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Endocannabinoid modulation of TLR4-induced neuroinflammation: implications for autism spectrum disorders

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Abstract

Neuroinflammation is a key component of various neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis and psychiatric and neurodevelopmental disorders such as depression and autism. Accumulating evidence indicates that the endocannabinoid system may play a significant role in modulating immune function and represent an important therapeutic target in the treatment of inflammatory disorders. Thus, one of the main objectives of the work presented in this thesis was to enhance our understanding of the role of the endocannabinoid system in the modulation of peripheral and central acute inflammatory responses *in vivo*. The data presented herein demonstrated for the first time that systemic administration of URB597, an inhibitor of fatty acid amide hydrolase (FAAH), the primary enzyme responsible for the metabolism of the endocannabinoid anandamide, enhanced N-acylethanolamine levels (including anandamide), an effect associated with an attenuation of the TLR4-induced expression of the pro-inflammatory cytokine IL-1 β in the hypothalamus. In comparison, URB597 did not alter TLR4-induced cytokine or corticosterone levels peripherally, indicating that the anti-inflammatory effect observed in the hypothalamus is centrally mediated. The data presented also demonstrate that systemic administration of JZL184, an inhibitor of the 2-arachidonoyl glycerol catabolising enzyme, monoacylglycerol lipase (MAGL), to rats, inhibits MAGL activity and enhances 2-AG levels peripherally, but not centrally. Despite this, JZL184 attenuated TLR4-induced increases in cytokine expression/levels both peripherally (TNF- α and IL-10) and in the brain (IL-1 β , IL-6, TNF- α and IL-10). These data indicate that endocannabinoid modulators such as JZL184 may not need to cross the blood brain barrier to induce potent anti-inflammatory effects centrally. This has important therapeutic implications because such a treatment strategy would avoid the potential psychotropic side effects associated with direct central CB₁ receptor activation.

The aforementioned studies demonstrate an important role for FAAH and MAGL in modulating neuroinflammatory responses following TLR4 stimulation, thus increasing our knowledge of endocannabinoid-neuroimmune interactions *in vivo*. Given that endocannabinoids elicit such profound effects on inflammatory processes, a further aim of this work was to characterise immune and endocannabinoid changes in a clinically relevant animal model of autism, the valproic acid (VPA) rat model. Data presented in this thesis demonstrate for the first time that rats prenatally exposed to VPA exhibit increased neuroinflammatory responses, namely enhanced IL-1 β and CCL2 mRNA expression in the cortex, TNF- α expression in the hippocampus and GFAP and IL-1 β expression in the cerebellum, following TLR4 stimulation, data correlating with previously published clinical findings. In addition, VPA-exposed rats were found to exhibit alterations in various components of the endocannabinoid system, including reduced expression of the 2-AG synthesising enzyme DAGL α in the cerebellum, reduced expression and increased activity of MAGL in the hippocampus, reduced expression of the endocannabinoid targets PPAR α and GPR55 in the frontal cortex, and reduced expression of PPAR γ and GPR55 in the hippocampus. Thus, VPA-exposed animals exhibit alterations in various aspects of the endocannabinoid system in key brain regions that modulate social and emotional processing. Furthermore, the data presented indicate that VPA-exposed animals display deficits in social behaviour on exposure to a novel conspecific animal, an effect associated with increased levels of anandamide, OEA and PEA in the hippocampus. Increases in N-acylethanolamines in the hippocampus may be a compensatory mechanism in VPA-exposed animals to overcome the stress associated with social exposure. Thus, alterations in neuroinflammatory processes and/or the endocannabinoid system may underlie some of the behavioural abnormalities observed in the VPA model. Endocannabinoid-neuroimmune interactions may provide a novel therapeutic target for some of the symptoms associated with autism.

In conclusion, the data presented in this thesis have enhanced our understanding of endocannabinoid modulation of neuroimmune processes, effects which may have important implications for the treatment of a host of neuroinflammatory disorders including autism.

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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 apart from the contributions acknowledged below the work presented was entirely my own. Dr Nikita Burke assisted with tissue collection and analysis of gene expression. Brendan Harhen and Dr Gemma Forde contributed to the development and validation of mass spectrometry protocols (Chapter 3-4). Dr Tom Connor provided invaluable training, assistance and advice with qRT-PCR (Chapter3). Dr Bright Okine established the MAGL and FAAH activity assays in the laboratory (Chapter 4). Dr Michelle Roche, Lisa Downey and Michelle Conboy conducted the behavioural characterization of the valproic acid animal model of autism (presented in Chapter 6).

This thesis, or any part of, had not been submitted to the National University of Ireland Galway, or any other institution, in connection with any other 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
ABHD12, α/β -hydrolase 12
ABHD6, α/β -hydrolase 6
AC, adenylyl cyclase
AEA, anandamide
AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide
AM630, [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)-methanone
ASD, autism spectrum disorder
ATP adenosine triphosphate
BAFF, B-cell activating factor
BDNF, brain derived neurotrophic factor
bFGF, basic fibroblast growth factor
c-AMP, cyclic adenosine monophosphate
CB₁, cannabinoid receptor 1
CCL4, Chemokine (C-C motif) ligand 4
CCL5, RANTES, Chemokine (C-C motif) ligand 5
CD11b, cluster of differentiation molecule 11b
CD14, cluster of differentiation 14
CIS, cytokine-inducible SH2 proteins
c-JNK, c-jun N-terminal kinase
c-JUN, c-jun family of transcription factors
Con A, concanavalin A
COX2, cyclooxygenase 2
Ct, copy threshold
CXCL10, C-X-C motif chemokine 10
DAG, diacylglycerol
DAGL, diacylglycerol lipase
DAMPs, damage-associated molecular patterns

DSM IV, Diagnostic and Statistical Manual of Mental Disorders IV
EIA, Enzyme Immunoassay
ELISAs, enzyme-linked immunosorbent assays
EPM, elevated plus maze
ERK1/2, extracellular signal-regulated kinase 1/2
eV, electron volt
FAAH, fatty acid amide hydrolase
FAK, focal adhesion kinase
FLAT, FAAH-like AEA transporter
G(1), gestation day 1
GABA, gamma-aminobutyric acid
GDE1, glycerophosphodiester phosphodiesterase 1,
GDNF, glial-derived neurotrophic factor
GFAP, glial fibrillary acidic protein
GLT1, glial glutamate transporter 1
GM-CSF, Granulocyte-macrophage colony-stimulating factor
GPCRs, G-protein coupled receptors
GPR55, G protein-coupled receptor 55
GS, glutamine synthase
HPA axis, Hypothalamic-pituitary-adrenal axis
HPLC, high pressure liquid chromatography.
ICAMS, intercellular adhesion molecules
IFN, interferon
IkB α , inhibitor of NF-kB, inhibitor of k light chain gene enhancer in B cells (IkB)
IKK, IkB kinase
IL, interleukin
IL-1ra, IL-1receptor antagonist
iNOS, inducible nitric oxide synthase
IP10, Interferon gamma-induced protein 10
IRAK-4, IL-1 receptor-associated kinase-4
IRF, interferon regulatory transcription factor
JAK, janus kinase
JAK/STAT, Janus Kinase /signal transducer and activator of transcription

JZL184, 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy) methyl)piperidine-1-carboxylate methyl)piperidine-1-carboxylate

LBP, LPS binding protein

LC-MS/MS, liquid chromatography coupled to mass spectrometry.

LOX, lipoxygenases

LPS, lipopolysaccharide

Lyso-PLC, lysophospholipase C

m/z, mass charge ratios

MAGL, monoacylglycerol lipase

Mal, MyD88-adaptor-like

MAPK, mitogen-activated protein kinase

MCP-1 (CCL2), monocyte chemotactic protein-1 Chemokine (C-C motif) ligand 2

MIA, maternal immune activation

MIP-1 β , Macrophage Inflammatory Proteins-1 β MRM, multiple reaction monitoring

MS, multiple sclerosis

MyD88, Myeloid differentiation primary response gene 88

NAAA, N-acylethanolamine-hydrolyzing acid amidase

NADA, N-arachidonoyl-dopamine

NAEs, N-acylethanolamines

NAPE, N-arachidonoylphosphatidylethanolamine

NAPE-PLD, NAPE-phospholipase D

NAT, N-acyltransferase

NF- κ B, Nuclear factor kappa B

NGF, nerve growth factor

NO, nitric oxide

Noladin ether, 2-arachidonoyl glycerol ether

OEA, *N*-oleoylethanolamide

OFT, open field test

PA, phosphatidic acid

PAG, periaqueductal gray matter

PAMPs, pathogen-associated molecular patterns

PBMCs, peripheral blood mononuclear cells

PBS, phosphate-buffered saline

PC, phosphatidylcholine
PCR, polymerase chain reaction
PE, phosphatidylethanolamine
PEA, *N*-palmitoylethanolamide
PGD2, prostaglandin D2
PGE2, prostaglandin E2
PI(3)K, phosphatidylinositide 3-kinase
PI, Phosphatidylinositol
PKA, protein kinase A
PLA1, phospholipase A1
PLC, phospholipase C ,
PND(21), Post natal day 21
PPAR, peroxisome proliferator-activated receptor
PPARs, peroxisome proliferator-activated receptors
PPP, pattern recognition receptors
PPRE, PPAR-responsive elements
PTPN22, Protein tyrosine phosphatase, non-receptor type 22
qRT-PCR, quantitative Real-Time Polymerase Chain Reaction
QTOF, quadrupole time of flight
RIP1, receptor interacting protein 1
RXR, retinoid X receptors
SARM, Sterile α and heat-armorillo motifs-containing protein
SHIP, Src homology 2 domain-containing inositol-5-phosphatase1
SOCS, suppressor of cytokine signalling
STAT, signal transducer and activation of transcription
TAD, transcriptional activation domains
TAK1, transforming growth factor- β -activated kinase 1
TANK , TRAF family member-associated NF κ B activator
TBK1, TANK binding kinase 1
TE, Tris-EDTA
TGF β , transforming growth factor beta
TGF β , transforming growth factor beta
THC, tetrahydrocannabinol
TIR, Toll-interleukin-1 receptor

TIRAP, TIR adaptor protein

TLR, Toll-like receptors

TNF, tumour necrosis factor

TRAF6, TNF receptor-associated factor 6

TRAM, TRIF-related adaptor molecule

TRIF, TIR domain-containing adaptor inducing IFN- β

TRPVI, transient receptor potential vanilloid 1

UBC13, ubiquitin-conjugating enzyme13

UEV1A, ubiquitin-conjugating enzyme E2 variant1 isoform A

URB597, [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate

Virodamine, O-arachidonoyl ethanolamine

VPA, valproic acid

Publications

Peer reviewed papers:

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

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Data generated as part of PhD studies has been presented at the following local, national and international conferences

- *Galway Neuroscience Centre Research Day (Dec 2013)*
- *Neuroscience Ireland Annual Meeting, Cork (Sept 2013)*
- *International Behavioural Neuroscience Symposium, Dublin (June 2013)*
- *Royal Academy of Medicine in Ireland, Biomedical Science Section Annual Meeting, Cork (June 2013)*
- *NUIG-UL Engineering, Science and IT Research Day, Galway (April 2013)*
- *6th European Workshop on Cannabinoid Research, Dublin (April 2013)*
- *Galway Neuroscience Centre Research Day (Dec 2012)*
- *Neuroscience Ireland Annual Meeting, Dublin (Sept 2012)*
- *Royal Academy of Medicine in Ireland, Biomedical Science Section Annual Meeting, Galway (June 2012)*
- *22nd Annual Symposium on Cannabinoids, International Cannabinoid Research Society, Germany (June 2012)*
- *Galway Neuroscience Centre Research Day (Dec 2011)*
- *Neuroscience Ireland Annual Meeting, Maynooth, (Sept 2011)*
- *Cost Action: Inflammation in Brain Disease – Neuroinfnet UCD Dublin (June 2011)*
- *Neuroscience Ireland Annual Meeting, Dublin (Sept 2010)*
- *20th Annual Symposium on the Cannabinoids, International Cannabinoid Research Society, Sweden (June 2010)*

Chapter 1

General Introduction

1.1 Inflammation and the innate immune system

The innate immune system represents the first line of host defence following infection and therefore is critical for the early detection and subsequent triggering of a suitable pro-inflammatory response to invading organisms. Whereas the innate immune system is activated soon after the invasion of the host and is responsible for initiating an immunological response during the early stages of the infection, the adaptive or acquired immune response is responsible for the elimination of pathogens in the late phase of infection and in the production of immunological memory. The innate response was once considered to be non-specific, while specificity of the adaptive response is considered to be mediated by T- and B-lymphocytes that express highly diverse antigenic receptors which recognise a wide variety of invading pathogens and foreign substances. However, this view has now changed as it has been shown that cells of the immune system such as macrophages, fibroblasts, mast cells, neutrophils and dendritic cells, as well as circulating leukocytes, recognize pathogen invasion or cell damage via intracellular or surface-expressed pattern recognition receptors (PRRs). These receptors detect pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins and carbohydrates, or damage-associated molecular patterns (DAMPs) released from injured cells. Activated PRRs then oligomerise and initiate signalling cascades that trigger the release of pro-inflammatory cytokines, and inducible interferons (IFNs) and other inflammatory mediators that co-ordinate the elimination of the pathogens and infected cells. The ability of the innate immune system to recognise and eliminate pathogens employs complement activation, phagocytosis, autophagy and immune activation by different families of PRRs. [For reviews of innate immunity and pathogen host interaction see (Basset et al., 2003; Akira et al., 2006)].

One particular class of PRRs is that of the Toll-like receptors (TLRs), Type-I transmembrane glycol proteins originally discovered based on homology to the *Drosophila melanogaster* Toll protein (Medzhitov et al., 1997). To date, 10 TLRs have been identified in humans and they each recognise particular PAMPs associated with viruses, bacteria and fungi (Akira et al., 2006). TLRs may be divided into subgroups based on their ability to recognise particular PAMPs; TLR1, TLR2, TLR4, TLR5 and TLR6 are located at the plasma membrane and sense microbial membrane

components while the intracellular TLR3, TLR7, TLR8 and TLR9 signal at endosomes and sense microbial and viral nucleic acids (Fig 1.1) [For review of TLR signalling see (Akira et al., 2006; Gangloff, 2012)]. The most extensively studied TLR is TLR4, which recognizes lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, activation of which induces a robust immune response both peripherally and centrally. Overall, TLR signalling is crucial for the innate inflammatory response and, if not tightly controlled, can result in conditions such as sepsis, asthma and autoimmune disorders to name but a few (Lehnardt, 2010).

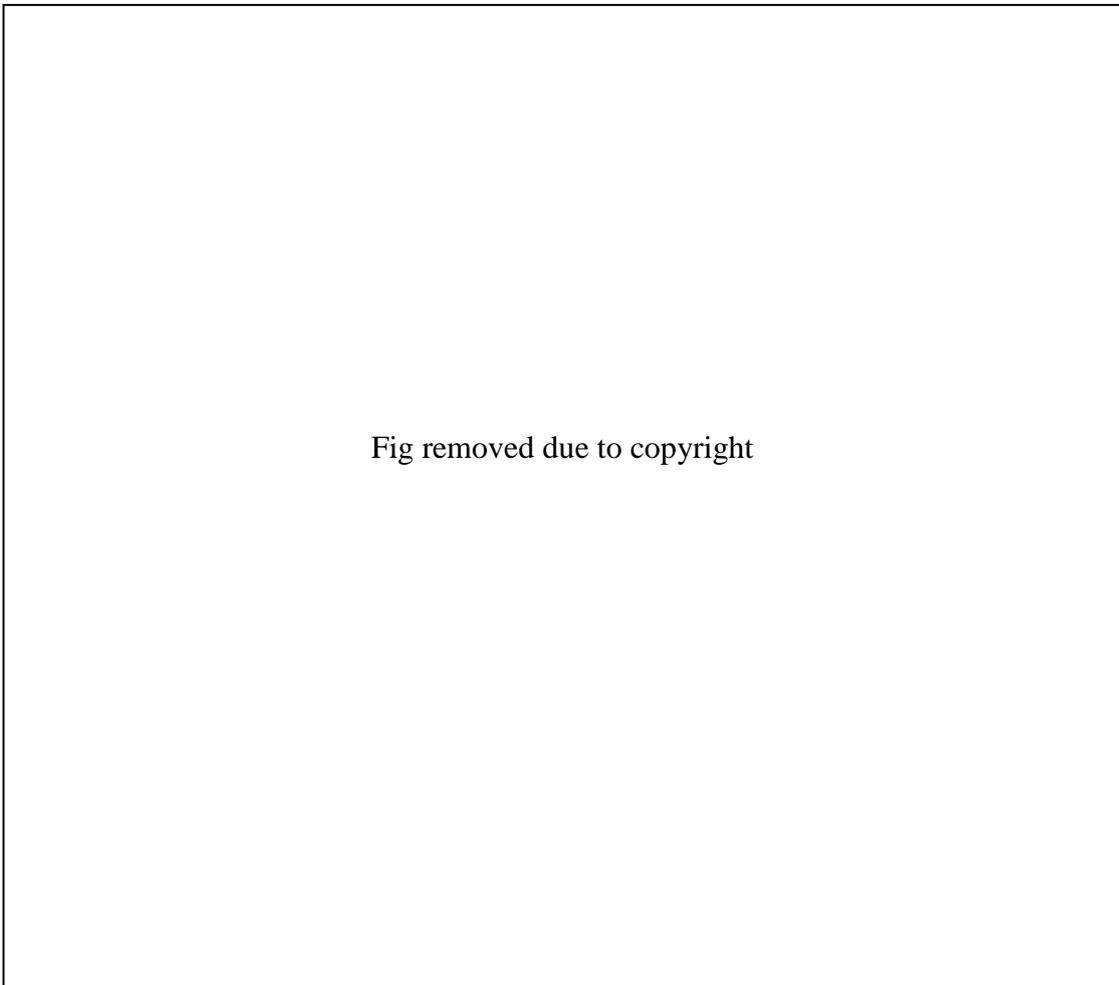


Fig 1.1 TLR Location: There are two types of TLRs, those located at the plasma membrane that sense microbial membrane components and the intracellular ones that sense microbial or viral nucleic acids. TLR4 can signal both at the plasma membrane and at endosomes, where it can be activated by viral envelope glycoproteins. All TLRs signal via the adaptor MyD88, except TLR3 that can function only via TRIF. Membrane signalling triggers an inflammatory response whereas intracellular TLR signalling leads to antiviral and adjuvant responses. (Gangloff, 2012)

1.2 LPS activation of TLR4

LPS stimulation of mammalian cells occurs through a series of interactions with several proteins including the LPS binding protein (LBP), cluster of differentiation 14 (CD14), MD-2 and TLR4 (Gioannini and Weiss, 2007; Miyake, 2007). LBP is a soluble protein which directly binds to LPS and enables the association between LPS and CD14 (Tobias et al., 1986; Wright et al., 1989). CD14 facilitates the transfer of LPS to the TLR4/MD-2 receptor complex and facilitates LPS recognition (Wright et al., 1990). On recognition of LPS, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the Toll-interleukin-1 receptor (TIR) domains which mediate protein–protein interactions between the TLRs and signal transduction adaptor proteins. There are five TIR domain-containing adaptor proteins: Myeloid differentiation primary response gene 88 (MyD88), TIR adaptor protein, also known as MyD88-adaptor-like (TIRAP) (Mal), TIR domain-containing adaptor inducing IFN- β (TRIF), TRIF-related adaptor molecule (TRAM), and Sterile α and heat-armorillo motifs-containing protein (SARM). [For a detailed review of TIR-domain-containing adaptors in TLR signalling see (O'Neill and Bowie, 2007)]. While TLRs use different combinations of adaptor proteins to facilitate downstream signalling, TLR4 is the only known TLR which utilizes all these adaptor proteins to induce an immune response to bacterial infection. Essentially, LPS-TLR4 signalling involves both MYD88-independent and MYD88-dependent pathways where the former was shown to be responsible for the expression of pro-inflammatory cytokines while the latter was shown to mediate the induction of Type I interferons and interferon-inducible genes (Kawai et al., 1999). These pathways have been well characterised [for reviews see (Akira and Takeda, 2004; Lu et al., 2008; Mogensen, 2009; Newton and Dixit, 2012)] and an overview of the signalling systems is presented below.

1.2.1 LPS-TLR4 induced activation of MyD88-dependent pathway

Following LPS stimulation, MyD88 recruits and activates IL-1 receptor-associated kinase-4 (IRAK-4) and IRAK-1/2 (Suzuki et al., 2002). Downstream of IRAK4, IRAK1/2 associate with another important adaptor protein, TNF receptor-associated factor 6 (TRAF6), which forms a complex with ubiquitin-conjugating enzyme13 (UBC13) and ubiquitin-conjugating enzyme E2 variant1 isoform A (UEV1A),

activating transforming growth factor- β -activated kinase 1(TAK1) (Lomaga et al., 1999; Gohda et al., 2004). TAK1 then activates downstream I κ B kinase (IKK) and mitogen-activated protein kinase (MAPK) pathways (Sato et al., 2005). An IKK α , β and γ complex then phosphorylates inhibitor of κ light chain gene enhancer in B cells (I κ B) resulting in the degradation of I κ B proteins and the translocation to the nucleus of the transcription factor NF- κ B which controls the expression of pro-inflammatory cytokines such as interleukin (IL)-1 α , IL-1 β , IL-6 and tumour necrosis factor (TNF) α and other immune related genes. In addition, concurrent activation of the downstream MAPK pathways results in the subsequent induction of the transcription factor AP-1 (Chang and Karin, 2001) which also induces the expression of pro-inflammatory cytokines (Fig 1.2). Binding of pro-inflammatory cytokines to their respective receptors can further activate various signalling cascades including NF- κ B, MAPK and JAK/STAT pathways, further modulating cytokine expression and inflammatory responses. [For detailed review of cytokine receptors and their signalling cascades see (Conti et al., 2008)].

