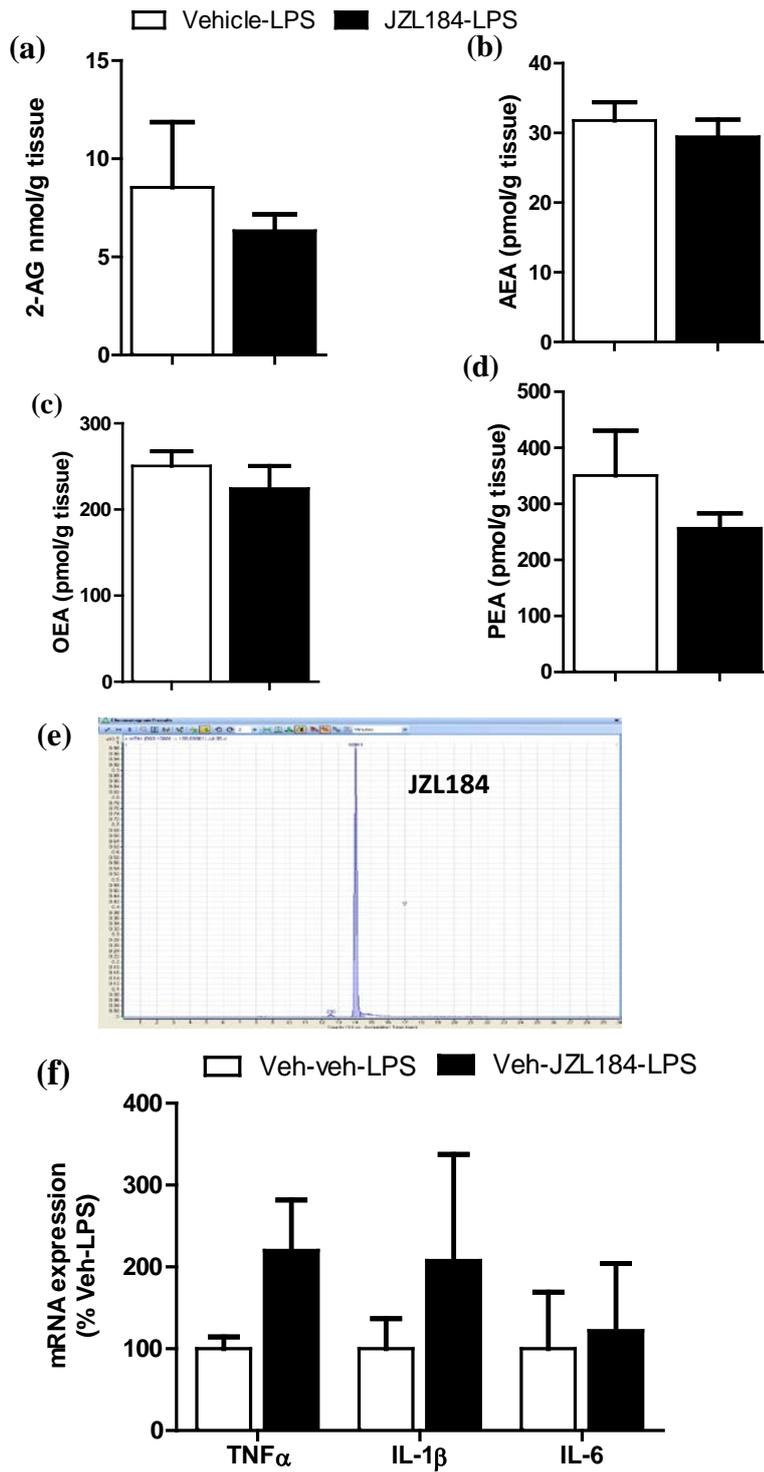


Figure 6.7 The effect of i.c.v. administration of JZL184 on hippocampal levels of 2-AG. I.c.v. administration of JZL184 does not significantly alter hippocampal levels of (a) 2-AG, or the (b-d) FAAH substrates, despite being (e) detectable within the hippocampus, 2 hours post LPS administration. I.c.v. administration of JZL184 does not alter hippocampal expression of (f) $TNF\alpha$, $IL-1\beta$ or $IL-6$, when compared to vehicle-LPS-treated counterparts, 2 hours post systemic LPS administration. Data expressed as mean + SEM, n = 8 per group.

6.3.8 Experiment 4: I.c.v. administration of MJN110 increases 2-AG levels in the rat hippocampus, 2 hours post administration

As i.c.v. administration of JZL184 failed to alter hippocampal levels of 2-AG, we next decided to examine if i.c.v. administration of the novel potent MAGL inhibitor MJN110 would increase 2-AG levels within the hippocampus, 2 hours post administration. MJN110 has been reported to be more potent for the rat form of MAGL when compared to JZL184 (Niphakis *et al.*, 2013). However, as with all MAGL inhibitors, MJN110 is difficult to dissolve and as such a suspension in a number of vehicles was examined. Accordingly, statistical analysis revealed that i.c.v. administration of MJN110; dissolved only in 100% dimethylformamide (DMF) significantly increased hippocampal levels of 2-AG [$t_{(6)}=3,412$, $p < 0.05$] (Fig 6.8a), with no significant effect on AEA, OEA or PEA levels, when compared to vehicle-treated counterparts (Fig 6.8b,c,d). Additional vehicle preparations examined included: MJN110 dissolved in 100% DMSO, 50% DMSO:50% PEG and 50% DMF:50% PEG; however, MJN110 prepared in these vehicle preparations failed to significantly alter hippocampal levels of 2-AG, when compared to their corresponding vehicle-treated counterparts (Fig 6.8e). It is also important to note that the solubility of MJN110 in saline-based vehicles was examined, these included PEG:saline and DMF saline based vehicles. However, upon addition of aqueous (saline) solution, MJN110 came out of solution and thus these vehicle combinations failed to offer a suitable vehicle for i.c.v. administration of MJN110.

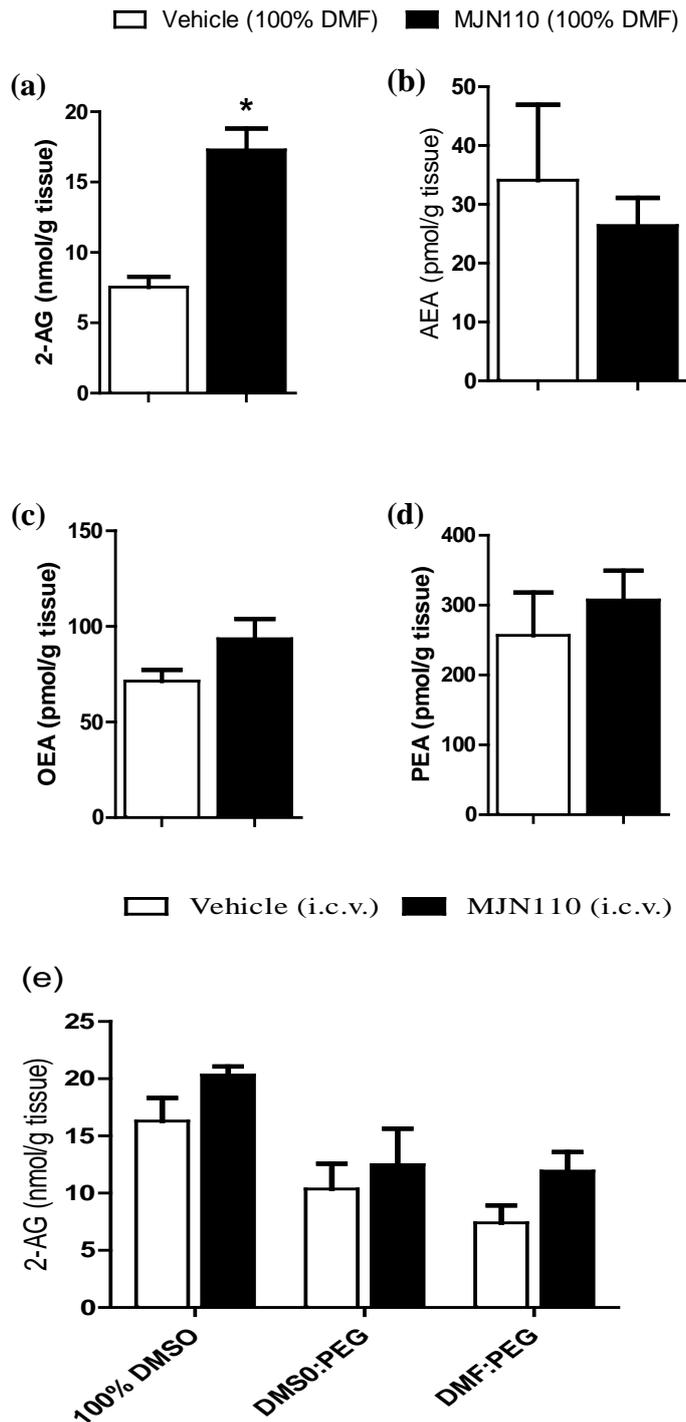


Figure 6.8 The effect of i.c.v. administration of MJN110 on hippocampal levels of 2-AG. (a) i.c.v. administration of MJN110 dissolved in 100% DMF significantly increases hippocampal levels of 2-AG, but not (b) AEA, (b) OEA or (c) PEA, 2 hours post microinjection. In comparison, (e) i.c.v. administration of MJN110 in other vehicles failed to alter hippocampal levels of 2-AG, when compared to vehicle-treated counterparts, 2 hours post microinjection. Data expressed as mean + SEM (n = 3 per group). * $p < 0.05$ vs. vehicle-treated animals.

6.3.9 Experiment 5: I.c.v. administration of MJN110 does not significantly alter TLR4-induced increases in hippocampal expression of NF- κ B-inducible genes, 2 hours post systemic LPS administration

We next wanted to determine whether increases in 2-AG levels directly within the brain modulated TLR4-induced increases in hippocampal expression of NF- κ B-inducible inflammatory genes. Statistical analysis revealed that i.c.v. administration of MJN110 significantly increased hippocampal levels of 2-AG [$t_{(13)}=2.534$, $p < 0.05$], when compared to their vehicle-LPS-treated counterparts, (Fig 6.9a). Although MJN110 appeared to attenuate TLR4-induced increases in mRNA expression of *TNF α* and *IL-1 β* , statistical analysis revealed that the hippocampal expression of *TNF α* , *IL-1 β* and *IL-6*, induced at 2 hours following systemic LPS administration were not significantly altered by i.c.v. administration of MJN110 (Fig 6.9b).

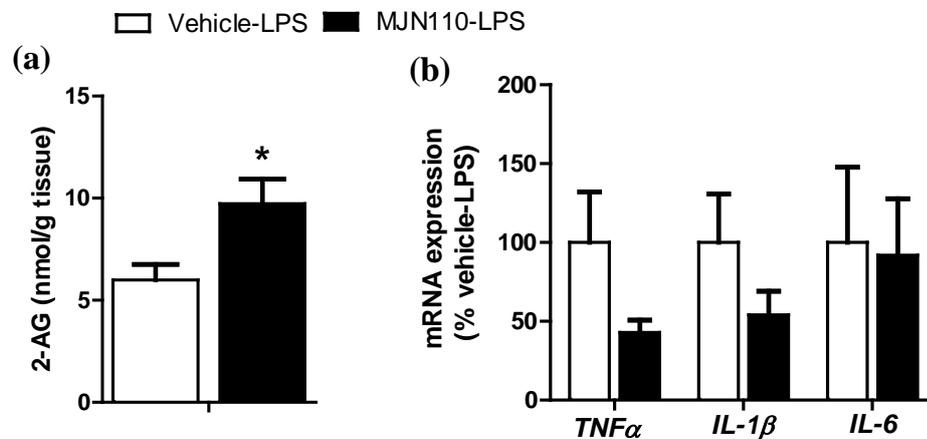


Figure 6.9 The effect of i.c.v. administration of MJN110 on hippocampal levels of 2-AG and hippocampal expression of NF- κ B-inducible genes, 2 hours post systemic LPS administration. (a) i.c.v. administration of MJN110 and subsequent increases in 2-AG does not alter (b) hippocampal expression of NF- κ B-inducible inflammatory cytokines, 2 hours following systemic LPS administration. Data expressed as mean + SEM ($n = 7-8$ per group). * $p < 0.05$ vs. vehicle-LPS-treated animals.

6.4 Discussion

The data presented herein demonstrate that systemic administration of the potent MAGL inhibitor MJN110 increases hippocampal levels of the MAGL substrate 2-AG. Such increases in hippocampal levels of 2-AG were in turn associated with modulation of TLR3-induced responses within the brain, but not in the periphery, at 4 hours post systemic poly I:C administration. Specifically, systemic administration of MJN110 enhanced TLR3-induced increases in type I IFN-induced signalling within the hippocampus, exemplified by increased expression of *STAT1*, *IRF7* and *SOCS1*. In addition to type I IFN-induced responses, systemic administration of MJN110 also enhanced NF- κ B-inducible genes within the hippocampus, including the pro-inflammatory gene *IL-1 β* and the anti-inflammatory cytokine *IL-10*. Contrastingly, systemic administration of MJN110 did not alter splenic expression of IFN- or NF- κ B-inducible inflammatory genes, induced following systemic TLR3 activation, highlighting that the effects of MAGL inhibition on TLR3-associated neuroinflammation are most likely mediated at the level of the CNS. Furthermore, we have demonstrated that i.c.v. administration of the MAGL inhibitor MJN110, but not JZL184, increased hippocampal levels of 2-AG, further highlighting that MJN110 is more efficient at inhibiting rat brain MAGL than JZL184. However, such increases in 2-AG levels directly within the brain were not associated with alterations in hippocampal expression of the NF- κ B-inducible inflammatory cytokines *TNF α* , *IL-1 β* or *IL-6* following systemic TLR4 activation. This data supports previous finding from the Roche group, indicating that 2-AG modulation of TLR4-induced neuroinflammation may not be directly mediated at the level of the brain but rather indirectly by modulation of peripheral immune responses (Kerr *et al.*, 2013b). Thus, taken together, these data suggest that increasing endogenous 2-AG levels modulates TLR-induced neuroinflammatory responses although the precise mechanism of action remains to be determined.

The synthetic dsRNA poly I:C is recognised by TLR3 (Alexopoulou *et al.*, 2001) and used by many researchers to examine the effects of type I IFNs both *in vitro* and *in vivo*. Accordingly, our previous data (Chapters 3-5) and other published reports (Cunningham *et al.*, 2007; Field *et al.*, 2010; Murray *et al.*, 2015a) have demonstrated that systemic administration of poly I:C is associated with increases in type I IFN-inducible genes

within the brain. In accordance with our initial data, no alterations in expression of the type I IFNs in the brain (hippocampus) or in the spleen, were observed in the current study. However, robust increases in several type I IFN-inducible genes including *STAT1*, *IRF7* and *IP-10* within the hippocampus and *IRF7* and *IP-10* within the spleen strongly suggests that type I IFNs were produced at the protein level and active in both the spleen and hippocampus, likely at an earlier time point than examined in the present study. Further support for this suggestion comes from the recent report that IFNAR1-deficient mice exhibit significantly less *STAT1* phosphorylation and expression and also decreased *IRF7* expression within the hippocampus following systemic poly I:C administration, when compared to their wild-type-treated counterparts (Murray *et al.*, 2015a) which thus suggests that type I IFN signalling is required for robust induction of these inflammatory genes. There are now several reports that type I IFNs and downstream genes are readily expressed within the CNS (Cunningham *et al.*, 2007; Wang *et al.*, 2008) and microglia are suggested to be the main source of such mediators under neuroinflammatory conditions (Costello *et al.*, 2013; Kallfass *et al.*, 2012). Although there are reports that type I IFNs exert pathogenic effects within the CNS (Owens *et al.*, 2014), a substantial amount of evidence demonstrates that in addition to their anti-viral properties, type I IFN-induced signalling plays a critical role in the regulation of inflammation within the CNS, primarily via their ability to limit immune cell entry into the CNS. Specifically systemic administration of IFN- β reduces chemokine-induced neutrophil infiltration in the rat brain (Veldhuis *et al.*, 2003) while IFNAR-, IRF7-, and type I IFN-deficient mice display increased leukocyte infiltration and exacerbation of symptoms in an experimental model of MS (Galligan *et al.*, 2010; Prinz *et al.*, 2008; Salem *et al.*, 2011; Teige *et al.*, 2003). Thus, modulation of endogenous type I IFN-induced signalling within the CNS may offer a novel therapeutic strategy for viral-induced neuroinflammation and associated neurodegeneration. Accordingly, the current findings demonstrate that systemic administration of MJN110 and subsequent increases in 2-AG levels within the brain, is associated with enhanced expression of type I IFN responses within the hippocampus, but not within the spleen, exemplified by an enhancement of TLR3-induced increases in hippocampal expression of *STAT1* and *IRF7*, and a strong trend for an increase in *IP-10*. Although enhanced 2-AG may have exerted modulation on TLR3-induced increases in peripheral mediators at an earlier time point than that examined in the present study, these current findings suggest that 2-AG-induced

modulation of TLR3-induced increases in type I IFN responses within the hippocampus is occurring directly at the level of the CNS, rather than via indirect modulation of peripheral cytokines. The 2-AG-induced increases in hippocampal expression of type I IFN signalling, following TLR3 activation, is in accordance with our previous findings which demonstrated that pharmacological inhibition of FAAH and subsequent increases in FAAH substrates within the brain were associated with an enhancement of the type I IFN, *IFN- α* , following TLR3 activation (Chapter 3). In accordance with these data, the synthetic CB₁/CB₂ receptor agonist WIN55,212-2 has previously been reported to strongly promote nuclear transcription of IRF3 and subsequent IFN- β production in primary astrocytes (Downer *et al.*, 2011). Thus, taken together, these findings provide strong evidence that cannabinoids/endocannabinoids exert positive effects on type I IFN signalling under TLR3-induced neuroinflammatory conditions which may in turn confer neuroprotective effects.

In addition to inducing type I IFN responses, activation of TLR3 also initiates activation of the transcription factor NF- κ B and subsequent cytokine production. Accordingly, our current findings demonstrate that systemic administration of poly I:C robustly increased splenic expression of *TNF α* , while additionally increasing hippocampal expression of the *IL-1 β* , *IL-6*, *IL-10* and *iNOS*, correlating with our previous findings (Chapter 4) and published reports in both rats and mice (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013). The present findings demonstrate that in addition to enhancement of type I IFN-inducible genes, systemic administration of MJN110 also exerted immunoregulatory effects on hippocampal expression of NF- κ B-inducible genes, but not the spleen, following systemic TLR3 activation. Specifically, systemic administration of MJN110 enhanced the TLR3-induced increases in hippocampal expression of the pro-inflammatory cytokine *IL-1 β* , with a strong trend for *IL-6* and *iNOS*. Thus, these findings indicate that 2-AG induces a heightened pro-inflammatory (M1) effect, following systemic TLR3 activation. Such findings are in contrast to published reports demonstrating that direct administration of 2-AG, or enhancement of endogenous 2-AG, via pharmacological inhibition of MAGL, is associated with an attenuation of TLR4-induced increases in pro-inflammatory mediators, both *in vitro* (Alhouayek *et al.*, 2013; Chang *et al.*, 2001; Gallily *et al.*, 2000; Krishnan *et al.*, 2012) and *in vivo* (Kerr *et al.*, 2013b; Nomura *et al.*, 2011). However, this is the first study to examine the effects of increased 2-AG levels on TLR3-induced neuroinflammation. In this respect, although

TLR3 and TLR4 induce activation of NF- κ B, differential signalling pathways are responsible for its induction, which as a result may account for the reported differences in 2-AG modulation on specific NF- κ B-inducible genes. However, the exact molecular mechanisms mediating such responses remained to be fully determined.

Additionally, although uncontrolled activation of pro-inflammatory mediators including IL-1 β and TNF α are known to exert detrimental effects on neuronal function and repair, there is now emerging evidence that such mediators are involved in restorative processes in CNS repair and recovery through interactions with neural stem/progenitor cells (NSCs) (Molina-Holgado *et al.*, 2010). Furthermore, the endocannabinoid system has been implicated in mediating such responses where, interestingly, TNF α -induced proliferation of NSC is reported to be mediated via endocannabinoid (2-AG) signalling through CB₁/CB₂ receptors (Rubio-Araiz *et al.*, 2008) while interactions between IL-1 β and endocannabinoid signalling has been implicated in NSC differentiation (Molina-Holgado *et al.*, 2010). Therefore, these findings indicate that cross-talk between pro-inflammatory cytokines (IL-1 β and TNF α) and NSCs, via interactions with the endocannabinoid system, may have important consequences for neural development and brain repair (Molina-Holgado *et al.*, 2010). Thus, it may be likely that in the present study 2-AG initially acts to increase TLR3-induced neuroinflammatory responses (increased hippocampal expression of *IL-1 β*) as a compensatory mechanism to induce CNS repair responses. However, further studies are required in order to elucidate if increased 2-AG exerts overall protective or detrimental effects on neuronal function, following TLR3 activation.

In addition to increasing hippocampal expression of *IL-1 β* , our current findings also report that systemic administration of MJN110 increases hippocampal expression of the anti-inflammatory cytokines *IL-10* and *SOCS1*. As previously described, *IL-10* is a potent anti-inflammatory cytokine and key negative regulator of microglial activation. Furthermore, *SOCS1* is known to have an essential role in regulation of immune function whereby *SOCS1* deficient mice die within three weeks of birth, mainly due to dysregulated IFN- γ responses (Alexander *et al.*, 1999; Naka *et al.*, 1998; Starr *et al.*, 1998), and over-expression of *SOCS1* protects against deleterious effects of IFN- γ (Balabanov *et al.*, 2006). There are now reports that direct administration of both AEA (Correa *et al.*, 2010; Krishnan *et al.*, 2012) and 2-AG (Krishnan *et al.*, 2012), in addition

to inhibiting pro-inflammatory cytokines, also induces increases in IL-10 production following TLR4-induced activation of microglia *in vitro*. Furthermore, it has been demonstrated that IL-10 plays a pivotal role in endocannabinoid (AEA)-induced neuroprotection both *in vitro* and *in vivo*, at least in part, by increasing expression of CD200 on neurons and amplifying interactions with CD200R1 on microglia, and limiting harmful neuroinflammatory processes (Hernangomez *et al.*, 2012). Our findings have demonstrated that increasing MAGL substrate (current study) and FAAH substrate (Chapter 4) levels are associated with increased hippocampal expression of *IL-10*, following systemic TLR3 activation. As such it is interesting to speculate that such endocannabinoid-induced increases in hippocampal expression of *IL-10* and other anti-inflammatory mediators (*SOCS1*) may confer neuroprotective effects; however this remains to be elucidated.

The second part of this chapter focused on examining the effect of increasing 2-AG levels directly within the brain, on TLR-induced neuroinflammatory responses. This is an important question to address as there are now several reports that 2-AG is increased in the CNS in several neuropathologies including MS, PD, AD and TBI (Baker *et al.*, 2001; Loria *et al.*, 2008; Panikashvili *et al.*, 2005; van der Stelt *et al.*, 2005) where it is suggested to be neuroprotective. Accordingly, there are now several reports from both *in vitro* (Chen *et al.*, 2011; Du *et al.*, 2011; Zhang *et al.*, 2008) and *in vivo* (Lourbopoulos *et al.*, 2011; Panikashvili *et al.*, 2005; Panikashvili *et al.*, 2006) studies that 2-AG exerts neuroprotective effects, at least in part via its anti-inflammatory effects. As such, modulation of endogenous 2-AG levels is now emerging as a promising therapeutic approach for the modulation of inflammatory responses within the CNS. In the first instance, we decided to examine the effect of i.c.v. administration of the MAGL inhibitor JZL184 on 2-AG levels and NF- κ B-inducible inflammatory genes in the hippocampus, following systemic administration of the TLR4 agonist LPS. The rationale for initially examining TLR4- rather than TLR3-induced responses was based on previous work carried out within our laboratory (Kerr *et al.*, 2013b) and others (Nomura *et al.*, 2011), demonstrating potent anti-inflammatory effects on TLR4-induced neuroinflammation, following systemic administration of JZL184. Our findings demonstrate that although JZL184 was present at high levels within the hippocampus following i.c.v. administration; this was not associated with increases in 2-AG levels within the hippocampus or changes in TLR4-induced increases in hippocampal

expression of *TNF α* , *IL-1 β* or *IL-6*. Given that JZL184 was evidentially present within the hippocampus, it was quite surprising that this was not associated with increases in 2-AG levels; however a possible explanation for the lack of increase in 2-AG may likely be due to the fact that JZL184 is reported to have much lower affinity (approx 10 fold) for rat MAGL compared to murine and human MAGL (Long *et al.*, 2009a; Long *et al.*, 2009b). In addition, it has been proposed that within the CNS, inhibition of MAGL may shunt the hydrolysis of 2-AG onto another pathway such as COX2 (Alhouayek *et al.*, 2014a), which may also account for the lack of increase in 2-AG in the current study.

With the advent of novel MAGL inhibitors that have been proposed to have greater affinity for rat MAGL compared to JZL184, we next examined if i.c.v. administration of MJN110 could enhance endogenous 2-AG levels in the brain. I.c.v. administration of MJN110 increased 2-AG levels within the hippocampus, although this effect was dependant on the type of vehicle in which the drug was dissolved in. As such there remains a need to continue to develop new more soluble, potent and selective MAGL inhibitors that can be used in *in vivo* studies to evaluate the physiological effects of enhanced 2-AG tone. However, the data in this chapter demonstrated that although i.c.v. administration of MJN110 increased 2-AG levels and although there was a trend for a decrease in TLR4-induced increases in *TNF α* and *IL-1 β* , this effect failed to reach statistical significance. Previous studies have shown that 2-AG inhibits NF- κ B activation (Panikashvili *et al.*, 2005) and decreases pro-inflammatory cytokines within the brain, in a CHI model of TBI (Panikashvili *et al.*, 2006). However, this is the first report to examine the effect of 2-AG directly within the brain on TLR4-induced neuroinflammation. As such, it is possible that a greater enhancement in 2-AG levels (>2 fold observed in the current study) is required in order to significantly inhibit NF- κ B activation and induce an attenuation in cytokine expression, following TLR4 activation. However, the concentration of MJN110 used in this study was the maximum that could be dissolved in the vehicle, again highlighting the need for the development of novel MAGL inhibitors. Future studies using more selective, potent and soluble MAGL inhibitors (when developed) will investigate the effect of increased 2-AG within the brain on other TLR-induced neuroinflammatory responses and the potential receptor and molecular mechanisms involved.

6.4.1 Conclusion

Overall, the data presented in the present chapter demonstrate that increased levels of 2-AG within the brain, following systemic administration MJN110, exert potent immunoregulatory effects on TLR3-induced signalling within the hippocampus, exemplified by an enhancement of type I IFN-induced signalling and NF- κ B-inducible cytokines. Such findings may have important implications for neurological disorders such as MS in which type I IFNs are known to exert protective effects.

Chapter 7

FAAH substrate-mediated modulation of TLR4-induced neuroinflammation; effects partially mediated via activation of brain TRPV1

7.1 Introduction

The endogenous cannabinoid AEA is known to play a key role in the modulation of neuro-immune responses, including that following TLR activation, and thus is now emerging as potential novel therapeutic target in neurodegenerative disorders with underpinning inflammatory mechanisms (Bisogno *et al.*, 2010; Centonze *et al.*, 2007; Correa *et al.*, 2009b; Downer, 2011). AEA is produced on demand whereupon it mediates many of its biological effects via CB₁ and CB₂ receptors, although it has also been shown to have affinity for additional receptor targets including the PPARs, TRPV1 and also the novel cannabinoid receptor, GPR55 (Di Marzo *et al.*, 2001; Mackie *et al.*, 2006; O'Sullivan *et al.*, 2010; Ryberg *et al.*, 2007b; Toth *et al.*, 2009). AEA and the related *N*-acylethanolamines OEA and PEA, are primarily metabolised by FAAH into ethanolamine and their respective fatty acids, arachidonic acid, palmitic acid and oleic acid (Cravatt *et al.*, 1996b). However, in addition to FAAH metabolism, AEA is also subject to COX2 oxygenation which generates metabolic products, the prostaglandin-ethanolamides (prostamides), which mediate effects independent of cannabinoid receptors (Ross *et al.*, 2002; Woodward *et al.*, 2008). Enhancement of endocannabinoid tone has been proposed as an alternative means of activating CB₁/CB₂ receptors devoid of the unwanted psychotropic effects associated with direct activation with potent cannabinoid receptor agonists. Accordingly, several *in vitro* studies have demonstrated that increasing AEA tone, either directly, via inhibition of FAAH, or inhibition of its reuptake, is associated with a decrease in TLR4-induced increases in NF- κ B-inducible pro-inflammatory cytokines and inflammatory mediators including nitric oxide (Facchinetti *et al.*, 2003b; Molina-Holgado *et al.*, 1997; Ortega-Gutierrez *et al.*, 2005; Puffenberger *et al.*, 2000; Tham *et al.*, 2007), while concurrently increasing production of anti-inflammatory cytokines including IL-10 and TGF- β (Correa *et al.*, 2009a; Krishnan *et al.*, 2012). In some cases these immunoregulatory effects are reported to be mediated via AEA-induced activation of CB₁/CB₂ receptor activation (Correa *et al.*, 2009a; Correa *et al.*, 2010; Krishnan *et al.*, 2012; Ortega-Gutierrez *et al.*, 2005). Mechanistically, both CB₁/CB₂ receptors and TLR4 are positively coupled to the MAPK signalling pathways and several studies have demonstrated that MAPK activation is a key molecular target in AEA-induced modulation of TLR4-induced inflammation. For example, AEA enhances IL-10 production, while concurrently inhibits I κ B α phosphorylation, via a CB₂-receptor-induced activation of ERK1/2 and

JNK MAPK signalling pathways, in microglia cultures (Correa *et al.*, 2010), while CB₁/CB₂ receptor-induced activation of MAPK (ERK1/2, JNK & p38) signalling cascades are reported to be essential in mediating AEA-induced modulation of TLR4-induced inflammatory mediators (Krishnan *et al.*, 2012). In addition to cannabinoid receptor-dependent effects, there are also studies indicating that AEA mediates anti-inflammatory effects independent of CB₁/CB₂ receptor activation (Facchinetti *et al.*, 2003b; Puffenbarger *et al.*, 2000; Tham *et al.*, 2007), thus possibly suggesting alternative receptor targets. Additionally, anti-inflammatory effects of endocannabinoids have been reported to be mediated via reduced AA and subsequent reductions in the production of prostaglandins within the CNS (Nomura *et al.*, 2011) and COX2-derived endocannabinoid metabolites (Alhouayek *et al.*, 2014b).