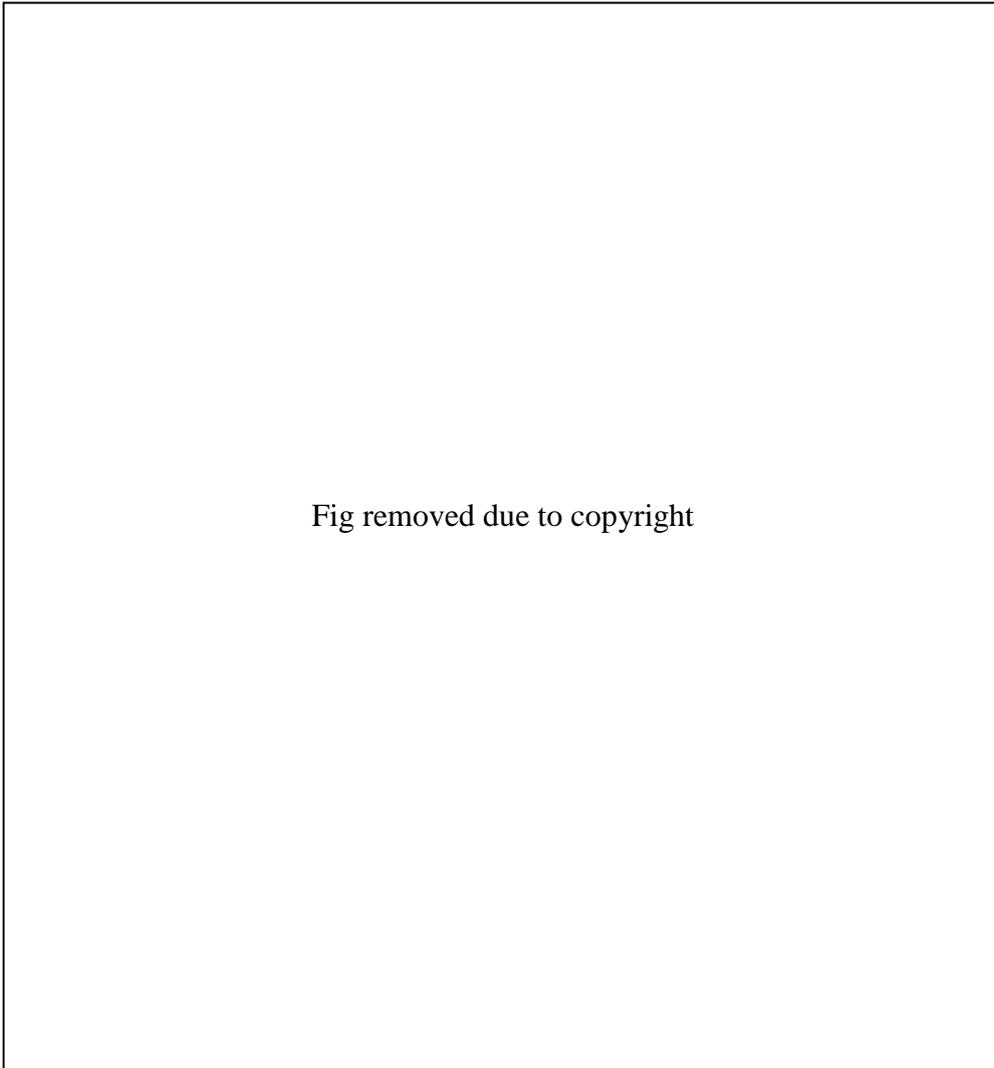


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Fig. 1.2 LPS activation of TLR4. TLR4 activates both the MyD88-dependent and MyD88-independent, TRIF-dependent pathways. The MyD88-dependent pathway is responsible for early-phase NF- κ B and MAPK activation, which control the induction of pro-inflammatory cytokines. The MyD88-independent, TRIF-dependent pathway activates IRF3, which is required for the induction of IFN- β and IFN- β inducible genes. In addition, this pathway mediates late-phase NF- κ B as well as MAPK activation, also contributing to inflammatory responses. (Mogensen, 2009)

1.2.2 LPS-TLR4 induced activation of the MyD88-independent pathway

In addition to activating the MyD88 dependant pathway, TLR4 activation results in stimulation of TRIF. TRIF is an important TIR-containing adaptor protein that mediates MyD88-independent signalling and is essential for the activation of transcription factor IRF3 and the later phase activation of NF-kB and MAPK. TRIF-dependent activation of NF-kB occurs via binding of TRAF6 to TRIF and subsequent recruitment and activation of TAK1 (Sato et al., 2003). To facilitate NF-kB activation, receptor interacting protein 1 (RIP1), involved in TNF-receptor mediated NF-kB activation, is also recruited to TRIF (Meylan et al., 2004). The RIP1-TRAF6, complex enables TAK1 activation, resulting in IKK-mediated activation of NF-kB and activation of the MAPK pathway (Cusson-Hermance et al., 2005). TRIF can also bind to TRAF3 which can associate with TRAF family member-associated NF-kB activator (TANK), TBK1 (TANK binding kinase 1) and IKK (Oganesyan et al., 2006; Guo and Cheng, 2007) to facilitate the phosphorylation and translocation of IRF3 to the nucleus resulting in the transcription of Type I interferons and interferon-inducible genes (Kawai et al., 1999) (Fig 1.2).

In order to limit the detrimental effects of increased levels of cytokines, several highly regulated mechanisms modulate cytokine bioavailability including increased glucocorticoid levels, the presence of soluble binding proteins or receptors, the synthesis of anti-inflammatory cytokines, chemokines (e.g. fractalkine) and suppressor of cytokine signalling proteins (SOCs). These may act in unison to return the immune system to a resting state. SOCs act to inhibit cytokine signalling and activation of the Janus Kinase signal transducer and activator of transcription (JAK/STAT) and NF-kB pathways, thereby down-regulating the expression of a host of pro-inflammatory genes. The NF-kB and JAK/STAT pathways, as well as their negative feedback regulation by SOCs and other mechanisms are described in detail below.

1.2.3 NF- κ B pathway

NF- κ B in particular plays a key role in the MyD88 pathway and is composed of homo- and heterodimers of five members of the Rel family including NF- κ B1 (p50), NF- κ B2 (p52), RelA (p65), RelB and c-Rel (Rel). Hetero- and homo-dimerization of NF- κ B proteins includes p50/RelA, p50/c-Rel, p52/c-Rel, p65/c-Rel, RelA/RelA, p50/p50, p52/p52, RelB/p50 and RelB/p52. Family members p65 (RelA), cRel, and RelB, contain transcriptional activation domains (TAD) at the C terminus and therefore are able to directly activate transcription. p50 and p52 lack a TAD and therefore form homodimers with no intrinsic ability to activate transcription, however, they form transcriptionally active heterodimers in association with p65, cRel, and RelB. This ability to diversely combine results in the regulation of distinct sets of genes. [For review of NF- κ B family of transcription factors see (Oeckinghaus and Ghosh, 2009)].

Activation of NF- κ B plays an important role in inflammation and upregulates the transcription of genes encoding inflammatory mediators such as the pro-inflammatory cytokines IL-1, IL-6, and TNF- α ; chemokines; adhesion molecules; growth factors; and inducible enzymes such as cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) (Baldwin, 1996). As stated earlier, inactive NF- κ B complexed to its inhibitor I κ B resides in the cell cytoplasm until phosphorylation of its inhibitor allows NF- κ B dimers to translocate from the cytoplasm to the nucleus thereby facilitating the transcription of various genes (Chen et al., 1995) (Fig 1.3).

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Fig 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, 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.

I κ B is one of the main genes transcribed following NF- κ B activation and consequently rebinds to NF- κ B dimers in the cytoplasm, thereby preventing its translocation to the nucleus. In this manner, I κ B acts as a negative-feedback regulator of the NF- κ B pathway (Fig 1.3). Another negative inhibitor of this pathway is A20, a cytoplasmic protein which has been shown to inhibit TNF α -induced NF- κ B activation and programmed cell death *in vitro* (Opipari et al., 1992; Cooper et al., 1996). It has been shown that A20 binds to the TNF receptor-associated factor-2 (TRAF2), an inhibitor of IKK γ and/or the A20 binding inhibitor of NF- κ B activation (ABIN), thereby providing a mechanism whereby A20 could regulate TNF receptor signalling (Song et al., 1996; Heyninck et al., 1999). In addition, it has been demonstrated in studies using A20 deficient mice that A20 is critically involved in attenuating inflammatory responses by terminating TNF-induced NF- κ B responses *in vivo* (Lee et al., 2000) (Fig 1.4).

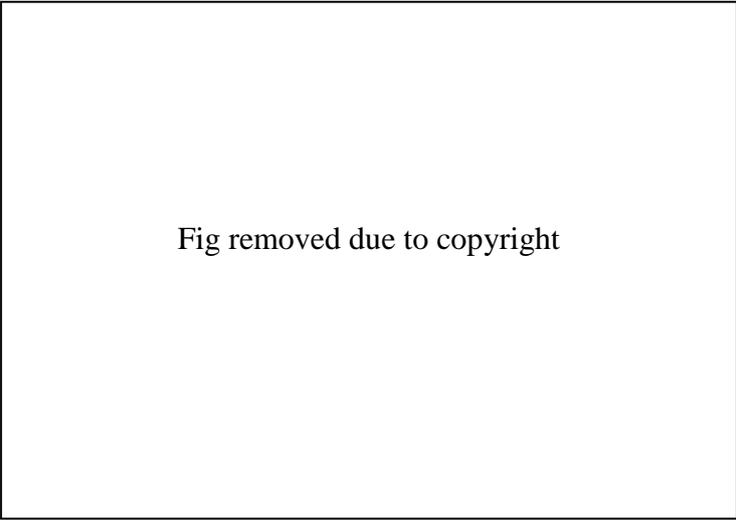


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Fig 1.4. Negative feedback regulation of NF- κ B. In this example, TNF α activates the IKK complex and then phosphorylates I κ B and liberates NF- κ B to the nucleus. I κ B and A20 are induced via NF- κ B transcriptional activity. Modified from (Hanada and Yoshimura, 2002)

1.2.4 JAK/STAT pathway

A further important cytokine signalling pathway is the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. A large number of cytokines, growth factors, and hormones activate the JAK/STAT signalling pathway including the interferon (IFN) family (IFN- α/β , IFN- γ), interleukins (IL-10, IL-19, IL-20, IL-22); the gp130 family (IL-6, IL-11, OSM, LIF, CT-1, G-CSF, IL-12, IL-23), and γ C family, (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) [reviewed in (Schindler and Strehlow, 2000)]. JAKs (janus kinases) represent a family of four tyrosine kinases, Jak1, Jak2, Jak3 and Tyk2 (Ihle, 1996). These kinases phosphorylate STATs, a family of seven structurally and functionally related proteins: STAT1, STAT2, STAT3, STAT4, STAT5a and STAT5b and STAT6, on a single tyrosine residue (Ihle, 1996; Darnell, 1997). Activated STATs dissociate from the receptor, dimerize, translocate to the nucleus and bind to specific response elements in promoters of target genes, thereby mediating the transcription of various genes including suppressors of cytokine signalling (SOCS1 and SOCS3), pro- and anti-inflammatory cytokines, chemokines, intercellular adhesion molecules (ICAMS) and growth factors. Each STAT protein responds to a specific set of cytokines and consequently regulates, with other transcription factors, a specific group of genes [For review on this signalling cascade and STAT target genes see (Heim, 1999; Hanada and Yoshimura, 2002; Yu et al., 2009)]. Once activated, STATs then play a critical role in regulating innate and acquired host immune responses.

Alterations in the JAK/STAT signalling pathway have been identified in a number of human inflammatory diseases including cutaneous T-cell lymphomas, chronic myelocytic leukaemia and immunodeficiency syndromes to name but a few [for detailed review see (Heim, 1999)].

A family of negative regulators of this pathway has also been described, namely the JAK kinase inhibitor proteins, SOCS, or cytokine-inducible SH2 proteins (CIS). The best characterised SOCS family members are CIS, SOCS1, SOCS2 and SOCS3 which act in a classical negative feedback loop to inhibit cytokine signal transduction. [Role in inflammation reviewed in (Yasukawa et al., 2000; Alexander, 2002)]. SOCS1 has been found to be essential for IL-2, IL-4 and IFN- γ signal suppression

and T-cell activation. SOCS1^{-/-} mice die within 3 weeks of birth with severe lymphopenia, activation of peripheral T cells, necrosis of the liver, and macrophage infiltration of major organs, effects attributed to aberrant IFN- γ signalling (Naka et al., 1998; Starr et al., 1998). In comparison, SOCS3 expression is induced by several cytokines including the pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10, increased levels of which act to inhibit JAKs associated with the IL-6, but not IL-10, receptor, thus negatively regulating signalling via this receptor. Therefore, IL-10 may mediate some of its anti-inflammatory effects on IL-6 signalling via enhancing SOCS3 expression (Fig 1.5). For comprehensive reviews of SOCS signalling see (Alexander, 2002; Kubo et al., 2003; Babon and Nicola, 2012).

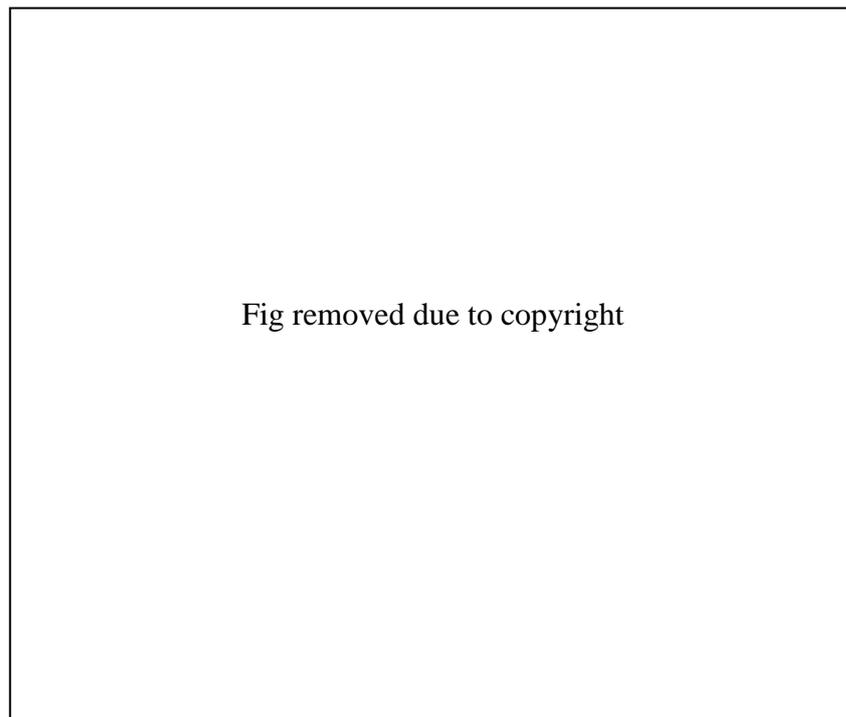


Fig 1.5 IL6 and IL-10 JAK/STAT signalling. IL-6 and IL-10 family ligands activate the gp130 and IL10R receptors, respectively, which subsequently phosphorylate and activate STAT3 through JAK. Activated and dimerized STAT3 translocates into the nucleus to promote the transcription of various molecules, including SOCS3. SOCS3 inhibits JAK and STAT3 activation thereby inhibiting Il-6 signalling. Diagram modified from (Wang et al., 2011).

1.2.5 Physiological consequences of TLR4 activation

Binding of LPS to TLR4 results in NF- κ B activation and translation of pro-inflammatory cytokines including IL-1 α , IL-1 β , TNF α , and IL-6 within 2 hours. This has been consistently demonstrated in several cell lines and *in vivo* (Pitossi et al., 1997; Ulmer et al., 2000; Turrin et al., 2001; Rossol et al., 2011). Cytokines can then induce the production of other cytokines resulting in an inflammatory cascade. IL-1 can induce its own synthesis as well as TNF α and IL-6. These cytokines can then limit their production/action by further inducing the synthesis of anti-inflammatory cytokines such as the IL-1 receptor antagonist (IL-1ra), IL-10 and IL-4 (Chung and Benveniste, 1990; Lee et al., 1993; Weber et al., 2010). Once released, these cytokines act on their respective receptors and mediate their effect by the transcription of a large number of genes which may modulate the inflammatory response. For example, IL-1 binds to Toll-like family receptor IL-1R1, binds MydD88, activates mitogen activated protein (MAP) kinase and NF- κ B transcription. TNF α binds to TNFR-1 and 2 activating transcription via c-JUN and NF- κ B. IL-10 binds to the IL-10R and IL-6 to its IL-6R receptor via the gp130 protein dimer, both of which result in the activation of signal transducers and activators of transcription (STAT)3.

The activation of TLR4 signalling cascades and the production of inflammatory mediators is an important component of the acute inflammatory response to eliminate the pathogen and restore homeostasis. Enhanced levels of pro-inflammatory cytokines IL-1, IL-6 and TNF α and mediators such as prostaglandins act to resolve inflammation in a number of ways, primarily by activation of pathways resulting in the transcription of various genes (described above) which control inflammation. Once transcription factors such as NF- κ B or AP-1 are upregulated, the cell responds either by phagocytosis, transcription of genes producing pro- or anti-inflammatory cytokines, upregulation of co-stimulatory molecules or maturation of antigen presenting cells (Caamano and Hunter, 2002; Tato and Hunter, 2002). Pro-inflammatory cytokines released by activated macrophages induce chemokines and adhesion molecules on the walls of vascular endothelial cells to which neutrophils, monocytes and lymphocytes adhere before migration into tissue. These cytokines also increase coagulation and vascular permeability and in association with IL-8 and

IFN- γ they induce additional effects such as increased chemotaxis for leukocytes and increased phagocytosis. In addition, enhanced pro-inflammatory cytokines upregulate the acute phase response with the production of complement and C-reactive protein, all of which act to resolve infection and return the immune system to a resting state [for a comprehensive review of innate immunity and pathogen-host interaction see (Basset et al., 2003)].

An important component of the immune response is the induction of increases in body temperature (fever) and “sickness” behaviour. Circulating pro-inflammatory cytokines can communicate with the brain via several routes (described in detail below) which further induce cytokine synthesis within the CNS. Increased pro-inflammatory cytokines within the brain can then result in fever (via the production of prostaglandins), hypothalamic-pituitary-adrenal (HPA) axis activation (Rivest et al., 2000; Romanovsky, 2000) and behavioural alterations such as anorexia, anhedonia, hypolocomotion and depressed mood. 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, 2004). While acute inflammation is beneficial and limiting, excessive or persistent inflammation causes tissue destruction and disease. In order to limit the detrimental effects of enhanced cytokines, several highly regulated mechanisms modulate cytokine bioavailability including increased glucocorticoid levels, the synthesis of anti-inflammatory cytokines (such as IL-1ra and IL-10) and release of SOCS proteins. These may act in unison to restore homeostasis.

Thus, the innate immune response is composed of intricate specific signalling cascades which regulate immunomodulatory functions on encountering an invading pathogen. As stated earlier, this cascade is not limited to peripheral responses but also induces alterations at the level of the brain. Cytokines and other immune mediators communicate with the brain and induce a neuro-immune response which, if not tightly regulated, may underlie the development or exacerbation of neurodegenerative and psychiatric disorders.

1.3 Neuroinflammation

Neuroinflammation may be defined as the activation of the immune response in the central nervous system and is characterised by a variety of cellular processes including activation of microglia and astrocytes, increased levels of pro-inflammatory cytokines, chemokines, eicosanoids, complement activation and acute phase proteins. This response is critical to the body's response to fight infection; however, excessive neuroinflammation is associated with several neurodegenerative and psychiatric diseases.

Following infection, increased circulating pro-inflammatory cytokines communicate with the brain via several routes such as via the circumventricular organs; transport across the blood brain barrier or the blood-cerebrospinal fluid barrier after binding to transporter molecules expressed in brain endothelium; binding of cytokines to cerebral vascular endothelium including perivascular macrophages and inducing the production of secondary mediators such as cytokines, prostaglandins and nitric oxide within the brain and; activation of the vagal communication pathway via peripheral and visceral afferent nerve terminals resulting in relayed cytokine-induced signals to the brain [(For review of brain-immune communication pathways see (Dantzer, 2004; Quan and Banks, 2007)].

The immune cells of the brain or glia are comprised of oligodendrocytes, astrocytes and microglia. Microglial cells play a pivotal role in the modulation of the CNS inflammatory network. They are macrophage-like cells that originate from myeloid tissue and migrate into the developing CNS (Rezaie and Male, 1999). In comparison, astrocytes are reportedly derived from two sources: radial glia in the ventricular zone during early development and shortly after birth, and stem cells in the subventricular zone at later stages (Cameron and Rakic, 1991; Marshall et al., 2003; Kriegstein and Alvarez-Buylla, 2009). Similar to peripheral immune cells, glia also express pattern recognition receptors (PRRs) such as TLRs and have the ability to recognise PAMPs and DAMPS, and induce activation of downstream signalling pathways and increased transcription of immune mediators as described above. These cells also recognise proteins from bacterial membranes including peptidoglycans and heat shock proteins as well as adenosine triphosphate (ATP), released in high amounts by

dying cells (Moller et al., 2000; Kim et al., 2001) and various nucleic acid patterns (Kumar et al., 2011).

In the event of infection, trauma or disease, microglia become activated and induce a neuroinflammatory response. Two activation states or phenotypes have been described for microglia, the nature of which is dependent upon the stimulus. The M1 or pro-inflammatory state, observed following stimulation with pro-inflammatory ligands such as IFN- γ or LPS, results in microglia exhibiting high phagocytosis and proteolysis potential and releasing pro-inflammatory cytokines such as IL-1 β , TNF α and IL-6 (Martinez et al., 2008). In the M2 anti-inflammatory or tissue repair state, observed following stimulation by IL-4 or IL-13, microglia secrete anti-inflammatory cytokines IL-10 and adenosine triphosphate (ATP) chemokines and extracellular matrix proteins in order to limit inflammatory responses and promote tissue repair. [For comprehensive reviews on activation phenotypes of microglia see (Varnum and Ikezu, 2012; Boche et al., 2013)].

Astrocytes also play a crucial role in the immune response in the brain. They control endothelial function by the secretion of bioactive molecules such as VEGF, transforming growth factor beta (TGF β), basic fibroblast growth factor (bFGF), TNF α , IL-1 β , IL-3, IL-6, B-cell activating factor (BAFF), and glial-derived neurotrophic factor (GDNF) (Chung and Benveniste, 1990; Igarashi et al., 1999; Abbott et al., 2006; Farina et al., 2007). In addition, they have been shown to express TLR2/3/4/5/9 and synthesise TNF α following LPS stimulation (Chung and Benveniste, 1990). Under normal conditions, astrocytes play a supportive, protective and maintenance role in the brain. However, under inflammatory conditions activated astrocytes have also been shown to mediate both protective and toxic effects depending on the experimental conditions (Maragakis and Rothstein, 2006).

Although such an immune response mediated by microglia, astrocytes and other cells of the CNS is beneficial in controlling acute infection or injury, when uncontrolled, increased pro-inflammatory cytokines within the brain can result in neuroanatomical and neurochemical alterations that may underlie the pathophysiology associated with various CNS pathologies. In accordance with this, neuroinflammation is now a well-recognised component of neurodegenerative disorders such as Alzheimer's disease (McGeer and McGeer, 2001), multiple sclerosis (MS) (Compston and Coles, 2008),

Parkinson's disease (Nagatsu and Sawada, 2005) and amyotrophic lateral sclerosis (ALS) (McGeer and McGeer, 2002).

Furthermore, increasing evidence indicates that neuroinflammatory mechanisms may also play a role in the pathophysiology of neurodevelopmental and psychiatric disorders such as autism spectrum disorder (ASD). Peripheral and central immune function has been reported to be altered in autistic children when compared to healthy children. Enhanced levels of cytokines in plasma and blunted responses by peripheral immune cells to various TLR agonists have been reported (Croonenberghs et al., 2002; Jyonouchi et al., 2008; Ashwood et al., 2011b), as well as enhanced activation of microglia and astroglia, elevated cytokines and chemokines in the brain and CSF of autistic individuals (Vargas et al., 2005; Li et al., 2009).

Thus, aberrant immune responses may underpin, at least in part, some of the symptoms associated with ASD and other neuropsychiatric and neurodegenerative disorders. As such, modulation of such responses provides a therapeutic target for these conditions. Several lines of research now indicate that cannabis-like compounds (cannabinoids) and modulation of the body's own cannabinoids (endocannabinoids) may provide a novel therapeutic target for the treatment of disorders with an immune component [reviewed in (Di Marzo et al., 2004; Di Marzo, 2008c)]. However, while much of this research has been conducted *in vitro*, *in vivo* whole-system studies examining the responses and mechanisms underlying endocannabinoid modulation of immune responses have been lacking.

1.4 The Endocannabinoid system

Cannabis Sativa (cannabis) is one of the earliest plants grown by man, with its seeds initially used as food *circa* 6000 B.C. (Touw, 1981). There is evidence to suggest that 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.) (Li, 1974). Cannabis was considered sacred in Tibet (B.C.) where the plant was abundant and used by Buddhists to facilitate meditation. 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 was first reported in the world’s oldest pharmacopoeia, the *pen-ts’ao ching*, which was traditionally passed down orally from the time of Emperor Shen-Nung (2700 B.C.). The Chinese used cannabis for the treatment of rheumatic pain, constipation, disorders of the female reproductive system and malaria (Li, 1974). Similarly, in India, the medical properties of cannabis were reported around 1000 B.C., where it was recommended as an analgesic, anticonvulsant, hypnotic, anxiolytic and anti-inflammatory agent. Early Christendom sources also indicate that the plant was used with wine to anaesthetize patients during surgical operations (A.D. 110 – 207). The first reference to the use of cannabis as a psychoactive drug is also found in the *pen-ts’ao ching* which states that cannabis, if taken in excess, may precipitate “visions of devils” and make one “communicate with spirits”. 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 have been known for thousands of years, 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 and Gaoni, 1967) that extensive studies revealed the mechanisms

underlying the physiological and pharmacological effects of cannabinoids. This discovery of the active ingredient led to increased research interest in the field, and approximately 100 other cannabinoids have been identified in the cannabis plant to date. Because of the lipophilicity of Δ^9 -THC, it was assumed that it might mediate its biological effects by disrupting cell membrane fluidity rather than binding to a particular receptor. However this concept was challenged following the discovery of Δ^9 -THC's ability to inhibit adenylyl cyclase activity through Gi/o proteins, clearly indicating a receptor-mediated mechanism (Dill and Howlett, 1988). This discovery fuelled the synthesis of more potent THC analogues such as HU210 and CP55,940 which ultimately led to the molecular identification of two cannabinoid G-protein coupled receptors (GPCRs): cannabinoid type 1 (CB₁) (Devane et al., 1988; Matsuda et al., 1990) and cannabinoid type 2 (CB₂) receptors (Munro et al., 1993).

1.4.1 Distribution of CB₁ and CB₂ receptors.

CB₁ receptors are expressed highly throughout the human and rodent brain (Herkenham et al., 1991; Tsou et al., 1998; Mackie, 2008). CB₁ receptor mRNA has been detected in the human adrenal gland, heart, lung, liver, pancreas, prostate, muscle, ovary, bone marrow, thymus and tonsils (Bouaboula et al., 1993; Galiegue et al., 1995; Cota et al., 2003; Osei-Hyiaman et al., 2005; Cavuoto et al., 2007; Cota, 2007). Early autoradiography studies using the radiolabelled cannabinoid agonist [³H]CP-55,940 revealed that the expression of CB₁ receptors in the brain was extremely high and similar to that of ionotropic GABA and glutamate receptor expression (Herkenham, 1991). These early studies were important in that they demonstrated that CB₁ receptor expression was high in brain regions such as the basal ganglia, cerebellum, frontal cortex, amygdala and hippocampus (Herkenham, 1991), which would account for the psychoactive, motor coordination and short-term memory effects of Δ^9 -THC. Moderate expression levels of the CB₁ receptor are observed in the dorsal root of the spinal cord, the periaqueductal gray matter (PAG) and the hypothalamus (Herkenham, 1991).

CB₂ receptors are widely distributed in peripheral tissues, and particularly in immune tissues including the spleen, tonsils, thymus, mast cells and blood cells (Munro et al., 1993; Berdyshev, 2000; Sugiura et al., 2000). The highest expression in immune cells has been reported in B-cells followed by natural killer cells, monocytes,

neutrophils, CD8 leukocytes and CD4 leukocytes (Galiegue et al., 1995; Lee et al., 2001) Until recently, CB₂ receptors were not thought to be expressed to any appreciable extent in the brain, but recent data has identified CB₂ receptors on human cerebral microvascular endothelial cells (Golech et al., 2004), human foetal astrocytes (Sheng et al., 2005) and on limited populations of microglia (Benito et al., 2003; Nunez et al., 2004) in the healthy human brain. With the advancement of modern molecular biology and immunohistochemical techniques, low levels of CB₂ receptor expression have been demonstrated on neurons in the brain stem, cortex, hippocampus, striatum, hypothalamus, substantia nigra, amygdala and cerebellum of rats (Van Sickle et al., 2005; Onaivi et al., 2006). Since these seminal studies, various other groups have confirmed the presence of CB₂ receptors in these and other rodent brain regions (Ashton et al., 2006; Gong et al., 2006; Brusco et al., 2008; Suarez et al., 2008).

Initial characterization of the cannabinoid receptors has enabled a number of endogenous cannabinoid receptor agonists (endocannabinoids) to be identified, the best characterised of which are arachidonoyl ethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Other less well studied endocannabinoid ligands include 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) (Huang et al., 2001; Bisogno et al., 2005). [A detailed overview of the biosynthetic and degradative pathways of these endocannabinoids is provided below].

AEA has a higher affinity for both CB₁ and CB₂ receptors than 2-AG, however, the efficacy of 2-AG at these receptors is more pronounced than AEA (Howlett and Mukhopadhyay, 2000; Di Marzo, 2008b). AEA is considered to be a partial agonist for the CB₁ receptor, while 2-AG is a full agonist for both CB₁ and CB₂ (Sugiura et al., 1999; Sugiura and Waku, 2000).

1.4.2 Other receptor targets for the endocannabinoids

Several lines of evidence have now indicated that endocannabinoids can bind to receptors other than CB₁ or CB₂, namely the transient receptor potential vanilloid 1 (TRPV1), peroxisome proliferator-activated receptors (PPARs), GPR55 and GPR119 (Huang et al., 2002; Overton et al., 2006; Sun et al., 2006; Ryberg et al., 2007) (Table 1.1).

The transient receptor potential (TRP) receptors are ligand-gated non-selective ion channels which are expressed widely in the brain (Toth et al., 2005) and in the peripheral nervous system on small diameter primary afferent fibres (Caterina et al., 1997) where they act as a focal point for the summation of noxious stimuli such as high temperature and acidic pH. Their activation instigates an influx of cations into the cell (Patapoutian et al., 2009). Some cannabinoids and endocannabinoids bind directly to the vanilloid receptor VR1 (TRPV1) (Di Marzo et al., 2002). While AEA is a partial agonist at the CB₁ receptor, it is a full agonist at TRPV1 channels (thus it is referred to as an endovanilloid as well as an endocannabinoid) (Zygmunt et al., 1999; Smart et al., 2000). It has been proposed that under certain conditions such as during 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., 2002).

PPARs belong to the family of nuclear receptors and have three isoforms (i.e. α , δ , and γ). These receptors form heterodimers with retinoid X receptors (RXR) and bind to PPAR-responsive elements (PPRE) in the DNA, thereby promoting or suppressing the transcription of target genes once the receptor is activated (O'Sullivan, 2007). These nuclear receptors have been demonstrated to play an important role in regulating metabolism, inflammation, energy homeostasis, insulin sensitivity and food intake (Ferre, 2004; Glass and Ogawa, 2006; Stienstra et al., 2007). They can be activated by a large range of compounds including synthetic and endogenous cannabinoids including AEA, noladin ether and virodhamine (Sun and Bennett, 2007). In addition, 2-AG-induced suppression of the cytokine IL-2 has been proposed to be mediated through PPAR γ (Rockwell et al., 2006).

Until recently, there were no known endogenous ligands for the orphan G-protein coupled receptor GPR55. However, screening of the affinity of various cannabinoids revealed that this receptor was activated by several cannabinoid receptor agonists and antagonists, including AEA and 2-AG (Ryberg et al., 2007). As such, this receptor is tentatively considered to be the third cannabinoid receptor, however it has no affinity for the synthetic cannabinoid agonist WIN55,212-2 (Johns et al., 2007; Ryberg et al., 2007). Further studies are required in order to determine the functional significance of endocannabinoid modulation of this receptor. [For a comprehensive review of endocannabinoid receptor targets see (Alexander and Kendall, 2007; O'Sullivan, 2007)].

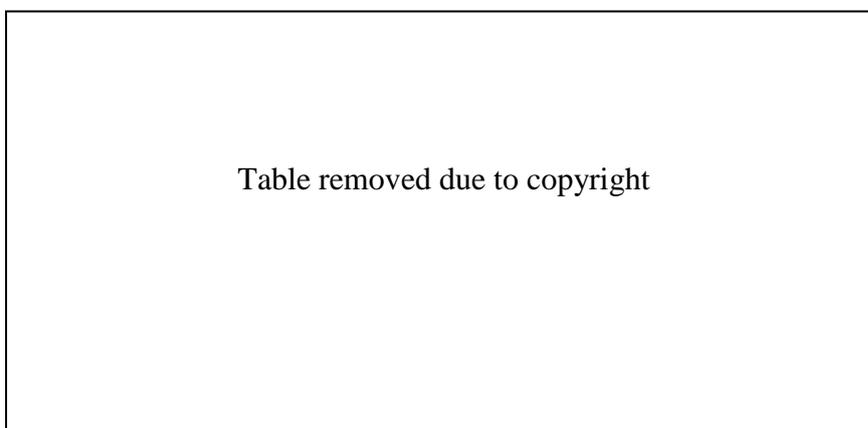


Table 1.1 Receptor targets for endocannabinoids and related compounds. Activity of endocannabinoids and related compounds at metabotropic receptors (CB1, CB2, GPR55, GPR119), ligand activated ion channel (TRPV1) and nuclear receptors (PPAR α and PPAR γ)
Key; – antagonist; + weak/no activity; ++ intermediate; +++ high relative activity; a blank space indicates the activity of the ligand has not been described. Modified from (Alexander and Kendall, 2007)

1.4.3 Endocannabinoid biosynthesis

The enzymes responsible for the production of AEA and 2-AG are present in all cells and tissues in the body including oligodendrocytes (Gomez et al., 2010), astrocytes, microglia (Hegyí et al., 2012) and neurons in the CNS. At a neuronal level, AEA and 2-AG are synthesised in postsynaptic neurons and travel to the presynaptic terminal to activate cannabinoid receptors in a process known as retrograde neurotransmission (Fig. 1.6).

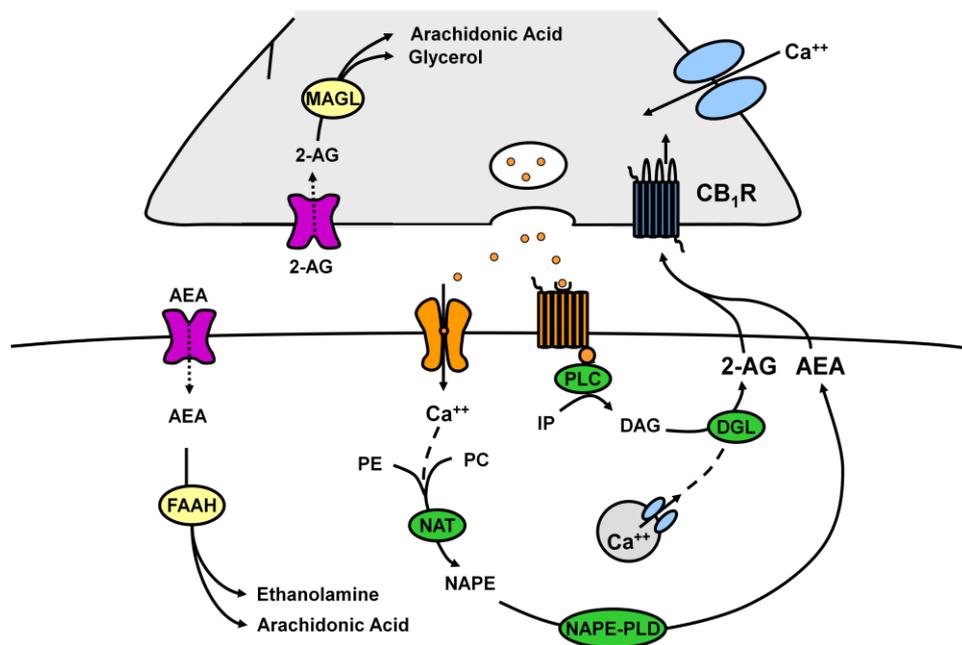


Fig. 1.6 Retrograde signalling by endocannabinoids

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 taken up 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. Modified with permission from (Rea et al., 2007).

1.4.3.1 Anandamide (AEA) Biosynthesis

Endocannabinoids are not stored in vesicles but rather their biosynthesis occurs on demand (Di Marzo and Deutsch, 1998) via hydrolysis of cell membrane phospholipid precursors - e.g. following post synaptic depolarisation, in a calcium-dependent manner (Di Marzo et al., 1994). For example, the initiation of AEA synthesis coincides with activation of dopaminergic, muscarinic and glutamate metabotropic post-synaptic receptors (Giuffrida et al., 1999; Varma et al., 2001; Kim et al., 2002). AEA is formed from the precursor N-arachidonoylphosphatidylethanolamine (NAPE) which is produced by N-acyltransferase (NAT) when it catalysis the transfer of arachidonic acid from phosphatidylcholine (PC) to the primary amino group of phosphatidylethanolamine (PE) (Di Marzo et al., 1994). In a second step, NAPE is subsequently hydrolyzed to AEA and phosphatidic acid (PA) via a phospholipase D enzyme NAPE-PLD (Di Marzo et al., 1994; Sugiura et al., 1996). It has also been shown that cyclic AMP (c-AMP) and Ca^{2+} can modulate N-acyltransferase (NAT) activity and therefore regulate the amount of substrate available for AEA synthesis (Cadas et al., 1996). There is also evidence to suggest that AEA may be synthesised via a phospholipase-C dependant pathway or via hydrolysis of NAPPs by α/β -hydrolase 4 (ABH4) (Varma et al., 2001; Liu et al., 2006) (Fig 1.6 & 1.7).

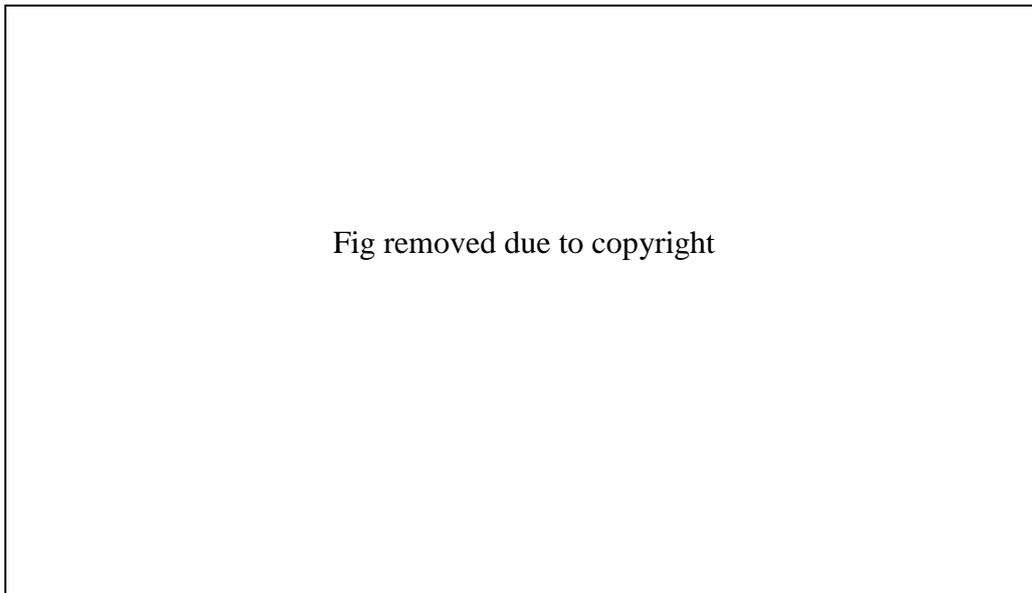


Fig. 1.7 Major pathway for synthesis of anandamide (AEA). (Fonseca et al., 2013)

1.4.3.2 2-arachidonoylglycerol (2-AG) biosynthesis

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 is then converted to 2-AG by diacylglycerol lipase (DAGL) (Prescott and Majerus, 1983; Sugiura et al., 1995). Inhibitors of the PLC and DAGL enzymes have been shown to prevent 2-AG production, thereby validating this pathway as one of the main biosynthetic routes for 2-AG synthesis (Stella et al., 1997).

An alternative pathway has also been proposed which postulates that phospholipase A1 (PLA1) may cause the hydrolysis of phosphatidylinositol to generate an intermediary 2-arachidonyl-lysophospholipid which may be hydrolysed to 2-AG by lysophospholipase C (Lyso-PLC) (Sugiura et al., 1995) (Fig 1.6 & 1.8).

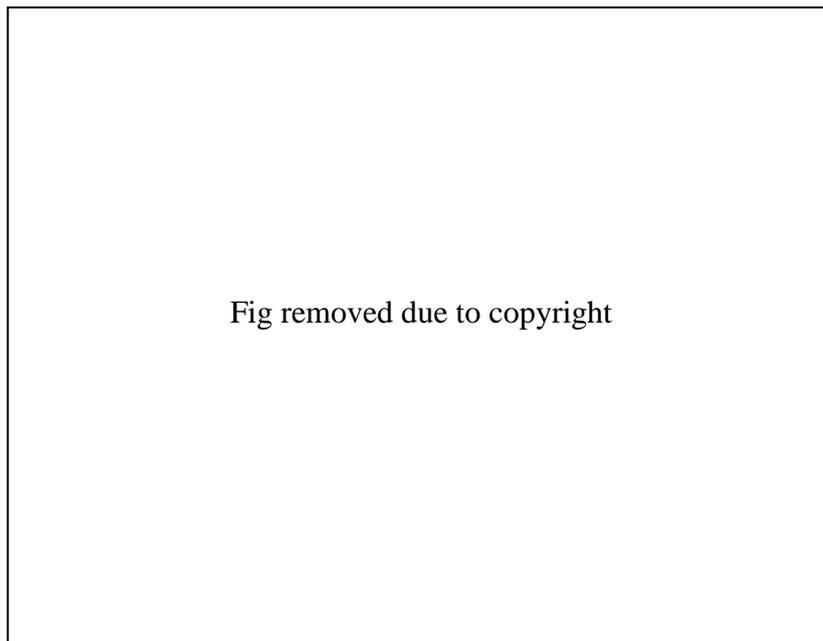


Fig. 1.8 Major pathway for synthesis of 2-arachidonoylglycerol (2-AG). Note the alternative pathway for 2-AG biosynthesis involving phospholipase A1 (PLA1) and then lyso-phospholipase C (lyso-PLC) activities. (Fonseca et al., 2013)

1.4 .3.3 N-Acylethanolamines

Two metabolically-related AEA analogues (N-acylethanolamines, NAEs) N-oleoylethanolamide (OEA) and N-palmitoylethanolamide (PEA) are also formed through the same biosynthetic pathways responsible for the formation of AEA. These lipid mediators may be formed from the phospholipid NAPE by several possible enzymatic pathways, involving glycerophosphodiester phosphodiesterase 1(GDE1), Src homology 2 domain-containing inositol-5-phosphatase1 (SHIP), Protein tyrosine phosphatase, non-receptor type 22 (PTPN22), and lysophospholipase D (lyso PLD) [reviewed in (Ahn et al., 2008)]. Unlike AEA, these compounds have little or no activity at the cannabinoid CB₁ and CB₂ receptors (Sheskin et al., 1997; Lambert et al., 1999; Griffin et al., 2000), however they display activity at other non-CB receptor targets such as PPARs and GPR55 (Table 1.1).

PEA and OEA are metabolised preferentially by the enzyme fatty acid amide hydrolase (FAAH), the primary enzyme responsible for the metabolism of AEA. As such, these N-acylethanolamines may induce an “entourage effect”, potentiating the effect of AEA on the CB or TRPV1 receptors (Mechoulam et al., 1997; De Petrocellis et al., 2001).

1.4.4 Endocannabinoid signalling in neurons

The actions of the endocannabinoids are realised via their activation of the cannabinoid receptors. Both CB₁ and CB₂ receptors are Gi/o protein-coupled receptors that are negatively coupled to adenylyl cyclase (AC) (Howlett et al., 1999) and positively coupled to mitogen-activated protein kinase (MAPK) (Bouaboula et al., 1995).

Therefore, activation of the CB₁ and CB₂ receptors inhibits AC and associated cAMP levels (Wade et al., 2004), thereby causing the inactivation of the protein kinase A (PKA) phosphorylation pathway or stimulation of MAPK (Hoffman and Lupica, 2000; Wilson et al., 2001) which results in major changes in cellular activity, including gene expression (Bouaboula et al., 1995). In addition, CB₁ receptors are coupled to ion channels through Gi/o proteins, positively for A-type and inwardly

rectifying potassium channels and negatively for N-type and P/Q-type calcium channels and D-type potassium channels (Pertwee, 1997; Mu et al., 1999; Pertwee, 1999). In this manner, CB₁ activation in neurons directly inhibits Ca²⁺ channels, reduces Ca²⁺ entry and activates inwardly rectifying potassium (K⁺) channels thereby inhibiting neurotransmitter release (McAllister et al., 1999) (Fig 1.9), including noradrenaline (Kathmann et al., 1999; Gobel et al., 2000), dopamine (Cadogan et al., 1997; Kathmann et al., 1999), serotonin (Nakazi et al., 2000), GABA (Vaughan et al., 1999; Irving et al., 2000; Takahashi and Linden, 2000; Katona et al., 2001; Ohno-Shosaku et al., 2001) and glutamate (Auclair et al., 2000; Vaughan et al., 2000; Robbe et al., 2001). Recent evidence has also identified CB₁ receptors on postsynaptic neurons, the activation of which results in increased kinase activity and associated gene expression (e.g. BDNF) (Mestre et al., 2005; Murphy et al., 2012). Furthermore, the activation of these CB₁ receptors results in activation of additional protein kinases, such as focal adhesion kinase (FAK) (Karanian et al., 2005), c-jun N-terminal kinase (c-JNK) (Rueda et al., 2000) and extracellular signal-regulated kinase (ERK) (Rueda et al., 2002).