In accordance with *in vitro* studies, there is now increasing evidence that modulation of AEA tone is associated with potent immunoregulatory effects following TLR4 activation, *in vivo*. Specifically, pharmacological inhibition of AEA uptake with the endocannabinoid re-uptake inhibitor, AM404, attenuates TLR4-induced increases in plasma levels of IL-6 and IL-1 β (Roche *et al.*, 2008). Furthermore, inhibition of AEA reuptake and pharmacological inhibition of FAAH via systemic administration of URB597 are associated with an increase in plasma levels of TNF α (Roche *et al.*, 2008), with similar augmentations in TLR4-induced plasma TNF α following i.c.v. administration of URB597 (De Laurentiis *et al.*, 2010). In addition, previous work from our laboratory has demonstrated that systemic administration of the FAAH inhibitor URB597 and subsequent increases in hypothalamic levels of AEA, OEA and PEA are associated with an attenuation of TLR4-induced increases in hypothalamic expression of *IL-1 β* and *SOCS3* (Kerr *et al.*, 2012). Thus, although enhancing AEA tone has been shown to modulate TLR4-induced inflammatory responses both peripherally and in the CNS (hypothalamus), it is unknown if such immunoregulatory effects of increased FAAH substrates on TLR4-induced neuroinflammation are mediated via indirect modulation of peripheral immune responses or occur directly within the brain. Understanding the potential role of FAAH substrates directly within the brain in mediating such neuroinflammatory responses is an important question to address as several neuropathologies including MS, ischemia and PD have all been reported to be associated with increased FAAH substrates within the brain (Baker *et al.*, 2001; Degen *et al.*, 2007; Franklin *et al.*, 2003; Loria *et al.*, 2008). Previous findings in this thesis

(Chapters 3-5) have reported that i.c.v. administration of the FAAH inhibitor URB597 and subsequent increases in FAAH substrates directly within the brain, elicited potent anti-inflammatory effects in the hippocampus following systemic TLR3-activation. These data support a pivotal role for FAAH substrates directly within the brain in modulating TLR3 associated neuroinflammation, and as such the studies contained in this chapter proposed to examine if similar effects occurred following the activation of other TLRs, namely TLR4. In addition, we (Chapter 3) and others (Gibney *et al.*, 2013) have previously demonstrated that systemic activation of TLR3 is associated with long-term alterations in neuro-immune responses including increases in glial activation and concurrent reductions in BDNF expression; effects which are partially modulated by systemic administration of the FAAH inhibitor URB597 (Chapter 3). However, no studies have examined if similar effects are observed following acute systemic TLR4 activation.

Hypothesis: Based on these data we hypothesised that increasing AEA tone directly within the brain would attenuate neuroinflammatory responses following TLR4 activation. Therefore, the aims of this chapter were to:

1. Examine the effect of systemic administration of a selective inhibitor of FAAH PF3845, on TLR4-induced increases in NF- κ B-inducible inflammatory genes within discrete regions of the rat brain
2. Examine the effect of inhibiting FAAH directly within the brain following i.c.v. administration of PF3845, on TLR4-induced increases in NF- κ B-inducible inflammatory genes within discrete regions of the rat brain
3. Examine if enhancing peripheral FAAH substrate levels alone using a peripherally restricted FAAH inhibitor URB937, would modulate TLR4-induced increases in NF- κ B-inducible inflammatory genes within discrete regions of the rat brain

4. Examine the possible receptor and molecular targets within the brain that may mediate the effects of FAAH substrate-mediated modulation of NF- κ B-inducible inflammatory gene expression within the frontal cortex following TLR4 activation. This was evaluated via a number of studies investigating the role of
 - a. CB₁ and CB₂ receptors and downstream molecular signalling via MAPK pathways (ERK, JUN and p38)
 - b. iNOS/COX2 mediators
 - c. Other FAAH substrate receptor targets namely PPAR- α , PPAR- γ , TRPV1 and GRP55

5. Examine the effect of FAAH inhibition on microglial activation and behavioural outcomes, 24 hours post systemic TLR4 activation

7.2 Methodology and Experimental design

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.

7.2.1 Experiment 1: Investigation of the effect of systemic administration of the potent FAAH inhibitor PF3845 on TLR4-induced increases in hippocampal and frontal cortical expression of neuroinflammatory mediators

Rats were randomly assigned into one of two treatment groups: Vehicle-LPS and PF3845-LPS (n = 8-10 per group). The potent FAAH inhibitor PF3845 (NIMH drug synthesis programme; 10mg/kg) or vehicle (ethanol:cremaphor:saline; 1:1:18) were administered in a single, acute i.p. injection, in an injection volume of 2ml/kg and was followed 30 min later by a single, acute i.p. injection of LPS (100ug/kg) or saline vehicle (sterile 0.89% NaCl) administered in an injection volume of 1ml/kg. The dose of PF3845 was chosen on the basis of previous published work which demonstrated that systemic administration of PF3845 at the same dose utilised in the present study increased brain levels of AEA, OEA and PEA (Ahn *et al.*, 2009). It is important to note that previous work within our laboratory has demonstrated that systemic administration of PF3845 has no effect on cytokine expression within the brain, in the absence of LPS immune stimulus (unpublished data). Furthermore, previous work from our group has demonstrated that LPS, at the same dose used in this study does not alter brain levels of endocannabinoids or related fatty acids (Kerr *et al.*, 2012; Kerr *et al.*, 2013b). As such non-LPS groups were not included in the present work. The dose and time of LPS administration was chosen on the basis of previous published work within our laboratory

demonstrating enhanced cytokine expression in the rat brain and periphery (Kerr *et al.*, 2012; Kerr *et al.*, 2013b; Roche *et al.*, 2006; Roche *et al.*, 2008). Animals were sacrificed by decapitation two hours post LPS/saline administration, the brain rapidly removed, the hippocampus and frontal cortex excised, snap-frozen on dry ice and stored at -80°C until assayed for the levels of endocannabinoids and *N*-acylethanolamines and cytokine expression. (The methodology was as described in Chapter 2).

7.2.2 Experiment 2: Investigation of the effect of i.c.v. administration of PF3845 on TLR4-induced increases in hippocampal and frontal cortical expression of neuroinflammatory mediators

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol in Chapter 2), rats were randomly assigned into one of two treatment groups: Vehicle-LPS and PF3845-LPS (n = 8-10 per group). Rats received a single, acute i.c.v. administration of PF3845 (500nmoles) or vehicle (100% DMSO) in an injection volume of 5ul infused over 1 min followed 15 min later by a single, acute i.p. injection of LPS (100ug/kg, i.p.) or sterile saline (0.89%) in an injection volume of 1mg/kg. The dose and time of PF3845 was chosen on the basis of pilot work within our laboratory demonstrating increases in AEA, OEA and PEA in discrete regions of the rat brain. Animals were returned to their home cages and sacrificed by decapitation at 2 hours following LPS administration, brain removed, hippocampus and frontal cortex excised, snap-frozen and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression.

7.2.2.1 Experiment 2a: Investigation of the effect of i.c.v. administration of PF3845 on cytokine expression within the brain, in the absence of systemic LPS administration

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol in Chapter 2), rats were randomly assigned into one of two treatment groups: Vehicle-saline and PF3845-saline (n = 4 per group). Rats received a single, acute i.c.v. administration of PF3845 (500nmoles) or vehicle (100% DMSO) in an injection volume of 5ul infused over 1 min followed 15 min later by a single, acute i.p. injection of sterile saline (0.89%) in an injection volume of 1mg/kg. Animals were returned to their home cages and sacrificed by decapitation at 2 hours following saline

administration, brain removed and frontal cortex excised, snap-frozen and stored at -80°C until assayed for cytokine expression.

7.2.3 *Experiment 3: Investigation of effect of the peripherally restricted FAAH inhibitor URB937 on inflammatory gene expression in the brain, following systemic LPS challenge*

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,) or vehicle (ethanol: cremophor: saline; 1:1:18) were administered in a single, acute i.p. injection in an injection volume of 1ml/kg and was followed 1 hour later by a single, acute i.p. injection of LPS (100ug/kg) or sterile saline (0.89% NaCl) in an injection volume of 1mg/kg. The dose of URB937 was chosen on the basis of previous published work demonstrating that at this dose, URB937 increased FAAH activity and AEA levels outside of the rodent CNS (Clapper *et al.*, 2010). Animals were returned to their home cages and sacrificed by decapitation at 2 hours following LPS administration, hippocampus, frontal cortex and spleen excised, snap-frozen and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines and cytokine expression.

7.2.4 *Experiment 4: Investigating the receptor and molecular mechanism underlying FAAH substrate-mediated modulation of NF- κ B-inducible inflammatory genes within the frontal cortex following systemic administration of LPS*

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol in Chapter 2), rats were randomly assigned into one of 8 treatment groups: Vehicle-vehicle-saline, Vehicle-vehicle-LPS, Vehicle-PF3845-LPS, AM251-PF3845-LPS, AM360-PF3845-LPS, MK886-PF3845-LPS, GW9662-PF3845-LPS, IRTX-PF3845-LPS and CID-16020046 (CID)-PF3845-LPS (n = 10-18 per group). Rats received a single, acute i.c.v. administration of either AM251 (2.5ug), AM360 (5ug), MK886 (300ng), GW9662 (50ug), IRTX (1nmol), CID (10 μ M) or vehicle (100% DMSO) in an injection volume of 5ul infused over 1 min, followed immediately by a single, acute i.p. injection of PF3845 (10mg/kg, i.p.) or vehicle (ethanol:cremaphor:saline; 1:1:18) in an injection volume of 2ml/kg. Animals were returned to their home cage and received a single, acute i.p. injection of LPS (100ug/kg,

i.p.) or sterile saline (0.89%) in an injection volume of 1mg/kg, 30 min post PF3845 injection. The doses of the antagonists were based upon previous published work demonstrating antagonistic activity *in vivo* (De Laurentiis *et al.*, 2010; dos Santos *et al.*, 2012; Fakhfour *et al.*, 2012; Morgenweck *et al.*, 2010; Rea *et al.*, 2013). Two hours following LPS or saline, animals were sacrificed by decapitation, the hippocampus and frontal cortex excised, snap-frozen and stored at -80°C until assayed for levels of endocannabinoids and *N*-acylethanolamines, cytokine, *COX2* and related metabolite expression and MAPK activation. (The methodology was as described in Chapter 2).

7.2.5 Experiment 5: Investigation of effect of systemic administration of PF3845 on hippocampal expression of markers of microglial activation, sickness- and depressive-like behaviour, 24 hours post systemic LPS administration

Rats were randomly assigned into one or four treatment groups: Vehicle-saline, Vehicle-PF3845, Vehicle-LPS and PF3845-LPS (n = 7-9 per group). Rats received a single, acute i.p. injection of PF3845 (10mg/kg) or vehicle (1:1:18; ethanol:cremaphor:saline) in an injection volume of 2ml/kg, followed 30 min later by a single, acute i.p. injection of LPS (100ug/kg) or saline vehicle (sterile 0.89% NaCl) administered in an injection volume of 1mg/kg. In the 24 hour period post administration of LPS, behavioural testing was carried out, including the sucrose preference test (SPT) and home cage activity (HCA), see below for protocols. Twenty four hours post LPS or saline, animals were sacrificed by decapitation, the hippocampus excised, snap-frozen and stored at -80°C until assayed for expression of immune mediators.

7.2.5.1 Home cage activity (HCA) monitoring

The Opto-M3 Dual Axis system (Columbus Instruments, Columbus, OH) was used to monitor horizontal and vertical locomotor activities. Home cage locomotor activity was recorded before the commencement of the study to ensure that animals were subdivided into groups with comparable activity. The system evaluated movement of animals in 2 horizontal planes 5 cm and 11.75 cm from the floor of the cage. Each plane was monitored by 16 beams spaced 2.54 cm apart, and there was an infrared emitter and detector per beam located across the width of the cage. The total number of beams broken per interval was calculated and data sent to a central computer, which displayed outputs as X-total/horizontal activity (total number of beams broken in the lower plane)

and Z-total/vertical activity (total number of beams broken in the upper plane) over 5-minute time bins, which were then totaled and analyzed in 2 hour time bins. Activity in the lower (X) plane represents horizontal locomotor activity, and activity in the upper (Z) plane represents vertical or rearing activity. Activity was recorded for 24 hours post LPS injection as described above, after which animals were immediately sacrificed by decapitation.

7.2.5.2 Sucrose preference test (SPT)

Animals that were used for measuring HCA also underwent the SPT in which animals were exposed to a 1% (w/v) sucrose solution and also to tap water in standard drinking bottles in the feeding compartment of their home cage. In order to obtain a baseline sucrose preference, animals underwent a 4 day training period prior to the test day. During this period the position of the sucrose and water bottles were alternated across the left and right side of each animal feeding compartment every 24 hours so as to avoid place preference. Once baseline preference was obtained, there was a 24 hour break period, following which animals received a single, acute i.p. injection of PF3845 or vehicle, followed 30 min later with a single, acute i.p. injection of LPS. Bottles were weighed at 24 hours post LPS administration, following which animals were immediately sacrificed by decapitation. Total fluid consumption was calculated by dividing the total amount of fluid consumed over the test period by the average total fluid consumption over training period. Sucrose preference scores were calculated by dividing the amount of sucrose consumed over test period by the average sucrose consumed over training period.

7.2.6 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-Wilk and Levene test, respectively. When comparing the means of two unrelated groups, parametric data were analysed using unpaired *t*-test. One-way ANOVA was used to compare the mean of more than two groups on one factor. Data were analysed using 2-way analysis of variance (ANOVA) when comparing the mean of more than two groups on two factors simultaneously. *Post-hoc* analysis was performed using Fisher's LSD test where appropriate. Data were considered significant when $p < 0.05$. Results are expressed as group means + SEM.

7.3 Results

7.3.1 Experiment 1: The effect of systemic administration of the FAAH inhibitor PF3845 on levels of endocannabinoids and related *N*-acylethanolamines in the frontal cortex and hippocampus

Systemic administration of the potent FAAH inhibitor PF3845 increased AEA [$t_{(16)}=27.847$, $p = 0.000$], OEA [$t_{(16)}=41.344$, $p = 0.000$] and PEA [$t_{(16)}=23.068$, $p = 0.000$] levels in the frontal cortex, when compared to vehicle-LPS-treated counterparts (Fig 7.1). Similarly, systemic administration PF3845 significantly increased hippocampal levels of OEA [$t_{(15)}=8.751$, $p = 0.000$] and PEA [$t_{(15)}=7.315$, $p = 0.000$], while concurrently significantly decreasing hippocampal levels of AEA [$t_{(15)}=2.137$, $p = 0.049$], when compared to vehicle-LPS-treated counterparts, at 2 hours post systemic LPS administration (Fig 7.1). Furthermore, PF3845 had no significant effect on 2-AG levels in either region examined, when compared to vehicle-LPS treated counterparts (Fig 7.1).

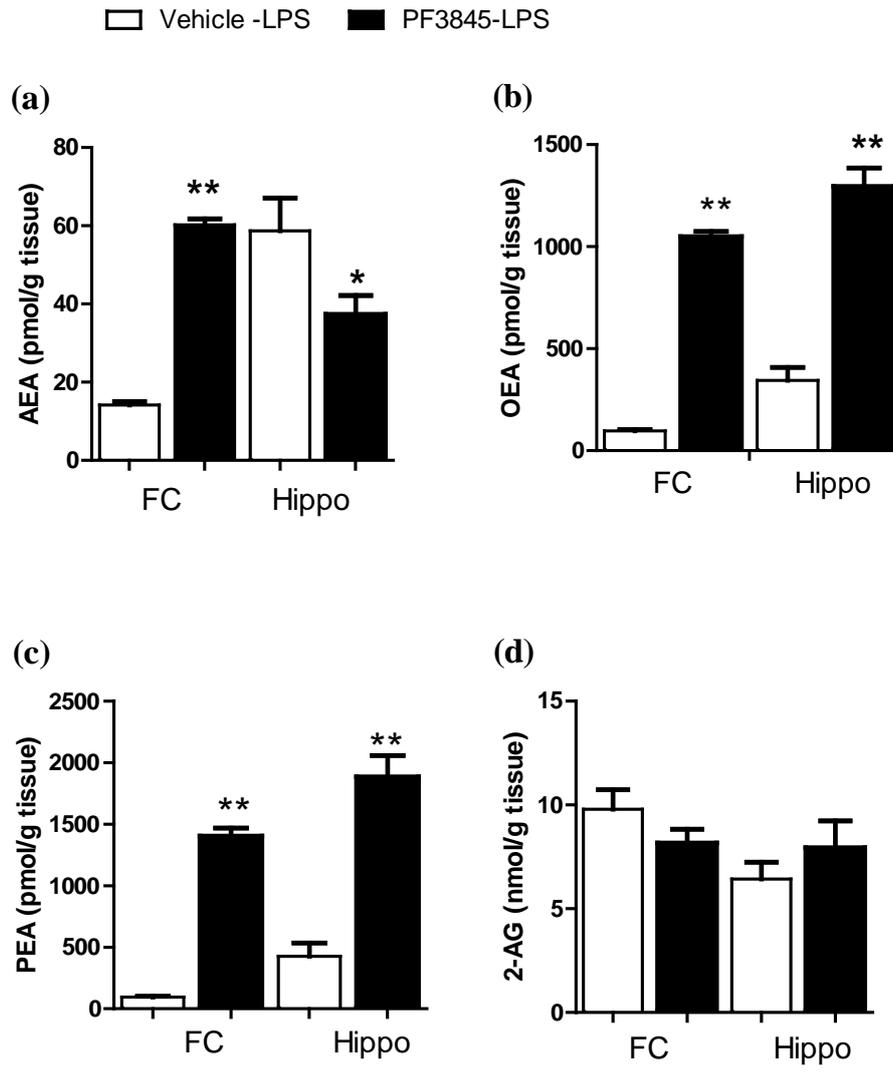


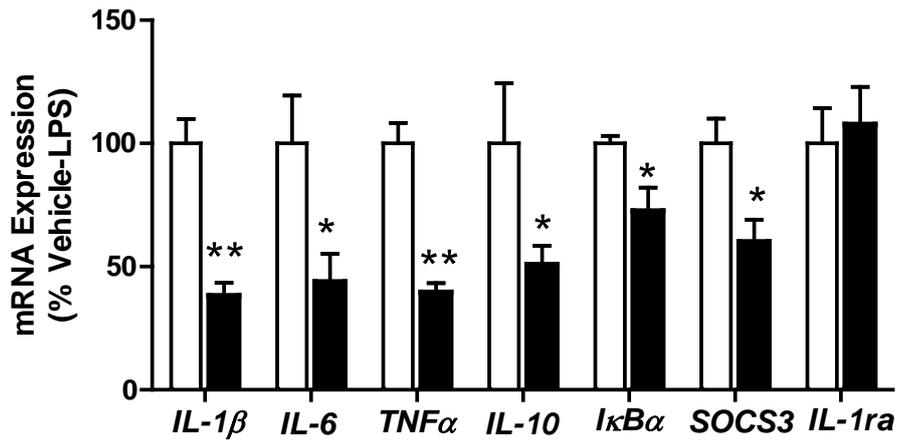
Figure 7.1 The effect of systemic administration of PF3845 on levels of endocannabinoids and *N*-acylethanolamines in the frontal cortex (FC) and hippocampus (Hippo). Systemic administration of PF3845 enhances both frontal cortical and hippocampal levels of (a) AEA (b) OEA, (c) PEA, but not (d) 2-AG, when compared to vehicle-LPS-treated counterparts, 2 hours post LPS challenge. Data expressed as mean + SEM (n = 8-10 per group). ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

7.3.2 Experiment 1: Systemic administration of PF3845 attenuates frontal cortical and hippocampal expression of NF- κ B-inducible inflammatory genes following systemic activation of TLR4

Systemic administration of PF3845 significantly attenuated the LPS-induced increases in mRNA gene expression of *IL-1 β* [$t_{(15)}=5.203$, $p = 0.000$], *IL-6* [$t_{(15)}=2.238$, $p = 0.041$], *TNF α* [$t_{(15)}=5.934$, $p = 0.000$], *IL-10* [$t_{(13)}=2.156$, $p = 0.05$], *I κ B α* [$t_{(14)}=2.858$, $p = 0.013$] and *SOCS3* [$t_{(16)}=2.887$, $p = 0.011$] in the frontal cortex (Fig 7.2a). A similar profile was reported in the hippocampus whereby systemic administration of PF3845 significantly attenuated the LPS-induced increases in mRNA gene expression of *IL-1 β* [$t_{(12)}=2.698$, $p = 0.019$], *IL-6* [$t_{(12)}=2.514$, $p = 0.027$], *TNF α* [$t_{(12)}=4.976$, $p = 0.000$] and *SOCS3* [$t_{(12)}=3.846$, $p = 0.002$], when compared to vehicle-LPS-treated counterparts (Fig 7.2b). In comparison, systemic administration of PF3845 did not significantly alter the expression of the anti-inflammatory cytokine *IL-1 α* in either the frontal cortex or hippocampus, nor did this treatment regime alter hippocampal expression of *I κ B α* or *IL-10*, when compared to vehicle-LPS-treated counterparts.

□ Vehicle-LPS ■ PF3845-LPS

(a) Frontal cortex



(b) Hippocampus

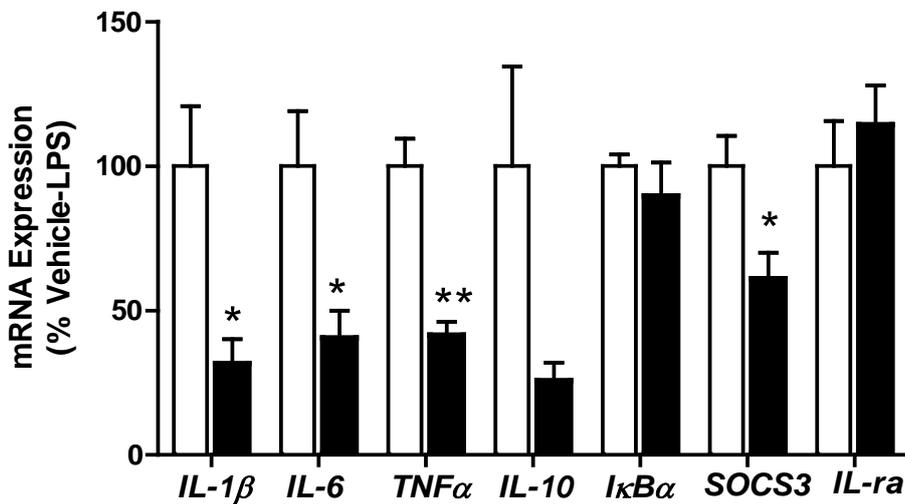


Figure 7.2 The effect of systemic administration of PF3845 on frontal cortical and hippocampal gene expression of NF- κ B-inducible inflammatory cytokines, 2 hours post systemic LPS administration. Systemic administration of PF3845 significantly attenuates the LPS-induced increases in mRNA expression of NF- κ B-inducible inflammatory genes in the (a) frontal cortex and (b) hippocampus. Data expressed as mean + SEM (n = 8-10 per group). * $p < 0.05$, ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

7.3.3 Experiment 2: I.c.v. administration of PF3845 increases AEA, OEA and PEA levels in the frontal cortex and hippocampus

I.c.v. administration of PF3845 increased levels of AEA [$t_{(25)}=6.490, p = 0.000$], OEA [$t_{(25)}=12.821, p = 0.000$] and PEA [$t_{(25)}=10.929, p = 0.000$] in the frontal cortex, when compared to vehicle-LPS-treated counterparts (Fig 7.3). Similarly, i.c.v. administration of PF3845 significantly increased hippocampal levels of AEA [$t_{(25)}=8.099, p = 0.000$], OEA [$t_{(25)}=11.307, p = 0.000$] and PEA [$t_{(25)}=9.939, p = 0.000$], when compared to vehicle-LPS-treated counterparts (Fig 7.3). Furthermore, i.c.v. administration of PF3845 did not significantly alter levels of 2-AG in either regions examined (Fig 7.3).

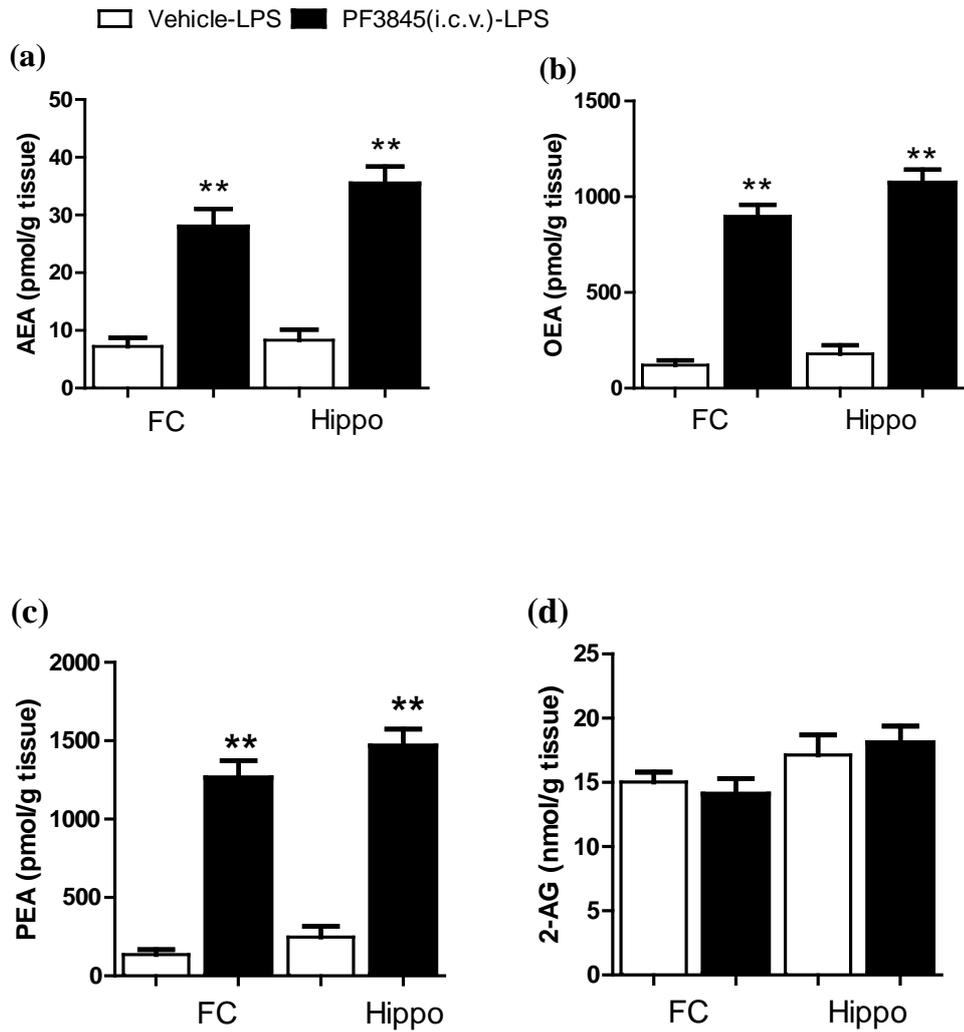


Figure 7.3 The effect of i.c.v. administration of PF3845 on levels of endocannabinoids and *N*-acylethanolamines in the frontal cortex (FC) and hippocampus (Hippo). I.c.v. administration of PF3845 significantly increases the levels of (a) AEA, (b) OEA, (c) PEA, but not (d) 2-AG in the frontal cortex and hippocampus, when compared to vehicle-LPS-treated counterparts, 2 hours post LPS challenge. Data expressed as mean + SEM (n = 8-10 per group). ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

7.3.4 Experiment 2: I.c.v. administration of PF3845 attenuates expression of NF- κ B-inducible inflammatory genes in the hippocampus and frontal cortex, following systemic TLR4 activation

Initial findings demonstrated that systemic administration of PF3845 increased FAAH substrates in the brain and this was associated with an attenuation of TLR4-induced increases in several NF- κ B-inducible genes in the frontal cortex and hippocampus. However, the contribution of FAAH substrates to these effects of PF3845 is not known. Accordingly, the findings here demonstrate that i.c.v. administration of PF3845 enhances FAAH substrate levels (AEA, OEA and PEA) directly within the brain (Fig 7.3) and this was associated with an attenuation of LPS-induced increases in NF- κ B-responsive genes in the frontal cortex (Fig 7.4a). Specifically, i.c.v. administration of PF3845 significantly attenuated LPS-induced increases in mRNA gene expression of *IL-1 β* ($t_{(15)} = 2.381$, $p = 0.03$), *TNF α* ($t_{(15)} = 3.684$, $p = 0.002$), *IL-6* ($t_{(13)} = 4.517$, $p = 0.01$), *IL-10* ($t_{(10)} = 2.439$, $p = 0.03$] and *SOCS3* ($t_{(15)} = 3.311$, $p = 0.0065$) in the frontal cortex, when compared to vehicle-LPS-treated counterparts (Fig 7.4a). Unlike the robust anti-inflammatory effects reported in the frontal cortex, i.c.v. administration of PF3845 only significantly attenuated the LPS-induced increases in *IL-1 β* ($t_{(16)} = 2.476$, $p = 0.025$) and *IL-10* ($t_{(11)} = 2.789$, $p = 0.018$) within the hippocampus, with a trend for a reduction in hippocampal expression of *IL-6* and *SOCS3* also observed, when compared to vehicle-LPS-treated counterparts (Fig 7.4a).

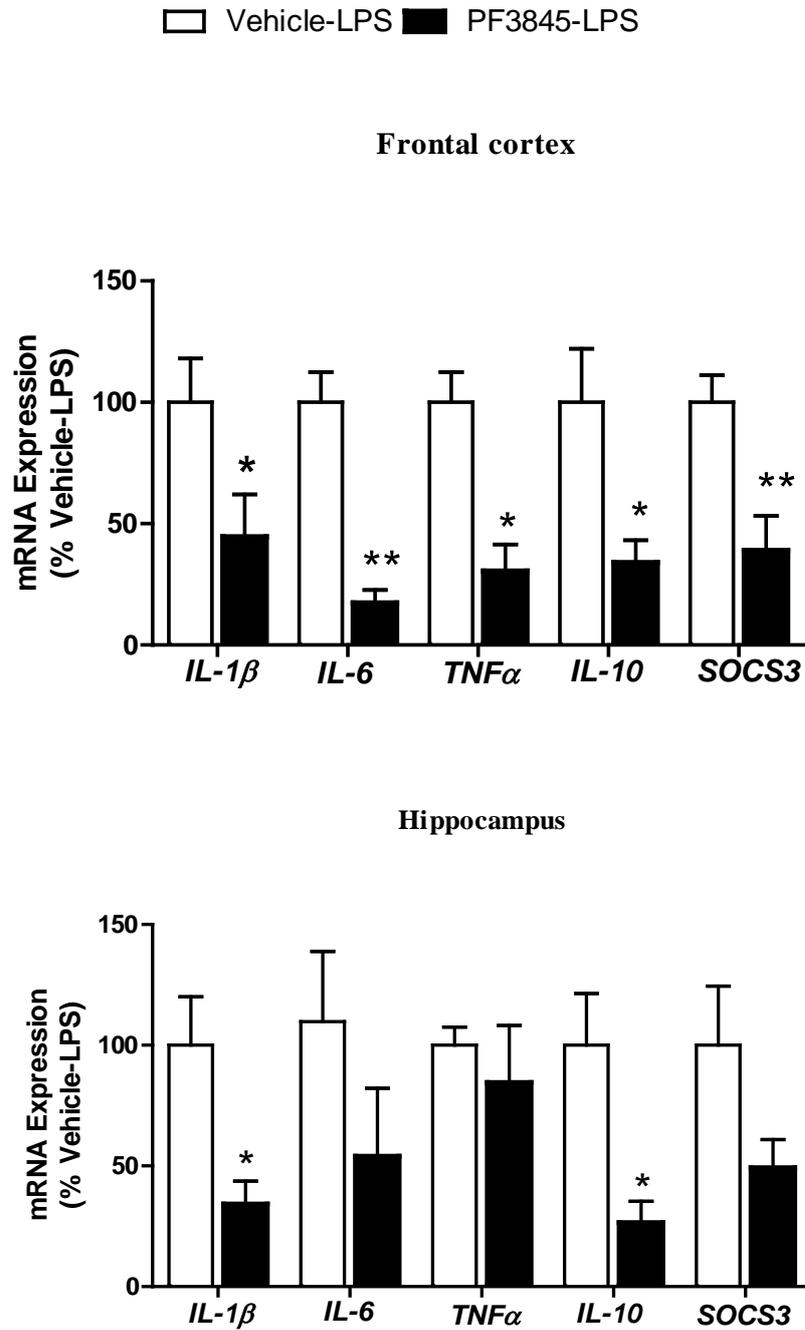


Figure 7.4a The effect of i.c.v. administration of PF3845 on frontal cortical and hippocampal expression of NF- κ B-inducible genes, 2 hours post systemic LPS administration. I.c.v. administration of PF3845 significantly attenuates the LPS-induced increases in the expression of several NF- κ B-inducible inflammatory genes in the (a) frontal cortex and (b) the hippocampus. Data expressed as mean + SEM (n = 5-11 per group). * $p < 0.05$, ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

7.3.4.1 Experiment 2a: I.c.v. administration of PF3845 does not alter the expression of NF- κ B-inducible inflammatory cytokines in the frontal cortex, in the absence of TLR4 activation

Data from Chapter 4 demonstrated that i.c.v. administration of the FAAH inhibitor URB597 did not alter hippocampal expression of inflammatory cytokines in the absence of an immune stimulus. Similarly, this study confirmed that i.c.v. administration of PF3845 did not significantly alter frontal cortical expression of *TNF α* , *IL-1 β* or *IL-6*, at 2 hours post saline administration, when compared to vehicle-saline-treated counterparts (Fig 7.4b).

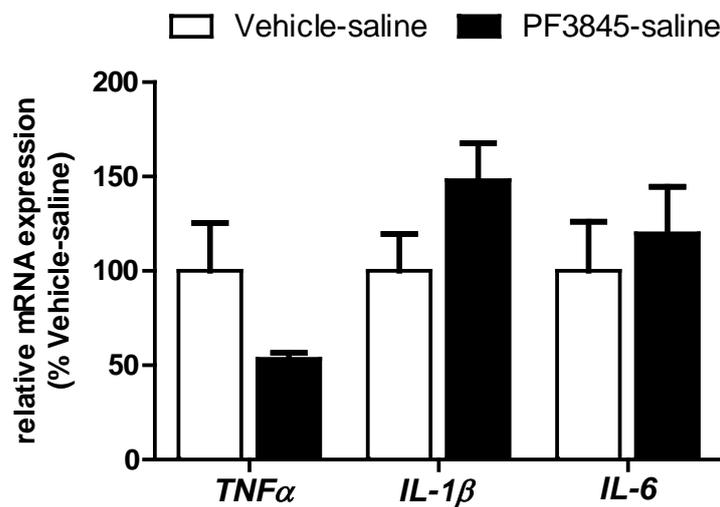


Figure 7.4b The effect of i.c.v. administration of PF3845 on frontal cortical expression of NF- κ B-inducible genes, in the absence of systemic LPS administration. I.c.v. administration of PF3845 does not significantly alter expression of NF- κ B-inducible inflammatory cytokines in the frontal cortex in the absence of LPS. Data expressed as mean + SEM (n = 4 per group).

7.3.5 Experiment 3: The peripherally restricted FAAH inhibitor URB937 does not alter TLR4-induced increases in NF- κ B-responsive genes in the frontal cortex or hippocampus

In order to confirm the role of the brain's FAAH substrates in the modulation of TLR4-induced neuro-immune responses a separate group of animals were administered the peripherally restricted FAAH inhibitor URB937 and its effects on TLR4-induced neuroinflammatory responses were examined. Statistical analysis revealed that although systemic URB937 had no significant effect on the endocannabinoids, AEA or 2-AG (Fig 7.5a), levels of OEA and PEA in both regions [FC; $t_{(14)} = 2.415$, $p = 0.03$; Hippo; $t_{(13)} = 3.198$, $p = 0.007$] and [FC; $t_{(14)} = 2.399$, $p = 0.03$; Hippo; $t_{(13)} = 2.831$, $p = 0.14$] were increased, respectively, when compared to vehicle-LPS-treated counterparts, (Fig 7.5a). However, it should be noted that the magnitude of the increase in OEA and PEA levels following systemic URB937 was significantly less than that observed following systemic or i.c.v. administration of PF3845 (Fig 7.1 and 7.3). Furthermore, assessment of FAAH substrate levels in the spleen revealed that AEA (Vehicle: 8.27pmol/g tissue vs. URB937: 15.71pg/g tissue, $p = 0.000$) OEA (Vehicle: 101.14pmol/g tissue vs. URB937: 169.16pmol/g tissue, $p = 0.000$) and PEA (Vehicle: 147.27pmol/g tissue vs. URB937: 233.01pmol/g tissue, $p = 0.000$) were significantly elevated in this peripheral organ following systemic administration of URB937 (data not shown). Despite these increases in OEA and PEA within the brain, systemic administration of URB937 failed to significantly alter either frontal cortical or hippocampal mRNA gene expression of any of the NF- κ B-responsive genes including *TNF α* , *IL-1 β* and *IL-6* induced following TLR4 activation (Fig 7.5b).

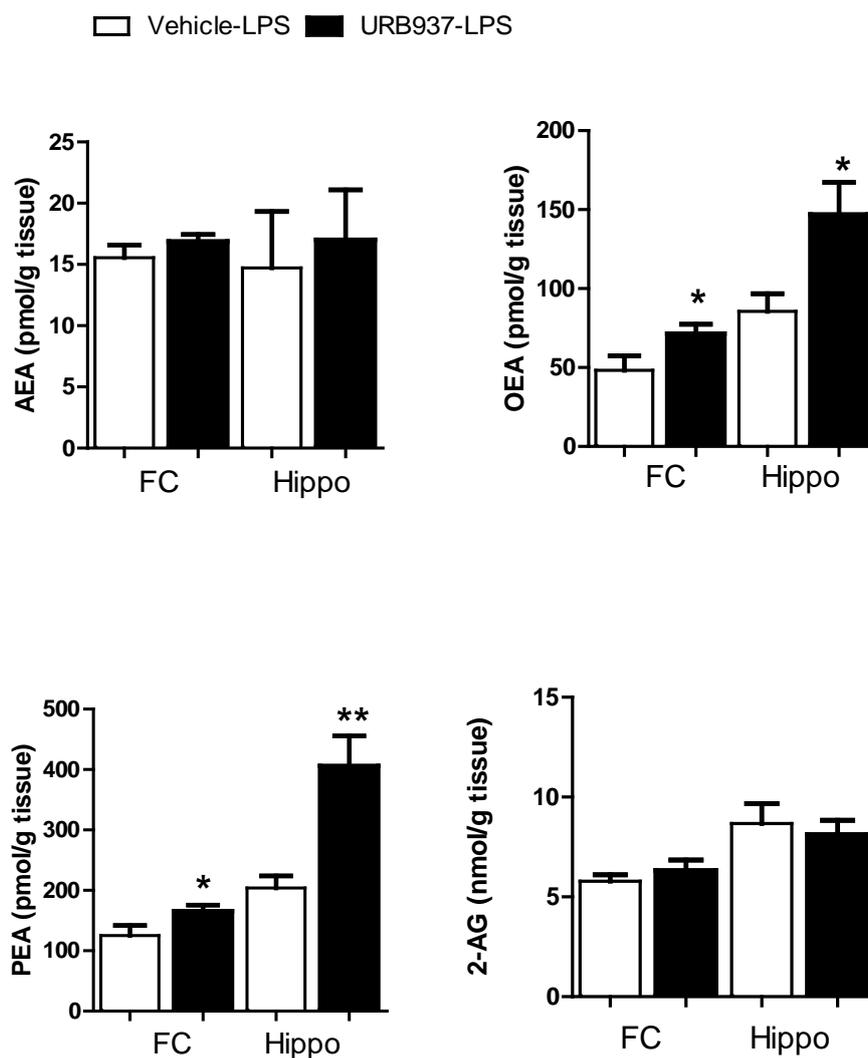


Figure 7.5a The effect of systemic administration of URB937 on levels of endocannabinoids and *N*-acylethanolamines in the frontal cortex (FC) and hippocampus (Hippo). Systemic administration of URB937 does not significantly alter frontal cortical or hippocampal levels of AEA or 2-AG, but increases levels of OEA and PEA in both the FC and Hippo, compared to vehicle-LPS-treated counterparts. Data expressed as mean + SEM (n = 8 per group). * $p < 0.05$, ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

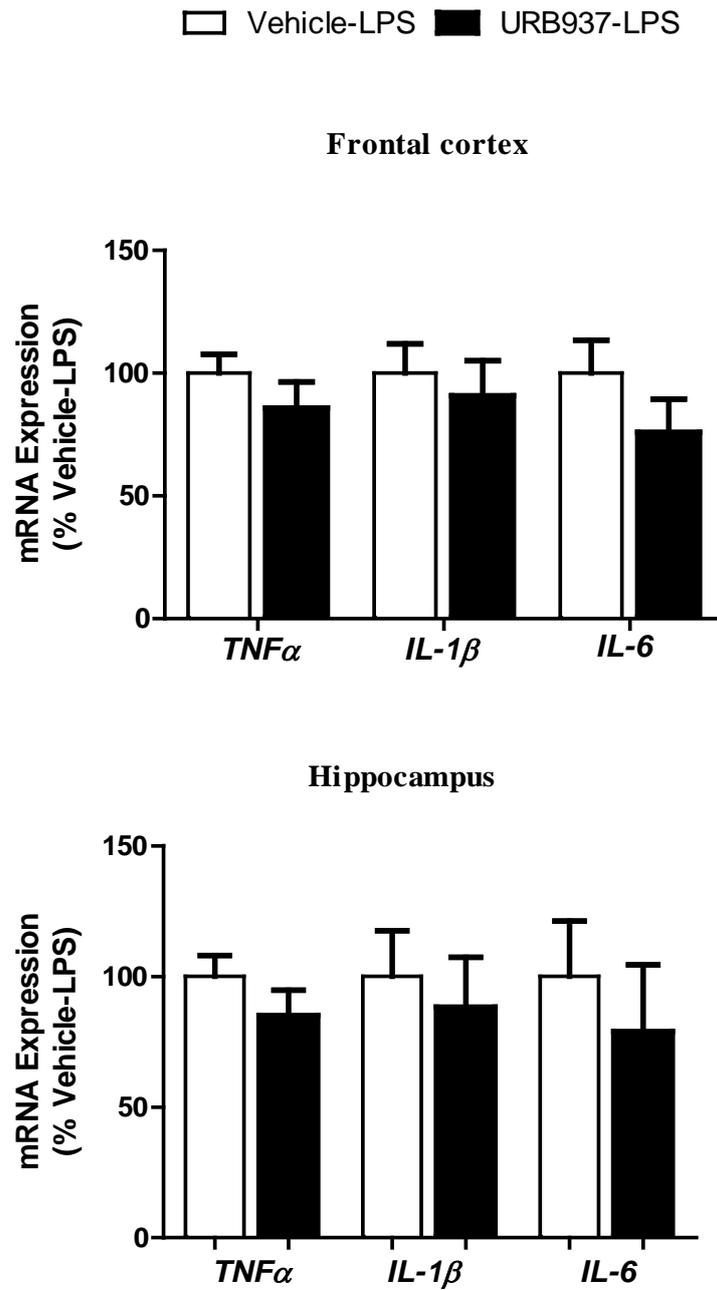


Figure 7.5b The effect of systemic administration of URB937 on gene expression of NF- κ B-inducible genes in the frontal cortex and hippocampus, 2 hours post systemic LPS administration. Systemic administration of URB937 does not significantly alter either frontal cortical or hippocampal expression of NF- κ B-inducible genes, when compared to vehicle-LPS-treated counterparts. Data expressed as mean + SEM (n = 8 per group).

7.3.6 Experiment 4: The anti-inflammatory effects of systemically administered PF3845 on TLR4-induced inflammation within the frontal cortex are not mediated by CB₁ or CB₂ receptors directly within the brain

Our data have demonstrated that increasing levels of FAAH substrates directly within the brain exerts potent anti-inflammatory effects on TLR4-induced increases in inflammatory responses within the frontal cortex and hippocampus. As such, the next part of this study aimed to examine the potential receptor mechanisms within the brain responsible for mediating such responses. In the first instance the potential involvement of brain CB₁ and CB₂ receptors were investigated. Statistical analysis revealed a significant effect of treatment on mRNA gene expression of the NF- κ B-inducible genes *TNF α* [$F_{3,56}=4.481$, $p < 0.01$], *IL-1 β* [$F_{3,56}=4.976$, $p < 0.01$] and *IL-6* [$F_{3,56}=5.496$, $p < 0.01$] in the frontal cortex at 2 hours post systemic LPS administration (Fig 7.6). Systemic administration of PF3845 significantly attenuated frontal cortical expression of *IL-1 β* and *IL-6*, but not *TNF α* , when compared to vehicle-vehicle-LPS-treated counterparts (Fig 7.6). I.c.v. administration of the selective CB₁ and CB₂ antagonists AM251 or AM630, respectively, did not alter the frontal cortical expression of *IL-1 β* or *IL-6* when compared to vehicle-PF3845-LPS-treated counterparts [AM251-PF-LPS vs. Vehicle-PF-LPS $p > 0.05$; AM630-PF-LPS vs. Vehicle-PF-LPS $p > 0.05$] (Fig 7.6b,c). However, AM630-PF3845-LPS-treated animals did exhibit significantly reduced expression of *TNF α* , when compared to vehicle-PF3845-LPS-treated counterparts (Fig 7.6a).

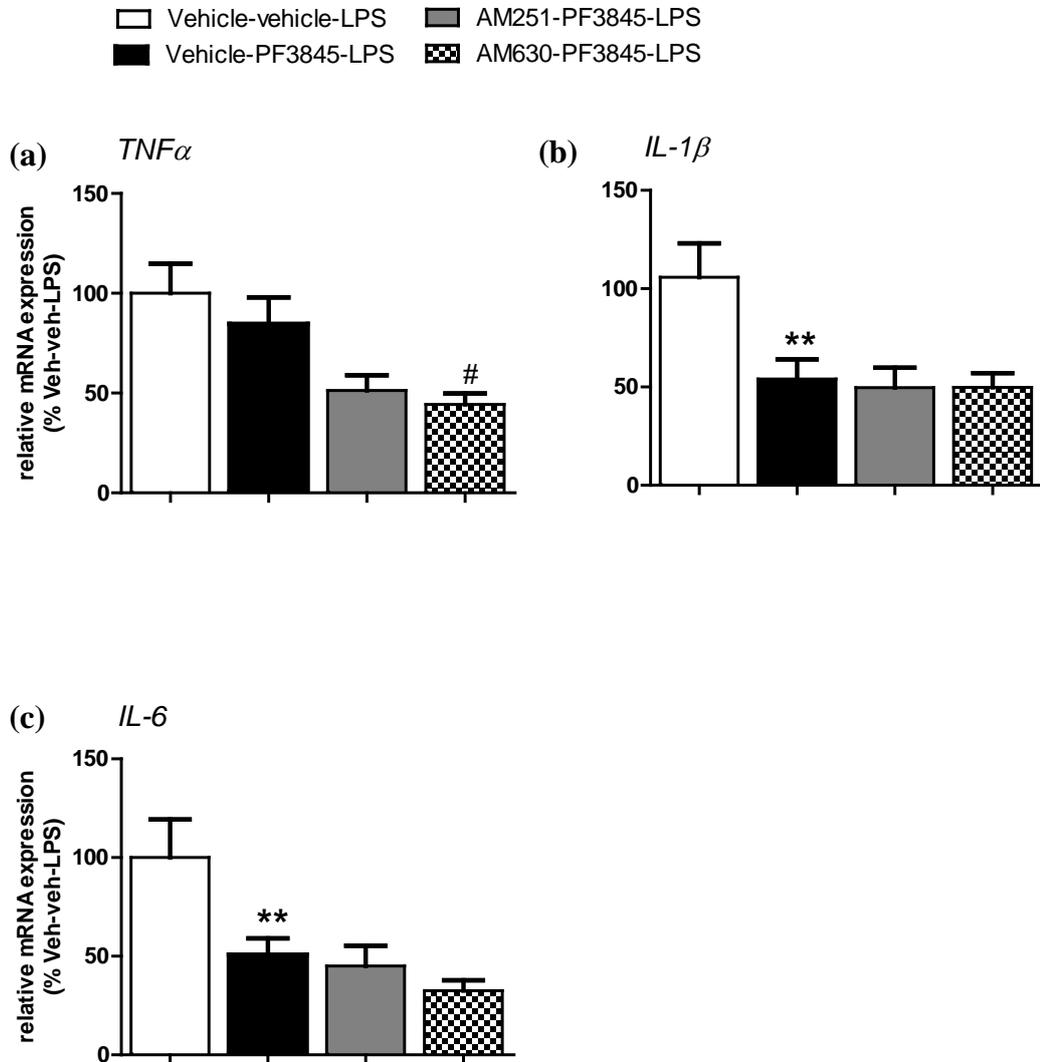


Figure 7.6 The effect of i.c.v. administration of selective CB₁/CB₂ antagonists, in the presence of systemic administration of PF3845, on NF- κ B-inducible gene expression within the frontal cortex, 2 hours post systemic LPS administration. Antagonism of the CB₁ or CB₂ receptors within the brain does not alter PF3845-induced attenuation of cortical expression of (b) *IL-1 β* and (c) *IL-6*, 2 hours post LPS administration. In addition, there was no effect of treatment on frontal cortical expression of (a) *TNF α* . Data expressed as mean + SEM (n = 10-18 per group) ** $p < 0.01$ vs. vehicle-vehicle-LPS-treated animals, # $p < 0.05$ vs. vehicle-PF3845-LPS-treated counterparts.

7.3.7 Experiment 4: Systemic administration of PF3845 does not alter MAPK activation in the rat frontal cortex, following systemic TLR4 activation

Activation of CB₁ and CB₂ receptors are positively coupled to MAPK activation which in turn are known to be important in modulating NF-κB-inducible cytokines in activated microglia (Correa *et al.*, 2010; Krishnan *et al.*, 2012). Thus, in order to further confirm that the reported anti-inflammatory effects in the current study were independent of cannabinoid receptor activation, MAPK signalling pathways were examined. Accordingly, statistical analysis revealed that PF3845-LPS-treated animals did not display any significant alterations in total ERK, JNK or p38, when compared to vehicle-LPS-treated counterparts (Fig 7.7). Additionally, ERK1/2 phosphorylation was not altered in PF3845-LPS-treated, when compared to their vehicle-LPS-treated counterparts (Fig 7.7a). Although there is a trend for a decrease in JNK phosphorylation in PF3845-LPS-treated animals, when compared to vehicle-LPS-treated counterparts, this effect failed to reach significance (Fig 7.7b). Furthermore, phosphorylated levels of p38 in the frontal cortex were undetectable at 2 hours post systemic LPS administration (Fig 7.7c). Overall, these findings indicated that PF3845 did not alter MAPK (ERK or JNK) activation at 2 hours following LPS administration.

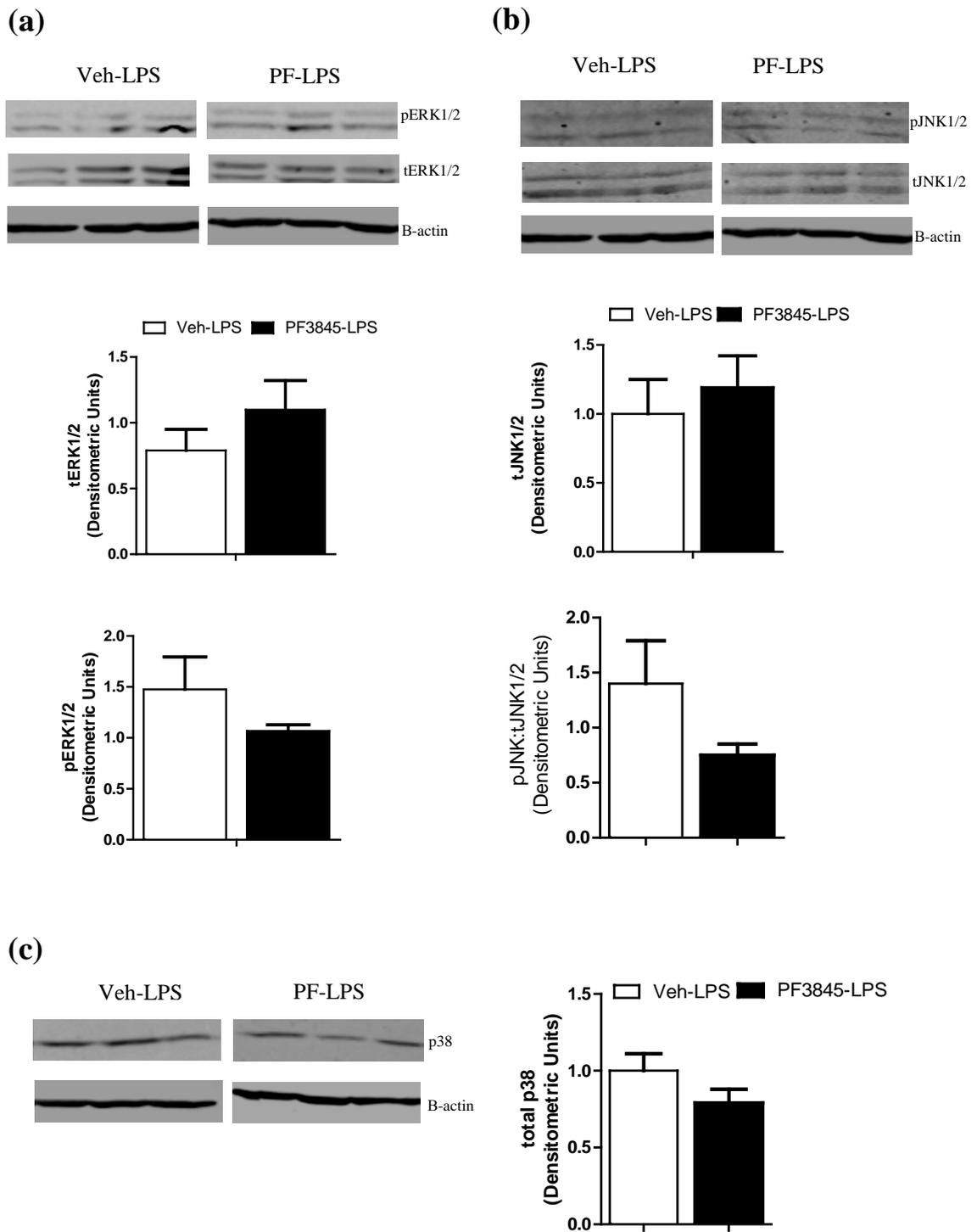


Figure 7.7 The effect of systemic administration of PF3845 on MAPK activation in the frontal cortex, 2 hours post systemic LPS administration. Systemic administration of PF3845 does not significantly alter total (a) ERK1/2, (b) JNK or (c) p38, when compared to vehicle-LPS-treated counterparts. In addition, PF3845 does not alter phosphorylation of (a) ERK1/2 or (b) JNK, when compared to vehicle-LPS-treated counterparts. Data expressed as mean + SEM (n = 5-6 per group).

7.3.8 Experiment 4: Systemic administration of PF3845 attenuates TLR4-induced frontal cortical expression of *COX2* and *iNOS* expression

In addition to mediating immunoregulatory effects via CB₁/CB₂ receptors, there are now reports that endocannabinoids mediate anti-inflammatory effects via alternative mechanisms including reductions in AA and subsequent production of prostaglandins within the CNS (Nomura *et al.*, 2011) or via COX2-derived endocannabinoid metabolites (Alhouayek *et al.*, 2014b; Correa *et al.*, 2008). Thus, based on the initial findings suggesting that FAAH substrates mediate anti-inflammatory effects independent of CB₁/CB₂ receptor activation, we next examined the effect of systemic administration of PF3845 on frontal cortical expression of *COX2*, *iNOS* and *mPGE-s*, a key enzyme involved in prostaglandin (PGE₂) synthesis. Statistical analysis revealed that systemic administration of PF3845 attenuated the LPS-induced increases in mRNA expression of *COX2* [$t_{(22)} = 2.274, p = 0.03$] and the neurotoxic metabolite *iNOS* [$t_{(21)} = 2.896, p = 0.009$] in the frontal cortex, 2 hours following LPS administration (Fig 7.8). In contrast, this treatment regime failed to alter frontal expression of the enzyme *mPGE-s* (Fig 7.8). Based on this finding and previous preliminary work from our laboratory demonstrating that PGE₂ was unaltered in the frontal cortex, 2 hours following systemic LPS administration, we did not measure prostaglandin levels in the current study.

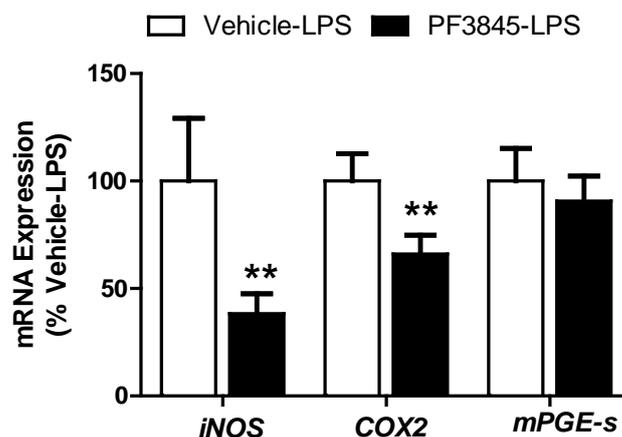


Figure 7.8 The effect of systemic administration of PF3845 on frontal cortical expression of *COX2*, *iNOS* and *mPGE-s*, 2 hours post systemic LPS administration. PF3845 significantly attenuates LPS-induced increases in expression of *iNOS* and *COX2*, but not frontal cortical expression of *mPGE-s*, when compared to vehicle-LPS-treated counterparts. Data expressed as mean + SEM (n = 10-15 per group). ** $p < 0.01$ vs. vehicle-LPS-treated counterparts.

7.3.9 Experiment 4: Blockade of TRPV1 within the brain attenuates the FAAH substrate-mediated attenuation of LPS-induced *IL-6* expression within the frontal cortex

In addition to CB₁ and CB₂ receptors, AEA is known to directly activate additional receptor targets including PPARs (Rockwell *et al.*, 2004), TRPV1 (Smart *et al.*, 2000; Zygmunt *et al.*, 1999) and GPR55 (Ryberg *et al.*, 2007b). Furthermore, it is now well recognised that neither OEA nor PEA directly activates CB_{1/2} receptors; rather they are known to activate PPARs (Fu *et al.*, 2003; LoVerme *et al.*, 2005). Earlier studies in this thesis reported a role for brain CB₁ receptor and PPAR- α/γ in FAAH substrate-mediated modulation of TLR3-induced neuroinflammation (Chapter 5). As such we decided to examine the potential role of brain PPAR- α , PPAR- γ , TRPV1 and GPR55 in FAAH substrate-mediated modulation of TLR4-induced increases in neuroinflammatory responses within the frontal cortex. Statistical analysis revealed a significant effect of treatment on mRNA expression of *TNF α* [$F_{5,73}=2.502$, $p < 0.05$], *IL-1 β* [$F_{5,74}=4.027$, $p < 0.01$] and *IL-6* [$F_{5,73}=2.276$, $p < 0.05$] in the frontal cortex (Fig 7.9). Systemic administration of PF3845 significantly attenuated LPS-induced increases in the frontal cortical expression of *IL-1 β* and *IL-6*, but not *TNF α* , [Vehicle-PF3845-LPS vs. Vehicle-vehicle-LPS] (Fig 7.9). I.c.v. administration of IRTX (TRPV1 antagonist) but not MK886 (PPAR- α antagonist), GW9662 (PPAR- γ antagonist), or CID (GPR55 antagonist) prevented the PF3845 induced decrease in frontal cortical expression of *IL-6* following LPS administration [IRTX-PF3845-LPS vs. Vehicle-PF3845-LPS] (Fig 7.9c). In comparison, none of the antagonists altered the PF3845-induced decrease in frontal cortical expression of *IL-1 β* (Fig 7.9b). However, MK886-PF3845-LPS-treated animals exhibited a significant decrease in expression of *TNF α* , when compared to vehicle-PF3845-LPS-treated counterparts (Fig 7.9a). In addition, IRTX-vehicle-LPS-treated animals do not display altered frontal cortical expression of *IL-6*, when compared to vehicle-vehicle-LPS-treated counterparts (Fig 7.9d). CGRP is a neuropeptide released following TRPV1 activation and is reported to modulate immune function including activation of NF- κ B activation (Ding *et al.*, 2007). As such, frontal cortical mRNA expression of *CGRP* was measured, however statistical analysis revealed no significant effect of treatment on expression (Vehicle-LPS: 100 + 14.14 vs. PF3845-LPS: 96.32 + 17.99, $p > 0.05$), data not depicted graphically.