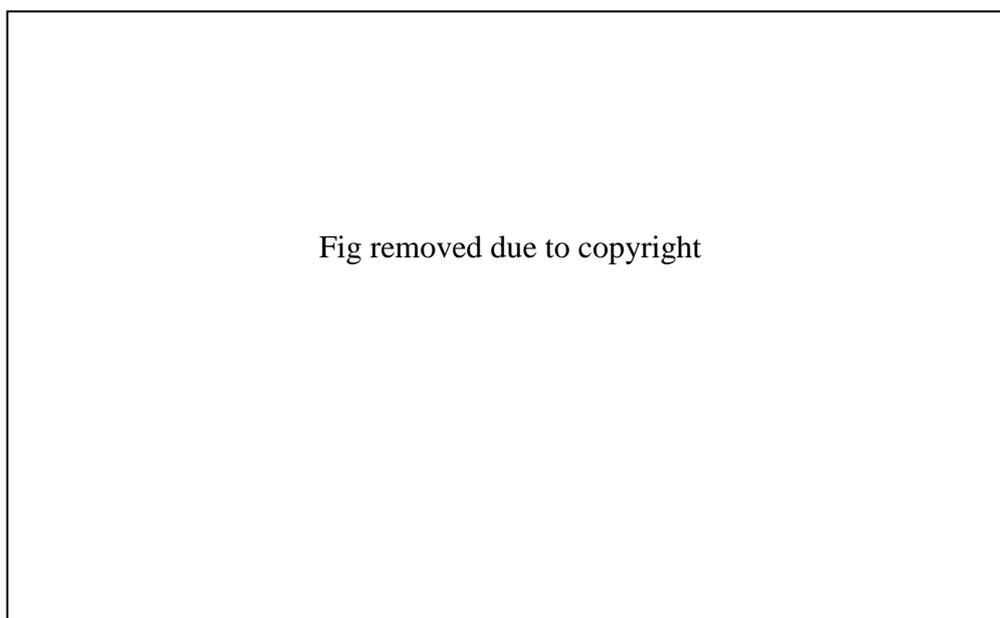


Fig. 1.9 Signalling pathways associated with cannabinoid receptor activation. Signalling pathways associated with cannabinoid receptor activation by agonists. (ATP) adenosine triphosphate. (Di Marzo et al., 2004)

1.4.5 Endocannabinoid signalling in (neuro)immune cells

Endocannabinoid signalling also occurs in cells of the immune system, primarily macrophages, astrocytes and microglia that have been shown to express cannabinoid receptors, of which CB₂ is predominant (Galiegue et al., 1995; Caterina et al., 1997; Carlisle et al., 2002; Di Marzo et al., 2002; Sheng et al., 2005; Ashton et al., 2006). Under normal conditions, microglia express low levels of cannabinoid receptors however, during neuroinflammation, CB₂ receptors are up-regulated (Stella, 2009, 2010). Furthermore, it has been demonstrated *in vitro* that rodent peritoneal macrophages and microglia, following stimulation with either IFN γ or LPS, exhibit levels of CB₂ mRNA and protein that are regulated differently in relation to cell phenotype or activation states. CB₂ mRNA was low/undetectable in resident cells, present at high levels in thioglycolate-elicited inflammatory and IFN γ -primed cells, and detected at significantly diminished levels in bacterial LPS-activated cells (Carlisle et al., 2002). It has been proposed (Stella, 2009) that neurons, microglia and astrocytes signal in an autocrine and paracrine manner (described below) so as to resolve inflammation and limit tissue damage. In brief, neurons damaged by injury, toxins or pathogens secrete large amounts of glutamate which in turn activate glutamatergic receptors on neighbouring neurons resulting in a sustained rise in postsynaptic calcium levels and enhanced endocannabinoid production. CB₁ receptor expression is also up-regulated on damaged neurons and the aforementioned endocannabinoids, acting at presynaptic CB₁, reduce neurotransmitter release and at postsynaptic CB₁ receptors increase kinase activity and associated gene expression (e.g. BDNF) (Coull et al., 2005). Astrocytes may also be stimulated by ATP from damaged cells and produce endocannabinoids that may also bind to pre- and post-synaptic CB₁ receptors. Endocannabinoids produced by these damaged neurons and activated astrocytes also act on CB₂ receptors up-regulated on microglia which results in a switch from a M1 (pro-inflammatory) to a M2 (anti-inflammatory) phenotype. Furthermore, ATP released by neurons enhances the sustained release of endocannabinoids from microglia which continue to stimulate pre- and post-synaptic CB₁ receptors in order to return the immune system to a state of homeostasis. Signalling via this autocrine and paracrine manner acts to try to resolve inflammation and limit cell damage. [For comprehensive reviews of the role of the CB₂ receptor in

immune regulation and endocannabinoid signalling in microglia cells see (Cabral and Griffin-Thomas, 2009; Stella, 2009)].

As stated earlier, cannabinoid receptors are responsible for activating signalling cascades including adenylate cyclase and cAMP, MAPK and modulation of levels of intracellular calcium (Fig 1.9). One of the proposed mechanisms by which activation of CB₂ receptors on immune cells, including microglia, results in a decrease in the production of pro-inflammatory cytokines and reactive oxygen species involves the inhibition of cAMP production and consequent inhibition of protein kinase A (PKA). Multiple kinases including PKA, PKC, p38 and Raf-1 have been shown to phosphorylate IκBα, allowing NF-κB to translocate to the nucleus (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990; Li and Sedivy, 1993; Han et al., 1994). Therefore, inhibition of PKA in immune cells following CB₂ receptor activation ultimately results in inhibition of NF-κB translocation and subsequent transcription of pro-inflammatory mediators (Fig 1.10).

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Fig 1.10. CB₂ receptor signalling in immune cells. CB₂ receptor activation by endocannabinoids 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). IκB-α remains unphosphorylated due to PKA inactivation, thus preventing activation, nuclear translocation and DNA binding of NF-κB and other transcription factors. These events ultimately lead to the down-regulation of pro-inflammatory mediator gene expression. Modified from (Cabral and Griffin-Thomas, 2009).

1.4.6 Endocannabinoid uptake

The actions of endocannabinoids are terminated following hydrolysis by intracellular catabolic enzymes and as such these lipid mediators must be translocated across the cell membrane to cease their signalling. The mechanism responsible for AEA and/or 2-AG transport across the membrane remains controversial [for review see (Yates and Barker, 2009)]. It has been proposed that these lipophilic endocannabinoids readily cross the cell plasma membrane via simple diffusion through the lipid bilayer (Glaser et al., 2003; Kaczocha et al., 2006). In comparison, others suggest a protein-facilitated transport process (Hillard et al., 1997; Beltramo and Piomelli, 2000), the nature and existence of which until recently remained elusive. Fu *et al* recently described a cytosolic variant of the AEA-degrading enzyme fatty acid amide hydrolase-1 (FAAH-1), termed FAAH-like AEA transporter (FLAT), that bound AEA and facilitated its translocation into cells (Fu et al., 2012). Support for this transport process arises due to the ability of putative known transport inhibitors AM404 and OMDM-1, and the newly characterised FLAT inhibitor ARN272, to inhibit this transporter and increase extracellular AEA levels (Fu et al., 2012). In addition, Oddi and colleagues found that cytosolic heat shock protein 70 (Hsp70) and albumin could bind AEA and overexpression of Hsp70 increased AEA uptake into cells (Oddi et al., 2009).

In comparison, relatively little is known about the uptake mechanism for 2-AG. FLAT does not appear to contribute to the transport of 2-AG across the membrane, however, 2-AG has been shown to inhibit AEA uptake in human astrocytoma cells, indicating that these endocannabinoids probably compete for the same transporter (Beltramo and Piomelli, 2000; Bisogno et al., 2001), which has yet to be identified. However, in neurons, that AEA is taken up into the presynaptic neuron while 2-AG is transported into the post-synaptic neuron, indicate that separate transporter mechanisms may mediate the transport of these endocannabinoids in certain cells/neurons.

1.4.7 Endocannabinoid Degradation

A number of degradation pathways have been identified for the endocannabinoids. Degradation of anandamide, to arachidonic acid and ethanolamine, is primarily

mediated by FAAH (Cravatt et al., 1996). It should be noted that FAAH has specificity for other substrates including 2-AG (Di Marzo et al., 1998) and the N-acylethanolamines OEA and PEA (Cravatt et al., 1996; Ahn et al., 2008). Thus, these compounds may compete with AEA for the catalytic site on FAAH, resulting in attenuated metabolism of AEA and enhanced AEA signalling (entourage effect) (Cravatt et al., 1996; Cravatt et al., 2001; Walker et al., 2002). Furthermore, a second isoform of FAAH has been identified (FAAH2) in humans however this isoform has not been identified in rodents or dogs (Wei et al., 2006), and its functional significance remains unknown.

AEA can also be metabolised by a further NAE-hydrolyzing enzyme, N-acylethanolamine-hydrolyzing acid amidase (NAAA). This enzyme is highly expressed in immune cells, specifically in macrophages, and localized in lysosomes. It has been proposed that metabolism of AEA via NAAA may be important when N-acylethanolamines accumulate at the site of inflammation and tissue degeneration (Tsuboi et al., 2005; Tsuboi et al., 2007). A recent study comparing the involvement of FAAH and NAAA in the degradation of AEA and other N-acylethanolamines demonstrated that both enzymes cooperatively degraded these substrates in macrophages, however in brain tissue, the AEA hydrolysing activity was mostly attributed to FAAH (Sun et al., 2005).

In comparison to AEA, 2-AG is catabolised to arachidonic acid and glycerol, primarily by the enzyme monoacylglycerol lipase (MAGL) (Dinh et al., 2002). While up to 85% of brain 2-AG hydrolase activity can be ascribed to MAGL, the remaining 15% has been mostly attributed to two relatively uncharacterised enzymes, ABHD6 and ABHD12 (Blankman et al., 2007). However AEA, and 2-AG, can also be metabolised by several of the enzymes that oxidise arachidonic acid including COX2 and the lipoxygenases (LOX) (Di Marzo, 2008a). COX2 (Kozak et al., 2000; Kozak et al., 2001) catalyses the oxidation of 2-AG and AEA into the various prostaglandin-ethanolamides (prostamides and prostaglandin-glycerol esters (Woodward et al., 2008), compounds which in turn have specific effects often opposite to their parent endocannabinoid (Fig. 1.11) [reviewed in (Ricciotti and FitzGerald, 2011)].

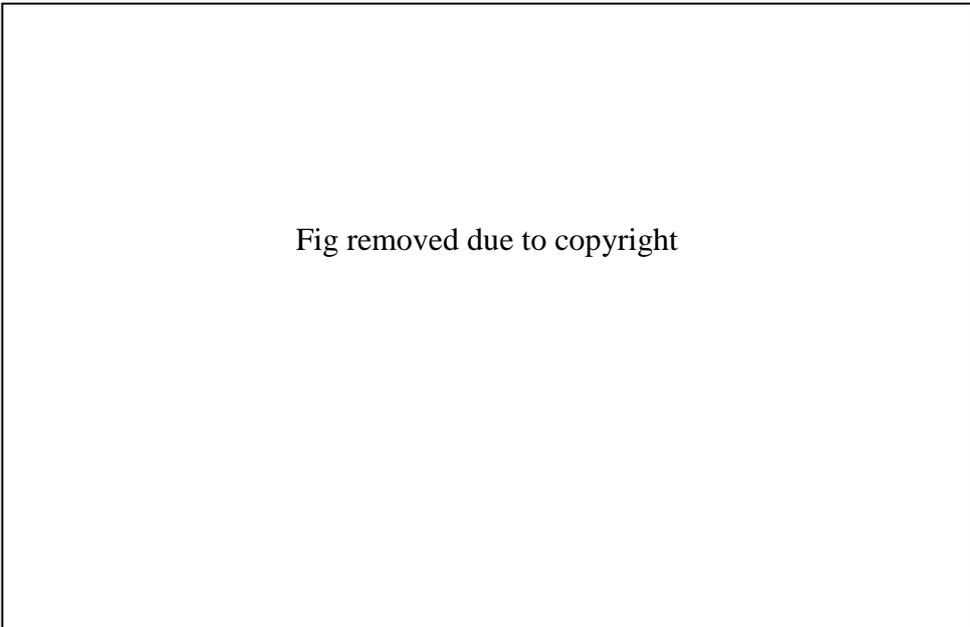


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Fig 1.11. Degradative pathways of 2-AG. (Ueda et al., 2011).

In summary, the endocannabinoid system which comprises receptors, endogenous ligands, transporters and enzymes for ligand biosynthesis and inactivation form the framework of a complex signalling system, all elements of which are densely expressed in the mammalian immune system and brain (Herkenham et al., 1990; Galiegue et al., 1995; Onaivi et al., 2006) .

1.5 Endocannabinoid modulation of inflammatory responses

The endocannabinoid system is implicated in a broad range of physiological processes including appetite, GI function, respiration, cardiovascular, liver and neural function to name but a few. Alterations in the endocannabinoid system, predominantly changes in levels of AEA and 2-AG, have been reported in various disorders including obesity, neurological and neuropsychiatric conditions, cancer and diseases of the cardiovascular system. [For review see (Di Marzo, 2008c)]. In addition, *in vitro* and *in vivo* data have demonstrated that cannabinoids modulate immune function and responses to inflammation in various models of disease (Yoshihara et al., 2005; Storr et al., 2009; Tschop et al., 2009; Yu et al., 2010).

Endocannabinoid modulation of neuroinflammation and consequently neurological disorders including Alzheimer's disease, Parkinson's disease and multiple sclerosis, has received considerable attention over the past 10 years (McGeer and McGeer, 2001; Nagatsu and Sawada, 2005; Compston and Coles, 2008). Furthermore, pre-clinical models of these conditions exhibit alterations in the endocannabinoid system (Di Marzo et al., 2000; Baker et al., 2001; Maccarrone et al., 2003; Walsh et al., 2010; Mulder et al., 2011) and modulation of immune responses in these models has resulted in therapeutic-like effects in many cases. The endocannabinoid system regulates many aspects of the brain's neuroinflammatory responses, including the release of pro-inflammatory cytokines and regulation of microglial activation, thereby providing a target for the treatment of diseases associated with neuroinflammation [For review see (Ullrich et al., 2007; Nagarkatti et al., 2009; Stella, 2009)].

1.5.1 *In vitro* studies demonstrating a modulatory role of endocannabinoids on immune responses

Several *in vitro* studies have demonstrated that treatment of LPS-stimulated macrophages, microglia or astrocytes with endogenous as well as exogenous cannabinoids and/or inhibitors of endocannabinoid uptake or degradation modulates the release of pro-inflammatory and anti-inflammatory mediators (Table 1.2).

Table 1.2 Endocannabinoid modulation of immune mediators (*in vitro studies*)

Endocannabinoid/ drug	Modulator	Cell type	Stimulus	Response	Receptor/ signalling pathway	References
AEA	AEA	J774 Macrophages	LPS	↓ LPS-induced NO, IL-6, PGE2,	-	(Chang et al., 2001)
	AEA	Rat microglia	LPS	↓ LPS-induced TNF- α	Non CB ₁ /CB ₂	(Facchinetti et al., 2003)
	AEA	Rat microglia	LPS	↓LPS-induced IL-1 α , IL-1 β , IL-6, TNF α	Non CB ₁ /CB ₂	(Puffenbarger et al., 2000)
	URB597	Rat Microglia	LPS	↓LPS-induced COX2, iNOS, PGE2, NO TNF α	↓PGE2, Non CB ₁ /CB ₂	(Tham et al., 2007)
	UCM707	Rat astrocytes	LPS	↓ LPS-induced iNOS, NO, TNF α and IL-1 β , ↑ IL-6	CB ₁ /CB ₂ mediated	(Ortega-Gutierrez et al., 2005)
	AEA	Murine astrocytes	TMEV	↑ IL-6	CB ₁ mediated	(Molina-Holgado et al., 1998)
	AEA JWH-015	Human peripheral T lymphocytes	PMA/Ionomycin	↓proliferation, IL-2, TNF α , IFN- γ , IL17	CB ₂ mediated	(Cencioni et al., 2010)
	AEA	Murine and human microglia	LPS /IFN γ mix	↓ induced IL-12 and IL-23	CB ₂ mediated ERK1/2 and JNK MAPK pathway	(Correa et al., 2009)
	AEA	Murine and human microglia	LPS /IFN γ mix	↑induced IL-10 inhibition of IKK α	CB2 ERK1/2 and JNK MAPK pathway, NF-kB	(Correa et al., 2010)
	AEA	Human eye Muller glia cultures.	LPS	↓LPS-induced cell death, ↑CB ₁ and CB ₂ expression on glia. ↑LPS-induced IL-10, TGF β ↓IL-6, IL-1 β , TNF α , IL-2, IFN γ , IL-15, IL-12. ↓ IL-8	↑LPS-induced IL-10, TGF β ↓ IL-6, TNF α , IL-2, IFN- γ CB ₁ and CB ₂ mediated. ERK1/2,JNK, p38, inhibit NF-kB	(Krishnan and Chatterjee, 2012)
	UCM707	Brain endothelial cells	TMEV	↓VCAM	CB ₁ mediated	(Mestre et al., 2011)

	AEA	Murine neuron/microglia cultures	LPS/IFN- γ	↓LPS/IFN- γ induced neuronal death. Reversed LPS/IFN- γ downregulation of CD200R ↑IL-10 by microglia	CB2 mediated Neuroprotection via IL-10 induced increase in CD200 on neurons	(Hernangomez et al., 2012)
2-AG	2-AG	J774macrophages	LPS	↓LPS induced IL-6 and ↑ induced NO	-	(Chang et al., 2001)
	2-AG	Human eye Muller glia cultures	LPS	↓LPS-induced cell death, ↑CB ₁ and CB ₂ expression on glia. ↑ LPS-induced IL-10, TGF β ↓IL-6, IL-1 β , TNF α , IL-2, IFN γ , IL-15, IL-12. ↑ IL-8	↑LPS-induced IL-10, TGF β , ↓IL-6, TNF α , IL-2, IFN- γ CB ₁ and CB ₂ mediated. ERK1/2, JNK, p38, inhibit NF- κ B	(Krishnan and Chatterjee, 2012)
	2-AG	Rat microglia	LPS	↓ LPS induced TNF α	Non CB ₁ /CB ₂	(Facchinetti et al., 2003)
	2-AG	Jurkat cells	PMA Ionomycin(Io)	↓COX2	Possibly via PPAR γ	(Raman et al., 2011)
	2-AG	Murine Neurons and astroglia	LPS	↓LPS induced COX2	Mediated by CB ₁ and MAPK/NF- κ B signalling pathway	(Zhang and Chen, 2008)
	2-AG	Murine jurkat cells	PMA ionomycin	↓ IL-2	Mediated via PPAR γ and NF- κ B and NFAT	(Rockwell et al., 2006)
	2-AG	Murine macrophages	LPS or LPS/IFN γ	↓ LPS induced TNF α	-	(Gallily et al., 2000)
	2-AG	Murine splenocytes	PMA/Io	↓PMA/Io induced IFN γ secretion. Same response in CB ₁ (-/-) and CB ₂ (-/-)	Partly inhibition of NFAT nuclear translocation	(Kaplan et al., 2005)
	2-AG, URB602 JZL184	Hippocampal neurons	β -amyloid (A β)	↓COX2, ↓apoptosis, ↓caspase activity ↓ neurodegeneration	CB ₁ mediated ERK1/2 NF- κ B	(Chen et al., 2011)
	2-AG	Caudate nucleus neurons	LPS	↓ LPS-induced COX2	CB ₁ mediated ERK1/2, p38 MAPK, NF- κ B	(Lu et al., 2014)

	2-AG URB602 JZL184	Hippocampal neurons	IL-1 β LPS	↓ IL-1 β - and LPS-induced increase in COX2 and NF κ B p65 phosphorylation. ↑ IL-1 β and LPS induced reduction in PPAR γ expression.	Mediated by PPAR γ . PPAR γ expression mediated by CB $_1$	(Du et al., 2011)
Synthetic and exogenous cannabinoids						
Synthetic cannabinoids	THC /IMMA	J774 macrophages	LPS	↓ LPS induced NO, IL-6, PGE2, COX2	-	(Chang et al., 2001)
	WIN55,212-2 CP55940 HU210	Rat microglia	LPS	↓LPS induced TNF α	Non CB $_1$ /CB $_2$	(Facchinetti et al., 2003)
	THC	Rat microglia	LPS	↓LPS induced IL-1 α , IL-1 β , IL-6, TNF α	Non CB $_1$ /CB $_2$	(Puffenbarger et al., 2000)
	THC CBD	Murine microglia	LPS	↓LPS induced IL-1 β , IL-6, IFN β	CBD ↑STAT3 ↓NF- κ B, CBD and THC ↓SOCS3 ↓STAT1	(Kozela et al., 2010)
	WIN55,212-2	Brain derived endothelial cells	TMEV Upregulated COX2	↑COX2, PGE2	P38 MAPK PI(3) K pathway	(Mestre et al., 2006)

Delta-9 tetrahydrocannabinol (THC); Cannabidiol (CBD); Imma indomethacin morpholinylamide (IMMA); vascular cell adhesion protein 1(VCAM1); c-Jun N-terminal kinase(JNK); p38 mitogen activated protein kinase(p38);WIN55,212-2 , CP55940, HU210 JWH-015 (synthetic cannabinoid agonists); nuclear factor of activated T cells (NFAT); Theilers virus encephalomyelitis virus (TMEV); URB597,UCM707 (FAAH inhibitors); JZL184, URB602 (MAGL inhibitors); Phorbol 12-myristate-13-acetate(PMA).

Several *in vitro* studies have demonstrated that modulation of the endocannabinoids AEA and 2-AG, either directly or indirectly by inhibiting their degradation, attenuates the production of pro-inflammatory mediators such as nitric oxide, prostaglandins, interleukins, (IL-1, IL-6, TNF α) and chemokines while enhancing anti-inflammatory cytokine production (IL-10) in LPS-stimulated cells (Table 1.2). The data indicate that the endocannabinoids are capable of inhibiting COX-2 and iNOS, with concomitant attenuation of the release of pro-inflammatory cytokines, PGE2 and NO, respectively (Puffenbarger et al., 2000; Ortega-Gutierrez et al., 2005; Tham et al., 2007). While similar effects have been demonstrated with potent synthetic cannabinoids (Table 1.2), the cannabinoid receptor agonist WIN55,212-2 has been shown to enhance COX-2 expression in endothelial cells infected with Theiler's murine encephalomyelitis virus (TMEV) (Mestre et al., 2006). This virus has been shown to result in chronic primary immune-mediated demyelination, which resembles multiple sclerosis. Further analysis revealed that while TMEV upregulation of COX-2 was shown to be via the p38 MAPK pathway, WIN55,212-2 induced COX2 upregulation required the phosphatidylinositide 3-kinase PI(3)K pathway (Mestre et al., 2006). However, there are certain instances where endocannabinoids induce an increase in pro-inflammatory cytokine expression. For example, AEA and UCM707, a potent and selective AEA uptake inhibitor, have been shown to enhance IL-6 expression in rodent astrocytic cultures treated with TMEV or LPS (Molina-Holgado et al., 1998; Ortega-Gutierrez et al., 2005). While many of the endocannabinoid effects on immune mediators could be attributed to activation of CB_{1/2} receptors, non-CB receptor effects have also been demonstrated. For example, 2-AG-induced inhibition of IL-2 has been shown to be mediated by PPAR γ and not CB_{1/2} receptors (Rockwell et al., 2006).

Further involvement of the endocannabinoids in the modulation of immune function is evident in the following studies. AEA has been shown to attenuate LPS/IFN γ -induced expression of IL-12 and IL-23 in microglia cells, cytokines known to play a crucial role in the pathogenesis of multiple sclerosis. This study demonstrated that the effects of AEA were partially mediated by CB₂ receptor activation via the ERK1/2 and JNK pathways and increased expression of the anti-inflammatory cytokine IL-10 (Correa et al., 2009; Correa et al., 2010). Furthermore, 2-AG has also been shown to protect neurons from β -amyloid (A β)-induced neurodegeneration.