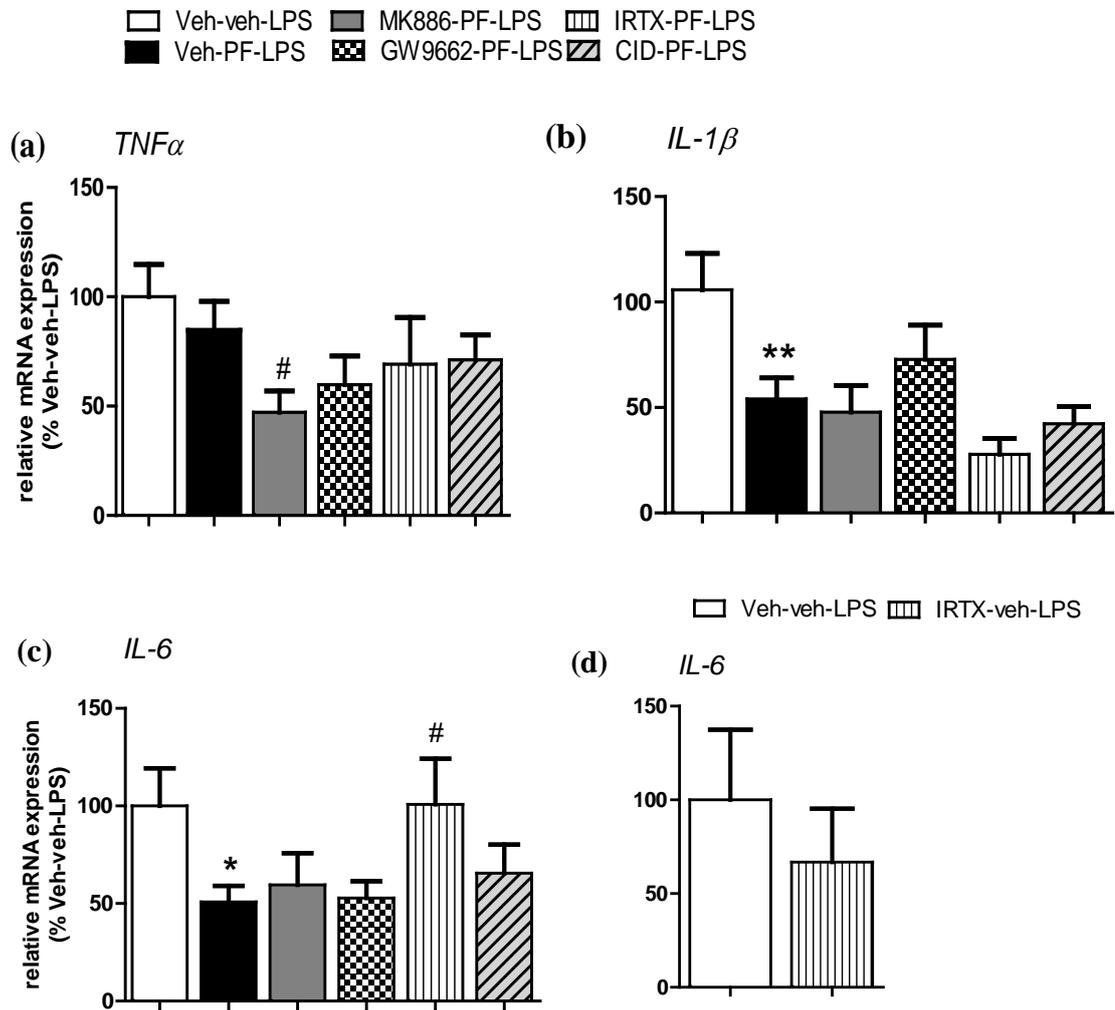


Figure 7.9 The effect of i.c.v. administration of selective PPAR, TRPV1 and GPR55 antagonists, in the presence of systemic administration of PF3845, on NF- κ B-inducible gene expression within the frontal cortex, 2 hours post systemic LPS administration. Antagonism of TRPV1, but not PPAR- α/γ or GPR55, directly within the brain attenuates the PF3845-induced decreases in cortical expression of (c) *IL-6*, when compared to vehicle-PF3845-LPS-treated counterparts. In addition, none of the antagonists examined altered frontal cortical expression of (a) *TNF α* or (b) *IL-1 β* , when compared to vehicle-PF3845-treated counterparts. In addition, i.c.v. administration of the TRPV1 antagonist IRTX does not alter (d) *IL-6* expression in the frontal cortex, when compared to vehicle-LPS-treated counterparts. Data expressed as mean + SEM (n = 10-18 per group) * $p < 0.05$, ** $p < 0.01$ vs. vehicle-vehicle-LPS-treated counterparts, # $p < 0.05$ vs. vehicle-PF3845-LPS-treated counterparts.

7.3.10 Experiment 5: Systemic administration of LPS increases hippocampal expression of *CD11b*, *GFAP* and *CD68*, 24 hours post LPS administration

Previous findings demonstrated that systemic administration of the TLR3 agonist poly I:C altered hippocampal and frontal cortical expression of markers of glial activation and *BDNF*, effects of which were partially modulated by systemic administration of the FAAH inhibitor URB597, 24 hours post systemic poly I:C administration (Chapter 3). As such, this study examined if similar long-term alterations in neuro-immune function occurred within the hippocampus and if systemic administration of PF3845 altered such responses. Statistical analysis revealed a significant main effect of LPS, but not PF3845 or LPS-PF3845, on mRNA expression of *CD11b* [$F_{(1,26)}=49.726$, $p = 0.000$] and *CD68* [$F_{(1,26)}=4.526$, $p = 0.043$](Fig 3), 24 hours following LPS administration (Fig 7.10). *Post hoc* analysis revealed that both vehicle-LPS- and PF3845-LPS-treated animals displayed significant increases in hippocampal expression of *CD11b* (marker of microglial activation) (Fig 7.10a), while only vehicle-LPS-treated animals displayed significant increases in expression of *CD68* (marker of M1 microglia activation), compared to vehicle-saline-treated counterparts (Fig 7.10c). In addition, there was no significant effect of treatment on hippocampal expression of *GFAP* (marker of astrocyte activation), *MRC2* (marker of M2 microglia activation) or *BDNF* (neurotrophic factor), 24 h post LPS challenge (Fig 7.10d,e). Furthermore, systemic administration of PF3845 in the absence of LPS did not alter hippocampal expression of any of the genes examined, when compared to vehicle-saline-treated counterparts (Fig 7.10).

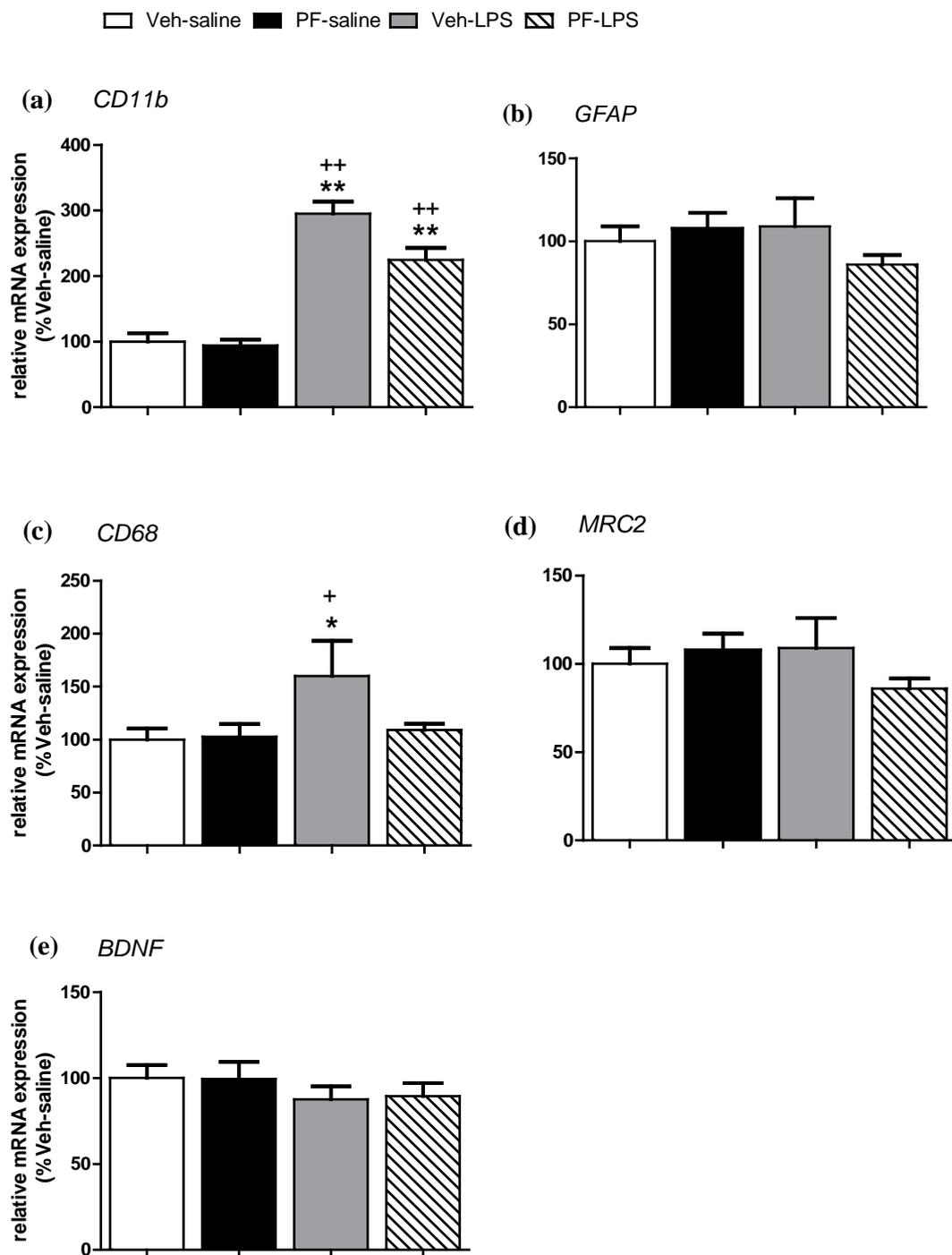


Figure 7.10 The effect of systemic administration of PF3845 on hippocampal expression of long-term neuro-immune mediators, 24 post systemic LPS administration. Systemic administration of LPS increases hippocampal expression of (a) *CD11b* and (c) *CD68*, but not (b) *GFAP*, (d) *MRC2* or (e) *BDNF*, when compared to vehicle-saline-treated counterparts. Prior systemic administration of PF3845 did not alter the LPS-induced increases in any of the genes examined. Data expressed as mean + SEM (n=7-9 per group). * $p < 0.05$, ** $p < 0.01$ vs. vehicle-saline-treated counterparts, + $p < 0.05$, ++ $p < 0.01$ vs. PF3845-saline-treated counterparts.

7.3.11 Experiment 5: Systemic administration of PF3845 does not alter LPS-induced reductions in homecage locomotor activity

Statistical analysis revealed a significant main effect of LPS [$F_{(1,26)}=46.882, p=0.000$], but not PF3845 or PF3845-LPS on home-cage horizontal activity during the 24 hour period following LPS administration (Fig 7.11a). *Post hoc* analysis revealed that both vehicle-LPS- and PF3845-LPS-treated animals displayed significantly less home cage horizontal activity between 10-22 hours post LPS administration, compared to their vehicle-saline-treated counterparts, which returned to baseline levels by 24 hours post LPS injection (Fig 7.11c). In addition, statistical analysis revealed a significant main effect of LPS on homecage vertical activity [$F_{(1,26)}=48.890, p=0.000$] but not PF3845 or PF3845-LPS on home-cage horizontal activity during the 24 hour period following LPS administration (Fig 7.11b). *Post hoc* analysis revealed that both vehicle-LPS- and PF3845-LPS-treated animals displayed significantly less home cage vertical activity between 10-22 hours post LPS administration, compared to their vehicle-saline-treated counterparts, which returned to baseline levels by 24 hours post LPS injection (Fig 7.11d).

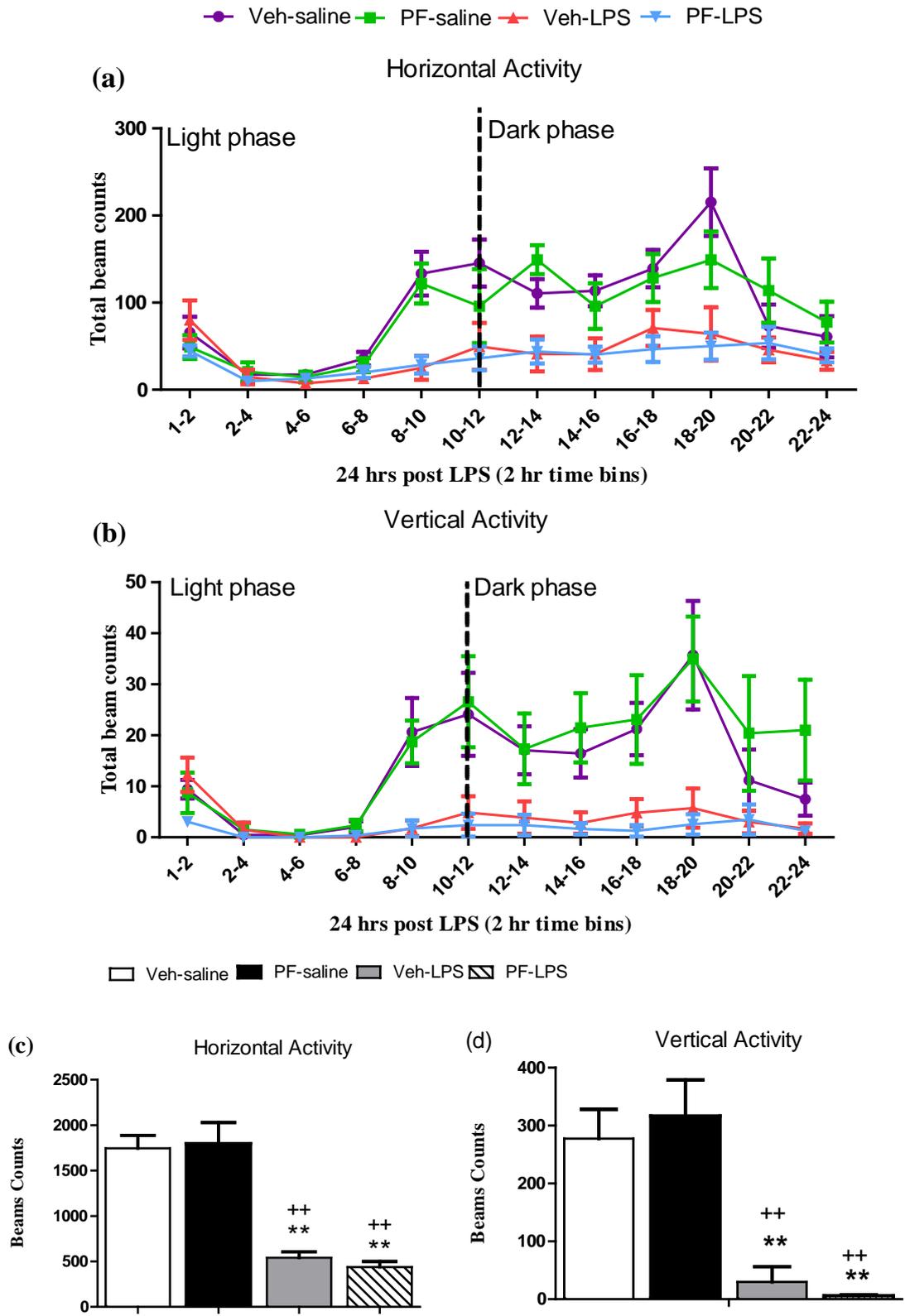


Figure 7.11 The effect of systemic administration of PF3845 on homecage locomotor activity in rats, during the 24 hour period post systemic LPS administration. (a) Horizontal activity and (b) vertical activity assessed as number of beam breaks over 24 hours post LPS; (c) Horizontal and (d) Vertical activity over the dark phase period from 10 to 22 hours post LPS. Data expressed as mean + SEM (n=7-9 per group). ** $p < 0.01$ vs. vehicle-saline-treated counterparts, ++ $p < 0.01$ vs. PF3845-saline treated counterparts.

7.3.12 Experiment 5: Systemic administration of PF3845 does not alter LPS-induced reductions in total fluid intake

Systemic administration of LPS has previously been reported to be associated with a depressive-like anhedonic response, 24 hours post LPS administration (Frenois *et al.*, 2007; Salazar *et al.*, 2012; Wang *et al.*, 2014). Thus, we aimed to examine if similar effects occurred in our study and if this was modulated by systemic PF3845 administration. Statistical revealed no significant effect of LPS or LPS-PF3845 on sucrose preference, 24 hours following LPS challenge. However, analysis revealed a significant effect of PF3845 treatment on sucrose preference [$F_{(1,27)}=6.598$, $p = 0.016$]. *Post hoc* analysis revealed that PF3845-saline-treated animals displayed significantly greater intake of sucrose when compared to their vehicle-LPS-treated counterparts ($p = 0.005$) (Fig 7.12a). In addition, statistical analysis revealed a significant effect of LPS [$F_{(1,28)}=17.109$, $p = 0.000$], but not PF3845 or LPS-PF3845 on total fluid intake (Fig 7.12b). *Post hoc* analysis revealed that both vehicle-LPS- and PF3845-LPS-treated animals displayed significantly less fluid intake (mls) compared to vehicle-saline-treated counterparts, 24 hours following LPS administration (Fig 7.12b).

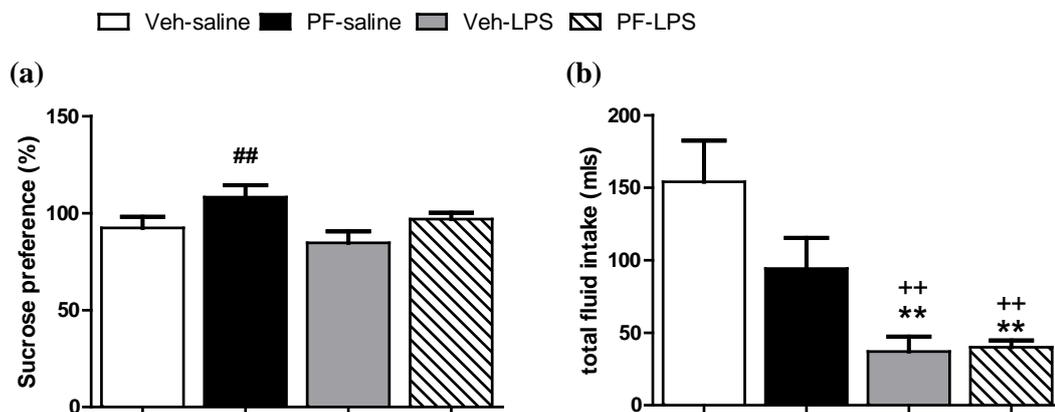


Figure 7.12 The effect of systemic administration of PF3845 on (a) % sucrose preference and (b) total fluid intake (mls), during the 24 hour period post systemic LPS administration. Data expressed as mean + SEM (n=7-9 per group). ## $p < 0.01$ vs. vehicle-LPS-treated counterparts, ** $p < 0.01$ vs. vehicle-saline-treated counterparts, ++ $p < 0.01$ vs. PF3845-saline-treated counterparts.

7.4 Discussion

The data presented herein have demonstrated that both systemic and i.c.v. administration of the potent FAAH inhibitor PF3845 increases AEA, OEA and PEA levels in discrete regions of the rat brain. Increased levels of FAAH substrates (AEA, OEA and PEA) within the frontal cortex and hippocampus following either systemic or i.c.v. PF3845 administration, were associated with an attenuation of TLR4-induced increases in several NF- κ B-inducible genes. Furthermore, systemic administration of the proposed peripherally restricted FAAH inhibitor URB937 resulted in a slight elevation in OEA and PEA levels in the CNS, however this was not associated with any alteration in TLR4-induced increases in NF- κ B-inducible cytokines in either brain region examined. Taken together, these data suggests that FAAH substrate-mediated modulation of TLR4-induced neuroinflammation occurs at the level of the CNS, rather than indirect modulation of peripheral inflammatory responses. Evaluating the receptor and molecular mechanism that underpin this effect revealed that FAAH substrate-mediated modulation of TLR4-induced NF- κ B-mediated responses occurs independent of brain CB_{1/2} receptors or MAPK activation. However, blockade of the TRPV1 directly within the brain reversed the FAAH substrate-mediated attenuation of TLR4-induced increases in frontal cortical expression of *IL-6*. Although systemic administration of PF3845 was not associated with alterations in TLR4-induced increases in hippocampal expression of *CD11b*, PF3845-LPS-treated animals displayed a lack of increase in M1 activated microglia, as measured by *CD68* expression, 24 hours post LPS. The reported immunoregulatory effects associated with increased FAAH substrates on TLR4-induced neuroinflammation were not accompanied with a PF3845-induced modulation of LPS-induced sickness behaviour over the 24 hour period following LPS administration. Furthermore, although LPS treatment failed to induce depressive-like anhedonic responses at 24 hours post LPS, PF3845-saline-treated animals exhibited an increase in sucrose preference. Overall these findings demonstrate an important role for FAAH substrates directly within the brain, in the modulation of TLR4-induced neuroinflammatory responses within the frontal cortex and hippocampus. Furthermore, these data indicate an important role for activation of TRPV1 within the brain in FAAH substrate-mediated modulation of TLR4-induced responses in the frontal cortex.

The present findings demonstrate that both systemic and i.c.v. administration of the potent FAAH inhibitor PF3845 increases levels of AEA, OEA and PEA in discrete regions of the rat brain. These findings are in accordance with previous reports which have demonstrated that systemic administration of PF3845, at the same dose utilized in the current study, increased levels of AEA in the brain and spinal cord of mice (Booker *et al.*, 2012). However, to our knowledge this is the first report to demonstrate that i.c.v. administration of PF3845 is associated with increases in brain (frontal cortex and hippocampus) levels of AEA, OEA and PEA. Systemic and i.c.v. administration of PF3845 and subsequent increases in FAAH substrates were associated with potent immunoregulatory effects on TLR4-induced increases in several NF- κ B-inducible cytokines including the pro-inflammatory cytokines *TNF α* , *IL-1 β* and *IL-6*, in both the frontal cortex and hippocampus. These data are in accordance with previous work demonstrating evident immunoregulatory effects of FAAH substrates on TLR4-induced neuroinflammatory responses; specifically, systemic administration of the FAAH inhibitor URB597 and subsequent increases in hypothalamic levels of AEA, OEA and PEA was shown to be associated with an attenuation of TLR4-induced increases in hypothalamic expression of *IL-1 β* and *SOCS3* (Kerr *et al.*, 2012). Furthermore, our previous data demonstrated that systemic and i.c.v. administration of URB597 and subsequent increases in FAAH substrates within the brain, were associated with robust immunoregulatory effects on TLR3-induced neuroinflammatory responses within the hippocampus (Chapters 3 & 4). Our current findings now extend on these findings and demonstrate that an alternative selective FAAH inhibitor PF3845 is capable of increasing FAAH substrates within the brain and in turn modulating TLR4-induced neuroinflammatory responses in discrete rat brain regions. These findings are important because as previously mentioned in Chapter 3, the use of URB597 has been somewhat compromised by reports that in addition to FAAH, there are several off-target targets including the serine hydrolases in peripheral tissues (Alexander *et al.*, 2005; Lichtman *et al.*, 2004; Zhang *et al.*, 2007) and also acts as a agonist at the transient receptor potential (TRP) ion channel TRPA1 (Niforatos *et al.*, 2007). As such, the use of PF3845, which is reported to selectively inhibit FAAH activity *in vivo* (Ahn *et al.*, 2007; Ahn *et al.*, 2009) may offer a more appropriate pharmacological tool to examine the effects of FAAH substrates *in vivo*. Furthermore, to our knowledge this is the first report to demonstrate

that increasing FAAH substrates directly within the brain is associated with potent anti-inflammatory effects on TLR4-induced neuroinflammation.

Given that increasing FAAH substrates directly within the brain is associated with anti-inflammatory effects in a manner comparable to that reported following systemic administration of PF3845 strongly suggests that FAAH substrate-mediated immunoregulation of TLR4-induced neuroinflammatory responses occurred directly within the brain, rather than indirect modulation of peripheral immune responses. Further supporting that such actions of FAAH substrates on neuroinflammatory processes occurred within the brain is provided by the data that systemic administration of the putative peripherally restricted FAAH inhibitor URB937 (Clapper *et al.*, 2010) failed to alter TLR4-induced increases in frontal cortical and hippocampal expression of *TNF α* , *IL-1 β* or *IL-6*. However, it should be noted that although AEA levels in either the frontal cortex or hippocampus were not elevated following systemic administration of URB937, increases in the levels of both OEA and PEA in these regions were observed. Thus, although URB937 has previously been shown to be incapable of penetrating the BBB (Clapper *et al.*, 2010), it is possible that under the current experimental conditions some of the drug did cross the BBB and inhibit FAAH activity. This may be due to the species of animals used (mice vs. rats), brain regions examined (hypothalamus vs. frontal cortex and hippocampus), the presence of an inflammatory state or possibly the different time point examined (1 hour vs. 3 hours post URB937) between studies. However, despite the significant increases in frontal cortical and hippocampal levels of OEA and PEA induced following systemic administration of URB937, the levels were significantly lower than those observed following systemic or i.c.v. administration of PF3845 [OEA: PF3845 i.p. 10 fold; PF3845 i.c.v. 7 fold; URB937 i.p. 1.47 fold increase vs. LPS-treated counterparts, PEA: PF3845 i.p. 15 fold; PF3845 i.c.v. 9 fold; URB937 i.p. 2 fold vs. vehicle-LPS-treated counterparts, within the frontal cortex and similar effects in hippocampus]. In addition, these increases were not associated with alterations in TLR4-induced increases in frontal cortical or hippocampal expression of pro-inflammatory cytokines. Taken together the data suggest that either high levels of FAAH substrates 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 potent immunoregulatory effects on TLR-induced neuroinflammatory responses within the frontal cortex and hippocampus.

In accordance with the latter, there are several lines of evidence indicating that AEA exerts potent anti-inflammatory effects *in vitro* (Correa *et al.*, 2009a; Correa *et al.*, 2010; Facchinetti *et al.*, 2003b; Krishnan *et al.*, 2012; Puffenbarger *et al.*, 2000) and *in vivo* (Booker *et al.*, 2012; Holt *et al.*, 2005; Jayamanne *et al.*, 2006).

Recent reports indicate that in addition to attenuating TLR4-induced increases in pro-inflammatory cytokines, AEA also increases glial production of the anti-inflammatory cytokine IL-10 (Correa *et al.*, 2010; Krishnan *et al.*, 2012), which has been proposed to underlie the anti-inflammatory effects of this endocannabinoid (Correa *et al.*, 2010). However, our findings demonstrate that increases in FAAH substrates are associated with an attenuation of TLR4-induced expression of *IL-10* in the frontal cortex and a strong trend for the hippocampus, which therefore suggest that the anti-inflammatory effects observed herein are not mediated by increased production of IL-10. Similarly, previous work from our lab has demonstrated that systemic administration of 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 current findings demonstrated that FAAH substrate-mediated anti-inflammatory effects on TLR4-induced responses within the brain are not likely mediated via increases in the IL-1 β receptor antagonist, IL-1ra or the negative regulator of IL-6, SOCS3. Thus, a likely explanation for the current findings is that PF3845-induced immunoregulation of TLR4-induced neuroinflammation occurs via FAAH substrate-mediated regulation of the transcription factor NF- κ B, exemplified by an attenuation of TLR4-induced increases in frontal cortical expression of *I κ B α* ; an indirect measure of NF- κ B activation (Read *et al.*, 1994). This in turn likely results in the inhibition of all downstream genes of NF- κ B, including *IL-10* and *SOCS3*.

A further important aim of this thesis was to examine the possible receptor mechanisms within the brain that may underlie the reported neuroimmuno-modulatory effects of FAAH substrates. Evidence from *in vitro* studies indicate that AEA-mediated modulation of TLR4-induced neuroinflammation may be mediated via a mechanism dependent (Correa *et al.*, 2010; Correa *et al.*, 2009b; Hernangomez *et al.*, 2012) or independent (Facchinetti *et al.*, 2003b; Puffenbarger *et al.*, 2000; Tham *et al.*, 2007) of CB₁/CB₂ receptor activation. Furthermore, a limited number of studies have examined the potential receptor mechanisms mediating such responses *in vivo*, where it has been

reported that systemic administration of the AEA reuptake inhibitor AM404 is associated with an attenuation of TLR4-induced increases in plasma IL-1 β via CB₁-receptor activation (Roche *et al.*, 2008), while TLR4-induced increases in plasma TNF α levels are reported following FAAH inhibition within the brain, and activation of hypothalamic CB₁ receptors was found to be critical in mediating this response (De Laurentiis *et al.*, 2010). However, to date no studies have examined the potential role of the brain's cannabinoid receptors on TLR-induced neuroinflammatory responses. Accordingly, the current findings demonstrate that antagonism of either CB₁ or CB₂ receptors directly within the brain does not alter the FAAH substrate-mediated attenuation of TLR4-induced increases in NF- κ B-inducible genes, thus suggesting that such immunoregulatory effects are occurring independent of CB₁ or CB₂ receptor activation directly within the brain. Both CB₁/CB₂ receptors and TLR4 are positively coupled to MAPK activation which in turn is known to play a key role in phosphorylation of transcription factors involved in cytokine production (Cao *et al.*, 2002; Liu *et al.*, 2006b). In this regard, it has previously been reported that AEA modulation of TLR4-induced neuroinflammatory responses is dependent on cannabinoid receptor activation of ERK1/2, JNK and p38 pathways (Correa *et al.*, 2010; Krishnan *et al.*, 2012). Therefore the present findings reporting unaltered levels of MAPK (ERK, JNK) activation within the frontal cortex, following systemic administration of PF3845, further suggest that FAAH substrate-mediated modulation of TLR4-induced neuroinflammation is independent of brain CB₁/CB₂ receptor activation. In this respect, endocannabinoids are reported to be capable of exerting anti-inflammatory effects on TLR4-induced neuroinflammation via alternative mechanisms including reductions in AA and consequent production of prostaglandins within the CNS (Nomura *et al.*, 2011) and COX2-derived endocannabinoid metabolites (Correa *et al.*, 2008). Accordingly, the present findings demonstrated that although systemic administration of PF3845 and subsequent increases in FAAH substrates were associated with an attenuation of TLR4-induced increases in frontal cortical expression of *COX2* and *iNOS*, this was not associated with alterations in frontal cortical expression of *m-PGE2-s*, an enzyme involved in the synthesis of PGE₂, thus suggesting that the reported anti-inflammatory effects associated with increased FAAH substrates are likely occurring independent of reductions in PGE₂ production, which is further supported by preliminary data within our group demonstrating unaltered levels of PGE₂ in the frontal cortex, 2 hours

following LPS. Furthermore, recent published work from our group has reported that 2-AG modulation on TLR4-induced neuroinflammation occurred independent of alterations in frontal cortical levels of PGE₂ (Kerr *et al.*, 2013b), at the same time point examined in the present study.

AEA and FAAH substrates, are known to also mediate effects via non-cannabinoid receptors including the PPARs (O'Sullivan *et al.*, 2010; Rockwell *et al.*, 2004), TRPV1 (Di Marzo *et al.*, 2001; Smart *et al.*, 2000; Zygmunt *et al.*, 1999) and GPR55 (Ryberg *et al.*, 2007b). AEA-induced activation of PPAR- γ has been shown to inhibit IL-2 release (Rockwell *et al.*, 2004). In addition, blockade of PPAR- γ is associated with an attenuation of AM404-induced increases in plasma TNF α , induced following systemic TLR4 activation (Roche *et al.*, 2008). However, the present findings demonstrated that blockade of either PPAR- α or PPAR- γ directly within the brain does not alter FAAH substrate-mediated attenuation of *IL-1 β* or *IL-6* within the frontal cortex, induced following systemic administration of LPS. Based on these findings, it is plausible to suggest that the reported immunomodulatory effects on TLR4-induced neuroinflammatory responses within the frontal cortex are occurring independent of either cannabinoid (CB_{1/2}) receptor or PPAR (PPAR- α/γ) targets within the brain and thus suggested an alternative receptor mechanism. However, it cannot be ruled out that effects may occur via activation of several receptors simultaneously, and that antagonism of only one is not sufficient to block the effects. However, blockade of TRPV1, but not GPR55, directly within the brain, attenuated the FAAH substrate-mediated attenuation of TLR4-induced increases in frontal cortical expression of *IL-6*. Thus, TRPV1 appears to mediate the effects of FAAH substrates, on frontal cortical expression of *IL-6* following systemic TLR4 administration of TLR4. Several lines of evidence indicate that TRPV1 activation exerts anti-inflammatory effects under several 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 TLR-induced neuroinflammatory responses. Mechanistically, activation of TRPV1 receptors results in production of neuropeptides including CGRP which modulates many aspects of immune function (Holzmann, 2013a; Holzmann, 2013b) including NF- κ B activation (Ding *et al.*, 2007). Given that we reported that increases in FAAH substrates were associated with an attenuation of TLR4-induced increases in frontal cortical expression of *I κ B α* , it may be likely that the

reported TRPV1-mediated immunoregulatory effects on TLR4-induced neuroinflammation may be mediated, at least in part, via CGRP-mediated regulation of NF- κ B activation. Examination of frontal cortical mRNA expression of *CGRP* revealed no significant effect of PF3845; although effects at earlier time points or protein levels cannot be ruled out. Taken together the data indicate a role for TRPV1 directly within the brain in mediating at least some of the effects of FAAH substrates on TLR4-induced neuroinflammation.

In addition to modulating acute neuroinflammatory responses (2 hours post LPS challenge) this study also examined the effect of systemic administration of PF3845 on long-term neuro-immune responses, 24 hours post LPS administration. Accordingly, acute systemic activation of TLR4 increased hippocampal expression of markers of glial activation including *CD11b* and *CD68*, but not GFAP or *MRC2*, 24 hours post administration. Such increases in these markers are likely mediated via a similar manner to that suggested in Chapter 3; briefly acute neuroinflammatory cytokines induced following TLR4 activation likely acted on corresponding receptors on microglia and astrocytes, inducing their activation and further cytokine production, which in turn led to a feed-forward neuroinflammatory response and increases in hippocampal expression of markers of glial activation, by 24 hours post LPS administration. Although prior administration of PF3845 did not alter the TLR4-induced increases in expression of the marker of microglial activation (*CD11b*), PF3845-LPS-treated animals exhibited a lack of increase in M1 microglia activation, characterised by a lack of increase in hippocampal expression of the M1 marker *CD68*, therefore indicating that FAAH substrates may act to down-regulate microglial activation under TLR4-induced neuroinflammatory conditions, which may in turn confer modulation of neuronal activity and thus exert neuroprotective effects under certain CNS neuropathologies. Accordingly, it has recently been reported that chronic administration of PF3845 was associated with a shift in M1 (decreased iNOS) to a M2 (increased Arg-1) microglia activation phenotype, which in turn was associated with a reversal of TBI-induced impairments in functional outcomes and neurodegenerative processes (Tchantchou *et al.*, 2014). Although we do not report an effect of treatment on hippocampal expression of *MRC2* (marker of M2 microglia phenotype), we cannot rule that alterations are not occurring at differential time points than examined in the present study. In addition, under the current treatment regime there was no significant effect on hippocampal expression of *BDNF*,

24 hours following LPS administration. However it is important to note that we have only examined mRNA expression and we cannot rule out the BDNF protein levels are not reduced/altered. Thus, further studies are required in order to determine the long-term significance of modulating neuroinflammatory responses to TLR4 activation *in vivo*.

In addition to examining long-term neuro-immune responses, behavioural outcomes were investigated in the present study. Accordingly, in the 24 hour period following acute LPS administration, rats displayed “sickness behaviour” characterised by reductions in locomotor activity and decreases in total fluid intake, which were not altered by prior administration of PF3845. In addition, although it has previously been demonstrated that acute administration of LPS is associated with depressive-like behaviour (Frenois *et al.*, 2007; Salazar *et al.*, 2012; Wang *et al.*, 2014), we did not report any depressive-like anhedonic response, exemplified by no effect of LPS treatment on sucrose preference, 24 hours post LPS. Despite the lack of effect of LPS on sucrose preference, findings do show that PF3845-saline-treated animals exhibited greater sucrose preference when compared to their vehicle-LPS-treated counterparts, thus indicating that FAAH substrates may exert effects on depressive-like behaviour, possibly via modulation of neuro-immune mediators. However, more detailed studies are required to evaluate the effects of such a treatment regime on long-term neuronal activity, neuroprotection and behavioural effects such as anxiety and depression which are known to be evident after the acute sickness behaviour resolves (24 hours +) (Gibney *et al.*, 2013; O'Connor *et al.*, 2009).

7.4.4 Conclusion

Overall, the data reported in this chapter have demonstrated an important role for the brain's FAAH substrates, in the modulation of TLR4-induced neuroinflammatory responses. Such potent immunoregulatory effects were shown to be independent of cannabinoid receptors (CB₁, CB₂, and GPR55) or PPARs (PPAR- α/γ) targets within the brain but rather the data demonstrate for the first time a role for TRPV1 within the brain in mediating FAAH substrate-mediated modulation of TLR4-induced neuroinflammation. Overall, these findings support an important role for FAAH substrates directly within the brain in mediating anti-inflammatory effects under TLR4-induced neuroinflammatory conditions.

Chapter 8

General Discussion

Toll-like receptors (TLRs) mediate the innate immune response to pathogens and are critical in the host defence, homeostasis and response to injury. However, uncontrolled and aberrant TLR activation can elicit potent effects on neurotransmission and neurodegenerative cascades, and has been proposed to trigger the onset of certain neurodegenerative disorders and elicit detrimental effects on the progression and outcome of established disease [for reviews see (Arroyo *et al.*, 2011; Lehnardt, 2010; Owens, 2009; van Noort *et al.*, 2009)]. As such, the need to develop a greater understanding of the neurobiological mechanisms mediating and regulating TLR-induced inflammatory responses is critical at a fundamental physiological level and for the development of novel, more efficacious treatments for neuroinflammatory disorders. All elements of the endocannabinoid system are widely and densely expressed in the mammalian immune system and CNS (Herkenham *et al.*, 1990; Onaivi *et al.*, 2006; Stella, 2009), thus endocannabinoid regulation of immune function is emerging as an important therapeutic target for a number of peripheral and central inflammatory disorders (Centonze *et al.*, 2007; Jean-Gilles *et al.*, 2010; Nagarkatti *et al.*, 2009; Stella, 2009). There is now a wealth of evidence from *in vitro*, and to a lesser extent from *in vivo* studies, that endocannabinoids exert potent immunoregulatory effects on TLR4-induced neuroinflammatory processes [see Table 1.1-1.2]. However, the mechanisms underlying these effects are largely unknown, particularly in *in vivo* model systems, and furthermore less is known about the role of the endocannabinoid system in the modulation of inflammatory responses following activation of other TLRs, including TLR3. Given that several neuropathologies including MS (Baker *et al.*, 2001; Loria *et al.*, 2008), PD (Ferrer *et al.*, 2003), ischemic stroke (Berger *et al.*, 2004; Franklin *et al.*, 2003; Schabitz *et al.*, 2002) and brain injury (Hansen *et al.*, 2001; Panikashvili *et al.*, 2001) are reported to be associated with increases in endocannabinoid levels within the CNS, it is also important to evaluate the role of the endocannabinoid system specifically within the brain in modulating neuroinflammatory and consequently neurodegenerative/regenerative responses. As such, the main objective of this thesis was to investigate the involvement of the brain's endocannabinoid system on acute neuroinflammatory responses, induced following systemic TLR3 and TLR4 activation.

8.1 Primary findings

This discussion focuses on appraising the most significant findings of this thesis and how these contribute to increasing our understanding of the contribution of the brain's endocannabinoid system in the modulation of TLR-induced neuroinflammatory responses. Taken together this thesis has contributed 3 major findings to the body of knowledge regarding endocannabinoid modulation of acute neuroinflammation namely that (1) increasing FAAH substrate levels within the brain is associated with immunoregulatory effects on TLR3-induced neuroinflammatory responses, particularly within the hippocampus, effects which were shown to involve activation of several receptor targets directly within the brain including the cannabinoid₁ (CB₁) receptor and PPARs (PPAR- α/γ), (2) enhancement of endogenous 2-AG tone following MAGL inhibition is associated with an augmentation of the expression of IFN- and NF- κ B-inducible inflammatory genes within the hippocampus, following systemic TLR3 activation. While further studies remain to be conducted including the site of action (peripheral vs. central) and the receptor and molecular mechanisms, these data indicate that in conditions where 2-AG levels may be elevated, the neuroinflammatory response to viral infection may be exacerbated (3) finally this thesis demonstrated that enhancement of FAAH substrates directly within the brain is associated with potent anti-inflammatory effects on TLR4-induced neuroinflammation; effects which were shown to be partially mediated via activation of TRPV1 directly within the brain. This, to our knowledge, is the first study to demonstrate a role for brain TRPV1 in modulating neuroinflammatory processes; although it is likely that other, as yet to be determined, receptor or molecular mechanisms are also involved in the effects observed. Overall, these findings demonstrate an important role for the brain's endocannabinoid system in the modulation of neuroinflammatory responses, induced following systemic TLR3 and TLR4 activation. The remainder of this discussion will provide an overview of the primary findings and how they have added to the body of knowledge. A schematic depicting the major finding of this thesis is presented in Figures 8.1 and 8.2.

8.1.1 Pharmacological enhancement of FAAH substrates within the brain is associated with potent anti-inflammatory effects on TLR3-induced neuroinflammation; effects which are partially mediated via activation of brain cannabinoid₁ (CB₁) receptor and PPAR (PPAR- α / γ) targets

Systemic administration of the TLR3 agonist poly I:C is known to induce increases in both type I IFN- and NF- κ B-inducible inflammatory genes in discrete brain regions including the hippocampus and frontal cortex (Cunningham *et al.*, 2007; Gibney *et al.*, 2013) which in turn are reported to be associated with sickness (Cunningham *et al.*, 2007; Fortier *et al.*, 2004; Gibney *et al.*, 2013; McLinden *et al.*, 2012; Murray *et al.*, 2015b), anxiety and depressive-like behaviours (Gibney *et al.*, 2013) and exacerbation of chronic neurodegenerative processes (Field *et al.*, 2010). Data presented in this thesis are in accordance with previous findings demonstrating that systemic administration of poly I:C is associated with increases in type I IFN- and NF- κ B-inducible inflammatory mediators; both in the periphery and within discrete rat brain regions, namely within the hippocampus and frontal cortex. Additionally, data presented in Chapter 3 are the first to demonstrate the effect of endocannabinoid modulators on both acute neuroinflammatory (4 hours post systemic TLR3 activation) and long-term neuro-immune (24 hours post systemic TLR3 activation) responses in an *in vivo* model system. Prior to this body of work, just one study has investigated cannabinoid modulation of TLR3-induced neuroinflammation. In this study, Downer and colleagues demonstrated that the synthetic cannabinoid WIN55,212-2 enhanced TLR3-induced increases in IFN- β production, while concurrently decreasing production of the pro-inflammatory cytokine TNF α , in primary astrocytes (Downer *et al.*, 2011). Data from Chapter 3 and 4 now build on and extend these findings to an *in vivo* model system and has provided evidence for the first time that pharmacological enhancement of FAAH substrates within discrete regions of the rat brain is associated with increases in hippocampal expression of the type I IFN, IFN- α and also the pleiotropic cytokine IL-6, while concurrently decreasing hippocampal expression of pro-inflammatory cytokines including TNF α and IL-1 β , following systemic TLR3 activation. There are several lines of evidence that type I IFNs elicit anti-inflammatory effects within the brain due to their ability to limit leukocyte infiltration (Prinz *et al.*, 2008) and reduce expression of pro-inflammatory cytokines (Teige *et al.*, 2006). Thus, increasing type I IFN expression in combination with a reduction in pro-inflammatory cytokines may limit the neuroinflammatory and possibly

neurodegenerative cascades in the hippocampus following TLR3 activation. In addition, Chapters 4 & 5 of this thesis investigated the potential role of modulation of the brain's endocannabinoid system on TLR3-induced neuroinflammatory responses within the rat hippocampus; a key brain structure within the cortico-limbic system affected in several neurological disorders (Hu *et al.*, 2014; Nikonenko *et al.*, 2009; Shankar, 2010; Sierra *et al.*, 2015). While global systemic inhibition of FAAH was associated with evident immunoregulatory effects on TLR3-induced neuroinflammation within the hippocampus, a more robust anti-inflammatory profile was evident when FAAH substrate levels were enhanced directly within the brain, exemplified by increases in expression of anti-inflammatory cytokines (*IL-10*) and concurrent decreases in pro-inflammatory cytokines. Furthermore, the peripherally restricted FAAH inhibitor URB937 (Clapper *et al.*, 2010) failed to alter neuroinflammatory responses following systemic TLR3 activation. Thus, taken together these findings demonstrated for the first time that FAAH substrates directly within the brain are associated with attenuation of TLR3-induced neuroinflammatory process. However, the precise receptor mechanism by which this was mediated remained unknown. Accordingly, Chapter 5 of this thesis investigated the potential role of both cannabinoid (CB₁/CB₂) and non-cannabinoid (PPAR- α/γ) receptor targets directly within the brain on FAAH substrate-mediated modulation of neuroinflammation. Our findings have provided evidence that both brain cannabinoid₁ (CB₁) receptor and PPAR- α/γ are involved in the regulation of TLR3-induced neuroinflammatory responses *in vivo*. Given that all three FAAH substrates are increased within the hippocampus following FAAH inhibition (Chapter 5), we cannot solely attribute the reported immunoregulatory effects on TLR3-induced responses to any one of these in particular, with further studies required in order to fully decipher the precise role of each of the FAAH substrates in such responses. In addition, while these findings indicate a role for activation of both brain CB₁ receptor and PPAR- α/γ targets, the receptor cellular location (neurons vs. glial cells) where responses are likely mediated is not known and remains to be determined. In addition, it is not possible at this stage to specifically attribute reported immunoregulatory effects to one specific receptor target, as it is possible that multiple receptors are acting in co-operation to mediate effects. In addition, it may also be possible that blocking actions at specific receptor targets (CB and PPAR) may unmask actions of AEA and related *N*-acylethanolamines actions at other receptor targets, including TRPV1, GPR55 and

GPR19, which may in turn be involved in mediating reported effects on TLR3-induced neuroinflammation.

Overall, the data presented in Chapters 3-5 of this thesis have demonstrated a key role for the brain's endocannabinoid system in the modulation of TLR3-induced neuroinflammatory responses *in vivo*, highlighting that increasing FAAH substrates directly within the brain is associated with potent immunoregulatory effects on TLR3-induced neuroinflammation within the hippocampus, via a mechanism likely involving both brain CB₁ receptor and PPAR- α/γ (see Figure 8.1 for schematic overview). As previously mentioned, prior to this body of work just one study has examined and reported immunoregulatory effects of cannabinoid-based drugs on TLR3-induced neuroinflammation (Downer *et al.*, 2011). The present data extend on these findings and demonstrate for the first time that modulation of the endocannabinoid system (specifically within the brain) is associated with potent immunoregulatory effects on TLR3-induced neuroinflammation in an *in vivo* model system. Such findings are of particular interest given reports that systemic activation of TLR3 is associated with alterations in long-term neuro-immune responses (Gibney *et al.*, 2013) and exacerbation of existing neurodegenerative processes within the hippocampus (Field *et al.*, 2010). Thus, FAAH substrate-mediated modulation of the TLR3-induced signalling pathway may offer novel therapeutic potential in neurodegenerative processes. In this regard, there are now reports of increased FAAH substrates in several CNS disorders including ischemic stroke (Degn *et al.*, 2007; Franklin *et al.*, 2003), PD (Pisani *et al.*, 2010) and MS (Loria *et al.*, 2008), whereby modulation, via inhibition of their degradation is associated with neuroprotective effects in *preclinical* models of disease processes (Degn *et al.*, 2007; Karanian *et al.*, 2005; Marsicano *et al.*, 2003). Such increases in FAAH substrates during neuropathological conditions are proposed to act as an endogenous compensatory mechanism to limit neuroinflammatory processes associated with CNS diseases and thus protect neuronal function. Thus, it is possible that modulation of the brain's FAAH substrates may also be neuroprotective via their ability to regulate TLR3-induced acute neuroinflammatory responses and protect against possible detrimental effects of systemic viral infection on CNS function. Furthermore, potent cannabinoid receptor agonists are reported to be associated with unwanted side effects including global immunosuppression, as such the use of such treatments in both

peripheral and CNS inflammatory conditions may result in increased susceptibility to systemic infection. Thus, increasing FAAH substrates directly within the brain and therefore avoiding such side effects may offer an alternative therapeutic approach in CNS inflammatory disorders.

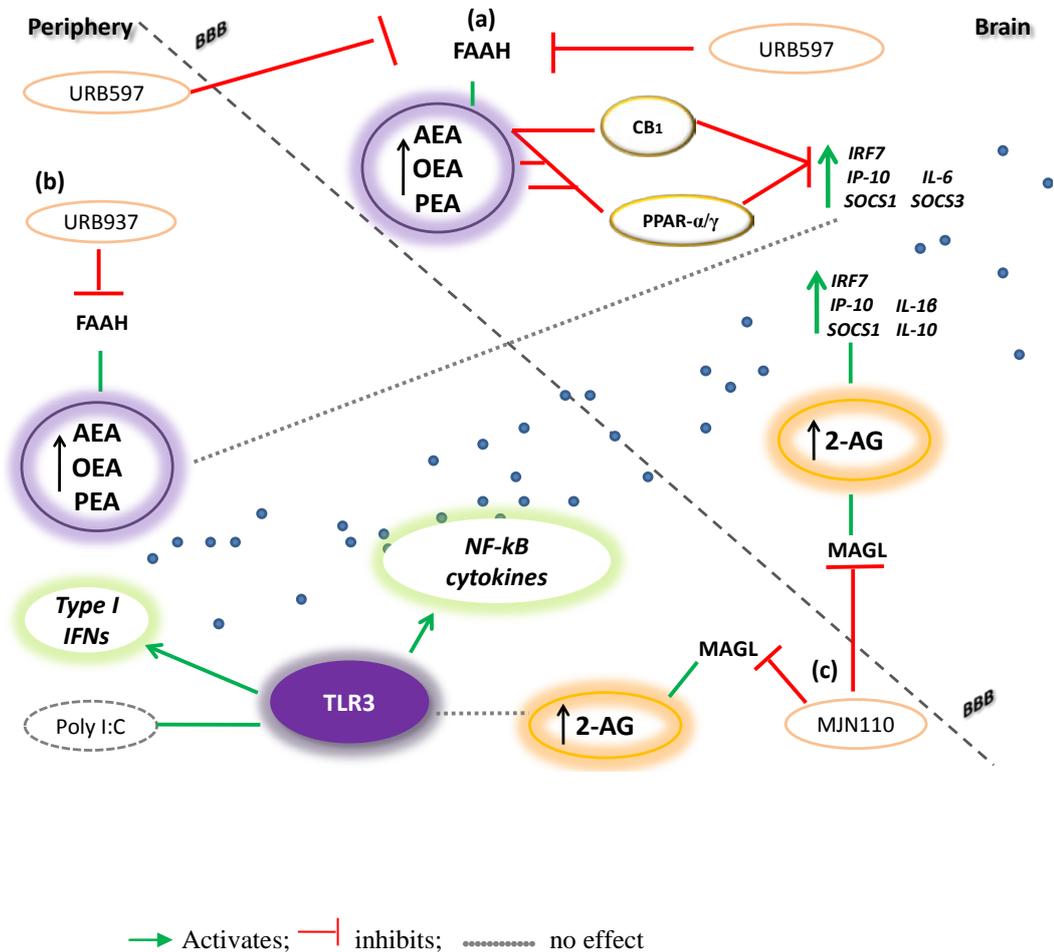


Figure 8.1 Schematic depicting primary findings in Chapters 3-6. TLR3 activation following peripheral administration of poly I:C results in activation of type I IFN- and NF-κB-inducible inflammatory genes. These communicate via various mechanisms with the CNS, ultimately resulting in enhanced expression of type I IFN- and NF-κB-inducible inflammatory gene expression within the brain. Both (a) systemic and central (i.c.v.) administration of the FAAH inhibitor URB597 results in increases in FAAH substrates within the brain (hippocampus), which in turn is associated with potent immunoregulatory effects on TLR3-induced neuroinflammation; effects of which are partially mediated via activation of cannabinoid (CB₁) receptor and PPAR (PPAR-α/γ) targets within the brain. Furthermore, (b) systemic administration of the peripherally restricted FAAH inhibitor URB937 does not alter cytokine expression within the hippocampus, following TLR3 activation. Additionally, (c) systemic administration of the MAGL inhibitor MJN110, increases 2-AG levels both peripherally and within the brain, which in turn is associated with modulation of TLR3-induced increases in inflammatory genes in the hippocampus, but not peripherally (spleen).

8.1.2 Pharmacological enhancement of endogenous 2-AG levels is associated with increases in hippocampal expression of TLR3-induced type I IFN- and NF- κ B-inducible inflammatory genes

Although 2-AG is the most abundant endocannabinoid within the mammalian brain, and also the preferred natural ligand for CB₁ receptors, our understanding of the role of this endocannabinoid in regulating physiological function has been somewhat limited due to the lack of pharmacological and genetic tools available. As such, over the last number of years there has been extensive efforts to develop novel tools to enable researchers to examine the role of endogenous 2-AG in both physiological and pathological processes. The data presented in Chapter 6 of this thesis demonstrate for the first time that pharmacological enhancement of 2-AG tone, following systemic administration of the novel MAGL inhibitor MJN110, is associated with enhanced TLR3-induced expression of both type I IFN- and NF- κ B-inducible genes in the hippocampus, therefore suggesting that 2-AG may exacerbate TLR3-induced neuroinflammatory responses and consequently impair neuronal functioning. There are now several reports from *preclinical* models of CNS disorders such as AD, PD, MS and TBI that 2-AG levels are increased within the brain (Baker *et al.*, 2001; Loria *et al.*, 2008; Panikashvili *et al.*, 2005; van der Stelt *et al.*, 2005), where it is suggested to mediate neuroprotective effects. However, our data indicate that 2-AG enhances TLR3-induced neuroinflammatory responses, thus suggesting that under certain conditions, including that of acute viral infections, increases in 2-AG levels may be associated with an exacerbation of the neuroinflammatory response, which if left uncontrolled, may lead to detrimental effects on neuronal function.

In addition, although the lack of effects of MAGL inhibition on the peripheral immune response to TLR3 activation suggest that the effects of increased 2-AG on TLR3-induced neuroinflammation reported in the hippocampus are likely mediated at the level of the brain, rather than indirect modulation of peripheral immune responses, further studies are required to definitely determine this. In an attempt to examine this, this thesis demonstrated that direct administration of the MAGL inhibitor JZL184 failed to enhance 2-AG levels in the brain (or modulate TLR4-mediated neuroinflammatory responses) while MJN110 elicited a slight (1.62 fold) increase in 2-AG levels. As such we feel that

to truly investigate the role of the brain 2-AG system vs. indirect modulation via peripheral mechanism will require the development of new more hydrophilic MAGL compounds.

Overall, data presented in Chapter 6 of this thesis have enhanced the body of knowledge regarding endocannabinoid (2-AG)-induced modulation of acute neuroinflammatory responses *in vivo*. To date, studies carried out in this area of research have focused on 2-AG modulation of TLR4-induced neuroinflammatory responses (Kerr *et al.*, 2013b; Nomura *et al.*, 2011), with no studies examining the effect of enhanced 2-AG tone on viral (TLR3) responses. Thus, these findings have shown for the first time that increases in 2-AG levels within the brain is associated with potent immunoregulatory effects on TLR3-induced neuroinflammatory responses, without altering systemic immune responses (See Figure 8.1). The reported differential effects of 2-AG (pro-inflammatory) vs. FAAH substrates (anti-inflammatory) on TLR3-induced neuroinflammatory responses indicates that the tone of the endocannabinoid system (AEA vs. 2-AG) is important in determining what effects on neuroinflammatory responses will be seen in the brain, effects of which could have important consequences on neuronal function and outcome of disease processes within the brain.

8.1.3 Pharmacological enhancement of FAAH substrates within the brain exerts potent anti-inflammatory effects on TLR4-induced neuroinflammation; effects of which are partially mediated via activation of TRPV1 within the brain

Chapters 3-6 of this thesis primarily focused on the examination of endocannabinoid (AEA and 2-AG) modulation of TLR3-induced neuroinflammation, highlighting a key role for the brain's endocannabinoid system in mediating such immunoregulatory effects in an *in vivo* model system. Previous work from our laboratory has demonstrated that systemic administration of the FAAH inhibitor URB597 and subsequent increases in hypothalamic levels of AEA, OEA and PEA are associated with an attenuation of TLR4-induced increases in hypothalamic expression of *IL-1 β* and *SOCS3* (Kerr *et al.*, 2012). However, it is unknown if such immunoregulatory effects of increased FAAH substrates on TLR4-induced neuroinflammation that occurs in other brain regions, are mediated via indirect modulation of peripheral immune responses or occur directly within the brain, and the receptor/molecular mechanisms underlying the effects. Accordingly, Chapter 7 of this thesis provides novel evidence that FAAH substrate-mediated modulation of

TLR4-induced neuroinflammation (in the frontal cortex) likely occurs at the level of the CNS, rather than via indirect modulation of peripheral immune responses. This was shown in a number of ways including: (1) direct increase in FAAH substrate levels within the brain following i.c.v. administration of the potent FAAH inhibitor PF3845 was associated with an attenuation of TLR4-induced increases in frontal cortical expression of NF- κ B-inducible genes and (2) increasing FAAH substrate levels only in the periphery, and slightly within the brain, following systemic administration of peripherally restricted FAAH inhibitor URB937 failed to alter frontal cortical or hippocampal expression of TLR4-induced NF- κ B- genes. Furthermore, these data demonstrate for the first time that the brain's FAAH substrates modulate TLR4-associated inflammatory effects independent of either cannabinoid (CB₁, CB₂, GRP55) receptors or PPARs within the brain, but rather are mediated, at least in part, by TRPV1 within the brain. To date, there has been a paucity of data relating to the potential role of TRPV1 in the brain on neuroinflammatory responses and to our knowledge this is the first report to demonstrate a role for TRPV1 within the brain, in the modulation of TLR4-induced increases in frontal cortical expression of *IL-6*. A possible explanation for the lack of effects of other receptor antagonists (CB and PPAR) on TLR4-induced neuroinflammatory responses, may be due to that as previously described, in that multiple receptor targets are acting co-operatively to mediate effects of FAAH substrates, thus blockade of any one single receptor may not be sufficient, but rather blockade of multiple receptors at once may be required for the effects to be fully observed.

Overall, these findings add to the body of knowledge relating to endocannabinoid modulation of TLR4-induced neuroinflammation, demonstrating for the first time that FAAH substrate-mediated modulation of TLR4-induced neuroinflammation likely occurs at the level of the brain and is mediated, at least in part, via TRPV1 within the brain (See Figure 8.2 for schematic overview).

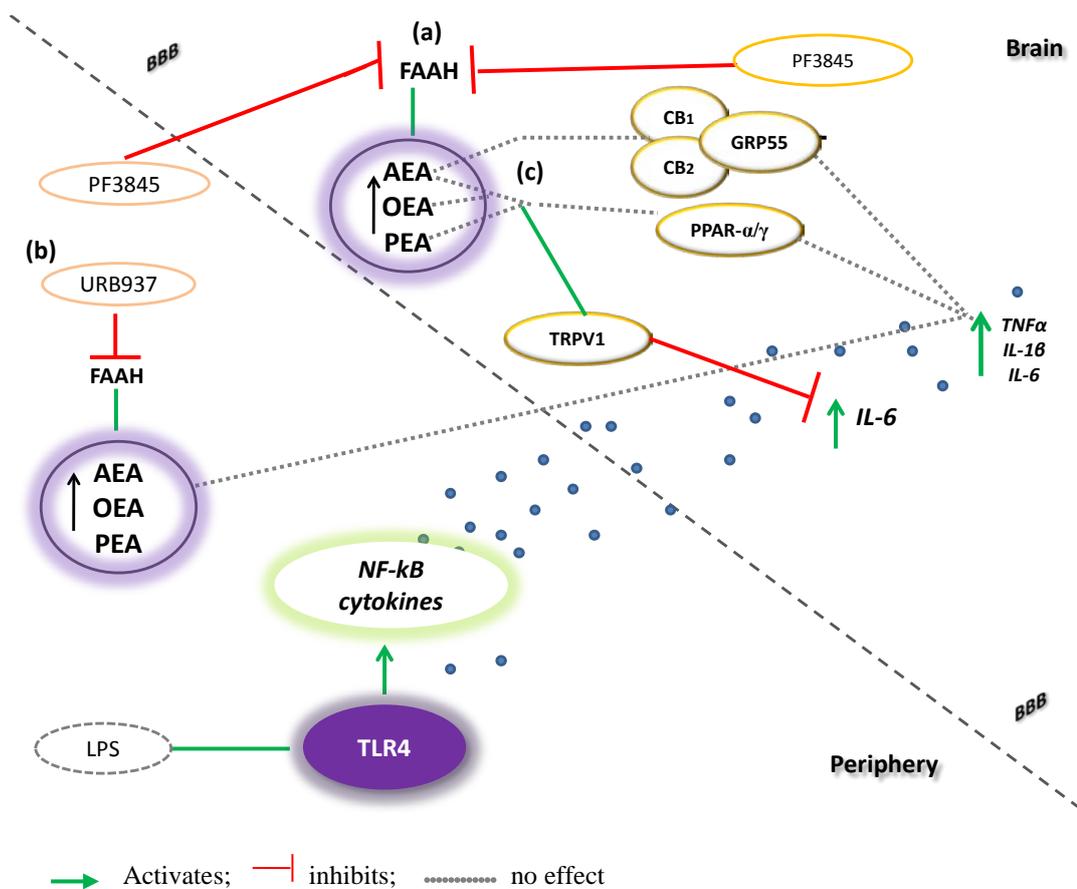


Figure 8.2 Schematic depicting primary findings in Chapter 7. TLR4 activation following peripheral administration of LPS results in activation of NF-κB-inducible inflammatory cytokines. These communicate via various mechanisms with the CNS, ultimately resulting in enhanced expression of pro-inflammatory cytokines in the brain. Both (a) systemic and i.c.v. administration of the FAAH inhibitor PF3845 increases FAAH substrates (AEA, OEA and PEA) within the brain. Increased levels of FAAH substrates within the frontal cortex and hippocampus following either systemic or i.c.v. PF3845 administration, is associated with an attenuation of TLR4-induced increases in several NF-κB-inducible genes. Furthermore, (b) systemic administration of the proposed peripherally restricted FAAH inhibitor systemic administration of URB937 does not alter the TLR4-induced increases in NF-κB in either brain region examined. Evaluating the receptor mechanisms revealed that FAAH substrate-mediated regulation of TLR4-induced neuroinflammation appears to be mediated (c) independent of both cannabinoid (CB₁, CB₂, GPR55) and PPAR- (α,γ) receptor activation within the brain, rather effects are shown to be partially mediated via activation of TRPV1 within the brain, at least in the case of FAAH substrate-mediated inhibition of *IL-6* within the frontal cortex.