Enhancing 2-AG levels in cultured hippocampal neurons treated with β -amyloid ($A\beta$), either by direct treatment with 2-AG or indirectly via inhibition of MAGL using the pharmacological tools URB602 and JZL184, significantly reduces ($A\beta$)-induced neurodegeneration and apoptosis. These 2-AG neuroprotective effects were mediated by CB_1 -induced suppression of ERK1/2 and NF- κ B and significant attenuation of β -amyloid induced COX-2 expression (Chen et al., 2011). These studies demonstrate that endocannabinoid modulation of neuroimmune function can protect neurons and neuronal function.

In summary, the data suggest that modulation of endocannabinoid tone *in vitro* exerts potent anti-inflammatory effects in various cell populations, effects which may be mediated by both CB and non-CB receptor mechanisms depending on the conditions under examination.

1.5.2 *In vivo* studies demonstrating a modulatory role of endocannabinoids on immune response

Enhancing endocannabinoid tone *in vivo* has been proposed as an alternative means of activating cannabinoid receptors, without concomitant overt psychotropic effects associated with potent synthetic cannabinoid receptor agonists. The recent advent of selective pharmacological and genetic tools has enabled more detailed *in vivo* studies to be carried out examining the role of the endocannabinoid system in various animal models of inflammation and neuroinflammation. Enhancing tone via FAAH or MAGL inhibition has been shown to elicit anti-inflammatory effects in several animal models (Mestre et al., 2005; Comelli et al., 2007; Alhouayek et al., 2011; Costola-de-Souza et al., 2013) (Table 1.3).

Enhancing AEA tone, either via inhibition of FAAH with URB597 or the AEA transporter using AM404, has been shown to elicit CB₁ or CB₂ receptor-mediated anti-inflammatory properties in various inflammatory models including neuropathic and inflammatory pain, colitis and neurodegenerative disorders (Holt et al., 2005; Mestre et al., 2005; Costa et al., 2006; Jayamanne et al., 2006; Storr et al., 2008). Furthermore, examination of the effects of enhancing AEA in acute inflammatory models has revealed that AEA, for the most part, elicits an anti-inflammatory effect by reducing TLR4-induced increases in pro-inflammatory cytokines both peripherally and centrally (Table 1.3). The AEA reuptake inhibitor AM404 has also been demonstrated to attenuate LPS-induced increases in plasma IL-1 β and IL-6 levels while concurrently augmenting TNF α levels (Roche et al., 2008), while similar plasma augmentations in LPS-induced TNF α were observed following URB597 i.c.v. administration (De Laurentiis et al., 2010), thereby indicating the possible involvement of AEA in LPS-induced TNF α production. However overall, enhancing AEA tone primarily elicits anti-inflammatory and neuroprotective effects.

It should be noted that the use of FAAH inhibitors to study the effects of enhanced AEA tone is complicated by the parallel increase in alternative FAAH substrates such as PEA and OEA, which have also been demonstrated to elicit potent anti-inflammatory effects (Berdyshev et al., 1998; Hoareau et al., 2009; Esposito et al., 2013; Luongo et al., 2013).

Studies that have investigated the role of 2-AG in the modulation of immune mediators *in vivo* are listed in Table 1.3. Similar to AEA, enhancing levels of 2-AG *in vivo*, directly or via administration of the MAGL inhibitors JZL184 or URB602, has been shown to elicit anti-inflammatory properties, in various inflammatory models of pain, colitis and closed head injury (Panikashvili et al., 2001; Comelli et al., 2007; Alhouayek et al., 2011; Guindon et al., 2011; Ghosh et al., 2013). 2-AG has been shown to be neuroprotective in a closed head injury (CHI) model, effects associated with reduced brain oedema, infarct volume and hippocampal death, (Panikashvili et al., 2001). Subsequent studies by these researchers demonstrated that 2-AG decreased BBB permeability and inhibited the acute cortical expression of the pro-inflammatory cytokines TNF α , IL-1 β and IL-6 in this model and confirmed that the beneficial effects of 2-AG were mediated by activity at the CB₁ receptor and modulation of NF-kB signalling pathway (Panikashvili et al., 2005; Panikashvili et al., 2006).

Recent development of the more potent and selective MAGL inhibitor JZL184 (Long et al., 2009b; Long et al., 2009a), has allowed researchers to investigate the role of 2-AG in a number of physiological and pathophysiological processes (Kinsey et al., 2009; Kinsey et al., 2010; Sciolino et al., 2011; Sticht et al., 2011; Ye et al., 2011). JZL184 has been shown to increase gastric levels of 2-AG in mice and inhibit nonsteroidal anti-inflammatory (NSAID)-induced gastric haemorrhage and associated increases in gastric expression of the pro-inflammatory cytokines, effects mediated by CB₁ receptor (Kinsey et al., 2011). Similarly, in the trinitrobenzene sulfonic acid (TNBS)-induced mouse model of colitis, JZL184-induced an increase in colonic 2-AG levels which resulted in a subsequent reduction of macroscopic and histological colon alterations, and attenuated TNBS-induced expression of pro-inflammatory cytokines TNF α , IL-6, IL-12 and the chemokine MCP-1, effects mediated by CB₁ and CB₂ receptors (Alhouayek et al., 2011). Enhancing central 2-AG levels has also been demonstrated to modulate neuroinflammatory processes. Nomura and colleagues demonstrated that enhanced brain levels of 2-AG following JZL184 significantly attenuated central LPS-induced levels of IL-1 β , IL-1 α , IL-6, TNF α and prostaglandin PGE₂ and arachidonic acid in mice. The neuroprotective effects of MAGL inactivation were not mediated by CB₁ or CB₂ receptors but rather attributed to a reduction in arachidonic acid and downstream arachidonic acid-

derived inflammatory eicosanoids (Kinsey et al., 2011). Similar anti-inflammatory and neuroprotective effects following central enhancement of 2-AG levels were observed in a MPTP mouse model of Parkinson's disease (Kinsey et al., 2011). Thus, while systemic peripheral anti-inflammatory effects of enhancing 2-AG tone appear to be mediated by CB_{1/2} receptors, data suggest that different mechanisms (attenuation of PGE₂) may underlie the anti-inflammatory effects in the CNS and consequently neuroprotective effects.

Thus, considerable data now exist demonstrating a role for the endocannabinoid system in modulating all aspects of inflammation. However, the effect may depend on the conditions under investigation, the timing of modulation and the type of endocannabinoid modulated. Further studies are required in order to examine the role of this system in acute inflammation such as following TLR4 activation and to decipher the receptor and molecular mechanism involved. Due to the topography of the extensive receptor and enzyme network of the endocannabinoid system, its manipulation may identify potential therapeutic targets for inflammatory disorders both centrally and peripherally

Table 1.3 Endocannabinoid modulation of immune mediators (*in vivo studies*)

Cannabinoid/ Endocannabinoid	Modulator	Species	Stimulus	Response	Receptor	References
AEA	URB597 1mg/kg Every 2 nd day for 28 days.	rat	Aged rats with ↑ hippocampal and cortical MHCII, CD11b, CD68, CD40, IL-1β, IL-6, TNFα compared to young rats	↑ AEA,OEA PEA in cerebellum ↓ MCHII expression in hippocampus and cortex ↓ CD11b, CD86, IL-1β, TNFα in hippocampus	-	(Murphy et al., 2012)
	AEA 1mg/kg s.c.	rat	LPS 100ug/kg i.p.	↓ LPS induced fever, hypophagia Abolished LPS-induced decreases in Fos expression in the hypothalamus. Both AEA and LPS increased Fos expression within the nucleus accumbens.	-	(Hollis et al., 2011)
	URB597 i.c.v. 50ng/5ul	rat	LPS 5mg/kg	↑ TNFα, Oxytocin Plasma (1 hour post LPS)	LPS induced increases reversed by CB ₁ antagonism and combination of CB ₁ +CB ₂ antagonism	(De Laurentiis et al., 2010)
	URB597	rats	LPS 100ug/kg	↑ plasma TNFα	-	(Roche et al., 2008)
	AM404	rats	LPS 100ug/kg	↑ plasma TNFα ↓ plasma IL-1, IL-6	↑ plasma TNFα, possibly PPARγ,CB ₁ ,CB ₂ TRPV1 ↓IL-1 is CB ₁ mediated	(Roche et al., 2008)
	AEA icv 50ug	rats	LPS 100ug/kg i.v.	LPS induced a hypothermic response. Plasma LPS-induced TNFα and ACTH attenuated by i.c.v. CB ₁ antagonist. i.c.v. AEA caused greater LPS- induced hypothermic response.	LPS hypothermia blocked by CB ₁ i.p. and i.c.v. administered antagonists.	(Steiner et al., 2011)
	URB597 (0.3- 0.6mg/kg)	rat	LPS Sepsis model	LPS-induced increase in leukocyte adhesion in intestinal venules and decrease in functional capillary density (FCD) (hallmarks of inflammation) were reversed by URB597	URB597 effects on leukocyte adhesion CB ₂ mediated.	(Kianian et al., 2013)

AA-5HT 5mg/kg AEA 3.5µl/h over 7 days	mice	TMEV	↑TMEV induced reduction in CD200 and CD200R in spinal cord. ↑motor co-ordination. ↑IL-10 ↓TMEV induced IL-1β and IL-6 in spinal cord.	-	(Hernangomez et al., 2012)
URB597 5mg/kg VDM11 5mg/kg	mice	Trinitrobenzene sulphonic acid (TBNS) (colitis model)	Inflammation was significantly reduced in the presence of URB597 and VDM11, as evaluated by macroscopic damage score, myeloperoxidase levels, and colon length Effects not observed in CB _{1/2} ^{-/-} mice	CB ₁ and CB ₂ mediated	(Storr et al., 2008)
AEA 10 ⁻⁸ M 30µl local inj	rats	Periodontitis induced by ligature. +immobilisation stress 4 hours daily for 7 days	↓plasma corticosterone, ↓TNF-α, IL-1β in in gingival tissue in stress group	CB ₁ and CB ₂ mediated	(Rettori et al., 2012)
OMDM1 OMDM2 AEA transport inhibitors.	mice	TMEV	↑ AEA spinal cord and ↓motor symptoms, ↓microglia/macrophage activation spinal cord (immunohistochemistry) Also in vitro in macrophages ↓MHCII, NOS-2, IL-1β, IL12	-	(Mestre et al., 2005)
AM404 10mg/kg Daily for 1 week	rats	CCI chronic constriction injury of the sciatic nerve	↓mechanical allodynia, hyperalgesia ↓ NO, nNOS in paw homogenates ↓TNFα, ↑IL-10 in the DRG ↓NF-κB activation in sciatic nerve nuclei	↓mechanical allodynia, hyperalgesia CB ₁ ,CB ₂ ,TRPV1 mediated Completely reversed by all 3 antagonists.	(Costa et al., 2006)
URB597 ip 0.1-3 mg/kg	mice	Interplantar carrageenan	↓Oedema	CB ₂ mediated	(Holt et al., 2005)
URB597 0.3mg/kg	Rat	CFA - complete Freund's adjuvant	↓mechanical allodynia, hyperalgesia	CB ₁ and CB ₂	(Jayamanne et al., 2006)

2-AG	WWL70 (ABHD6 inhibitor) 20 mg/kg i.p.	mice	LPS (300ug/kg) i.p.	2-AG unchanged in cerebellum ↓IL-1, IL-6, MCP-1 in cerebellum	Peripheral CB ₁ mediated and possibly COX2 metabolite of 2-AG i.e. prostaglandin D2-G	(Alhouayek et al., 2013)
	JZL184 40mg/kg i.p.	mice	LPS 20mg/kg	↑2-AG ↓IL-1β, IL-1α, IL-6, TNFα, PGE2 in brain	Non CB ₁ /CB ₂ Possible JZL reduction of AA ,and eicosanoid cascade	(Nomura et al., 2011)
	2-AG 3mg/kg i.p.	mice	LPS i.p.	↓LPS induced COX2 expression in hippocampus	CB ₁ mediated MAPK/NF-κB pathway	(Zhang and Chen, 2008)
	JZL184 16mg/kg i.p.	mouse	LPS 100ug/kg Intranasal Acute lung injury model	↓ LPS induced leukocyte count in Bronchoalveolar lavage fluid (BALF). ↓LPS-induced lung damage (histology). ↓LPS induced TNFα, IL-6, MCP-1 in BALF	CB ₁ and/CB ₂ mediated	(Costola-de-Souza et al., 2013)
	JZL184 40mg/kg i.p.	mice	MPTP (Parkinson model)	↑ 2-AG↓dopaminergic neuron loss ↓Dopamine reductions in substantia nigra and striatum ↓ MPTP induced AA,prostaglandins,IL-1β,IL-6, TNF(whole brain)	Neuroprotection Non CB ₁ /CB ₂ Possible JZL reduction of AA, eicosanoid cascade	(Nomura et al., 2011)
	JZL184 16mg/kg i.p.	mouse	Trinitrobenzene sulphonic acid (TBNS) (colitis model)	↑2AG↓histological alterations, ↓mucosal IL-12,IL-6, TNF-α,MCP-1. ↓IL-1β and IL-6 in the colon	CB ₁ and CB ₂ mediated	(Alhouayek et al., 2011)
	JZL184 40mg/kg i.p.	mice	NSAID (diclofenac sodium) induced gastric haemorrhage and ↑cytokines	↑gastric 2-AG, ↓ gastric haemorrhage ↓ gastric IL-1β, IL6, TNF, GCSF, IL-10	All effects CB ₁ mediated	(Kinsey et al., 2011)
	2-AG 5mg/kg	mice	CHI	↓ BBB permeability, ↓IL-1β, IL-6, TNFα cortex ↑antioxidants ascorbic acid, uric acid	-	(Panikashvili et al., 2006)
	2-AG 0.1-10mg/kg i.v.	mouse	CHI(closed head injury) increased 2-AG levels	↓brain odema, infarct volume, hippocampal apoptosis	CB ₁ mediated	(Panikashvili et al., 2001)
	2AG 5mg/kg/i.p	CB1-/- and WT mice	CHI	no improvement in odema or neurobehavioural function in CB1-/- as compared to WT. ↑NF-κB activation in WT only	Neuroprotection CB ₁ and NF-κB mediated	(Panikashvili et al., 2005)

	JZL184 4mg/kg	Mice	Intraplantar carrageen	↓oedema and mechanical allodynia	↓oedema CB ₂ mediated mechanical allodynia CB ₁ and CB ₂	(Ghosh et al., 2013)
	URB602 10-20mg/kg	mice	Intraplantar carrageen	↓oedema, nociception	All CB ₂ mediated	(Comelli et al., 2007)
Synthetic and exogenous cannabinoids						
Synthetic cannabinoids	HU210	rats	LPS 100ug/kg	↓IL-1β, TNFα in brain ↓IL-1β, TNFα, IL-6, IFNγ plasma	↓IL-1β brain CB ₁ ↓IL-1β, IL-6 plasma CB ₁ mediated	(Roche et al., 2006)
	WIN55,212-2 THC	mice	Intranasal LPS 100μg/ml	↓ LPS induced TNFα in Bronchoalveolar lavage fluid (BALF). ↓LPS induced neutrophil recruitment in BAL	-non CB ₁	(Berdyshev et al., 1998)
	WIN55,212-2	rats	LPS 250ng/h for 3 weeks i.c.v.	↓LPS activated microglia (immuno)	- non CB ₁	(Marchalant et al., 2007)
	WIN555,212-2 JWH-015 ACEA	Mice	TMEV	↓microglial activation, ↓CD4 infiltrating T cells, ↓MHCII expression in spinal cord. Also reduction in number of demyelinated axons in spinal cord.		(Arevalo-Martin et al., 2003)

Table 1.3. Monocyte chemoattractant protein (MCP-1), granulocyte macrophage colony-stimulating factor (G-CSF), blood brain barrier (BBB), (URB597) FAAH inhibitor (JZL184, URB602), MAGL inhibitors, (WWL70); ABHD6 inhibitor (OMDM1, OMDM2, AM404); AEA transport inhibitors (Win555,212-2, JWH-015, ACEA) synthetic cannabinoid agonists.

1.6 Autism

Autism is one of the groups of neurodevelopmental disorders known as pervasive developmental disorders characterised by impairment in social interaction, deficits in verbal and non-verbal communications, and restrictive, repetitive stereotyped patterns of behaviours, interests and activities (American Psychiatric Association, DSM-IV, 1994)

1.6.1 The Genetics of Autism

Increasing evidence suggests that autism is highly heritable. The concordance rate of between 0–37% has been reported in dizygotic twins as opposed to a 44–91% concordance in monozygotic twins (Ritvo et al., 1985; Steffenburg et al., 1989; Bailey et al., 1995; Kates et al., 2004), suggesting that genetic composition may contribute, at least in part, to increased risk of developing ASD. Attempts at determining the most likely genes to be involved have suggested genes encoding proteins mainly involved in the development of neurons and their synapses. A number of mutations and deletions in candidate genes have been identified see table 1.4. [For comprehensive reviews on the genetics of autism see (Geschwind, 2011; Wisniowiecka-Kowalnik et al., 2013)]. Moreover, a number of disorders known to result from single gene mutations including Rett’s syndrome (MeCP2), Fragile X (FMR1), tuberous sclerosis (either TSC1 or TSC2), Timothy syndrome (CACNA1C), Cowden’s syndrome (PTEN), and Angelman’s syndrome (UBE3A) are either included under the spectrum of autism disorders or exhibit some autistic-like behavioural traits (table 1.4). However, even with major advancements in identifying the genes involved, all identified genetic risk factors combined account for only 10–20% of the total population diagnosed with ASD (Abrahams and Geschwind, 2008). In addition a number of the genetic mutations listed have been identified in the general population (non-ASD individuals) and as such it has been proposed that such mutations may predispose to the development of ASD. Thus, environmental factors on the background of a genetic predisposition contribute to the development of neurodevelopmental disorders such as ASD. Environmental factors identified include thalidomide (Stromland et al., 1994), valproic acid (VPA) (Christianson et al., 1994; Williams and Hersh, 1997; Williams et al., 2001; Rasalam et al., 2005), and ethanol (Nanson, 1992; Miles et al., 2003; Landgren et al., 2010).

Table 1.4 Identified genes associated with ASD

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1.6.2 Neuroanatomical changes in Autism

Neuroimaging and anatomical analysis of post mortem brains of autistic patients have demonstrated various neuroanatomical abnormalities in ASD. Neuroimaging studies have revealed that the size of the brains of autistic individuals are increased when compared to non autistic counterparts with enlargement region specific and commonly including limbic regions of the hippocampus and amygdala. In comparison, the size of the cerebellum or brainstem structures does not differ from control when total brain size is taken into account. In addition, the volume of the corpus collosum is reduced while the caudate nucleus is enlarged in autistic brains. [For reviews of neuroanatomical abnormalities in autism see (Cody et al., 2002; Bauman and Kemper, 2005)]. In depth neuroanatomical analysis of the post mortem brain of autistic patients has revealed reduced neuronal cell size and increased cell packing density (increased numbers of neurons per unit volume) in limbic brain regions such as the the hippocampus, subiculum, entorhinal cortex, amygdala, mammillary body, anterior cingulate gyrus, septum and in the medial septal nucleus (MSN) (Bauman and Kemper, 1994). There is also indications that neuroanatomical alterations in autistic individuals may change over the course of development where increased neuron size has been observed in the nucleus of the diagonal band of Broca (NDB) of the septum of adolescent autistic patients, while neuron size and number has been observed to be reduced in this area in adult autistic brains (Kemper and Bauman, 1998). The most prominent abnormalities reported outside limbic regions have been in the cerebellum and related inferior olive. A significant decrease in the number of Purkinje cells, primarily effecting the posterolateral neocerebellar cortex and adjacent archicerebellar cortex of the cerebellar hemispheres have been consistently reported in autistic brains, regardless of age, sex or cognitive abilities (Ritvo et al., 1986; Arin et al., 1991; Bailey et al., 1998). In comparison, changes in cell number in the fastigial, globose and emboliform nuclei in the roof of the cerebellum, similar to the NDB, appear to be age dependant. Specifically, in these three nuclear groups, most adult brains have small pale neurons whose numbers are significantly decreased when compared to control subjects. In contrast, all childhood brains (ages 5–13 years) examined have neurons that are enlarged and increased in number (Bauman and Kemper, 1994).

Thus taken together, the data suggest a number of neuroanatomical alterations exist in several brain regions of autistic individuals, effects which most likely underlie the behavioural symptoms displayed by these individuals.

1.7 Endocannabinoid system, immune regulation and autism

While the neurobiology of autism remains largely unknown, a wealth of evidence indicates that immune or neuroimmune mechanisms may, at least in part, underlie the pathogenesis of this spectrum of disorders (see next section below). Given that the endocannabinoid system elicits a profound effect on immune processes and responding, it is possible that alterations in the endocannabinoid system may underlie or account for at least some of the behavioural changes observed in autism, possibly via modulation of neuroimmune processes. While this theory has not yet been examined directly, the endocannabinoid system is a well-recognised regulator of emotional behaviour [for reviews see (Viveros et al., 2005; Viveros et al., 2007; Lutz, 2009)]. It has been shown that mice lacking CB₁ receptors show strongly impaired deletion of emotionally aversive memories in auditory fear conditioned tests (Marsicano et al., 2002) and heightened anxiety and depressive-like behaviour (Haller et al., 2002; Martin et al., 2002). As well as modulating emotionality, there is evidence to suggest the endocannabinoid system modulates social behaviours. Recent data have demonstrated that interaction with a non-familiar social partner increases striatal levels of AEA in rats (Marco et al., 2011), an effect proposed as a management strategy in novel social encounters. Furthermore, the FAAH inhibitor URB597, enhances social play while direct CB₁ agonism using WIN55,212-2 has been shown to reduce social play in rats (Trezza and Vanderschuren, 2008). In addition, chronic persistent WIN55,212-2 administration induced object/social recognition deficits as well as aberrations in social play and social behaviour in pubertal and adult rats, effects shown to be more pronounced in pubertal rats following acute administration (Schneider et al., 2008a). Thus, AEA may enhance social interactive behaviour. Evidence for a role of the EC system in autism primarily arises from studies investigating the effects of cannabinoids on social or stereotyped behaviour in normal animals. While such evidence indicates a possible role for this system in these behaviours, until recently no studies have examined if

endocannabinoid tone was altered in animal models of autism and thus may be responsible for the behavioural aberrations observed in these models. Recent evidence has demonstrated that in a model of fragile X syndrome (the *fmr*^{-/-} mouse), the most common genetic form of autism, central activity of diacylglycerol lipase (DAGL) α and MAGL, the enzymes responsible for the synthesis and catabolism of 2-AG respectively, is enhanced (Maccarrone et al., 2010; Jung et al., 2012). In addition, pharmacological inhibition of MAGL and subsequent augmentation of endogenous 2-AG levels, results in the normalisation of locomotor and anxiety-related behavioural changes in *fmr*^{-/-} mice (Jung et al., 2012). Furthermore, endocannabinoid signalling has been shown to be impaired in two mouse models of autism associated with different mutations of the neuroligin-3 gene. In essence, while the neuroligin-3 functionality is different, tonic endocannabinoid signaling at the CB₁ receptor and consequent suppression of GABA release was found to be impaired in both models, effects which may account for the similar behavioural profile of these animals (Foldy et al., 2013). While these latter studies indicate a possible role for the endocannabinoid system in the pathogenesis of autism, no study to date had investigated if alterations also exist in a non-genetic animal model.