In addition to examining acute neuroinflammatory responses (2 hours post LPS), Chapter 7 also examined the effect of increased FAAH substrates on long-term neuro-immune and behavioural responses (24 hours post LPS). Thus, by attenuating acute neuroinflammatory response to TLR4 activation, PF3845 also prevented the increase in hippocampal expression of the M1 microglial marker *CD68*, therefore down-regulating long-term microglial activation under TLR4-induced neuroinflammatory conditions. These effects are not associated with changes in acute sickness behaviour, however more detailed studies are required to evaluate the effects on long-term neuronal activity, neuroprotection and behavioural effects such as anxiety and depression which are known to be evident after the acute sickness behaviour resolves (24 hours +) (Gibney *et al.*, 2013; O'Connor *et al.*, 2009). In any case, the current data have significantly built on our understanding of how the brain's endocannabinoid system modulates TLR4 associated neuroinflammation and provide a scaffold on which to build for future studies in relation to neuroinflammatory components associated with psychiatric and neurodegenerative disorders

In conclusion, the work presented in this thesis has provided further evidence for a role of FAAH and MAGL in the regulation of TLR-induced neuroinflammation. Importantly, the data in this thesis provide novel evidence that FAAH substrate-mediated modulation of both TLR3 and TLR4-induced neuroinflammation likely occurs at the level of the CNS, rather than indirect modulation of peripheral immune responses and highlight that effects are mediated by different receptor mechanisms depending on the inflammatory state under investigation. Furthermore, the data indicate that FAAH and MAGL substrates elicit differential effects on neuroinflammation due to TLR3/viral infection which could have important consequences on neuronal function. Given the increasing interest in exploring the development of FAAH and MAGL inhibitors for the treatment of various conditions, the data presented may indicate that patients taking such pharmacotherapies may have altered neuroinflammatory responses to bacterial and viral infection, the consequences of which remain to be determined. Overall the data has extended the body of knowledge regarding endocannabinoid modulation of neuroinflammatory responses to acute bacterial and viral infection, and may inform the development of novel therapeutics for acute and chronic neuroinflammatory disorders.

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Appendices

Appendix 1 Bradford Protein Assay

Standard protein solutions (0-1500 μ g/ml) were prepared from a stock (2mg/ml) solution of bovine serum albumin (BSA: cat no: A8022: Sigma-Aldrich, Ireland).

Conc μg/ml	0	100	250	500	750	1000	1500
μ l stock BSA	-	10	25	50	75	100	150
μ l H ₂ O or PBS	200	190	175	150	125	100	50
Tot Vol μ l	200	200	200	200	200	200	200

5 μ l of samples or standards were pipetted into designated wells on a 96-well plate followed by 250 μ l of Bradford reagent (B6916: Sigma-Aldrich, Ireland). The plate was left at RT for 10 mins to facilitate optimum colour development and absorbance then read at 595nm (Biotek instruments; Mason technology Dublin)

Protein content of samples was extrapolated from standard linear regression plots of protein concentration μ g/ml versus optical density (OD) at 595nm (GraphPad Prism).

Appendix 2 Preparation of SDS-PAGE gels

To prepare the 12 % resolving gel, specific quantities of each component (see table below) were added to a 50mL conical tube. Approximately 5.5ml of 12 % stacking gel was then added to the assembled plates and 0.1 % SDS solution was immediately added to prevent bubble formation. The gel was allowed to polymerise at room temperature for approximately 30 min following which the stacking gel (see table below) was prepared and added. Once set, gels were wrapped in damp tissue paper and cling film and stored overnight at 4°C.

Preparation of 12% resolving gel

12% SDS-PAGE RESOLVING GEL	1x (total volume 10ml)	4x (total volume 40ml)
H ₂ O	3.29ml	13.16
30% Acrylamide mix	4ml	16ml
1.5M Tris pH 8.8	2.5ml	10ml
10% SDS	100µl	400µl
10% APS (fresh)	100µl	400µl
TEMED (add last in fumehood)	10µl	40µl

Preparation of stacking gel

Stacking GEL	1x (total volume 4.04ml)	4x (total volume 16.016ml)
H ₂ O	2.74ml	10.96ml
30% Acrylamide mix	0.68ml	2.72ml
0.5M Tris pH 6.8	0.5ml	2.0ml
10% SDS	40μl	160μl
10% APS (fresh)	40μl	160μl
TEMED (add last in fume hood)	4μl	16μl

Appendix 3 Electrophoresis and immunoblotting

- Colourburst Electrophoresis maker (Sigma; C1992) was thawed, vortexed and placed on ice along with samples.
- Gels were removed from 4°C and the comb was gently removed from the stacking gel so as to ensure all lanes remained intact.
- Assembled plates were placed in the running tank with the loading plate facing inwards. The inner chamber of tank was immediately filled with 1X running buffer and allowed to overflow in order to avoid air bubbles (Chapter 2; Table 2.4).
- 5µL of the Colourburst Electrophoresis maker and 28µl of samples were pipetted into allocated lanes in accordance with assay plan.
- After all samples were loaded, the loading tank was filled with 1X running buffer (approximately 500ml per tank).
- The tanks were then connected to power supply (Biorad; Ireland) and run at 100V constant voltage for 2 hours.
- On completion of electrophoresis (bromophenol blue dye at bottom of gel), the gels were removed and the stacking gel was discarded. The resolving gel was gently lifted off the plate and placed in transfer buffer (Chapter 2; Table 2.4) until transfer.

Semi-Dry Transfer

The transfer was carried out using a CLP semi dry cassette type transfer apparatus (Medical Supply Co. Dublin), using either nitrocellulose membrane (0.2 µm; VWR International, UK 8cm x 4.6 cm) or PVDF membrane (0.45µm; Millipore, Cat# IPVH00010, Sigma Aldrich, Ireland) 1X transfer buffer (Chapter 2; Table 2.3) PVDF membrane, was pre-wetted in 100% methanol for approximately 10 min, before use. Filter papers were soaked in 1X transfer buffer.

A gel-membrane sandwich was prepared in the transfer apparatus as follows;

Cassette top
Filter paper
Gel
Nitrocellulose paper
Filter paper
Cassette bottom

- The entire cassette was soaked with 1X transfer buffer and connected to a power supply (Biorad; Ireland) which was operated at 10V constant voltage for 2 hours.
- Once transfer was complete, the membrane was placed back into transfer buffer.
- In order to visualise protein bands and to ensure that correct transfer occurred the membrane was stained with 0.1% Ponceau S (Sigma; P7170, Ireland) in 5% acetic acid for approximately 1 min and following examination was destained with dH₂O.

Peer-reviewed original research article

**FAAH-MEDIATED MODULATION OF TLR3-INDUCED
NEUROINFLAMMATION IN THE RAT HIPPOCAMPUS**

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Abbreviations: 2-AG 2-arachidonoyl glycerol, CB cannabinoid, FAAH fatty acid amide hydrolyase, i.c.v. intracerebroventricular, IFN interferon, IL interleukin, IP-10 Interferon gamma-induced protein 10 (also known as C-X-C motif chemokine 10 (CXCL10)), IRF interferon regulatory factor, NFκB nuclear factor kappa B, OEA oleoylethanolamide, PEA palmitoylethanolamide, Poly I:C polyinosinic:polycytidylic acid, PKR dsRNA-dependent protein kinase, TLR toll-like receptor, TNF tumour necrosis factor, TMEV Theiler's virus, SOCS suppressor of cytokine signalling

Abstract

The present study examined the effect of enhancing fatty acid amide hydrolase (FAAH) substrate levels *in vivo* on Toll-like receptor (TLR)3-induced neuroinflammation. Systemic and central (i.c.v.) administration of the FAAH inhibitor URB597 increased hippocampal levels of the *N*-acylethanolamines palmitoylethanolamide and oleoylethanolamide, but not anandamide. Systemic URB597 increased IFN α , IFN γ and IL-6 expression following TLR3 activation and attenuated TLR3-induced IL-1 β and TNF α expression. In comparison, central URB597 administration attenuated the TLR3-induced increase in TNF α and IFN γ expression (and associated downstream genes IP-10 and SOCS1), while concurrently increasing IL-10 expression. These data support an important role for FAAH-mediated regulation of TLR3-induced neuroinflammatory responses.

Key words: fatty acid amide hydrolase; cannabinoid; anandamide, cytokine; interferon, virus, brain

1. Introduction

Neuroinflammation is a key component underlying several neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis and psychiatric disorders including depression. Viral and bacterial infections induce systemic and central inflammation, an effect proposed to trigger the onset of some neurodegenerative disorders including multiple sclerosis (Deleidi *et al.*, 2012) and elicit detrimental effects on the progression and outcome of established disease (Holmes *et al.*, 2009; Perry, 2004; Teeling *et al.*, 2009). Toll-like receptors (TLRs) which recognize and induce an immune response to bacterial (TLR4) and viral (TLR3) infection, are expressed on neurons, astrocytes and microglia (Bsibsi *et al.*, 2002). While activation of TLR participates in host defences, homeostasis and response to injury, uncontrolled and aberrant TLR activation can elicit potent neurodegenerative cascades [for reviews see (Arroyo *et al.*, 2011; Lehnardt, 2010; Owens, 2009; van Noort *et al.*, 2009)] and TLR expression has been reported to be increased in the post-mortem brain of patients exhibiting neurodegenerative disorders (Brudek *et al.*, 2013; Salaria *et al.*, 2007). Thus, understanding the neurobiological mechanisms mediating TLR-induced

neuroinflammation is critical at a fundamental physiological level and for the development of novel, more efficacious treatments.

The endogenous cannabinoid (endocannabinoid) system has been shown to exhibit a potent modulatory role on neuroinflammatory responses, including those induced by TLR activation (Downer, 2011; Jean-Gilles *et al.*, 2010; Nagarkatti *et al.*, 2009). The endocannabinoid system comprises the cannabinoid₁ (CB₁) and cannabinoid₂ (CB₂) receptors, the naturally occurring endogenous ligands, the best characterised being anandamide and 2-arachidonoyl glycerol (2-AG), and enzymes involved in their synthesis and degradation. Anandamide, and related fatty acid amides, *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA), are primarily catabolised by the enzyme fatty acid amide hydrolase (FAAH). Anti-inflammatory and neuroprotective effects of the FAAH substrates anandamide and PEA, have been documented (Bisogno *et al.*, 2010; Correa *et al.*, 2009b; Esposito *et al.*, 2013; Murphy *et al.*, 2012; Scuderi *et al.*, 2013). *In vitro* studies have shown that increasing anandamide tone directly, or inhibiting FAAH activity and thereby elevating substrate levels, in microglial or astrocyte cultures, attenuates TLR4-induced increases in pro-inflammatory cytokines and inflammatory mediators such as TNF α , IL-1 β and nitric oxide, and enhances the release of the anti-inflammatory cytokine IL-10 (Correa *et al.*, 2009a; Correa *et al.*, 2010; Facchinetti *et al.*, 2003b; Molina-Holgado *et al.*, 1997; Puffenberger *et al.*, 2000; Tham *et al.*, 2007). *In vivo* studies have demonstrated that anandamide activation of hypothalamic CB₁ receptors facilitates (De Laurentiis *et al.*, 2010), while antagonism of the central CB₁ receptors attenuates (Steiner *et al.*, 2011), TLR4-induced increases in plasma TNF α levels. In addition, recent data from our group have shown that enhancing endogenous FAAH substrate levels attenuates the TLR4-induced increase in IL-1 β expression while concurrently augmenting the expression of suppressor of cytokine signalling (SOCS)-3 in the hypothalamus (Kerr *et al.*, 2012). However, there is a paucity of data regarding the effect of FAAH substrates on neuroinflammatory responses elicited by other TLRs. The potent cannabinoid receptor agonist WIN55,212-2 has been shown to attenuate TLR3-induced increases in TNF α while concurrently augmenting interferon regulatory factor (IRF)3 and the expression of the type 1 interferon IFN β , an effect necessary for the protective effects of WIN55212-2 in a rat model of multiple sclerosis (Downer *et al.*, 2012; Downer *et al.*, 2011). Theiler's virus (TMEV) induction of inflammatory

processes is thought to be primarily mediated via TLR3 activation (So *et al.*, 2006). *In vitro* data have demonstrated that anandamide downregulates pro-inflammatory cytokines IL-12, IL-17, TNF α and enhances IL-10 and IL-6 levels in TMEV-infected astrocytes (Correa *et al.*, 2011; Hernangomez *et al.*, 2012; Molina-Holgado *et al.*, 1997; Molina-Holgado *et al.*, 1998). Furthermore, PEA or FAAH inhibition attenuates microglial activation, the expression of pro-inflammatory cytokines and ameliorates motor symptoms in the TMEV-model of multiple sclerosis (Correa *et al.*, 2011; Hernangomez *et al.*, 2012; Loria *et al.*, 2010; Loria *et al.*, 2008; Mestre *et al.*, 2005; Ortega-Gutierrez *et al.*, 2005). Thus, modulation of TLR3-induced neuroinflammatory responses may have significant therapeutic benefit in neurodegenerative disorders.

The aim of this study was to investigate the effect of URB597, a potent FAAH inhibitor, administered systemically and centrally on the expression of neuroinflammatory mediators in the hippocampus, following TLR3 activation with the viral mimetic polyinosinic:polycytidylic acid (Poly I:C). Systemic poly I:C administration to rodents has been shown to enhance the expression of type 1 interferons and pro-inflammatory cytokines in several brain regions including the hippocampus (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013). Poly I:C-induced increases in IFN β signalling in the hippocampus have been shown to be associated with enhanced neuronal excitability (Costello *et al.*, 2013), impaired contextual and working memory (Galic *et al.*, 2009) and seizure susceptibility (Galic *et al.*, 2009). In addition, TLR3 deficient mice exhibit enhanced hippocampal-dependent working memory, increased hippocampal volume and neurogenesis (Okun *et al.*, 2010). Although we and others have demonstrated that FAAH substrate levels are enhanced in the brain following systemic administration of URB597 (Fegley *et al.*, 2005; Kerr *et al.*, 2012; Piomelli *et al.*, 2006), the contribution of central FAAH substrates to the neuro-immuno-modulatory effects of URB597 is unknown. Evaluating the role of central FAAH substrates on neuroinflammatory processes is of particular importance because several neuropathologies including ischemia, Parkinson's disease and multiple sclerosis are associated with increases in FAAH substrate levels within the brain (Baker *et al.*, 2001; Degn *et al.*, 2007; Franklin *et al.*, 2003; Loria *et al.*, 2008). Thus, a further aim of this study was to decipher the effects of FAAH inhibition specifically within the brain on modulation of TLR3-induced

neuroinflammatory responses, and to determine if this differed to the effects of global systemic inhibition of FAAH.

2. Materials and Methods

2.1 Animals

Experiments were carried out on male Sprague Dawley rats (weight 220-260g; Charles River, UK), housed singly in plastic bottomed cages (45 X 25 X 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 0800 to 2000 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 an intraperitoneal (i.p.) injection of sterile saline (0.89% NaCl) for 3-4 days prior to experimentation in order 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 licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609.

2.2 Experimental design

2.2.1 Experiment 1: Effect of systemic administration of URB597 on poly I:C-induced increases in inflammatory gene expression in the hippocampus

Rats were randomly assigned to one of three groups. Vehicle-Saline, Vehicle-poly I:C and URB597-poly I:C (n=6-8 per group). The FAAH inhibitor URB597 (1mg/kg, Cayman Chemicals, Estonia) or vehicle (ethanol:cremaphor:saline; 1:1:18) were administered i.p. in an injection volume of 2ml/kg followed 30 min later by an i.p. injection of poly inosinic:poly cytidylic acid (poly I:C: 3mg/kg, Fisher Scientific, Ireland) or saline vehicle (sterile 0.89% NaCl) administered in an injection volume of 1.5ml/kg. The dose of URB597 was determined based on previous published work demonstrating that systemic administration of URB597 at this dose enhanced levels of AEA and the related *N*-acylethanolamines in the brain (Fegley *et al.*, 2005; Kathuria *et*

al., 2003; Kerr *et al.*, 2012). Furthermore, we have previously demonstrated that systemic administration of this dose of URB597 does not alter peripheral or central cytokine expression in the absence of an immune stimulus (Kerr *et al.*, 2012), thus the effect of URB597 was only evaluated in the presence of poly I:C in the current studies. The dose and time of poly I:C administration were chosen on the basis of previous published (Gibney *et al.*, 2013; Katafuchi *et al.*, 2005; Katafuchi *et al.*, 2003) and pilot studies demonstrating enhanced cytokine expression in the brain. Animals were sacrificed by decapitation four hours post poly I:C/saline administration, the brain rapidly removed, hippocampus excised, snap frozen on dry ice and stored at -80°C until assayed for endocannabinoids and *N*-acylethanolamines levels and cytokine expression.

2.2.2 Experiment 2: Effect of central administration of URB597 on poly I:C-induced increases in inflammatory gene expression in the hippocampus

Following 1 week recovery after surgical implantation of guide cannulae into the lateral ventricle (see protocol below), rats were randomly assigned into one of three treatment groups. Vehicle-Saline, Vehicle-poly I:C and URB597-poly I:C (n= 6-10 per group). Rats received intracerebroventricular (*i.c.v.*) administration of URB597 (50µg) or vehicle (100% DMSO) in an injection volume of 5µl/min followed 30 min later by systemic administration of poly I:C (3mg/kg, *i.p.*) or sterile saline (0.89%) in an injection volume of 1.5ml/kg. The dose and time of URB597 was chosen on the basis of pilot work within our laboratory demonstrating increases in FAAH substrate levels. Animals were returned to their home cages and sacrificed 4 hours following poly I:C administration, brain removed, hippocampus excised, snap frozen and stored at -80°C until assayed for anandamide, OEA and PEA levels and cytokine expression.

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

A guide cannula (5mm, Plastics One Inc., Roanoke, Virginia, USA) was stereotaxically implanted into the right lateral ventricle (AP: -0.07mm; ML: -0.15mm relative to bregma, DV: -0.30mm from skull surface, Paxinos and Watson,1998), of rats under isoflurane anaesthesia (1-3% in O₂; 0.5L/min). The cannula was permanently fixed to the skull using stainless steel screws and dental acrylic cement. A stylet made from stainless steel tubing (Plastics One Inc., Roanoke, Virginia, USA) was inserted into the guide cannula to prevent blockage by debris. The broad spectrum antibiotic,

enrofloxacin (2.5mg/kg s.c.) (Baytril, Bayer Ltd., Dublin, Ireland), was administered during surgery to prevent post-operative infection. Following cannulae implantation, the rats were housed singly and administered enrofloxacin (2.5mg/kg s.c.) for a further 3 days. Rats were allowed to recover for at least 6 days prior to experimentation. During this period, the rats were handled and their body weight and general health monitored on a daily basis. Correct cannula placement was verified by the Angiotensin II drinking test 3 days prior to the experiment as previously described (De Fanti *et al.*, 2002). Briefly, Angiotensin II (Sigma, St Louis, MO) was dissolved in sterile phosphate buffered saline + calcium (PBS⁺) at a concentration of 20ng/ μ l. An increased drinking response of at least 3ml of water within 20 min following an i.c.v. injection of 100ng ANG II confirmed correct cannula placement.

2.4 Quantitation of endocannabinoids and N-acylethanolamine concentrations in the hippocampus using liquid chromatography – tandem mass spectrometry (LC-MS/MS)

Quantitation of endocannabinoids and N-acylethanolamines was essentially as described previously (Kerr *et al.*, 2012; Kerr *et al.*, 2013a; Kerr *et al.*, 2013b). In brief, samples were homogenised in 400 μ L 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 40 μ L 65% acetonitrile and separated on a Zorbax® C18 column (150 \times 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.

2.5 Expression of inflammatory mediators assessed using quantitative real-time PCR

RNA was extracted from 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; Kerr *et al.*, 2012; Kerr *et al.*, 2013a; Kerr *et al.*, 2013b). Assay IDs for the genes were, IFN α (Rn02395770_g1), IFN β (Rn00569434_s1), TNF α (Rn99999017_m1), IL-1 β (Rn00580432_m1), IL-6 (Rn00561420_m1), IL-12b (Rn00575112_m1), IL-10 (Rn00563409_m1), IFN γ (Rn00594078_m1), IP-10 (Rn00594648_m1), NOS2 (Rn00561646_m1), I κ B α (Rn01473658_g1), IRF7 (Rn01450778_g1), PKR (Rn00571015_m1), SOCS1 (Rn00595838_s1), SOCS3 (Rn00585674_s1) and Fas (Rn00685720_m1). PCR was performed using Taqman Universal PCR Master Mix 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. β -actin was used as an endogenous control to normalise gene expression data. Relative gene expression was calculated using the $\Delta\Delta$ CT method.

2.6 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-Wilk and Levene test, respectively. Data were analysed using one-way analysis of variance (ANOVA). *Post-hoc* analysis was performed using Fisher's LSD test when appropriate. Data were considered significant when $P < 0.05$. Results are expressed as group means + standard error of the mean (SEM).

3. Results

3.1 Systemic and central administration of URB597 enhances OEA and PEA levels in the hippocampus

Systemic administration of poly I:C did not alter levels of the endocannabinoids or *N*-acylethanolamine levels in the hippocampus 4 hours following administration. Systemic and central administration of URB597 increased OEA [i.p.: $F_{2,25}=16.198$, $p < 0.001$; i.c.v.: $F_{2,19}=15.695$, $p < 0.001$] and PEA [i.p. $F_{2,25}=24.835$, $p < 0.001$; i.c.v. $F(2,19)=9.036$, $p < 0.01$] levels in the hippocampus (Fig. 1). URB597 did not alter levels of AEA or 2-AG in the hippocampus of poly I:C-treated animals following either systemic or central administration.

3.2 Differential effects of systemic and central administration of URB597 on hippocampal expression of type 1 IFN responsive genes following poly I:C administration

Systemic administration of poly I:C did not significantly alter the expression of the type 1 interferons IFN α and IFN β , however expression of the interferon-induced transcription factor (IRF)-7 [i.p.: $F_{2,12}=4.137$, $p < 0.05$; i.c.v.: $F_{2,20}=8.866$, $p < 0.01$] and chemokine IP-10 [i.p. $F_{2,21}=18.121$, $p < 0.01$; i.c.v.: $F_{2,20}=21.090$, $p < 0.001$] were significantly increased when compared to saline-treated counterparts (Fig. 2). The robust transcription of IRF7 and IP-10 suggests that IFN α/β are produced at the mRNA and protein level in the hippocampus following systemic poly I:C administration, possibly at an earlier time point than that examined in the present study, and were active at the type 1 IFN receptor. Systemic administration of the FAAH inhibitor URB597 robustly increased IFN α expression [$F_{2,24}=3.981$, $p < 0.05$] in the hippocampus of poly I:C-treated animals, an effect not observed following central administration (Fig. 2). In comparison, central administration of URB597 partially attenuated the poly I:C-induced increase in IP-10 expression, with a similar effect also observed on IRF7 expression, although this just failed to reach statistical significance ($p = 0.059$).

3.3 Systemic and central administration of URB597 modulates the expression of poly I:C-induced increases in NF κ B-responsive pro-inflammatory cytokines in the rat hippocampus

TLR3 activation stimulates both IRF-3 and NFκB signal transduction pathways. As shown in Fig 3, systemic administration of poly I:C significantly increased hippocampal expression of NFκB-dependant pro-inflammatory cytokines TNFα, IL-1β and IL-6, but not IL-12, 4 hours post administration (vehicle-saline vs vehicle-poly I:C; Fig. 3). Both systemic [$F_{2,23} = 7.112$, $p < 0.01$] and central [$F_{2,19} = 4.550$, $p < 0.05$] administration of URB597 attenuated the poly I:C-induced increase in TNFα expression in the hippocampus (Fig 3). Poly I:C-induced increase in hippocampal IL-1β expression was attenuated by systemic [$F_{2,22} = 5.995$, $p < 0.01$], but not central administration of URB597 (Fig. 3). In comparison, the increase in hippocampal IL-6 expression following poly I:C administration was augmented in rats receiving systemic URB597 [$F_{2,20} = 6.009$, $p < 0.01$], while central administration of URB597 tended to attenuate this response (Fig. 3).

3.4 Evidence of further anti-inflammatory effects following central, but not systemic, administration of URB597

The expression of the anti-inflammatory cytokine IL-10 in the hippocampus following systemic administration of poly I:C tended to be increased (3-4 fold), although this was not statistically significant when compared to saline-treated counterparts (Fig. 4). However, central, but not systemic, administration of URB597 significantly increased the expression of IL-10 in the hippocampus of poly I:C-treated rats, when compared to both saline- and vehicle-poly I:C-treated counterparts [$F_{2,17} = 8.346$, $p < 0.01$].

Expression of the type 2 interferon IFNγ, was significantly increased in poly I:C-treated animals following systemic administration of URB597 [URB597-poly I:C vs Veh-poly I:C : $F_{2,10} = 5.310$, $p < 0.05$]. In comparison, central administration of URB597, attenuated the poly I:C-induced increase in IFNγ expression in the hippocampus [$F_{2,17} = 4.491$, $p < 0.05$] (Fig 4).

Poly I:C induced an increase in iNOS expression [$F_{2,18} = 5.579$, $p < 0.05$] in the hippocampus, that was unaltered by prior systemic or central administration of URB597 (Fig. 4).

3.5 Central administration of URB597 attenuates poly I:C-induced increases in SOCS1, but not SOCS3, expression in the hippocampus.

Inflammation associated increases in cytokines and chemokines are self-regulated by concurrent increases in the expression of suppressors of cytokine signalling (SOCS), namely SOCS1 which primarily acts to inhibit the biological activity of IFN α and IFN γ ; and SOCS3 which primarily acts to inhibit IL-6 signalling. Thus, a further aim of this study was to examine if such regulatory mechanisms were altered by systemic or central administration of URB597.

Systemic administration of poly I:C induced a robust increase in the expression of both SOCS1 [i.p. $F_{2,12}=3.759$, $p = 0.05$; i.c.v.: $F_{2,21}=10.736$, $p < 0.01$] and SOCS3 [i.p. $F_{2,11}=6.790$, $p < 0.05$; i.c.v.: $F_{2,19}=8.095$, $p < 0.05$] in the hippocampus. Prior systemic administration of URB597 did not alter the poly I:C-induced increase in the expression of either the SOCS 1 or 3 gene. In comparison, central administration of URB597 significantly attenuated the poly I:C-induced increase in the expression of SOCS1 (Vehicle-PolyI:C vs URB597-poly I:C) (Fig. 5).

3.6 URB597 does not alter poly I:C-induced increases in the pro-apoptotic gene Fas in the hippocampus

Activation of the type1 IFN receptor (IFNR1) by IFN α/β results in increased transcription of anti-viral genes including interferon-induced dsRNA-dependent protein kinase (PKR) which in turn can induce the expression of the pro-apoptotic genes Fas and Bax and activation caspase 3 signalling resulting in cell loss. As such, a further aim was to examine if the neuroimmunomodulatory effects of URB597 were associated with changes in the expression of the pro-apoptotic genes PKR and Fas. Systemic administration of poly I:C increased the expression of Fas [$F_{2,20}=4.686$, $p < 0.05$], but not PKR, in the hippocampus (Fig.6). Although systemic administration of URB597 tended to reduce the poly I:C-induced increase in Fas, the effect failed to reach statistical significance [$F_{2,13}=3.339$, $p = 0.068$]. Central administration of URB597 did not alter poly I:C-induced increases in Fas expression (Fig. 6).

4. Discussion and Conclusion

The present study demonstrated that both systemic and central administration of the FAAH inhibitor URB597 increased levels of the PEA and OEA, in the hippocampus, and modulated the expression of various TLR3-responsive genes. Specifically, systemic administration of URB597 increased the hippocampal expression of the type 1 and type 2 interferons, IFN α and IFN γ respectively, and attenuated the poly I:C-induced increase in the expression of NF κ B responsive pro-inflammatory genes, IL-1 β and TNF α , while concurrently augmenting the expression of IL-6. In comparison, central administration of URB597, attenuated the poly I:C-induced increase in IFN γ , the interferon-inducible chemokine IP-10 and interferon regulatory gene SOCS1. Furthermore, the poly I:C-induced increase in the expression of TNF α and IL-10 were attenuated and augmented respectively, by central administration of URB597. Taken together, these data provide evidence for an important modulatory role of FAAH substrates in neuroinflammatory responses to TLR3 activation.

TLR3 activation is associated with induction of anti-viral type 1 IFNs, namely IFN α/β , activation of the type1 IFN receptor (IFNR1) and increased transcription of genes including IRF-7 and other anti-viral genes including PKR. Although the hippocampal expression of IFN α/β was not increased following systemic poly I:C administration in the current study, the robust induction of IRF7, and the interferon inducible chemokine IP-10, suggests strongly that type 1 interferons were produced at the protein level and active in the hippocampus. Furthermore, although poly I:C did not alter the expression of the anti-viral gene PKR, the increased expression of the pro-apoptotic gene Fas suggests the activation of downstream IFN-mediated apoptotic events. In addition to inducing IFN α/β production and signalling, TLR3 stimulation also induces activation of the NF κ B signalling pathway and subsequent production of inflammatory cytokines. The current data demonstrate that systemic poly I:C administration robustly increases the expression of the NF κ B-responsive genes, TNF α , IL-1 β , IL-6, IL-10 and iNOS in the hippocampus, correlating with previous reports in both rats and mice (Cunningham *et al.*, 2007; Field *et al.*, 2010; Gibney *et al.*, 2013). Thus, poly I:C induced activation of TLR3 results in the induction of interferon, cytokine and apoptotic genes within the hippocampus which may modulate neuronal function. Recent work from our lab (unpublished) and others (Gibney *et al.*, 2013) has demonstrated that systemic

administration of poly I:C is associated with reductions in the expression of BDNF and concurrent increased expression of markers of glial (microglial and astrocyte) activation in the hippocampus, 24 hours post administration, indicating lasting alterations in neuro-immune processing following acute TLR3 activation. Furthermore, poly I:C-induced increases in hippocampal cytokine expression are accompanied by enhanced neuronal excitability in the hippocampus (Costello *et al.*, 2013), alterations in memory consolidation (Kranjac *et al.*, 2012) and depression and anxiety-like behaviour (Gibney *et al.*, 2013). Thus, TLR3-induced neuroinflammation modulates neuronal function, which, if uncontrolled, may result in aberrant physiological functioning and neurodegeneration.

Although the effects of cannabinoids and endocannabinoid modulators including URB597, on the expression of inflammatory cytokines in the brain following systemic administration of the TLR4 agonist and bacterial endotoxin LPS, has been demonstrated (Duncan *et al.*, 2013; Kerr *et al.*, 2012; Kerr *et al.*, 2013b; Roche *et al.*, 2008), there has been a paucity of studies examining if similar responses occur following activation of other TLRs. The synthetic cannabinoid WIN55,212-2 inhibits poly I:C-induced NF- κ B activation and the expression of TNF α , while concurrently enhancing IRF3 signalling and IFN β expression in a human astrocyte cell line (Downer *et al.*, 2011). Theiler's virus (TMEV) induction of cytokines such as IL-6, IL-10, IL-12 and IL-23 is primarily mediated by TLR3 (So *et al.*, 2006) and modulated by enhancing anandamide tone (Correa *et al.*, 2011; Molina-Holgado *et al.*, 1998). Furthermore, the inhibition of FAAH or direct administration of PEA has been shown to limit inflammation and ameliorate motor deficits in TMEV-infected rodents (Correa *et al.*, 2011; Hernangomez *et al.*, 2012; Loria *et al.*, 2010; Loria *et al.*, 2008; Mestre *et al.*, 2005; Ortega-Gutierrez *et al.*, 2005). Thus, modulation of type 1 interferons and inflammatory cytokines may provide a novel therapeutic strategy for viral-induced neuroinflammation and associated neurodegeneration. The current data are the first to demonstrate that enhancing FAAH substrate levels *in vivo* can modulate the expression of TLR3-induced inflammatory genes in the brain. Specifically, the current data demonstrate that enhancing FAAH substrate levels following systemic administration of URB597 was associated with an increase in the hippocampal expression of both type 1 and type 2 interferons, IFN α and IFN γ , and IL-6, while concurrently attenuating the expression of TNF α and IL-1 β .