1.7.1 Autism and the immune system

There has been increasing evidence over the past 20 years demonstrating altered immune function in autistic children and adults. Maternal immune activation, especially during the first or second trimesters of pregnancy has been identified, as a potential environmental risk factor for the development of autism [reviewed in (Patterson, 2009)]. Maternal exposure to rubella (Chess, 1971, 1977), measles (Singh and Jensen, 2003), herpes simplex virus (Greer et al., 1989; Ghaziuddin et al., 1992), cytomegalovirus (Markowitz, 1983; Ivarsson et al., 1990) or viral meningitis (Barak et al., 1999) have all been associated with increased risk of ASD. In addition, increased rates of autoimmune disorders such as rheumatoid arthritis, coeliac disease and psoriasis have been observed in the families of individuals with ASD (Croen et al., 2005; Atladottir et al., 2009), suggesting that foetal antibody exposure may affect development during gestation.

Anecdotal and empirical evidence suggest that children with ASD may be more susceptible to infection, with longer duration and accompanying exacerbation of

behavioural symptoms, than children without ASD. A study by Jyonouchi *et al.*, identified a subgroup of autistic children characterised by frequent infections and accompanying changes in behavioural symptoms and/or loss of cognitive skills following infection (Jyonouchi *et al.*, 2008). A study of a Danish cohort revealed that children admitted to hospital for infectious diseases, either bacterial or viral, had a significantly higher rate of autism diagnosis (Atladottir *et al.*, 2010). In addition, increased frequency of infection in the first 30 days of life, persistent viral infections in mucosal tissue, and increased frequency of otitis media and respiratory infections have all been documented in autistic children when compared to normal developing counterparts (Konstantareas and Homatidis, 1987; Mason-Brothers, 1993; Rosenhall *et al.*, 1999; Uhlmann *et al.*, 2002; Niehus and Lord, 2006; Rosen *et al.*, 2007).

Alterations in immune parameters have been reported in autistic patients. Plasma levels of cytokines such as IL-1 β , IFN α , IL-1ra, TNF α , IL-6, IL-8 and IL-12p40 have been reported to be increased in the blood of autistic children when compared to non-autistic children (Croonenberghs *et al.*, 2002; Ashwood *et al.*, 2011a). In a further study, plasma cytokine levels of IFN γ and IL-12 have been shown to be increased in autistic children while no changes were observed in IL-6, TNF α and IFN α , leading the authors to suggest a potential Th1 shift (Singh, 1996). Stimulation of PBMCs from autistic children with various TLR agonists revealed a blunted IL-6 and IL-10 response to TLR2/6 stimulation, a blunted IL-1 β response to TLR7/8 stimulation, and enhanced IL-23 response to TLR4 stimulation, overall indicating an altered immune response to bacterial/viral pathogens in autism (Jyonouchi *et al.*, 2008). In a similar study, stimulation of PBMCs from autistic patients revealed an enhanced IL-1 β , IL-6 and TNF α response following TLR2 activation and an enhanced IL-1 β response following TLR4 stimulation. Conversely, following TLR 9 stimulation there was a decrease in IL-1 β , IL-6, and TNF α responses in monocyte cell cultures from children with ASD compared with controls, overall indicating a differential innate immune response following TLR 2, 4, and 9 stimulation and an altered TLR signalling pathway in children with ASD (Enstrom *et al.*, 2010).

Dysfunction of the peripheral immune system has been demonstrated to be associated with increased severity of behavioural symptoms in autism. Lower plasma levels of active TGF β in autistic patients were associated with lower adaptive behaviours and worsening behavioural symptoms (Ashwood *et al.*, 2008). Elevated

plasma chemokines MCP-1 (CCL2) and RANTES (CCL5) levels have been shown to be associated with higher aberrant behaviour scores and more impaired developmental and adaptive function (Ashwood et al., 2011b). In addition, elevated plasma and stimulated monocyte levels of the pro-inflammatory cytokines IL-1 β and IL-6 were associated with aberrant behaviours such as lethargy, stereotypy, hyperactivity as well as impaired social behaviours and non-verbal communication (Enstrom et al., 2010; Ashwood et al., 2011b). These data suggest a role for inflammation in mediating the behavioural deficits in autism including social interaction, communication and stereotypy. [For review see (Onore et al., 2012)].

In addition to the altered inflammatory cytokine profile in the plasma of autistic patients, recent data suggest that enhanced or altered central neuroinflammatory processes are also present in this disorder. Pro-inflammatory cytokines (TNF α , IL-6 and GM-CSF), Th1 cytokine (IFN- γ) and chemokines (IL-8) have been reported to be increased in the brains of autistic patients while the Th2 cytokines (IL-4, IL-5 and IL-10) were unaltered, indicating a skewed Th1/Th2 ratio in these subjects (Li et al., 2009). An elaborate study by Vargas and colleagues investigated the profile of over 79 proteins including cytokines, chemokines and growth factors in post-mortem brains of autistic patients. The study revealed a marked activation of microglia and astrocytes in cortical and cerebellar regions, and elevated MCP-1 (CCL2) and TGF- β 1 in several cortical areas. A large spectrum of pro-inflammatory and modulatory cytokines were enhanced in the anterior cingulate gyrus, a brain region of significance as it is the neural centre for the processing of emotional responding and facial expression recognition (Jehna et al., 2011; Klumpp et al., 2012). In addition, analysis of the pro-inflammatory cytokine profile in the CSF of autistic patients demonstrated enhanced levels of MCP-1 IL-6, IL-8 and IFN γ when compared to controls (Vargas et al., 2005).

Thus, increasing evidence suggests altered peripheral and central inflammatory responses in autistic patients, effects which appear to be associated with more severe behavioural deficits. Due to the intricate nature of this disorder and the ability to obtain samples for testing, animal models have provided a means of investigating the neurobiology this disease.

A brief overview of some of the most widely used animal models are presented in Table 1.5.

Table 1.5 Rodent models of autism and associated social behaviour or communication deficits. Adapted from (Belzung et al., 2005).

Table removed due to copyright

1.7.2 Immune and neuroimmune alterations in animal models of autism

Increasing evidence suggests that there is correlation between prenatal exposure to pathogens, such as measles, rubella, viral meningitis and even influenza during gestation and the occurrence of autism. During normal development, cytokines function as important regulatory molecules in all phases of CNS development (Burns et al., 1993; Dame and Juul, 2000; Deverman and Patterson, 2009). Therefore, infections during this critical developmental period have been proposed to result in dysregulation of cytokine homeostasis which may affect neurodevelopment. Thus, a number of preclinical models have been developed, termed maternal immune activation (MIA) models, where activation of the maternal immune system results in exposure of the developing foetus to maternal cytokines resulting in immunological and behavioural abnormalities in the offspring similar to those observed in autism and various other neurodevelopmental disorders [for review see (Boksa, 2010)].

Table 1.6 shows a summary of central and peripheral immune changes in offspring following maternal immune activation with various immunogens.

Table removed due to copyright

Table 1.6 *Central and peripheral immune changes in offspring following maternal immune activation with various immunogens. (Boksa, 2010)*

Evidently, maternal infection results in an altered cytokine profile in the developing offspring which not only is evident 0-24 hour following acute infection but may persist throughout adolescence and adulthood depending on the profile of maternal exposure.

Various studies have demonstrated that prenatally exposed offspring also exhibit an altered immune response. Williams *et al* demonstrated that mouse maternal systemic inflammation following a high dose of LPS caused blunted immune responsiveness in LPS-challenged adult offspring. Post-LPS serum levels of pro- and anti-inflammatory cytokines IL-1 β , IL-9, IL-10 and chemokines CCL4, CCL5 and CCL2 (MCP-1) were significantly reduced when compared to their saline counterparts (Williams et al., 2011). Furthermore, Lasala *et al* demonstrated that 2 hours post-LPS stimulation, offspring born to LPS-treated dams exhibited diminished serum levels and brain expression of pro-inflammatory cytokines TNF α , IL-1 β , and IL-6 as compared to offspring born to saline-treated dams (Lasala and Zhou, 2007). Similar blunted responses following an immune challenge were observed in 20 day old LPS prenatally exposed rats, while 50 day old offspring had similar responses to control offspring (Hodyl et al., 2007).

Apart from MIA models there is a paucity of studies examining immune changes in other models of autism. Intracerebral administration of neonatal rats with the Borna disease virus has been shown to result in behavioural and neurodevelopmental abnormalities accompanied by elevations in expression of IL-1 α , IL-1 β , IL-6 and TNF α in multiple brain regions as well as sustained glial activation (Hornig et al., 1999; Sauder and de la Torre, 1999), again indicating that heightened neuroimmune activation can lead to neuronal alterations that mediate behavioural changes which resemble those observed in autism.

1.8 The valproic acid rodent model of autism

Few studies have examined (neuro)immune parameters in environmental models that do not involve administration of an immunogen prenatally or neonatally. Prenatal exposure to valproic acid (VPA) during gestation (G 9-12.5) induces symptoms in rat offspring which resemble those observed in autistic patients and has been

proposed as a preclinical model to study this disorder (Schneider and Przewlocki, 2005).

1.8.1 Valproic acid (VPA): clinical use and mechanism of action.

Valproic acid (2-propylvaleric acid, 2-propylpentanoic acid) is a branched short chain fatty acid derived from valeric acid which is naturally produced by the valerian plant *Valeriana officinalis*. It has been used clinically for over 20 years in the treatment of epilepsy and as a mood stabiliser in the treatment of bipolar disorder and depression (McElroy et al., 1989). The antiepileptic and mood elevating properties of VPA has been attributed to its ability to enhance the inhibitory actions of the neurotransmitter Gamma Amino Butyric Acid (GABA) by inhibition of the GABA catabolic enzyme 4-aminobutyrate aminotransferase (ABAT), increasing GABA synthesis and decreasing turnover (Mesdjian et al., 1982). In addition, VPA has been shown to attenuate N-Methyl-D-Aspartate (NMDA) receptor-mediated excitation (Zeise et al., 1991; Gean et al., 1994) and inhibit Na⁺, Ca²⁺ channels and voltage-gated K⁺ channels (VanDongen et al., 1986).

VPA is also a histone deacetylase (HDAC1) inhibitor, acting to modulate the acetylation of histones and chromatin structure and thereby regulating gene expression. As a HDAC inhibitor, VPA and associated analogues may have potential in modulating the biological processes underlying a wide range of other pathologies including mood disorders, narcotic addiction, muscle disorders and HIV and is presently the subject of over 200 clinical trials involving the pathologies mentioned, reviewed in (Chateauvieux et al., 2010). In particular, VPA has been shown to potently modulate the biology of diverse tumour cells by inhibition of proliferation and induction of differentiation and increasing apoptosis and thus decreasing metastatic potency, effects tightly coupled to its HDAC inhibitory activity (Gottlicher et al., 2001; Phiel et al., 2001; Blaheta and Cinatl, 2002). Thus VPA and other HDAC inhibitors have been proposed as potent anti-cancer agents [for review see (Tan et al., 2010)].

An important consideration of the clinical use of VPA is the recognised clinical and pre-clinical research demonstrating that the use of VPA during pregnancy can result in enhanced risk of development of major congenital malformations, developmental delay, reduced cognitive function, and increased risk of autism, [for review see

(Wlodarczyk et al., 2012)]. Thus careful consideration must be given to the clinical prescription of VPA and other HDAC inhibitors to women of childbearing age. However, preclinical studies have taken advantage of this observation in order to investigate how VPA elicits such effects during pregnancy and also the consequential effects on the offspring. This has led to the development of the VPA model of autism, a now well recognised and validated preclinical model in rodents.

1.8.2 Immune changes in the VPA model

To date, only one study has examined immune alterations in this model of autism, demonstrating that rats prenatally exposed to VPA exhibited decreased thymus weight, decreased proliferative capacity of splenocyte cultures to concanavalin A (Con A) and increased production of nitric oxide by peritoneal macrophages, both basally and following LPS stimulation (Schneider et al., 2008b). In addition, while basal or Con A-induced IFN γ and IL-10 production by splenic lymphocytes did not differ between controls and VPA-exposed animals, the Con A-induced IFN γ /IL-10 ratio was decreased in VPA rats, possibly indicating an imbalanced Th1/Th2 response in the model (Schneider et al., 2008b) similar to that observed clinically.

Overall, in accordance with the clinical data, various preclinical models of autism exhibit immune dysregulation and behavioural abnormalities similar to those observed in autistic patients. These models may provide a better understanding of the neuropathological, behavioural and possible genetic factors observed in the disorder and allow us to examine the role of the endocannabinoid and/or immune system in mediating behavioural changes associated with autism.

1.9 Research Objectives

Neuroinflammation is a key component of various neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis and psychiatric and neurodevelopmental disorders such as depression and autism. As such, the need to develop a greater understanding of the neurobiological mechanisms mediating neuroinflammation is critical at a fundamental physiological level and for the development of novel, more efficacious treatments. Accumulating evidence indicates that the endocannabinoid system plays a significant role in modulating the immune system and represents an important therapeutic target in the treatment of both central and peripheral inflammatory disorders. Thus, one of the main objectives of the work presented herein was to enhance our understanding of the role of the endocannabinoid system in the modulation of peripheral and central inflammation *in vivo* (Chapter 3-4).

A further objective was to investigate the extent to which these neuroinflammatory processes may be altered in a preclinical model of autism and to investigate if alterations in the endocannabinoid system was accompanied by behavioural changes in this model (Chapter 5-6).

Specific aims were:

1. To investigate the effect of URB597, a selective inhibitor of FAAH, the enzyme that catabolises the endocannabinoid AEA, on LPS-induced changes in cytokine expression in the hypothalamus, an important site of cytokine-mediated regulation of physiological function and stress responses. A further aim was to investigate if alterations in LPS-induced cytokine expression following FAAH inhibition were associated with changes in NF κ B or SOCS3 expression in the hypothalamus or alterations in plasma corticosterone levels.
2. To investigate the effects of JZL184, a selective inhibitor of MAGL, the enzyme that preferentially catabolises the endocannabinoid 2-AG, on LPS - induced increases in cytokine expression in the rat frontal cortex and plasma and examine the possible receptor and molecular mechanisms involved. The

role of arachidonic acid and prostaglandins on 2-AG-mediated alterations in LPS-induced cytokines were also evaluated.

3. To investigate if basal expression of immune mediators in the frontal cortex, hippocampus and cerebellum, regions implicated in autistic-like symptoms, were altered in the VPA rat model of autism. A further aim was to investigate if VPA-exposed animals elicited an altered neuroimmunological response to TLR4 stimulation.

4. To investigate if alterations in the endocannabinoid system was accompanied by behavioural changes in the VPA rat model of autism. Behavioural responding (social, anxiety, thermal nociceptive responding) was assessed in adolescent rats prenatally exposed to VPA. Endocannabinoid and *N*-acylethanolamine levels, the expression of genes regulating the synthesis and catabolism of AEA and 2-AG, the expression of CB₁ and CB₂ receptors and other targets of the endocannabinoid system were subsequently investigated in various brain regions implicated in mediating autistic symptoms.

Chapter 2

Methods

While methods sections are provided within each results chapters (paper format), due to word restrictions detailed methodologies could not always be provided. This section expands on those methodologies and techniques that were developed and used during the course of these studies.

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

2.1.1 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). 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). 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,

Dublin) followed by centrifugation at 14,000g for 1 minute. The eluted RNA was then stored at -80°C until quantification and reverse transcription.

2.1.2 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 OD₂₆₀/OD₂₈₀ 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.

2.1.3 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, Fannin, Dublin) 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.

2.1.4 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 (Assay ID: Rn00667869_m1) 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 1.25 μ l target primers, 1.25 μ l β -Actin (multiplex version) and 12.5 μ l TaqMan master mix (Cat # 4324018: Applied Biosystems, UK) per sample. cDNA samples were diluted 1:4 and 10 μ l of each diluted sample was pipetted in duplicate onto a MicroAmp® optical 96 well plate (Applied Biosystems, UK). 15 μ l of the relevant reaction mixture was then added to each well giving a total reaction volume of 25 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.

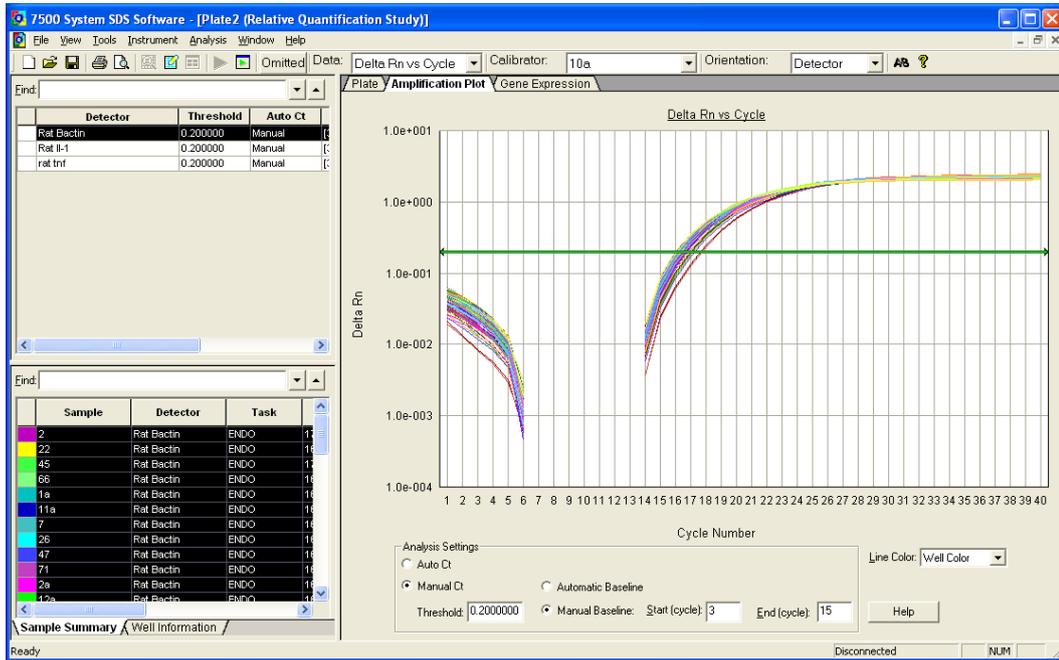
Target Gene	Assay number
Immune mediators	
IL-1 β	Rn00580432_m1
TNF α	Rn99999017_m1
IL-6	Rn00561420_m1
IL-10	Rn00563409_m1
I κ B α	Rn01473658_g1
SOCS3	Rn00585674_s1
CCL2	Rn00580555-m1
CCL5	Rn00579590
TLR4	Rn00569848-m1
MD-2	Rn01448830-m1
Markers of glial activation	
CD11b	Rn00709342-m1
GFAP	Rn00566603-m1
Endocannabinoid synthetic enzymes	
NAPE-PLD	Rn01786262_m1
DAGL α	Rn01454304_m1
DAGL β	Rn01453775_m1
Endocannabinoid catabolic enzymes	
FAAH	Rn00577086_m1
MAGL	Rn00593297_m1
Cannabinoid receptors	
CB ₁	Rn00562880_m1
CB ₂	Rn03993699_s1
PPAR α	Rn00566193_m1
PPAR γ	Rn00440945_m1
GPR55	Rn03037213_s1
Endogenous control	
β -actin	Rn4352340E

Table 2.1. List of TaqMan gene expression assays used.

2.1.5 Analysis of qRT-PCR Data

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 and Schmittgen, 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. In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.

(a)



(b)

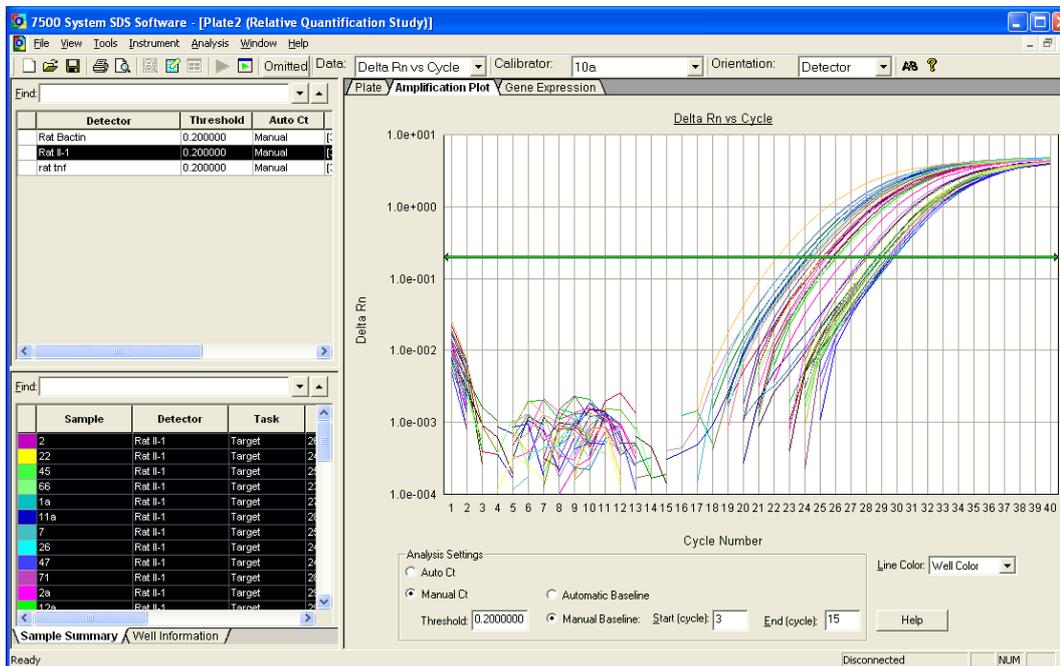


Fig 2.1. Sample Amplification Plots for (a) the endogenous control β -actin and (b) IL-1 β

2.2 Determination of cytokine protein levels using ELISA

Plasma and spleen TNF α (cat# DY510), IL-1 β (cat# DY501), IL-6 (cat# DY506) and IL-10 (cat# DY522) protein concentrations were determined using specific rat enzyme-linked immunosorbent assays (ELISAs) performed using antibodies and standards obtained from R&D Systems Europe Ltd, UK.

Plasma was isolated from whole blood by centrifugation at 5000g for 15min, plasma removed and stored in aliquots at -80°C until analysis. Spleen tissue (50-100mg) was sonicated in 1ml of phosphate-buffered saline [PBS: NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 8.1 mM, KH₂PO₄ 1.5 mM; pH 7.4] using a Branson sonicator (Mason Technology, Ireland) followed by centrifugation at 14,000g for 15 mins. The supernatant was removed and stored at -80°C until cytokine and protein determination.

2.2.1 ELISA Assay protocol

- Maxisorb Nunc microtitre plates (Biosciences Ltd. Dublin) were coated with 100 μ l of goat or mouse anti-rat cytokine antibodies (0.8–4 μ g/ml in PBS), covered with an adhesive plate cover and left at RT for 20 hours.
- Plates were then aspirated washed three times with 400 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 RT with 300 μ l reagent diluent (1% BSA in PBS, pH 7.4).
- Standards for each cytokine were prepared (5000-0 pg/ml) in reagent diluent (1% BSA in PBS, pH 7.4); with the addition of 2% v/v heat inactivated goat serum for IL-1 β and IL-6 assays.
- Following blocking, the plates were washed a further three times, following which 100 μ l aliquots of samples or standards were added, and plates were incubated at RT for 2 hours.
- After three washes, 100 μ l of specific biotinylated anti-goat or -mouse detection antibody (100-400ng/ml) was added to each well and plates incubated for 2 h at RT.
- After three washes, 100 μ l of horseradish peroxidase conjugated to streptavidin (1/200) was added to each well, and plates were incubated at 22 °C for 20 minutes.

- Following three washes, 100 μl of tetramethylbenzidine substrate solution containing H_2O_2 (T0440: Sigma-Aldrich, Dublin) was added to each well and the plate was placed in reduced light until adequate development of a blue colour, following which 50 μl of 2M H_2SO_4 was added per well to stop the reaction.
- Absorbance was read immediately at 450nm on a μQuant Bio-Tek plate reader (Mason Technology, Ireland).
- Cytokine levels were extrapolated from standard curves of cytokine concentration (pg/ml) versus optical density at 450nm (GraphPad Prism).
- The Bradford protein assay was used to determine protein concentration in spleen samples.
- Cytokine levels in plasma were expressed as pg/ml or in the spleen as pg/mg protein. Assay detection limits were 62.5pg/ml for IL-1 β , IL-10 and TNF α and 125pg/ml for IL-6.

2.3 Bradford Protein Assay

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

Conc $\mu\text{g/ml}$	0	100	250	500	750	1000	1500
μl stock BSA	-	10	25	50	75	100	150
μl H_2O or PBS	200	190	175	150	125	100	50
Tot Vol μl	200	200	200	200	200	200	200

Table 2.2. BSA standards.

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

Protein content of samples were extrapolated from standard linear regression plots of protein concentration $\mu\text{g/ml}$ versus optical density (OD) at 595nm (GraphPad Prism).

2.4 Corticosterone Enzyme Immunoassay (EIA)

Quantification of corticosterone in serum samples was performed with the Corticosterone EIA Kit according to the manufacturer's instructions (Item no. 500651, Cayman Chemical Company, Cambridge Biosciences, UK). Plasma samples were defrosted on ice and spun at 14,000g at 4°C for 15 minutes. To the 96-well plate provided, 100µl of EIA buffer was added to Non-Specific Binding (NSB) wells, and 50µl of EIA buffer was added to Maximum Binding (B_0) wells. 50µl of S1-S8 corticosterone EIA standards (10,000, 4,000, 1,600, 640, 256, 102, 41, and 16 pg/ml) were added in duplicate into corresponding wells on the plate, and 50µl of each sample was added in duplicate into designated wells. 50µl of Corticosterone AChE Tracer was added to each well except the Total Activity (TA) and the Blank (Blk) wells, and 50µl of Corticosterone EIA Antiserum was added to each well except the TA, Blk, and NSB wells. The plate was covered with an adhesive plate cover and incubated for two hours at RT on an orbital shaker, after which wells were washed five times with wash buffer. Thereafter, 200µl of Ellman's Reagent was added to each well, and 5µl of tracer to the TA well. The plate was covered with adhesive cover and incubated on an orbital shaker in the dark to allow optimum development which was obtained when the absorbance of the B_0 wells >0.3 units (blank subtracted). The plate was then read at a wavelength of 412nm after 60 minutes on a µQuant Bio-Tek plate reader. NSB average readings were subtracted from all sample and standard readings to obtain the amount of bound protein B (sample or standard bound) and from B_0 average readings to obtain corrected B_0 . Sample corticosterone levels were subsequently determined from a standard curve of $\ln(B/B_0/(1-B/B_0))$ versus log corticosterone concentration (pg/ml) (GraphPad Prism). The detection limit for the assay was 30pg/ml and all sample readings were above this limit of detection.

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

2.5.1 Preparation of Standards

Non-deuterated stock solutions were prepared in 100% acetonitrile for N-arachidonyl ethanolamide (AEA), 2-arachidonylglycerol (2AG), N-oleoyl ethanolamide (OEA) and N-palmitoyl ethanolamide (PEA) (Cayman chemicals, Cambridge Biosciences, UK) at concentrations of 2.5mg/ml for AEA, PEA, OEA and 0.5mg/ml for 2AG. A single stock solution of all the above standards was then prepared containing 2AG at a concentration of 5µg/ml and AEA, PEA and OEA at 0.5µg/ml in 100% acetonitrile.

Stock solutions of the deuterated form of AEA(d8), 2AG(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 2AG(d8) at 100ng/400µl and AEA(d8), OEA(d2) and PEA(d4) at 5ng/400µl. The deuterated homogenizing buffer was then dispensed into 20ml aliquots and stored with non-dueterated standards at -80°C.

2.5.2 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 (Ford et al., 2011). 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). Lyoph ilised 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 2.3. 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 (Table 2.4).

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 2.4. 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. (Peak Area analyte_(undeuterated)/Peak area analyte_(deuterated)) vs (Conc analyte_(undeuterated)/Conc analyte_(deuterated)) (Fig 2.2). 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 2.3).

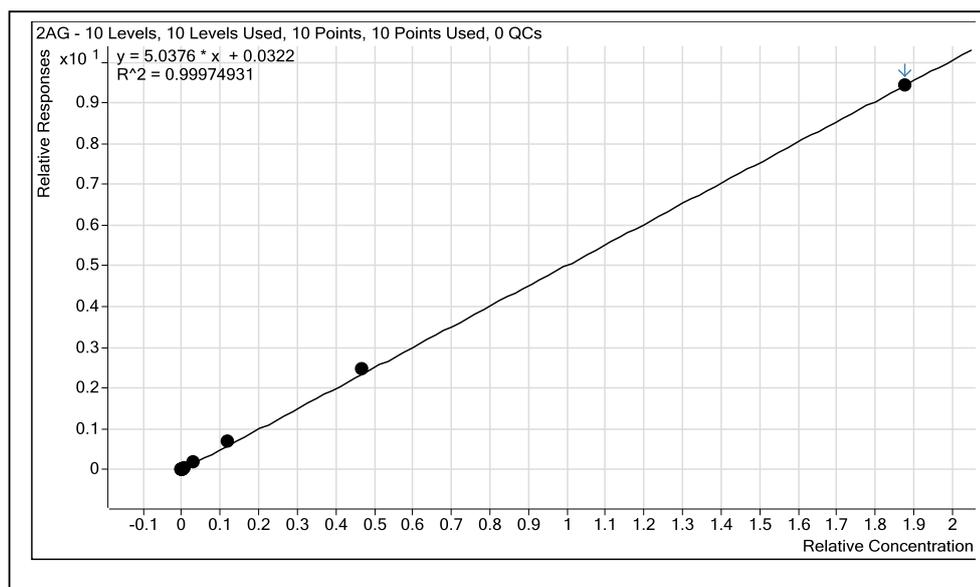


Fig 2.2. Plot of Relative Response vs. Relative Concentration for 2-AG.

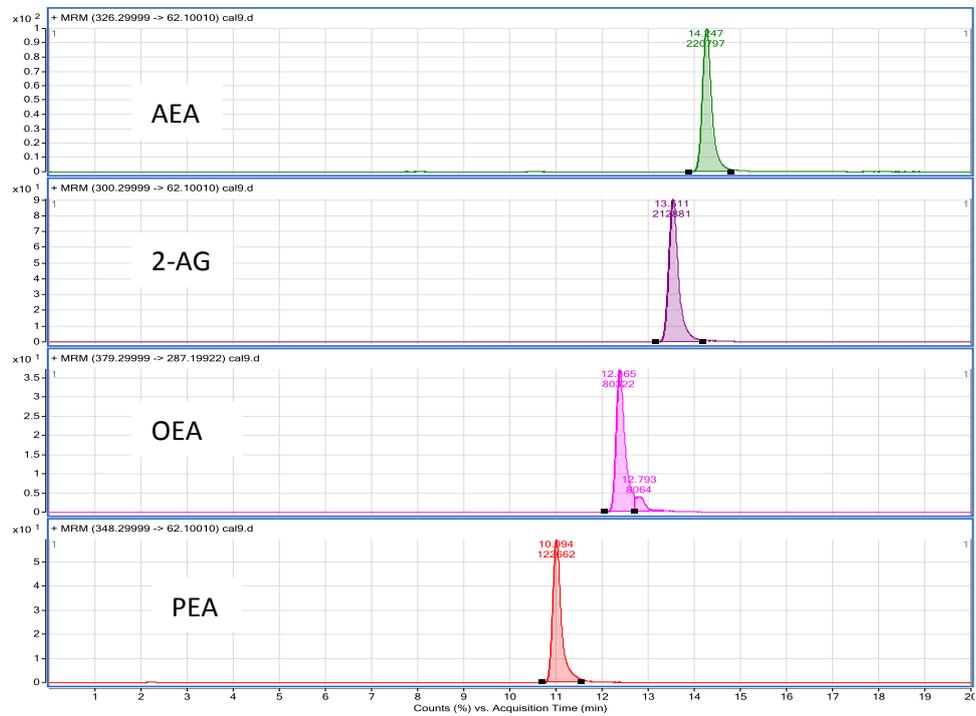


Fig 2.3. Chromatograms of AEA, 2AG, PEA and OEA.

2.6 Prostaglandin PGE2 and PGD2 tissue levels determination using LC-MS/MS

Homogenising buffer was prepared containing tetra deuterated labelled internal standards (600 pg/400µl (1.7pmoles) PGE2(d4) and PGD2(d4)) (Cayman chemicals, Cambridge Biosciences, UK). An undeuterated stock solution containing both PGE2 and PGD2 at a concentration 500ng/ml (Cambridge Biosciences, UK) was prepared in acetonitrile and then serially diluted in acetonitrile to yield a 7 point standard curve with a concentration range of 6.66ng to 27.3 pg for both PGE2 and PGD2. 400µl of the deuterated homogenising buffer was added to each point of the standard curve.

Spleen or cortical samples (50-70mg) were homogenised in 400µl of homogenising buffer and centrifuged at 14,000g for 15 minutes at 4°C and the supernatant was collected and evaporated to dryness along with standards in a centrifugal evaporator (Thermo SPD131DDA-230, Fischer Scientific, Ireland). Lyophilised samples and standards were resuspended in 40µl 25% acetonitrile and 4µl injected onto a Zorbax® SB C18 column (150 × 0.5 mm internal diameter) from a cooled autosampler maintained at 4 °C. 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. Analytes were eluted under gradient elution (Table 2.5).

Time(min)	%B(CH ₃ CN, 0.1% formic acid)	%A (H ₂ O, 0.1% formic acid)
0	25	85
20	50	50
25	100	0
30	100	0
31	STOP	

Table 2.5. Gradient used to elute analytes

Under these conditions, PGE2 and PGD2 eluted at 19.8 and 21 minutes respectively. Analyte detection was carried out in electrospray-negative ionisation and multiple reaction monitoring (MRM) mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6410 mass spectrometer (Agilent Technologies Ltd. UK). As PGE2 and PGD2 are structural isomers, the transitions monitored were identical for

both, and for their respective internal standards i.e parents 351 >daughter 271 and 355>275 respectively with a collision energy of 10 eV. Quantitation of each analyte was performed by determining the peak height response of each target analyte against its corresponding deuterated internal standard. The amount of analyte in unknown samples was calculated from a standard curve of Relative response vs Relative concentration for each analyte i.e. $(\text{Peak height analyte}_{(\text{undeuterated})} / \text{Peak height analyte}_{(\text{deuterated})})$ vs. $(\text{Conc analyte}_{(\text{undeuterated})} / \text{Conc analyte}_{(\text{deuterated})})$ (Fig 2.4). Linearity was determined over a range of 6.66ng to 27.3pg for both PGE2 and PGD2. Results were expressed as pmol/g of wet tissue. Sample chromatograms are shown below (Fig 2.5).

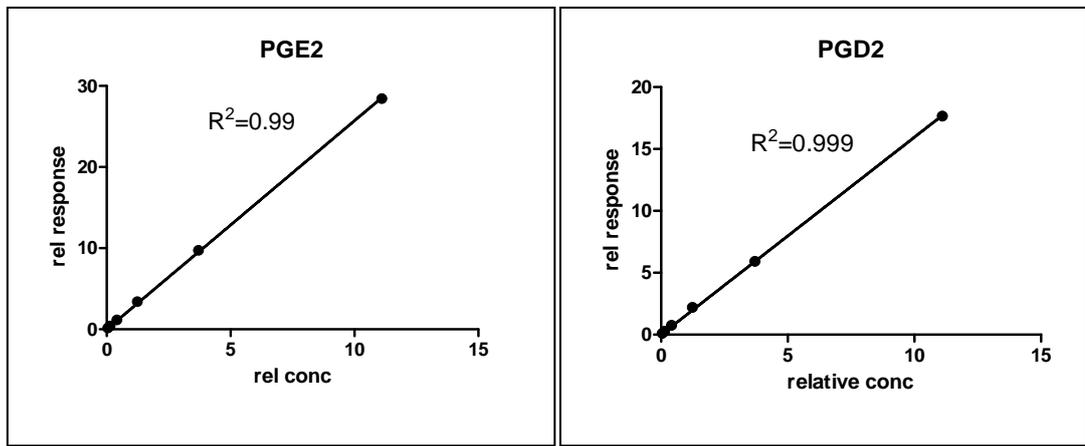


Fig 2.4. Plots of Relative response vs. Relative Concentration for PGE2 and PGD2

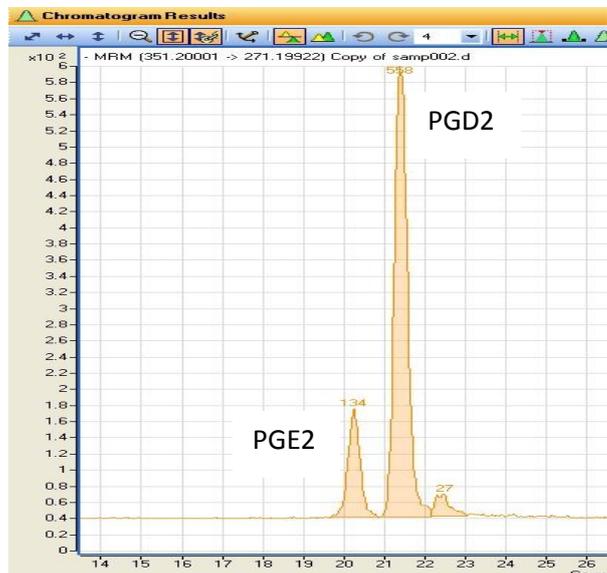


Fig 2.5. Chromatograms of PGE2 and PGD2

2.7 Qualitative detection of JZL184 in cortical and spleen tissue following systemic administration using LC-MS/MS

A standard picomolar solution of JZL184 (generously received from Prof Benjamin Cravatt, USA) 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 a 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: Cortical or spleen samples previously prepared and analysed for endocannabinoid determination were resuspended in 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 (Table 2.6).

Time (min)	%B(CH ₃ CN, 0.1% formic acid)	%A (H ₂ O, 0.1% formic acid)
0	25	75
10	100	0
20	100	0
21	25	75
25	STOP	

Table 2.6 Elution gradient

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 2.6).

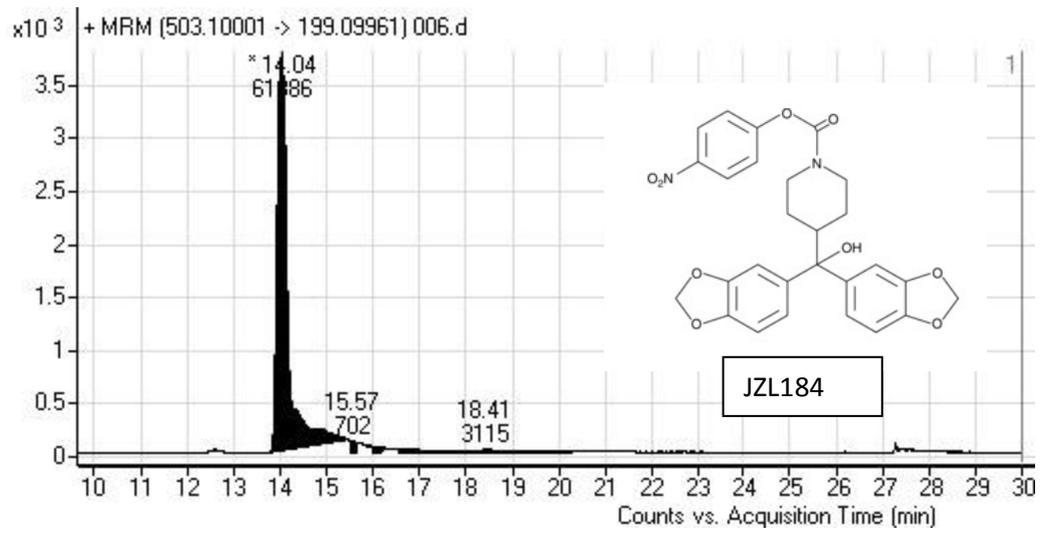


Fig 2.6. Chromatogram of JZL184 in rat spleen.

2.8 HPLC-UV determination of arachidonic acid (AA) concentration

Determination of arachidonic acid (AA) was carried out by HPLC with modifications to the method as described by (Lang et al., 1996). Pre-weighted cortical or spleen samples (60-100mg) were sonicated using a Branson sonicator in 1ml of mobile phase (8.5% phosphoric acid/acetonitrile (10:90 v/v) spiked with 50ng/20µl biphenyl as internal standard. Samples were then centrifuged at 14,000g for 15 minutes at 4°C and 20µl of the supernatant or standard mix (200ng/20µl AA spiked with 50ng/20µl biphenyl (Sigma-Aldrich, Ireland) prepared in mobile phase was then injected onto the Shimadzu HPLC system (Mason Technology, Ireland) consisting of a CTO-10Avp oven, LC-10ATvp pump, SIL-10Axl autosampler and SPD-10Avp UV detector. Separation was carried out at 30°C on a Synergie 4µm, 250x4.6mm reverse phase column (Cat#:00G-4337-E0, Phenomenex, UK) at a flow rate of 1ml/minute (isocratic elution) and detected on the SPD-10A UV detector at a wavelength of 204nm. The standard mix was run intermittently between samples. Chromatograms were recorded and integrated by Shimadzu CLASS-VP software (Mason Technology, Dublin) (Fig 2.7). Quantification of arachidonic acid levels was based on the ratiometric analysis of sample and standard peak heights at 204nm. The linearity between AA concentration and peak height was also determined. (Fig 2.8) The peak heights for AA and the internal standard were recorded for each sample and the standard mix. The average value of peak heights for the standard mixes was then used to determine the relative retention factor (RRF) for AA.

$$\mathbf{RRF_{AA} \text{ in the standard mix} = (CONC_{IS \text{ MIX}} \times PH_{AA \text{ MIX}}) / (CONC_{AA \text{ MIX}} \times PH_{IS \text{ MIX}})}$$

Assuming under identical operating conditions that RRF_{AA} in the standard mix = RRF_{AA} in the sample then;

$$\mathbf{CONC_{AA} \text{ in the sample} = (PH_{AA \text{ SAMPLE}} \times CONC_{IS \text{ SAMPLE}}) / (PH_{IS \text{ SAMPLE}} \times RRF_{AA} \text{ in the standard mix) where;}$$

- $CONC_{IS \text{ MIX}}$: Weight of the internal standard in the vol of standard mix injected
- $CONC_{IS \text{ sample}}$: Weight of the internal standard in the vol of sample injected
- $CONC_{AA \text{ MIX}}$: Weight of the AA in the vol of standard mix injected
- $CONC_{AA \text{ SAMPLE}}$: Weight of the AA in the vol of sample injected
- $PH_{AA \text{ sample}}$: Peak height of the AA in the sample
- $PH_{AA \text{ MIX}}$: Peak height of the AA in the standard mix
- $PH_{IS \text{ MIX}}$: Peak height of the internal standard in the standard mix
- $PH_{IS \text{ SAMPLE}}$: Peak height of the internal standard in the sample

The above calculation yielded AA concentration in ng/20 μ l, i.e. per volume injected. Final concentrations were expressed as nmol/g of tissue.

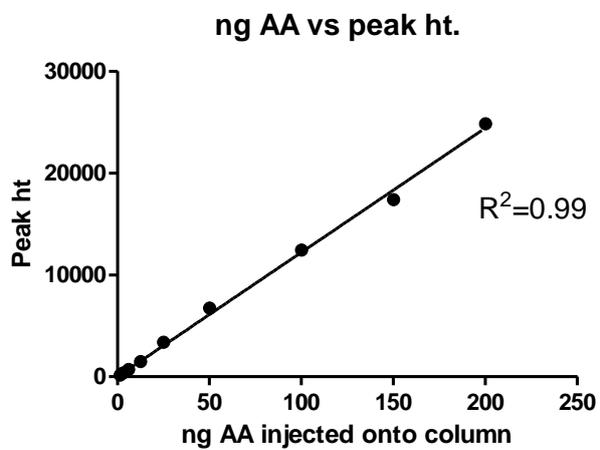


Fig 2.7 Linearity of AA concentration vs. Peak height.