Although IFNs have been shown to elicit pro-inflammatory effects and deleterious effects on neuronal function, several lines of evidence also indicate anti-inflammatory effects associated with these immune modulators. For example, type 1 interferons have been regarded as anti-inflammatory within the brain due to their ability to limit leukocyte infiltration (Prinz *et al.*, 2008) and reduce expression of pro-inflammatory cytokines including IL-1 β and TNF α (Teige *et al.*, 2006). IFN γ has also been shown to elicit anti-inflammatory effects (Muhl *et al.*, 2003), protect neurons from damage initiated by viral infection (Geiger *et al.*, 1997; Rodriguez *et al.*, 2003), protect cultured hippocampal neurons against glutamate-induced excitotoxic death (Lee *et al.*, 2006b) and alleviate status epilepticus-induced neuronal damage in the hippocampus of rats (Ryu *et al.*, 2010). Furthermore, enhancement of both type 1 and 2 interferons limits inflammation and disease progression in models of multiple sclerosis (Bowen *et al.*, 2013; Lin *et al.*, 2007; Naves *et al.*, 2013). Thus, increasing interferon expression in combination with a reduction in pro-inflammatory cytokines may limit the neuroinflammatory and possibly neurodegenerative cascades in the hippocampus following TLR3 activation. Although an increase in IL-6 expression may seem at odds with this hypothesis, IL-6 activates STAT3 signalling cascades. Recent evidence has demonstrated that endocannabinoids released in response to electroacupuncture mediate, via CB₁ receptor activation of STAT3, neuroprotection in a model of ischemic reperfusion (Wang *et al.*, 2009; Zhou *et al.*, 2013). Furthermore, the present study demonstrated that the poly I:C-induced increase in hippocampal Fas expression was not observed following systemic administration of URB597, although it cannot be ruled out that an effect may occur at a later time point or via an alternative mechanism. Taken together, the data suggest that enhancing FAAH substrates following systemic URB597 alters the expression of neuroinflammatory genes in the hippocampus following TLR3 activation, which may in turn modulate TLR3-associated alterations in neuronal function in this brain region.

A key aim of this research was to decipher the role of FAAH substrates within the brain on TLR3-induced neuroinflammation. Alterations in TLR3-induced interferon and cytokine expression in the hippocampus following systemic URB597 administration may have occurred due to indirect modulation of peripheral immune responses. Evaluating the role of central FAAH substrates on neuroinflammatory processes is of

particular importance as several neuropathologies including ischemia, Parkinson's disease and multiple sclerosis are associated with increases in FAAH substrate levels within the brain (Baker *et al.*, 2001; Degn *et al.*, 2007; Franklin *et al.*, 2003; Loria *et al.*, 2008). The data presented here indicate that central administration of URB597 is associated with a pronounced anti-inflammatory effect, exemplified by an attenuation of poly I:C-induced increase in IFN γ , IP-10, SOCS1, TNF α and an increase in the expression of the anti-inflammatory cytokine IL-10. IL-10 is a potent anti-inflammatory cytokine and key negative regulator of microglial activation. The inhibition of FAAH or direct administration of anandamide has been shown to increase TLR4- and TMEV-induced IL-10 levels in microglial cells (Correa *et al.*, 2011; Correa *et al.*, 2010). Follow-up studies from the same group have demonstrated that IL-10 plays a pivotal role in anandamide-induced neuroprotection both *in vitro* and *in vivo*, at least in part, by increasing expression of CD200 on neurons and amplifying interactions with CD200R1 on microglia, and limiting harmful inflammatory processes (Hernangomez *et al.*, 2012). Thus, it is possible that the FAAH substrates increase the expression and levels of IL-10 which in turn stabilise microglial activation and inhibit the expression of TNF α and IFN γ (and the associated downstream genes IP-10 and SOCS1), thus limiting TLR3-induced neuroinflammatory processes in the hippocampus. Thus, although the effect of systemic vs central FAAH inhibition on TLR3-induced neuroinflammatory responses differs in terms of individual cytokines modulated, taken together the present data demonstrate a key role for central FAAH substrates in limiting TLR3 associated pro-inflammatory responses in the hippocampus.

Although the receptor and molecular mechanism underlying the effect of URB597 on TLR3-induced neuroinflammatory responses remain to be determined, recent evidence has demonstrated that WIN212-2-induced increases in IFN β levels are mediated by PPAR α activation, that PPAR α ligands inhibit the induction of the NF κ B reporter gene (Downer *et al.*, 2011) and that anandamide-induced inhibition of NF κ B activation is not mediated via cannabinoid receptors (Sancho *et al.*, 2003). In comparison, PPAR γ activation attenuates TLR-induced inflammatory signals in glial cells (Gurley *et al.*, 2008) including attenuation of IFN β induction in response to TLR3 activation (Zhao *et al.*, 2011). Furthermore, in the current study both systemic and central administration of URB597 increased hippocampal levels of the FAAH substrates and potent PPAR ligands

OEA and PEA, but not anandamide. However, it is possible that anandamide may have been increased at an earlier time point to that examined, as previously reported (Kerr *et al.*, 2012). Thus, it is possible that the modulation of TLR3-induced cytokine/interferon expression in the hippocampus by URB597 may be mediated via FAAH substrate modulation of PPAR α activity. However, due to the multiple receptor (CB₁, CB₂, TRPV1, PPAR α , PPAR γ and GPR55) and molecular (STATs, ERK, MAPK, JUN, AP-1) targets of FAAH substrates, further in depth studies are required to determine the precise mechanism by which FAAH substrates modulate TLR3-induced inflammatory responses in the brain.

In conclusion, the data presented herein demonstrated that systemic and central inhibition of FAAH potently modulates the expression of TLR3-responsive inflammatory genes in the hippocampus. As TLR3-induced inflammatory mediators in the hippocampus are associated with learning, memory and seizure susceptibility, these data may have important implications for targeting FAAH in the treatment of neuroinflammatory and neurodegenerative disorders.

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For whom the endocannabinoid tolls: modulation of innate immune function and implications for psychiatric disorders

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Abbreviations: 2-AG 2-arachidonyl glycerol; AEA anandamide; CB cannabinoid receptor; CBD cannabidiol; COX-2 cyclooxygenase 2; ERK1/2 extracellular signal-regulated kinase 1/2; FAAH fatty acid amide hydrolase; IFN interferon; IL interleukin; iNOS inducible nitric oxide synthase; IP10 Interferon gamma-induced protein 10; IRF interferon regulatory transcription factor; LPS lipopolysaccharide; MAGL monoacylglycerol lipase; MAPK mitogen-activated protein kinase; NF- κ B Nuclear factor kappa B; NO nitric oxide; PAMPs pathogen-associated molecular patterns; PBMCs peripheral blood mononuclear cells; Poly I:C Polyinosinic:polycytidylic acid; PPAR peroxisome proliferator-activated receptor; THC, Δ^9 -tetrahydrocannabinol; TLR Toll-like receptors; TMEV Theiler's encephalomyelitis virus; TNF tumour necrosis factor; TRIF TIR domain-containing adaptor inducing IFN- β

Classes of cannabinoid-based pharmacological agents cited in the review

Nonselective CB₁/CB₂ agonists: Δ⁹-THC, HU210, CP55940, WIN55,212-2

Selective CB₂ agonists: JWH-015

FAAH inhibitors: URB597, AA-5HT

MAGL/ABHD6 inhibitors: JZL184, MJN110, KML129, WWL70

Endocannabinoid reuptake inhibitors: UCM707, OMDM1/2, AM404

Abstract

Toll-like receptors (TLRs) mediate the innate immune response to pathogens and are critical in the host defence, homeostasis and response to injury. However, uncontrolled and aberrant TLR activation can elicit potent effects on neurotransmission and neurodegenerative cascades and has been proposed to trigger the onset of certain neurodegenerative disorders and elicit detrimental effects on the progression and outcome of established disease. Over the past decade, there has been increasing evidence demonstrating that the endocannabinoid system can elicit potent modulatory effects on inflammatory processes, with clinical and preclinical evidence demonstrating beneficial effects on disease severity and symptoms in several inflammatory conditions. This review examines the evidence supporting a modulatory effect of endocannabinoids on TLR-mediated immune responses both peripherally and centrally, and the implications for psychiatric disorders such as depression and schizophrenia.

Keywords: Endocannabinoid; Anandamide; 2-AG; TLR3; TLR4; LPS; Poly I:C; depression; schizophrenia

Introduction

The endocannabinoid system is an important lipid signalling system involved in modulation of a host of physiological responses ranging from appetite, respiration, metabolism, inflammation, pain and neurotransmission to name but a few. Of particular interest over the past decade has been the discovery that cannabinoids (plant-derived, synthetic and endogenous) elicit potent modulatory effects on inflammatory processes, with clinical and preclinical evidence demonstrating beneficial effects on disease severity and symptoms in several inflammatory conditions (Storr *et al.*, 2009; Tschop *et al.*, 2009; Yoshihara *et al.*, 2005; Yu *et al.*, 2010). However, the precise mechanisms by which cannabinoids modulate immune function depend on the conditions under investigation, and in many cases remain to be determined. There has been increasing data to suggest that one mechanism by which cannabinoids influences innate immune function may be by interacting with a superfamily of pattern recognition receptors (PRR) namely toll-like receptors (TLRs). Activation of TLRs participates in host defences, homeostasis and response to injury however, uncontrolled and aberrant TLR activation can elicit potent effects on neurotransmission and neurodegenerative cascades [for reviews see (Arroyo *et al.*, 2011; Lehnardt, 2010; Owens, 2009; van Noort *et al.*, 2009)]. Furthermore, viral and bacterial induced activation of TLRs results in systemic and central inflammation, an effect proposed to trigger the onset of some neurodegenerative disorders (Deleidi *et al.*, 2012) and elicit detrimental effects on the progression and outcome of established disease (Holmes *et al.*, 2009; Perry, 2004; Teeling *et al.*, 2009). As TLRs are expressed on neurons, astrocytes and microglia within the CNS (Bsibsi *et al.*, 2002), and TLR expression has been reported to be increased in the post-mortem brain of patients with neurodegenerative and psychiatric disorders (Brudek *et al.*, 2013; Salaria *et al.*, 2007), modulation of TLR-associated innate inflammatory responses by cannabinoids may provide a novel therapeutic target for such disorders.

The endocannabinoid system

The endocannabinoid system is widely expressed in all tissues of the body and comprises the cannabinoid (CB)₁ and CB₂ receptors, the naturally occurring endogenous receptor agonists or so-called endocannabinoids, the best characterised of which are arachidonyl ethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) (Devane *et al.*, 1992; Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995), and the enzymes involved in their synthesis and degradation. It should be noted that other endocannabinoid ligands including oleamide (Leggett *et al.*, 2004), O-arachidonoyl ethanolamine (virodamine) (Porter *et al.*, 2002), 2-arachidonoyl glycerol ether (noladin ether) (Hanus *et al.*, 2001) and N-arachidonoyl-dopamine (NADA) (Bisogno *et al.*, 2005; Huang *et al.*, 2001b) have been identified however, the role of these ligands in physiological processes has not been examined in detail. Endocannabinoids are not stored in vesicles but rather their biosynthesis occurs on demand via hydrolysis of cell membrane phospholipid precursors. AEA, and two related analogues *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA), formed from the precursor *N*-arachidonoylphosphatidylethanolamine (NAPE), with AEA formed due to the hydrolytic activity of the phospholipase D enzyme NAPE-PLD (Di Marzo *et al.*, 1994; Sugiura *et al.*, 1996). The main biosynthetic pathway for 2-AG involves the hydrolysis of the membrane phospholipid phosphatidylinositol (PI) by phospholipase C (PLC), producing 1,2-diacylglycerol (DAG), which in turn is then converted to 2-AG by diacylglycerol lipase (DAGL) (Prescott *et al.*, 1983; Sugiura *et al.*, 1995).

Once release, endocannabinoids elicit their effect primarily via CB₁ and/or CB₂ receptors. CB₁ receptors are G-protein coupled receptors that are highly expressed throughout the human and rodent brain, with particularly high density on the pre-synaptic terminals of GABA and glutamate neurons (Herkenham *et al.*, 1991; Mackie, 2008; Tsou *et al.*, 1998). Activation of CB₁ receptors results in inhibition of cyclic AMP, activation of MAPK and inhibition of N- and P/Q- type voltage-activated Ca²⁺ channels while concurrently activating the inwardly rectifying K⁺ currents, effects which result in the inhibition of central neurotransmitter release. Although at lower density than on neurons, CB₁ receptors have also been shown to be expressed on glia and on a wide range of peripheral tissues (Carlisle *et al.*, 2002; Cavauto *et al.*, 2007; Cota, 2007; Galiegue *et al.*, 1995; Osei-Hyiaman *et al.*, 2005). In comparison, CB₂ receptors, also a G-protein coupled receptor, is widely distributed in peripheral tissues, particularly in

immune tissues including the spleen, tonsils, thymus, mast cells and blood cells (Berdyshev, 2000; Munro *et al.*, 1993; Sugiura *et al.*, 2000) and on activated glia within the brain (Carlisle *et al.*, 2002; Nunez *et al.*, 2004; Rock *et al.*, 2007). Accumulating evidence has also indicated that CB₂ receptor protein and mRNA is also expressed on subsets of neurons within the brain (Baek *et al.*, 2008; Gong *et al.*, 2006; Onaivi *et al.*, 2006; Van Sickle *et al.*, 2005; Zhang *et al.*, 2014) and thus this receptor may also directly modulate neurotransmission. In addition to CB₁ and CB₂, endocannabinoids are now known to also elicit activity at other receptors, namely the transient receptor potential vanilloid 1 (TRPV1), PPARs, GPR55 and GPR119 (Huang *et al.*, 2002; Overton *et al.*, 2006b; Ryberg *et al.*, 2007a; Sun *et al.*, 2006b). Activity at these receptors has been proposed to account, at least partially, for some of the differential effects observed with potent selective cannabinoid agonists and modulation of endocannabinoid tone.

A number of enzymes have been identified that are involved in the catabolism of endocannabinoids. Fatty acid amide hydrolase (FAAH) has been identified as the enzyme primarily responsible for the metabolism of AEA, exhibiting similar distribution to CB₁ receptors (2001; Cravatt *et al.*, 1996a; Walker *et al.*, 2002). In comparison, monoacylglycerol lipase (MAGL) is considered the primary enzyme involved in 2-AG inactivation, responsible for approximately 85% of its metabolism (Dinh *et al.*, 2002a; Long *et al.*, 2009a). The remaining 15% is thought to be broken down by FAAH, cyclooxygenase-2 (COX2), ABDH6 (serine hydrolase α/β -hydrolase domain) and ABDH12 (Blankman *et al.*, 2007). Moreover, both COX-2 and lipoxygenase (LOX) catalyse the oxidation of AEA and 2-AG into metabolic products which mediate biological effects independent of cannabinoid receptors (Ueda *et al.*, 2011; Urquhart *et al.*, 2014).

Due to the topography of this lipid signalling system, the endocannabinoid system is in a unique position to regulate a host of physiological activities. Over the past decade there has been increased interest in cannabinoid modulation of immune function in both health and disease, which has been examined in detail by several excellent reviews (Jean-Gilles *et al.*, 2010; Nagarkatti *et al.*, 2009; Rom *et al.*, 2013; Stella, 2010; Stella, 2009). The general consensus is that cannabinoid modulation of inflammatory processes provides a novel therapeutic target for central and peripheral inflammatory disorders. We propose that one of the mechanisms by which cannabinoids (both exogenous and endogenous)

influence immune function is via modulation of TLR-mediated responses and thus the aim of this review is to examine the evidence supporting a modulatory effect of cannabinoids on TLR-mediated immune responses both peripherally and centrally, and review the implications for psychiatric disorders such as depression and schizophrenia.

Toll-like Receptors and innate immune function

The innate immune system is critical in mediating the body's physiological response to invading pathogens and self antigens [For reviews of innate immunity and pathogen host interaction see (Akira *et al.*, 2006; Basset *et al.*, 2003)]. Comprised of cells including monocytes/macrophages, fibroblasts, mast cells, neutrophils, natural killer and dendritic cells, as well as other circulating leukocytes, the innate immune response is mediated and orchestrated by a diverse range of pattern recognition receptors (PRRs) located on these cells which recognise pattern associated molecular patterns (PAMPs), the molecular signatures of microbes. The most widely studied of the PRRs is a class of type-I transmembrane glycoprotein's known as Toll-like receptors (TLRs). Thirteen TLRs have been identified to date; TLRs 1-9 are conserved among humans and mice, TLR10, 12 and 13 are found in humans only while TLR11 is expressed only in mice. TLRs are divided into subgroups based on their ability to recognise particular PAMPs; TLR1-2, TLR4-6 and TLR10-12 sense microbial membrane components while TLR3, TLR7-9 and TLR13 sense microbial and viral nucleic acids. A comprehensive review of TLR signalling is beyond the scope of this article and has been extensively covered in several excellent reviews (Akira, 2011; Akira *et al.*, 2006; Gangloff, 2012). As such, provided here is an overview of the main classes of TLRs and their primary mechanism of action. Extracellular membrane bound TLRs include TLR4 and the associated MD-2 molecule which recognizes lipopolysaccharide (LPS) present on the cell wall of gram negative bacteria; TLR2 in conjunction with TLR1, TLR6 or TLR10 recognizes bacterial associated triacyl and diacyl portions of lipoproteins; and TLR5 and TLR11 recognizes flagellin, the major component of bacterial flagella. In comparison, the intracellular TLRs, located in the endosome, include TLR3 which recognizes double-stranded RNA released from viruses; TLR7-8 recognizes single-stranded RNA; TLR9 recognizes bacterial and viral DNA and TLR13 which recognizes bacterial ribosomal RNA. In addition, TLRs are known to be activated by several damage-associated

molecular patterns (DAMPs) released from stressed cells such as heat shock proteins (e.g. HSP70) and ATP; and environmental factors such as ozone and toluene [reviewed in (Asea, 2008; Lucas *et al.*, 2013; Schaefer, 2014)]. Following binding of the ligand, TLRs oligomerise and signal via various adaptor molecules such as myeloid differentiation primary response gene 88 (MyD88), Toll-interleukin 1 receptor (TIR)-domain-containing adaptor-inducing interferon- β (TRIF), TIR-domain containing adaptor protein (TIRAP) and TRIF-related adaptor molecule (TRAM). MyD88 is involved in all TLR signalling except for TLR3 which signals via the MyD88-independent pathway TRIF, resulting in the activation of the transcription factor interferon regulatory factor 3 (IRF3) and subsequent production of the type 1 interferons, IFN α and IFN β [Figure 1]. In comparison, activation of the MyD88 pathway results in activation of several signalling cascades, the translocation of the transcription factor NF κ B to the nucleus and the enhanced expression of chemokines, interferons and pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF α [Figure 1] [for reviews of signalling mechanisms of TLRs see (Akira *et al.*, 2004; Mogensen, 2009)]. Taken together, activation of TLRs induces an innate inflammatory state that acts to recruit macrophages and neutrophils and phagocytose invading pathogens, limit infection and promote healing. In addition, peripheral inflammatory mediators produced in response to TLR activation can communicate with the central nervous system via several routes, including entering the brain via the circumventricular organs, transported across the blood brain barrier via transport molecules expressed on brain endothelial cells, or cytokine-induced activation of the vagal communication pathway [for review of brain-immune communication pathways see (Dantzer, 2004; Quan *et al.*, 2007)]. This ultimately results in activation of microglia, the production of further chemokines and cytokines that then mediates a sickness response characterised by fever, hypolocomotion, hyperalgesia, anorexia, anhedonia and activation of the stress-hypothalamic-pituitary adrenal (HPA) axis. These symptoms are collectively considered to represent a highly organised strategy of an organism to fight infection, which acts to conserve energy, reduce bacterial/pathogen replication, stimulate proliferation of immune cells and minimize thermal loss in the host (Dantzer, 2001; Dantzer, 2004). In addition, microglia, astrocytes, neurons and oligodendrocytes also express multiple functional TLRs (van Noort *et al.*, 2009) which are important in mounting immune responses against microbial invasion of the CNS. Thus, TLR signalling is crucial for

peripheral and central innate immune responses, however if not tightly controlled can contribute to and/or exacerbate various diseases and disorders [reviewed in (Kawai *et al.*, 2010; Lehnardt, 2010; O'Neill *et al.*, 2009)]. Modulation of TLR-associated inflammatory responses has therefore been highlighted as a therapeutic target for a range of disorders. To date, numerous negative regulators of TLR signalling have been identified which down regulate TLR expression, block recruitment of signalling molecules, cause degradation of target proteins and negatively regulate transcription [reviewed in (Lucas *et al.*, 2013; Takeda *et al.*, 2007)]. Provided herein is the evidence that the endocannabinoid system may provide a further means of modulating in TLR-associated inflammatory responses and the possible implications for psychiatric disorders.

Endocannabinoid modulation of TLR4-induced inflammatory responses

TLR4 is the most characterised TLR, responsible for inducing inflammatory responses to gram negative bacterial antigens. In order to activate TLR4, lipopolysaccharide (LPS), a component of the wall of gram-negative bacteria, interacts with circulating LPS binding protein (LBP) which in turn enables the association between LPS and CD14 and consequently facilitates the transfer of LPS to the TLR4/MD-2 receptor complex. Binding of LPS to TLR4, causes the receptor to dimerise and activate the MyD88 dependant pathway resulting in translocation of NFkB to the nucleus with consequent enhancement of transcription and translation of pro-inflammatory mediators such as chemokines and cytokines including IL-1 β , TNF- α and IL-6, which mediate a concerted physiological response to fight infection. Due to the well recognised molecular mechanism underpinning TLR4-induced inflammatory responses, LPS is a very useful pharmacological tool with which to investigate peripheral and central immune processes and their modulation.

Some of the first evidence demonstrating a possible immunomodulatory role for the endocannabinoid system emerged from research investigating the effects of cannabinoids on TLR4-induced inflammatory responses *in vitro*. For example, potent non-selective cannabinoid receptor agonists such as Δ^9 -THC, HU210, CP55940 and WIN55,212-2 have been shown to inhibit TLR4-induced pro-inflammatory cytokine and nitric oxide release, induce apoptosis and inhibit migration of macrophages (Chang *et al.*, 2001; Jeon *et al.*, 1996; Klegeris *et al.*, 2003). Furthermore, these compounds have

also been demonstrated to inhibit TLR4-induced inflammatory responses in microglial and astrocyte cultures (Facchinetti *et al.*, 2003a; Puffenbarger *et al.*, 2000), highlighting an important role in modulation of neuroinflammatory responses. Due to the high expression of CB₂ receptors on immune cells and activated glia it was not surprising that many researchers attributed the anti-inflammatory effects of cannabinoids to activity at this receptor. However, while some of these studies demonstrated that modulation of TLR4-induced inflammation was mediated by activation of CB₂ receptors (Correa *et al.*, 2005; Germain *et al.*, 2002; Gui *et al.*, 2013; Merighi *et al.*, 2012; Zhao *et al.*, 2010), a role for CB₁ receptors in mediating effects of some cannabinoids was also noted (Cabral *et al.*, 2001; Esposito *et al.*, 2001; Germain *et al.*, 2002) and a significant proportion of studies indicated non-CB_{1/2} receptor mediated anti-inflammatory effects (Chiba *et al.*, 2011; Chiurchiu *et al.*, 2014; Facchinetti *et al.*, 2003a; Puffenbarger *et al.*, 2000; Ribeiro *et al.*, 2013; Verhoeckx *et al.*, 2006). As discussed above, it is now recognised that cannabinoids also exhibit activity at other receptor targets such as PPARs and GPR55, effects at which may underlie, at least in part, the anti-inflammatory activity of these compounds in certain cell types.

Enhancing endocannabinoid tone has been proposed as an alternative means of activating cannabinoid receptors without concomitant overt psychotropic effects associated with potent synthetic CB₁ receptor agonists. *In vitro* studies suggest that endocannabinoids elicit anti-inflammatory effects comparable to those of synthetic cannabinoids. Increasing AEA tone, either directly, via inhibition of its primary catabolic enzyme, FAAH, or by inhibiting its uptake, has been demonstrated to reduce TLR4-induced increases in the levels of pro-inflammatory cytokines and inflammatory mediators such as TNF α , IL-1 β and nitric oxide, and enhance the release of the anti-inflammatory cytokine IL-10 *in vitro* [see Table 1] (Chang *et al.*, 2001; Correa *et al.*, 2009a; Correa *et al.*, 2010; Facchinetti *et al.*, 2003a; Molina-Holgado *et al.*, 1997; Ortega-Gutierrez *et al.*, 2005; Puffenbarger *et al.*, 2000; Tham *et al.*, 2007). However, it should be noted that enhancing AEA tone has also been shown to enhance LPS-induced IL-6 levels in astrocytes (Ortega-Gutierrez *et al.*, 2005), thus effects of AEA may depend on the inflammatory mediators and cell type under investigation. Similarly, enhancing 2-AG tone has also been found to induce suppressive effects on TLR4-induced immune activation, namely by reducing proinflammatory cytokines such as IL-6, TNF- α , and expression of COX2 in macrophages and glia [Table 1] (Chang *et al.*, 2001; Facchinetti

et al., 2003b; Gallily *et al.*, 2000; Zhang *et al.*, 2008). Similar to the effects observed with synthetic cannabinoids, the effects of enhancing endocannabinoid tone have been attributable to CB_{1/2} and non-CB_{1/2} receptor activation (Correa *et al.*, 2008; Correa *et al.*, 2009a; Correa *et al.*, 2010; Facchinetti *et al.*, 2003a; Lu *et al.*, 2014a; Puffenbarger *et al.*, 2000) [Table 1]. However, regardless of the receptor mechanism, endocannabinoids have been shown, for the most part, to inhibit TLR4-induced NFκB activation (Correa *et al.*, 2010; Du *et al.*, 2011; Lu *et al.*, 2014a; Zhang *et al.*, 2008). TLR4 and CB_{1/2} receptors share common molecular targets such as MAPK and several studies have demonstrated that this is a key pathway for endocannabinoid modulation of TLR4-induced inflammatory responses. For example, AEA has been shown to augment and attenuate LPS-induced IL-10 and IL-12p70 expression respectively, in mixed glial cultures, effects mediated by CB₂ receptor activation of ERK1/2 and JNK pathways (Correa *et al.*, 2009a; Correa *et al.*, 2010). Similarly, AEA and 2-AG have been shown to up-regulate CB_{1/2} receptors and enhance IL-10 and TGFβ expression while concurrently reducing pro-inflammatory cytokine expression in primary muller glial cultures (Krishnan *et al.*, 2012). Thus, the anti-inflammatory effects of endocannabinoids following TLR4 activation has been proposed to be due to enhanced production of the anti-inflammatory cytokine IL-10 (Correa *et al.*, 2010). Furthermore, it has recently been shown that AEA activation of CB₂ receptors blocks the LPS-induced reduction in CD200R1 on microglia (Hernangomez *et al.*, 2012). Activation of CD200R1 was shown to attenuate LPS-induced pro-inflammatory and enhance IL-10 production, and IL-10 increases neuronal expression of CD200, an effect which consequently reduced neuronal cell death (Hernangomez *et al.*, 2012). Thus, AEA-induced up regulation of CD200R1 and IL-10 expression acts to attenuate TLR4-induced microglial activation, limiting the neuroinflammatory response and inducing neuroprotection.

In vivo studies support *in vitro* data demonstrating the immunomodulatory effects of enhanced endocannabinoid tone on TLR4-mediated effects [see Table 2]. Some of the first *in vivo* data demonstrating a modulatory role for the endocannabinoid system in TLR4-induced inflammatory responses arose from our data demonstrating that systemic administration of the AEA reuptake inhibitor AM404 attenuates LPS-induced increases in plasma IL-1β and IL-6 levels (Roche *et al.*, 2008). However, it was also noted that

LPS-induced plasma TNF α levels were augmented by systemic administration of either AM404, or the FAAH inhibitor URB597 (Roche *et al.*, 2008). Similar augmentations in LPS-induced plasma TNF α levels were observed following central FAAH inhibition, and activation of hypothalamic CB₁ receptors was found to be critical in mediating this response (De Laurentiis *et al.*, 2010). Thus, AEA activation of hypothalamic CB₁ receptors appears to facilitate the production and release of TNF α in the plasma in response to LPS. Hypothalamic IL-1 β has been shown to mediate fever (Murakami *et al.*, 1990) and hypophagia (Kent *et al.*, 1994) in response to LPS, effects which can be attenuated by AEA (Hollis *et al.*, 2011). Furthermore, recent studies from our group have demonstrated that systemic administration of the FAAH inhibitor URB597 increased AEA levels, an effect associated with the attenuation of LPS-induced IL-1 β expression in the hypothalamus (Kerr *et al.*, 2012) and CB₁ receptors have been shown to be critical in mediating the temperature response to LPS (Steiner *et al.*, 2011; Duncan *et al.*, 2013). Thus taken together, enhancing AEA tone, possibly via CB₁ receptor activation, attenuates TLR4-induced IL-1 β expression in the hypothalamus which may in turn inhibit associated sickness behaviour.

The relatively recent development of potent and selective MAGL inhibitors such as JZL184, KLM129 and MJN110 (Chang *et al.*, 2012; Ignatowska-Jankowska *et al.*, 2014; Long *et al.*, 2009a; Long *et al.*, 2009b; Niphakis *et al.*, 2013) has facilitated more detailed investigation of the role of 2-AG in a number of physiological and pathophysiological processes. Consistent with the *in vitro* data, enhancing 2-AG levels following MAGL inhibition also modulates peripheral and neuroinflammatory responses following TLR4 activation [Table 2], however the exact mechanisms underlying these effects remain unclear. MAGL inhibition has been shown to result in an attenuation of LPS-induced TNF α , IL-6 and MCP-1 levels in bronchoalveolar fluid (BALF) from a mouse model of acute lung injury, effects shown to be mediated by CB₁ and CB₂ receptors (Costola-de-Souza *et al.*, 2013). Furthermore, Alhouayek and colleagues demonstrated that MAGL inhibition was associated with a significant attenuation of colitis-induced increases in endotoxemia as measured by serum LPS levels, circulating inflammatory cytokines and the expression of TNF α and IL-1 β in the liver and brain. The anti-inflammatory effects of MAGL inhibition on mucosal and peripheral inflammation was shown to be partially mediated via CB₁ and CB₂ receptors

(Alhouayek *et al.*, 2011). In a subsequent study from this group, the authors demonstrated that inhibition of 2-AG metabolising enzyme ABHD6 attenuated LPS-induced increases in IL-1 β , IL-6 and MCP-1 expression in the cerebellum, lungs and liver of mice (Alhouayek *et al.*, 2013). However, 2-AG levels were increased in the peripheral tissues, but not in the cerebellum, and only in the liver were the anti-inflammatory effects were partially attenuated by CB₁ receptor antagonism. The authors went onto conduct further studies, demonstrating that central increases in 2-AG are not responsible for the anti-inflammatory effects of ABHD6 inhibition in the brain and that these are most likely attributed to PGD₂-G (a prostaglandin D₂-glycerol ester), a COX2 metabolite of 2-AG (Alhouayek *et al.*, 2013). Thus, enhancing 2-AG tone may modulate TLR4-induced inflammation via differential mechanisms depending on the tissue in question. Similarly, Nomura and colleagues demonstrated that systemic administration of the MAGL inhibitor JZL184, enhanced 2-AG levels both centrally and peripherally and attenuated LPS-induced IL-1 β , IL-1 α , IL-6, TNF α , prostaglandin PGE₂ and arachidonic acid levels in the brain of mice. The central anti-inflammatory effects of MAGL inactivation were shown not to be mediated by CB₁ or CB₂ receptors, but rather attributed to a reduction in arachidonic acid and downstream prostaglandins (Nomura *et al.*, 2011). Recent work from our laboratory further indicate that the mechanisms by which MAGL inhibition results in modulation of TLR4-induced inflammation may be different in the periphery and CNS. Our data demonstrated that systemic administration of JZL184 attenuated LPS-induced increases in cytokine expression in the rat frontal cortex and plasma, effects partially attenuated by pharmacological blockade of the CB₁ receptor (Kerr *et al.*, 2013b). However, 2-AG levels were only enhanced peripherally and central effects were not accompanied by reduced arachidonic acid and prostaglandin synthesis. Thus, the attenuation of TLR4-induced inflammatory responses in the brain following MAGL inhibition may be mediated by modulation of peripheral systemic innate immune responses that then communicate with the CNS to induce a state of neuroinflammation. Taken together the data to date indicate that while effects of enhancing 2-AG tone on TLR4-induced inflammatory responses in the periphery may be CB_{1/2} mediated, this does not appear to be the mechanism of action in the central nervous system. Such an effect may be significant, as this would allow for modulation of neuroinflammatory processes without the potential for adverse psychotropic effects that would be associated with central CB₁ receptor activation by 2-AG. Further studies are

required in order to determine if this is a possible therapeutic target for neuroinflammatory disorders.

Endocannabinoid modulation of TLR3-induced inflammatory responses

Although a wealth of evidence has demonstrated a role for endocannabinoid modulation of TLR4-induced inflammation, less is known about the role of this system in the modulation of inflammatory responding to other TLRs. TLR3 receptors are found mainly in the endosomal compartments of both immune and non-immune cells where they serve to recognise double stranded RNA (dsRNA) the molecular pattern associated with viral infection (West et al., 2006). Activation of TLR3 (and also TLR4) induces a MyD88-independent signalling response. Upon ligand binding, the adaptor protein TRIF) recruits a signalling complex leading to increased transcriptional activation of interferon regulatory factor (IRF3) and late phase NFkB activation. TRIF is the main adaptor molecule in the MyD88-independent pathway and once activated forms a complex with TRAF family-member-associated NFkB activator (TANK) binding kinase 1 (TBK1) and the inhibitor of NFkB (Ikb) kinase (IKK). This induces the phosphorylation of IRF3 and subsequent production of type I interferons (IFNs) which play an essential role in mediating the host's anti-viral responses and also induces late phase NFkB-inducible inflammatory genes.

TLR3 activation following systemic administration of the viral antigen polyinosinic:polycytidylic acid (poly I:C) results in enhanced production of the type I IFNs and NFkB-inducible inflammatory genes in the CNS (Cabral *et al.*, 2001; Cunningham *et al.*, 2007; Germain *et al.*, 2002; Gibney *et al.*, 2013). This enhanced neuroinflammatory profile is associated with sickness (Cunningham *et al.*, 2007; Dantzer *et al.*, 2011; Gibney *et al.*, 2013; McLinden *et al.*, 2012), anxiety- and depressive-like behaviour (Gibney *et al.*, 2013) and has been shown to exacerbate chronic neurodegenerative processes in a model of prion disease (Field *et al.*, 2010). Furthermore, poly I:C-induced increases in IFN β signalling in the hippocampus have been shown to be associated with enhanced neuronal excitability (Costello *et al.*, 2013), impaired contextual and working memory (Galic *et al.*, 2009) and seizure susceptibility (Galic *et al.*, 2009). In addition, TLR3 deficient mice exhibit enhanced hippocampal-dependent working memory, increased hippocampal volume and neurogenesis (Okun *et al.*, 2010). Thus, modulation of TLR3-induced inflammatory responses may provide novel therapeutic approaches for viral-induced neuroinflammation and associated neuronal alterations.

Some of the first data demonstrating a direct role of cannabinoids in modulating TLR3-induced inflammatory responses were reported by Downer and colleagues [Table 3]. This group demonstrated that the synthetic cannabinoid receptor agonist WIN55,212-2 enhances TLR3-induced IRF3 nuclear translocation and subsequent IFN β expression, while concurrently attenuating TLR3-induced NF κ B activation and TNF α expression in astrocytes cultures. These effects are in contrast to the WIN55,212-2 induced attenuation of both IFN β and TNF α following TLR4 activation (Downer *et al.*, 2011). Furthermore, enhanced IFN β was necessary for the protective effects of WIN55212-2 in a mouse model of multiple sclerosis (Downer *et al.*, 2011). Examination of the receptor mechanisms underpinning the augmentation of IFN β by WIN55,212-2 revealed that the effects were independent of CB $_1$ /CB $_2$ receptor activation, but rather mediated by peroxisome proliferator-activated receptor (PPAR) α -induced activation of JNK, activator protein-1 and positive regulatory domain (PRD) IV and subsequent IFN β transcriptional activation (Downer *et al.*, 2012; Downer *et al.*, 2011). Thus, cannabinoids appear to induce differential effects on the expression of type 1 interferons following TLR3 or TLR4 activation. However, until recently it was unknown what effect (endo)cannabinoids would have on TLR3-induced inflammatory processes *in vivo* [Table 4]. In an effort to address this question, we have recently examined the effect of the inhibition of FAAH activity on the expression of both IFN and NF κ B-inducible genes in the rat hippocampus following poly I:C-induced activation of TLR3 (Henry *et al.*, 2014). Data from this study show that systemic administration of the FAAH inhibitor URB597 increased the hippocampal expression of the type I and type II IFN, IFN α and IFN γ , respectively and IL-6, while concurrently attenuating the TLR3-induced increases in the NF κ B-responsive genes, TNF α and IL-1 β . Although IFNs have been shown to elicit pro-inflammatory effects and deleterious effects on neuronal function, several lines of evidence also indicate anti-inflammatory effects associated with these immune modulators. For example, enhancement of both type 1 and 2 interferon's limits inflammation and disease progression in models of multiple sclerosis (Bowen *et al.*, 2013; Lin *et al.*, 2007; Naves *et al.*, 2013). Thus, increasing interferon expression in combination with a reduction in pro-inflammatory cytokines may limit the neuroinflammatory cascade, at least in the hippocampus, following TLR3 activation. It should be noted that systemic administration of URB597 elicits minimal effects on TLR3-induced peripheral inflammatory responses (unpublished data) indicating that

enhancing levels of AEA and related *N*-acylethanolamines elicits more profound effects on TLR3-induced neuroinflammatory responses. In order to decipher the role of FAAH substrates within the brain on TLR3-induced neuroinflammation, the effects of central administration of the FAAH inhibitor URB597 on neuroinflammatory processes following systemic TLR3 activation was also evaluated. Our findings demonstrate that selective increases in FAAH substrates in the brain elicited a potent anti-inflammatory effect, exemplified by attenuation of TLR3-induced increases in IFN γ , the IFN-inducible chemokine IP-10, the IFN regulatory gene SOCS1 and the NF κ B responsive pro-inflammatory gene TNF α , with concurrent enhancement of the expression of the anti-inflammatory cytokine IL-10 (Henry *et al.*, 2014). Thus, while systemic administration of URB597 was associated with enhanced interferon and reduced pro-inflammatory gene expression, central administration elicits a more profound attenuation of TLR3-induced pro-inflammatory genes. Although the precise molecular and receptor mechanisms underpinning FAAH substrate-induced modulation of TLR3-mediated neuroinflammatory responses remains to be determined, we propose that one possible mechanism is via increased expression of the anti-inflammatory cytokine IL-10 which in turn may act to stabilise microglia (possibly via CD200-CD200R1 interactions; (Hernangomez *et al.*, 2012)), reducing NF κ B activation and decreasing expression of pro-inflammatory cytokines and downstream mediators, thereby limiting TLR3-induced neuroinflammation.

Further indirect evidence supporting an immunoregulatory role of the endocannabinoid system on TLR3-induced inflammation is evident from studies examining the effect of Theiler's murine encephalomyelitis virus (TMEV) which has been shown to induce an inflammatory response primarily via activation of TLR3 (So *et al.*, 2006). To date, several studies have demonstrated that the endocannabinoid system modulates such immune responses to TMEV *in vitro* [Table 3]. Administration of AEA or the endocannabinoid reuptake inhibitor OMDM1 or UCM707 attenuates TMEV-induced IL-1 β and IL-12p40 production in macrophages via CB $_1$ /CB $_2$ receptor activation (Mestre *et al.*, 2005), decreases NOS $^+$ and TNF α release in astrocytes (Molina-Holgado *et al.*, 1997) and VCAM-1 production in brain endothelial cells via CB $_1$ receptor activation (Mestre *et al.*, 2011). Furthermore, a *in vivo* data has demonstrated that administration of the endocannabinoid transport inhibitor UCM707 reduces TMEV-induced VCAM-1 expression and microglial activation in the brain, an effect partially mediated by CB $_1$

receptors (Mestre et al., 2011) [Table 4]. Although Mestre and colleagues did not directly investigate or discuss the role of TLR3, given the early timepoint of pharmacological intervention it is likely that enhancing anandamide tone may modulate TMEV-induced inflammatory responses via TLR3. Several other studies have revealed beneficial effects of endocannabinoid modulation on inflammatory and behavioural responses in the chronic phases of TMEV-induced demyelating disease, however the role of TLR3 in mediating effects at this stage is unknown (Correa *et al.*, 2011; Hernangomez *et al.*, 2012; Mestre *et al.*, 2005). Taken together, the data suggest that cannabinoids (exogenous and endogenous - AEA) modulate TLR3-induced inflammatory responses both peripherally and possibly more potently in the central nervous system. This may have important implications for neurodegenerative disorders such as multiple sclerosis where enhancing IFN β with concurrent attenuation of pro-inflammatory cytokines has been shown to be therapeutically beneficial (Javed *et al.*, 2006; Severa *et al.*, 2014). Further research is required in order to decipher the effects of modulating 2-AG and the receptor and molecular mechanisms underlying the effects of enhancing endocannabinoid tone on TLR3-induced inflammatory responses and the functional consequences of such.

Endocannabinoid modulation of inflammatory responses induced by other TLRs

A limited number of studies have examined the effects of endocannabinoid modulation on inflammatory responding following activation of TLRs other than from TLR3/4 [see Table 5]. Peptidoglycans, the main cell wall components of gram-positive bacteria, induce inflammatory processes via stimulation of TLR2 receptors. Echigo and colleagues recently reported that 2-AG suppressed TLR2-induced NF κ B phosphorylation in U87MG glioblastoma cells via CB₁ receptor activation (Echigo *et al.*, 2012) while in lymph node cells, 2-AG attenuated TLR2-induced IL-4 production via CB₂ activation (Maestroni, 2004). Thus, 2-AG may act at different receptors in different cell types in order to modulate TLR2-induced inflammatory responses. Recent data has demonstrated that the endocannabinoid/endovanilloid, N-arachidonoyl dopamine (NADA) attenuates TLR2/6-induced increases in IL-6 and IL-8 secretion, adhesion of neutrophils and the surface expression of E-selectins in human endothelial cells, effects partially mediated via a CB₁/CB₂ mechanism (Wilhelmsen *et al.*, 2014). Although further studies are required to determine the effects of modulating AEA tone on TLR2-induced inflammatory responding, the possible receptor and molecular mechanisms involved, or effects of modulating endocannabinoid tone *in vivo*, the data so far indicate that the endocannabinoid system is capable of modulating TLR2-induced inflammation.

TLR7 and 8 recognise and are activated in response to ssRNA and thus play an important role in mediating the host's anti-viral responses. To our knowledge, only one study has examined the role of the endocannabinoid system on TLR7/8-induced immune activation (Chiurchiu *et al.*, 2013). Pre-treatment with either AEA or the selective CB₂ receptor agonist JWH-015 attenuated TLR7/8-induced increases in pro-inflammatory cytokine release from myeloid dendritic cells (mDCs) isolated from both healthy donors and multiple sclerosis patients, effects which were completely abolished in the presence of CB₂ receptor antagonism in both cohorts (Chiurchiu *et al.*, 2013). In contrast, pre-treatment with AEA or JWH-015 had no significant effect on TLR7/8-induced cytokine production in isolated plasmacytoid dendritic cells (pDCs) from multiple sclerosis patients. However, it should be noted that pDCs isolated from MS patients exhibited a marked elevation in FAAH levels (Chiurchiu *et al.*, 2013) and thus, the authors suggest that the lack of effects of AEA on TLR7/8-induced cytokine production in pDCs is due

to rapid metabolism of AEA due to increased levels of FAAH. Supporting this hypothesis, the authors report that pharmacological inhibition of FAAH restored AEA-induced decreases in TNF α in TLR7/8 stimulated pDCs (Chiurchiu *et al.*, 2013). While the effects of 2-AG, the receptor mechanisms, and effects in other cell types and *in vivo* remain to be determined, these findings demonstrate that AEA modulates TLR7/8-induced immune responses, effects which differ depending on cell type and endogenous tone of the system.

Endocannabinoid regulation of TLR-induced inflammation: possible implications for treatment of depression

The role of the innate immune system in major depressive disorder (MDD) has generated a great amount of interest over the past two decades, with increasing evidence indicating that excessive inflammation may at least be partly involved in disease pathogenesis [for detailed reviews see (Berk *et al.*, 2013; Dantzer, 2006; Dantzer *et al.*, 2008; Maes, 2011)]. A role of altered immune responding in MDD is supported by reports in which up to 70% of patients receiving cytokine therapy for specific cancers and malignancies develop depressive symptomatology (Capuron *et al.*, 2004a; Capuron *et al.*, 2004b; Musselman *et al.*, 2001). Additionally, increases in serum and cerebrospinal (CSF) levels of pro-inflammatory cytokines (Dantzer *et al.*, 2008; Dowlati *et al.*, 2010; Raison *et al.*, 2006) and a concurrent decrease in levels of the anti-inflammatory cytokine IL-10 (Dhabhar *et al.*, 2009) has been widely reported in MDD patients. Furthermore, successful antidepressant therapy is associated with a normalisation of cytokine levels (Gazal *et al.*, 2013), thus indicating that immune alterations may be a trait marker for MDD. Despite the wealth of data indicating altered immune functioning both basally and in response to TLR activation in MDD, there has been a lack of studies directly examining if such alterations are also associated with altered TLR expression. Recent data examining the expression of TLRs in PBMCs revealed higher expression of TLR3, 4, 5 and 7 and lower expression of TLR1 and 6 in depressed patients. Furthermore, regression analysis revealed that TLR4 expression was an independent risk factor relating to the severity of MDD (Hung *et al.*, 2014). Additional studies have revealed that the expression of TLR3 and TLR4 mRNA is enhanced in post-mortem tissues from the dorsolateral prefrontal cortex of depressed subjects, and protein expression of these receptors increased in depressed suicide victims

(Pandey *et al.*, 2014). Similarly, preclinical studies have shown that in a stress-based model of depression, TLR4 expression in the prefrontal cortex is enhanced; effects associated with NF κ B activation and enhanced iNOS and COX2 expression (Garate *et al.*, 2014). Thus, depression appears to be associated with alterations in central and peripheral expression of TLRs which may account for the heightened inflammatory state associated with the disorder [for excellent review of role of TLR4 in depression see (Liu *et al.*, 2014)]. A wealth of preclinical evidence has demonstrated that activation of TLR4 is associated with depressive-like behaviour, effects attenuated by antidepressant and anti-inflammatory agents (O'Connor *et al.*, 2009; Salazar *et al.*, 2012; Wang *et al.*, 2011). Similarly, recent studies have demonstrated that TLR3 activation is associated with neuroinflammation, acute sickness behaviour and prolonged depressive-like behaviour (Gibney *et al.*, 2013). The TLR-induced neuroinflammatory cascade has been shown to modulate various glial and neuronal proteins, including increased indoleamine 2,3-dioxygenase (IDO) activation, a rate limiting enzyme in tryptophan production, reduced serotonin production and enhanced formation of the neurotoxins quinolinic acid and kynurenine. Furthermore, activation of this signalling pathway is associated with increased glutamate neurotransmission and excitotoxicity, reduced BDNF and neurogenesis, activation of neurodegenerative cascades and altered HPA axis functionality [for review (Dantzer *et al.*, 2011; Maes, 2011; Song *et al.*, 2011; Zunszain *et al.*, 2013)]; effects may underlie pathophysiology of inflammation-associated depression. Thus, modulation of TLR-induced innate immune responses may provide a novel therapeutic target for depression, and as highlighted previously, the endocannabinoid system may provide a route towards such modulation.

In accordance, dysregulation of the endocannabinoid system has also been demonstrated in MDD. For example, CB₁ receptor density has been shown to be increased in the prefrontal cortex of depressed suicide victims (Hungund *et al.*, 2004), while reduction in CB₁ receptor density has been reported in grey matter glia (Koethe *et al.*, 2007). Serum levels of endocannabinoids have been reported to be reduced in patients with major depression (Hill *et al.*, 2008). Recent studies have also indicated that genetic variations in the CB₁ and CB₂ receptor and FAAH may influence depressive symptoms and antidepressant treatment responding (Domschke *et al.*, 2008; Juhasz *et al.*, 2009; Monteleone *et al.*, 2010; Onaivi *et al.*, 2008). Similarly, genetic deletion of the FAAH or overexpression of CB₂ receptor in mice elicits a stress-resilient (antidepressant-like)

phenotype, while in comparison, CB₁ receptor knockout mice are particularly susceptible to stress-related impairments in emotional responding [see (McLaughlin *et al.*, 2012) (Garcia-Gutierrez *et al.*, 2010)]. Furthermore, several reports have shown stress-induced alterations in the endocannabinoid system and that modulation of the endocannabinoid function exerts anti-depressant-like effects in several animal models of depression [for detailed reviews see (Micale *et al.*, 2013; Saito *et al.*, 2010; Zajkowska *et al.*, 2014)]. However, to our knowledge there have been no studies to date examining if altering TLR-associated inflammatory processes may underlie the antidepressant-like effects of endocannabinoid modulation. Indirect support of this as a possible mechanism is provided by the research demonstrating that endocannabinoids modulate TLR-induced inflammatory responding both peripherally and centrally (see earlier sections). Central CB₁ receptors are critical in mediating TLR4-induced hypothermic/fever response, HPA axis activation and enhanced circulating levels of TNF α (Duncan *et al.*, 2013; Steiner *et al.*, 2011) and TLR3/4 activation is known to induce depressive symptomology, an effect dependant on neuroinflammatory processes (Gibney *et al.*, 2013; Salazar *et al.*, 2012). Furthermore, repeated immobilisation/acoustic stress elicits a neuroinflammatory response that is mediated by TLR4 (Garate *et al.*, 2014) and results in depressive-like behaviour (Kiank *et al.*, 2006). Pharmacological activation of CB₁ or CB₂ receptors attenuates, while genetic deletion of these receptors augments repeated stress-induced pro-inflammatory responses and cellular oxidation in the frontal cortex (Zoppi *et al.*, 2014; Zoppi *et al.*, 2011) and cannabinoids attenuate the reduction in hippocampal neurogenesis and depressive-like behaviour induced by chronic stress (Segev *et al.*, 2014; Zhong *et al.*, 2014). Thus, while the evidence is primarily anecdotal to date with further studies required, endocannabinoid-modulation of TLR-associated neuroinflammation may provide a novel antidepressant strategy for MDD.

Endocannabinoid regulation of TLR-induced inflammation: possible implications for the treatment of schizophrenia

Schizophrenia is a chronic and debilitating psychiatric disorder affecting approximately 1% of the world's adult population. Over the last number of years there has been increased focus on the role of immune-inflammatory responses in the disease pathophysiology (Bergink *et al.*, 2014; Monji *et al.*, 2009; Na *et al.*, 2012; Zakharyan *et al.*, 2014). In addition, a recent study has demonstrated that TLR3 and TLR4 expression is enhanced on monocytes from schizophrenic patients (Muller *et al.*, 2012). However, conflicting data have been reported on TLR-induced inflammatory responses in schizophrenic patients. For example, Muller and colleagues demonstrated a blunted enhancement in the expression of TLR3 and TLR4 receptors, and IL-1 β release following the stimulation of monocytes (Muller *et al.*, 2012) while in comparison, McKernan *et al.*, showed that TLR4-stimulated whole blood cultures from schizophrenic patients exhibited augmented IL-1 β release when compared to controls (McKernan *et al.*, 2011). These discrepant findings may relate to the methodological differences between the studies, however when taken together, the data suggest that schizophrenia is associated with an altered innate immune response. Further support for a possible role of TLRs in the pathogenesis of schizophrenia arises from the considerable data demonstrating that early prenatal exposure to TLR agonist's results in neuroinflammatory, neurodevelopmental and behavioural alterations in the offspring that resemble those observed in schizophrenia. Detailed consideration of the mechanisms and role of TLRs in the development of these alterations has been covered in detail elsewhere [see (Ibi *et al.*, 2011; Patterson, 2009; Venkatasubramanian *et al.*, 2013)] and is beyond the scope of this review. However, the data indicated that anti-inflammatory and/or anti-cytokine drugs may represent novel therapeutics in psychiatric disorders including schizophrenia. Accordingly, administration of the COX-2 inhibitor celecoxib, has been shown to be associated with improvements in both positive and negative symptoms in patients suffering from first episode of schizophrenia (Muller *et al.*, 2010), and also improved positive symptoms in patients with prolonged schizophrenia (Akhondzadeh *et al.*, 2007). As COX inhibitors are known to be associated with cardiovascular and gastrointestinal toxicities, alternative anti-inflammatory therapies need to be explored. So the question arises as to whether the endocannabinoid system could be a potential treatment target for schizophrenia.

Over the last number of years, there has been increased interest in the potential relationship between the endocannabinoid system and schizophrenia which has been examined in detail in a number of recent reviews (Fernandez-Espejo *et al.*, 2009; Muller-Vahl *et al.*, 2008; Saito *et al.*, 2013; Tan *et al.*, 2014). Several studies have examined the interaction between exposure to potent synthetic or plant-derived cannabinoids during critical stages of development such as during adolescence, and how this may affect brain functioning and behaviour relevant to schizophrenia in vulnerable populations (double-hit theory; see (Realini *et al.*, 2009)). However, it is unknown if exposure to cannabis (or other synthetic cannabinoids) is an independent risk factor for schizophrenia or a means of self medication. What is known though is that schizophrenia is associated with altered endocannabinoid tone, with reports of enhanced CB₁ receptor binding in the prefrontal cortex of schizophrenic patients (Dean *et al.*, 2001; Zavitsanou *et al.*, 2004), although studies demonstrating no change (Deng *et al.*, 2007; Koethe *et al.*, 2007) or a reduction in density or expression (Eggan *et al.*, 2008; Eggan *et al.*, 2010; Uriguen *et al.*, 2009) have also been reported. Recent studies have indicated that schizophrenia is associated with two SNPs in the CB₂ receptor gene, mutations responsible for reduced receptor expression and functionality (Ishiguro *et al.*, 2010), and patients with first-episode psychosis have been shown to exhibit a decreased expression of CB₂ receptors in isolated PBMCs in comparison to healthy controls (Bioque *et al.*, 2013). Similarly, CB₂ receptor knockout mice exhibit a schizophrenia-related behavioural phenotype (Ortega-Alvaro *et al.*, 2011). Increased levels of AEA in the CSF of patients have been shown to negatively correlate with psychotic symptoms (Giuffrida *et al.*, 2004; Koethe *et al.*, 2009) and schizophrenic subjects have lower post-mortem levels of AEA in the cerebellum, hippocampus and prefrontal cortex and higher levels of 2-AG (Muguruza *et al.*, 2013). In addition, several clinical and preclinical studies have demonstrated beneficial effects of non-selective cannabinoid receptor agonists, CB₁ receptor antagonists/inverse agonists or CB₂ receptor agonists on both positive and negative symptoms of schizophrenia [for review see (Kucerova *et al.*, 2014; Roser *et al.*, 2012)]. Additionally, the antipsychotic effects of the phytocannabinoid CBD has been extensively reported in preclinical models and is currently being evaluated in a number of ongoing clinical trials [for detailed review see (Zuardi *et al.*, 2012)]. While endocannabinoids may directly influence neuronal functioning and plasticity, modulation of immune function has been proposed as the link between cannabinoids and

psychosis [for review see (Suarez-Pinilla *et al.*, 2014)]. However, as in the case of MDD, no studies to date have examined if modulation of TLR-induced inflammation underlies the anti-psychotic effects of cannabinoids. Recent data has demonstrated a beneficial effect of CB₂ receptor agonism on MK-801-induced deficits in prepulse inhibition (Khella *et al.*, 2014), and although the authors propose that this is likely mediated by direct CB₂-induced changes in neurotransmission, it is also likely that given the high expression of these receptors on immune cells that modulation of inflammatory process may play a role. Accordingly, we have provided an overview of the data indicating that enhancing endocannabinoid tone is associated with a decrease in TLR-induced pro-inflammatory cytokines and a concurrent increase in the anti-inflammatory cytokines such as IL-10 (see above sections). Similarly, antipsychotics are known to also modulate TLR-induced inflammation (increasing IL-10 and reducing TNF α) (Sugino *et al.*, 2009; Szuster-Ciesielska *et al.*, 2004) and genetic enhancement of IL-10 has been demonstrated to be associated with an attenuation of pre-pulse inhibition and latent inhibition in adult offspring of dams exposed to the TLR3 agonist poly I:C (Meyer *et al.*, 2008). Thus, it is possible that endocannabinoid modulation of TLR-immune responses may provide a novel therapeutic target for schizophrenia.

Conclusion

Under normal physiological conditions, TLRs play a pivotal role in mediating host defences against invading pathogens and maintaining homeostasis however, aberrant or uncontrolled TLR signalling is associated with acute and chronic inflammation which may predispose or exacerbate existing disorders. Furthermore, accumulating evidence indicates that uncontrolled TLR signalling in the CNS may underlie, at least in part, the pathophysiology of neurodegenerative and psychiatric disorders [for reviews see [(Hung *et al.*, 2014; Pandey *et al.*, 2014; Trotta *et al.*, 2014)]. The studies reviewed herein demonstrate that the endocannabinoid system modulates TLR-induced inflammatory responses, with the greatest evidence supporting a role in TLR4-mediated events. The effects observed are often bi-directional, depend on the investigative conditions, the timing of modulation and the type of (endo)cannabinoid/receptor modulated. Furthermore, effects observed in studies examining endocannabinoid modulation of TLR4 immune responses cannot be generalised to those elicited by other TLRs. For example, while (endo)cannabinoids, for the most part, attenuate TLR-induced NF κ B activation in a variety of experimental setting [see table 1-6], differential effects of canabinoids are observed in relation to IRF3 activation in response to TLR3/4. The synthetic cannabinoid WIN55,212 and the FAAH inhibitor URB597 were found to augment TLR3-induced type 1 interferon expression/production, while WIN55,212 attenuated IRF3 activation in response to TLR4 activation (Downer *et al.*, 2011; Henry *et al.*, 2014). Furthermore, fever in response to LPS, but not poly I:C, is blocked in CB₁^{-/-} mice, indicating a role of CB₁ receptors in mediating the hyperthermic response to TLR4, but not TLR3 activation (Duncan *et al.*, 2013). Thus, while evidence indicated that the endocannabinoid system modulates TLR-induced inflammatory responses, further studies investigating receptor and molecular mechanism underlying the effects on TLR-induced immune responses are required. In addition, this review has highlighted the lack of direct evidence for endocannabinoid modulation of TLR-neuroinflammatory responses as a possible treatment strategy for psychiatric conditions such as MDD and schizophrenia. This is an area ripe for further investigation, particularly given the wide array (>150 over the past decade) of

cannabis-based entities in clinical trials for a variety of psychiatric and neurodegenerative disorders [International Clinical Trials Registry Platform], disorders known to have a neuroinflammatory component. Currently three synthetic cannabinoids have been licenced and are used clinically; Cesamet® (nabilone) prescribed for the relief of chemotherapy-induced nausea and vomiting, Marinol® (dronabinol; THC) for appetite stimulation and Sativex® (THC:cannabidiol) for control of cancer/neuropathic pain and spasticity in patients with multiple sclerosis. However, as these agents induce their activity via modulation of central CB₁ receptors, there is particular interest in the development of cannabinoid-based pharmaceuticals that are not associated with adverse CB₁ receptor associated psychoactive effects. Peripherally restricted CB₁ receptor agonists/antagonists have been developed and demonstrated to modulate nociceptive responding and metabolism (Cluny *et al.*, 2010a; Cluny *et al.*, 2010b; Yu *et al.*, 2010), however the effects on TLR-associated inflammation, peripherally or centrally, remains to be evaluated. Given the high expression of CB₂ receptors on immune cells it is not surprising that CB₂-selective agonists are considered to have multiple therapeutic applications for the relief of symptoms of neurodegenerative, immunological, and cardiovascular diseases [reviewed in (Pacher *et al.*, 2006; Pertwee, 2012)], however global immunosuppression will need to be considered in the use of these agonists. As endocannabinoids are synthesised on demand, preventing the breakdown of endocannabinoids at sites/tissues where they can elicit the most potent effects may have significant therapeutic benefit with less adverse side effects (Pertwee, 2014). Highlighted throughout this review, this approach has been shown to modulate TLR-induced inflammatory responses both peripherally and centrally, and elicit antidepressant and antipsychotic effects in several preclinical model systems (see previous sections). Of particular note was the data demonstrating that enhanced AEA tone following FAAH inhibition appears to modulate TLR-induced responses at the level of the CNS, thus possibly involving CB₁ receptor activation (Henry *et al.*, 2014; Kerr *et al.*, 2012). However, several lines of evidence indicate that MAGL/ABHD6 inhibitors can potently inhibit neuroinflammatory processes in a mechanism alternate to 2-AG associated central CB₁ receptor activation (Alhouayek *et al.*, 2013; Kerr *et al.*, 2013b; Nomura *et al.*, 2011), thus providing a means of

treating neuroinflammatory disorders that would be devoid of the potential adverse psychological effects. While this area of research is at a relatively early stage of investigation, the data to date indicate that targeting the endocannabinoid system may provide a novel and more efficacious treatment target for various diseases, in particular psychiatric and neurodegenerative conditions, where an accompanying TLR-mediated inflammatory component may be evident.

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