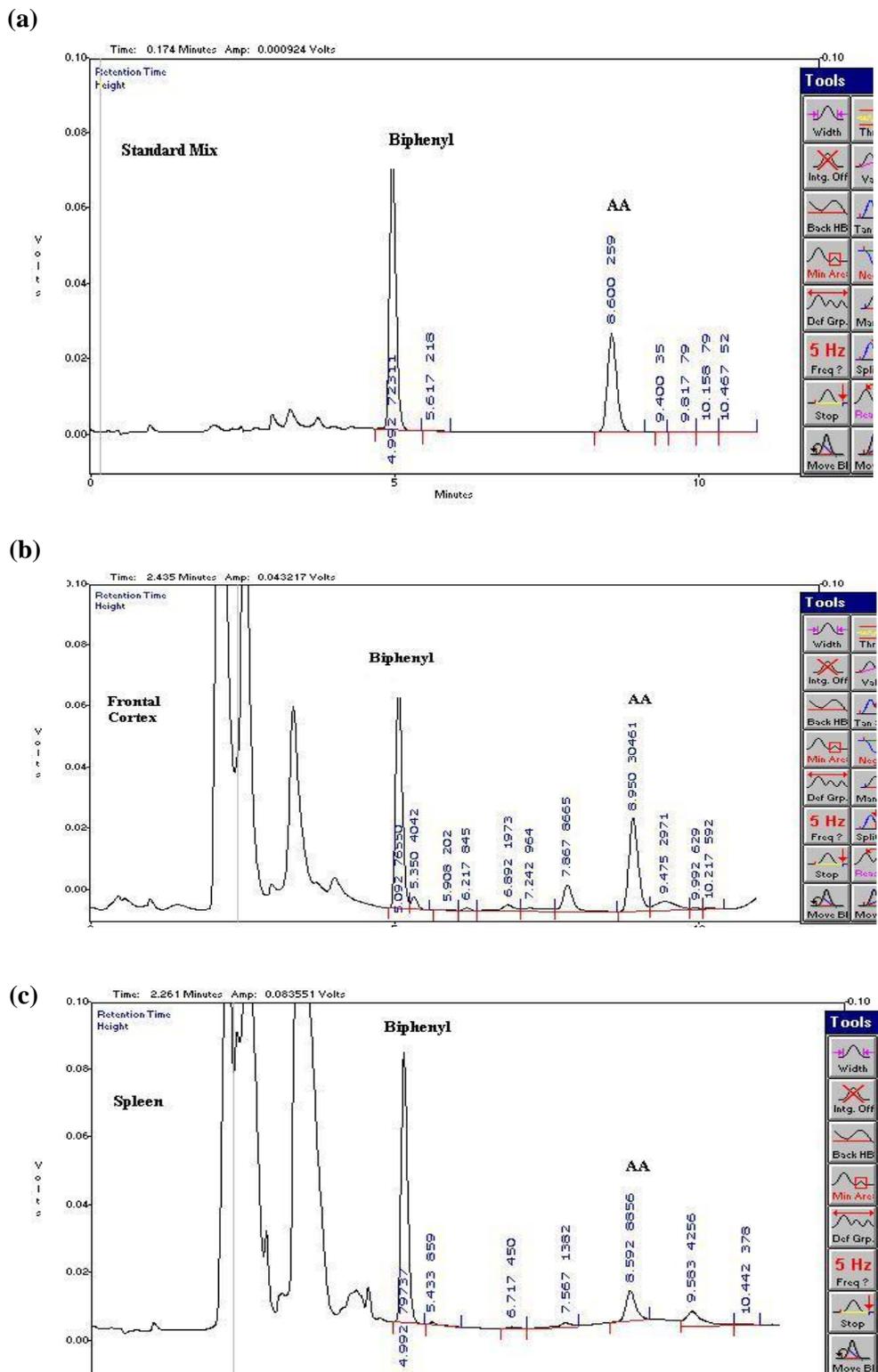


Fig 2.8 Chromatogram of (a) standard mix, (b) frontal cortex and (c) spleen

2.9 FAAH and MAGL enzyme activity assay

2.9.1 Reagents /solutions

ART 0400 2-Monooleoyl glycerol, [glycerol-1,2,3- ³ H], (50μCi) at a concentration of 1 mCi/ml American Radiolabelled Chemicals Inc), stored at -20°C in radiation suite		
ART 0626 Anandamide, [ethanolamine-1- ³ H, (50μCi) at a concentration of 1mCi/ml (American Radiolabelled Chemicals Inc), stored at -20°C in radiation suite		
TE buffer	50 mM TrisHCl/EDTA pH 7.4.	7.6 g TRIZMA [Tris/HCl], 380 mg EDTA per litre of distilled water. (Sigma-Aldrich, Ireland)
HH Incubation buffer	Hanks/Hepes with 1 mg/ml defatted albumin pH 7.4	NaCl 116 mM [6.5g/l], KCl 5.4mM [0.4g/l] CaCl ₂ .2H ₂ O 1.8 mM [0.26 g/l], HEPES 25 mM [5.9g/l], MgSO ₄ 0.8 mM [0.2 g/l], NaH ₂ PO ₄ .2H ₂ O 1 mM [0.296g/l] de-fatted albumin was added at 1mg/ml as required. (Sigma-Aldrich, Ireland)
Anandamide	Tocris UK	Stock solution 10 mg/mL in ethanol. 40 μM working solution was prepared in HH incubation buffer.
2-Oleoylglycerol	Sigma-Aldrich, Ireland	10 mM working solution was prepared in ethanol and stored in 100 μL aliquots at -80 °C
[³H]-AEA (FAAH Substrate)	2 μl [³ H]-AEA (equivalent to 2μCi or 74kBq) was added to 200 μl of 40 μM AEA (described above).	This solution was stored in glass vials at -20°C for short periods (less than 1 month). Note [³ H] atom substituted in the ethanolamine moiety.
[³H]-2OG (MAGL Substrate)	3.75 μl (equivalent to 3.75μCi or 138.75kBq) 2-oleoyl-[³ H]-glycerol is added to 100 μl of 10 mM 2OG and then diluted with 400 μL ethanol.	This solution was stored in glass vials at -20°C for short periods (less than 1 month). Note [³ H] atom substituted in the glycerol moiety.
Stop solution (activated acidified charcoal)	HCl/8 % w/v charcoal	2 g of activated charcoal (Sigma-Aldrich, Ireland) was added to 25 ml of 0.5 M HCl. This solution was prepared just prior to the experiment and constantly stirred until use.

Table 2.7 Reagents /solutions

Enzyme activity assays were conducted essentially as previously described (Boldrup et al., 2004; Ghafouri et al., 2004; Cable et al., 2011)

2.9.2 Sample preparation

Hippocampal, frontal cortical or spleen tissue was weighed (~20 mg), homogenized in 1mL of TE buffer using an Ultra-Turrax Polytron tissue disrupter (Fisher Scientific, Dublin) and centrifuged at 14,000g for 15 min. The pellet was resuspended in 1mL of TE buffer, centrifuged and resuspended in a final volume of TE buffer so as to give a 1 in 1000, 1 in 5000 or 1 in 500 dilution of the initial wet brain or spleen tissue weights (e.g. if weight of original tissue = 20mg, pellet was first suspended in 200µl of TE buffer to give an initial 1/10 dilution and subsequent dilutions then prepared from this). An appropriate dilution of the tissue was required to give between 5-15% conversion of substrate over blank to ensure linearity of enzyme kinetics. A 1 in 5000 and 1 in 1000 dilution was sufficient for determination of MAGL and FAAH activity in brain tissue respectively while a 1 in 500 dilution was required for the determination of MAGL activity in spleen tissue.

2.9.3 Enzyme Assay

90 µl of sample aliquots or blanks were pre-incubated with 5 µl of Hanks/Hepes buffer containing 1 mg/ml defatted albumin for 30 min at 37 °C. After pre-incubation, 5 µl of either FAAH or MAGL substrate was added to the samples or blanks to give a final [³H]-AEA concentration of 2 µM or [³H]-2-OG concentration of 100 µM. The reactions were allowed to proceed for 15 min and 30 min at 37 °C for MAGL and FAAH activity, respectively, following which 300 µl of stop solution (8% w/v charcoal in 0.5 M HCl) was added with mixing. Samples were allowed to stand for 20 min, centrifuged at 14,000 × g for 5 min to pellet the charcoal, to which unhydrolysed ³H-substrate and unlabelled substrate was bound and 200 µl of the supernatant was placed in scintillation vials (Sarstedt Ltd, Ireland), to which 3 ml of scintillation fluid was added. Vials were then counted on a Perkin Elmer 1409 Wallac scintillation counter (Perkin Elmer, Ireland) programmed to give an average ³H count in cpm for each sample over 3 mins. Homogenates were assayed in triplicate. 5µl of each substrate were also counted in triplicate. Data were expressed as pmol/min/g for FAAH activity or nmol/min/g for MAGL activity.

2.9.4 Calculations

The % substrate conversion rate (SCR) was determined for various tissue dilutions prior to the assay and was calculated as follows:

$$\frac{[(\text{sample count-blank count})/(\text{standard count-blank count})] \times [(\text{final volume of stopped solution})/(\text{volume counted})]}{100} \times 100.$$

Dilutions giving an SCR of between 5-15% were used in the assay.

Each sample is incubated with 10 nmoles of [³H] 2-OG in the MAGL assay and 200 pmoles of [³H] AEA in the FAAH activity assay. Multiplying this original [³H] substrate amount by the conversion rate allows calculation of product amount. Thereafter, dividing by time (15 or 30') and then tissue equivalent weight in the sample volume used allows standard units to be obtained.

Thus:

$$\text{MAGL activity in nmoles/min/g tissue} = \frac{(\text{sample conversion rate} \times 10 \text{ nmoles})}{(15 \text{ min} \times (\text{sample vol} \times \text{tissue wet weight equivalent}))}$$

$$\text{FAAH activity in pmoles/min/g tissue} = \frac{(\text{sample conversion rate} \times 200 \text{ pmoles})}{(30 \text{ min} \times (\text{sample vol} \times \text{tissue wet weight equivalent}))}$$

2.10 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. Where appropriate, when comparing the means of two unrelated groups, parametric data were analysed using unpaired *t*-test and non-parametric data were analysed using an independent sample Mann-Whitney U test. One-way ANOVA was used to compare the mean of more than two groups on one factor, whereas two-way ANOVA was used to compare the mean of more than two groups on two factors simultaneously. *Post-hoc* analysis was performed using Fisher's LSD test and Duncan's test where appropriate. 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

Pharmacological inhibition of endocannabinoid degradation modulates the expression of inflammatory mediators in the hypothalamus following an immunological stressor

Published Reference:

Kerr DM, Burke NN, Ford GK, Connor TJ, Harhen B, Egan LP, Finn DP, Roche M. (2012) Pharmacological inhibition of endocannabinoid degradation modulates the expression of inflammatory mediators in the rat hypothalamus following an immunological stressor. *Neuroscience* 204: 53-63.

ABSTRACT

The endocannabinoid system is an important regulator of the nervous, neuroendocrine and immune systems, thus representing a novel therapeutic target for stress-related neuroinflammatory and psychiatric disorders. However, there is a paucity of data relating to the effects of endocannabinoids on neuroinflammatory mediators following an immune stress/challenge *in vivo*. This study investigated the effects of URB597, a selective inhibitor of fatty acid amine hydrolyase (FAAH), the enzyme that preferentially metabolises anandamide, on lipopolysaccharide (LPS)-induced increases in the expression of immune mediators in the hypothalamus. Systemic administration of URB597 increased the levels of anandamide and the related *N*-acylethanolamines, *N*-palmitoyl ethanolamide and *N*-oleoyl ethanolamide, but not 2-arachidonoyl glycerol, in the hypothalamus and spleen. URB597 attenuated the LPS-induced increase in interleukin (IL)-1 β expression while concurrently augmenting the LPS-induced increase in suppressor of cytokine signalling (SOCS)-3 expression. In addition, URB597 tended to enhance and reduce the LPS-induced increase in IL-6 and IL-10 mRNA expression respectively. LPS-induced increases in peripheral cytokine levels or plasma corticosterone were not altered by URB597. The present study provides evidence for a role for FAAH in the regulation of LPS-induced expression of inflammatory mediators in the hypothalamus. Improved understanding of endocannabinoid-mediated regulation of neuroimmune function has fundamental physiological and potential therapeutic significance in the context of stress-related disorders.

Keywords: Endocannabinoid, anandamide, 2-AG, cytokine, HPA axis

Introduction

The endocannabinoid system comprises the CB₁ and CB₂ receptors, the naturally occurring endogenous ligands, anandamide (AEA) and 2-arachidonyl glycerol (2-AG); and the enzymes involved in their synthesis and degradation. The enzyme fatty acid amide hydrolyase (FAAH) preferentially metabolises AEA and although 2-AG also acts as a substrate for FAAH, monoacylglycerol lipase (MAGL) is considered the primary enzyme involved in 2-AG inactivation (Dinh et al., 2002; Long et al., 2009). By competing with AEA for the catalytic site of FAAH, fatty acid amides such as the *N*-acylethanolamines, *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) are capable of enhancing endocannabinoid signaling (Cravatt et al., 1996; 2001; Walker et al., 2002). All elements of this lipid signaling system are widely and densely expressed in the mammalian immune system and brain (Herkenham et al., 1990; Onaivi et al., 2006; Stella, 2009). As such, endocannabinoid regulation of immune function represents an important therapeutic target for a number of peripheral and central inflammatory disorders (Di Marzo et al., 2004; Baker et al., 2007; Centonze et al., 2007; Finn, 2009; Orgado et al., 2009).

Enhancing endocannabinoid tone has been proposed as an alternative means of activating cannabinoid receptors without concomitant overt psychotropic effects associated with potent synthetic cannabinoid receptor agonists. Enhancing endocannabinoid tone via FAAH or MAGL inhibition elicits anti-inflammatory effects in several animal models (Holt et al., 2005; Jayamanne et al., 2006; Comelli et al., 2007; Alhouayek et al., 2011; Booker et al., 2011). *In vitro* studies suggest that endocannabinoids elicit anti-inflammatory effects comparable to those of exogenous cannabinoids. Increasing AEA tone, either directly or via inhibition of its degradation or uptake, has been demonstrated to reduce the levels of pro-inflammatory cytokines and inflammatory mediators such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and nitric oxide, and enhance the release of the anti-inflammatory cytokine IL-10 *in vitro* (Puffenbarger et al., 2000; Chang et al., 2001; Facchinetti et al., 2003; Ortega-Gutierrez et al., 2005; Tham et al., 2007; Correa et al., 2009; Correa et al., 2010). However, AEA has also been demonstrated to enhance IL-6 in astrocyte cultures (Molina-Holgado et al., 1998; Ortega-Gutierrez et

al., 2005). Previous studies from our group have reported that the endocannabinoid re-uptake inhibitor AM404, attenuates lipopolysaccharide (LPS)-induced increases in plasma IL-1 β and IL-6 levels while concurrently augmenting TNF α levels (Roche et al., 2008). Comparably, De Laurentiis and co-workers recently demonstrated that AEA activation of hypothalamic CB₁ receptors facilitates LPS-induced increases in plasma TNF- α levels (De Laurentiis et al., 2010). However, the effects of endocannabinoids on neuroinflammatory responses *in vivo* have not been examined in detail. Panikashvili and colleagues showed that 2-AG activation of CB₁ receptors inhibits TNF- α , IL-1 β and IL-6 mRNA in the brain and protects against closed head injury (Panikashvili et al., 2005; 2006). Enhanced AEA levels following inhibition of endocannabinoid re-uptake attenuated pro-inflammatory responses in the spinal cord and ameliorated motor symptoms in an animal model of multiple sclerosis (Mestre et al., 2005). The present study investigated the effect of the selective FAAH inhibitor URB597 (Kathuria et al., 2003; Fegley et al., 2005; Piomelli et al., 2006), on LPS-induced changes in cytokine expression in the hypothalamus, an important site of cytokine-mediated regulation of physiological function and stress responses, and compared those to effects observed in the periphery (plasma and spleen).

The mechanisms by which endocannabinoids mediate their neuroimmunomodulatory effects remain largely unknown. The neuroprotective and anti-inflammatory effect of 2-AG following closed head injury has been reported to be associated with a CB₁ receptor-mediated decrease in nuclear factor-kappa B (NF- κ B) activation (Panikashvili et al., 2005). *In vitro* studies have suggested that the anti-inflammatory effects of AEA may be mediated by inhibition of NF- κ B activation and enhanced production of the anti-inflammatory cytokine IL-10 (Correa et al., 2010). Cannabinoid ligands have also been reported to enhance suppressor of cytokine signaling (SOCS)-1 and SOCS3 gene expression in the periphery (Lavon et al., 2003; Caraceni et al., 2009) and inhibit SOCS3 activation in microglial cell culture (Kozela et al., 2010). However, to date, no studies have reported on the effects of endocannabinoids on SOCS signaling in the brain. In addition, it is well known that glucocorticoids are potent inhibitors of inflammatory responses and recent studies have demonstrated that the endocannabinoid system is an important regulator of stress-related neuroendocrine activity (for review see (Cota, 2008; Steiner and Wotjak, 2008; Gorzalka and Hill, 2009). Thus, a further aim of this study was to

examine if alterations in LPS-induced cytokine expression in the hypothalamus following the inhibition of FAAH were associated with changes in NF- κ B or SOCS3 expression in the hypothalamus or alterations in plasma corticosterone levels.

EXPERIMENTAL PROCEEDURES

Animals

Experiments were carried out on male Sprague Dawley rats (weight 220-260g; Harlan, 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 0830 h and 1500 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 minimize 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 license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609.

Experimental Design

Rats were randomly assigned to one of four treatment groups: Vehicle + Saline (n = 5); Vehicle + LPS (n = 8); URB597 + Saline (n = 7); URB597 + LPS (n = 8). Rats were injected with URB597 (1 mg/kg i.p. Cayman Chemicals, Estonia) or vehicle (ethanol: cremophor: saline; 1:1:18) in an injection volume of 2 ml/kg. The dose of URB597 was chosen based on its effectiveness at attenuating stress-induced activation of the HPA axis and nociceptive behaviour (Patel et al., 2004; Holt et al., 2005). The time of URB597 administration was determined based on the finding that inhibition of FAAH peaks 1 hour post i.p. injection and decreases thereafter (Kathuria et al., 2003; Fegley et al., 2005). LPS (100 $\mu\text{g}/\text{kg}$; B0111:B4; Sigma Aldrich, Dublin, Ireland) or saline vehicle (sterile, 0.89% NaCl) was administered at a volume of 1 ml/kg, 30 minutes following URB597/vehicle. The dose and time of LPS administration were chosen on the basis of previous work within our laboratory demonstrating enhanced cytokine levels in the periphery and brain (Roche et al.,

2006; 2008). Blood samples were taken 2 hours post LPS or saline administration, via cardiac puncture into a heparinized syringe under CO₂ anaesthesia. Blood samples were centrifuged at 14,000g for 15 min at 4°C to obtain plasma which was removed and stored at -80°C until cytokine and corticosterone determination. In addition, spleen and hypothalamus were excised, dissected in half, weighed and snap-frozen and stored at -80°C until assayed for endocannabinoids, N-acylethanolamines and cytokines.

Quantitation of endocannabinoids and entourage N-acylethanolamines in hypothalamic and spleen tissue using liquid chromatography - tandem mass spectrometry (LC-MS/MS)

Quantitation of endocannabinoids and N-acylethanolamine was essentially as described previously (Olango et al., 2011). In brief, each hypothalamic or spleen sample was first homogenised in 400µL 100% acetonitrile containing known fixed amounts of deuterated internal standards (0.014 nmol AEA-d8, 0.48nmol 2-AG-d8, 0.016nmol PEA-d4, 0.015nmol OEA-d2). Homogenates were centrifuged at 14,000g for 15 minutes at 4°C and the supernatant was collected and evaporated to dryness. Lyophilized samples were re-suspended in 40µl 65% acetonitrile and 2µl were injected onto a Zorbax® C18 column (150 × 0.5mm internal diameter) from a cooled autosampler maintained at 4°C (Agilent Technologies Ltd., Ireland). Mobile phases consisted of A (HPLC grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 12µl/min. Reversed-phase gradient elution began initially at 65% B and over 10min was ramped linearly up to 100% B. At 10min, the gradient was held at 100% B up to 20min. At 20.1min, the gradient returned to initial conditions for a further 10mins to re-equilibrate the column. The total run time was 30min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 11.4min, 12.9min, 14.4min and 15.0min 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 Ltd., Cork, Ireland). Instrument conditions and source parameters including fragment or voltage and collision energy were optimized 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. Precursor and product ion mass-to-charge (m/z) ratios for all analytes and their corresponding deuterated forms were as follows: PEA ($m/z = 300.3-62.1$); PEA-d4 ($m/z = 304.3-62.1$); OEA ($m/z = 326.3-62.1$); OEA-d2 ($m/z = 328.3-62.1$); AEA ($m/z = 348.3-62.1$); AEA-d8 ($m/z = 356.3-63.1$); 2-AG ($m/z = 379.3-287.2$); 2-AG-d8 ($m/z = 387.3-294.2$). Quantitation 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 Ltd., Ireland). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard. The values obtained from the Masshunter Quantitative Analysis Software are initially expressed in ng/g of tissue by dividing by the weight of the tissue. To express values as nmol/g or pmol/g of tissue, the corresponding values are then divided by the molar mass of each analyte. Linearity (regression analysis determined R^2 values of 0.99 or greater for each analyte) was determined over a range of 18.75ng to 71.5fg except for 2-AG which was 187.5ng-715fg. The limit of quantification was 1.32pmol/g, 12.1pmol/g, 1.5pmol/g, 1.41pmol/g for AEA, 2-AG, PEA and OEA respectively.

Analysis of inflammatory mediators using real-time PCR

RNA was extracted from hypothalamic tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany). Genomic DNA contamination was removed with the addition of DNase to the samples according to the manufacturer's instructions. 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 polymerase chain reaction (PCR) was performed using an ABI Prism 7500 instrument (Applied

Biosystems, UK), as previously described (O'Sullivan et al., 2009; McNamee et al., 2010). Assay IDs for the genes examined were as follows: IL-1 β (Rn00580432_m1), TNF- α (Rn99999017_m1), IL-6 (Rn00561420_m1), IL-10 (Rn00563409_m1), I κ B α (Rn01473658_g1) and SOCS3 (Rn00585674_s1). PCR was performed using Taqman Universal PCR Master Mix (Applied Biosystems, UK) 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 normalize gene expression data. Relative gene expression was calculated using the $\Delta\Delta$ CT method.

Determination of cytokine protein levels

Plasma and spleen TNF- α , IL-1 β , IL-6 and IL-10 concentrations were determined using specific rat enzyme-linked immunosorbent assays (ELISAs) performed using antibodies and standards obtained from R & D Systems, UK as previously described (Roche et al., 2006; 2008). Briefly, Maxisorb microtitre plates were coated with goat or mouse anti-rat cytokine antibodies (0.8-4 μ g/ml in phosphate-buffered saline [PBS: NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 8.1 mM, KH₂PO₄ 1.5 mM; pH 7.4]) for 20 h at 22°C. Plates were then washed three times with wash buffer (0.05% Tween 20 in PBS, pH 7.4) and blocked at room temperature for an hour using reagent diluent (1% BSA in PBS, pH 7.4). Following three washes, 100 μ l aliquots of samples or standards (0–5000 pg/ml) were added and plates were incubated at 22°C for 2 h. After three washes, 100 μ l of specific biotinylated anti-goat or mouse antibody (1:1000) was added to each well and incubated for 1 h at 22°C. After three washes, 100 μ l horseradish peroxidase conjugated to streptavidin (1:200) was added to each well, and plates were incubated at 22°C for 20 minutes. Following three washes, 100 μ l of tetramethylbenzidine substrate solution was added per well and after for 20 min, 50 μ l of 1 M H₂SO₄ was added per well to stop the reaction and to facilitate color development. Absorbance was read immediately at 450 nm. Bradford protein assay was used to determine protein concentration in spleen samples. Cytokine levels in plasma are expressed as pg/mL or in the spleen as pg/mg protein.

Corticosterone determination

Plasma corticosterone was assessed as previously described (Roche et al., 2006) using a Corticosterone EIA kit (Cayman Chemicals, Tallin, Estonia) and was carried out in accordance with manufacturer's instructions. The limit of detection of the assay was 30pg/ml and data were expressed as ng/mL plasma.

Statistical Analysis

SPSS statistical package was used to analyze all data. Data were analyzed using two-way analysis of variance (ANOVA) with the factors of URB597/vehicle and LPS/Saline. *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).

Results

Enhanced AEA, OEA and PEA in the hypothalamus following systemic administration of URB597 is accompanied by alterations in the expression of inflammatory mediators

Levels of AEA [$F_{1,22} = 44.51$ $P < 0.001$], OEA [$F_{1,22} = 97.74$ $P < 0.001$] and PEA [$F_{1,22} = 62.36$ $P < 0.001$], but not 2-AG, were significantly increased in the hypothalamus following systemic administration of URB597 (Figure 1). LPS did not significantly alter the concentration of the endocannabinoids or the related *N*-acylethanolamine compounds, nor did it alter the URB597-induced increase in the levels of AEA, OEA or PEA.

LPS significantly enhanced cytokine (IL-1 β : $F_{1,22} = 54.62$ $P < 0.001$, TNF- α : $F_{1,23} = 29.48$ $P < 0.001$; IL-6: $F_{1,22} = 19.63$ $P < 0.001$ and IL-10: $F_{1,22} = 30.20$ $P < 0.001$), I κ B α [$F_{1,20} = 349.38$ $P < 0.001$] and SOCS3 [$F_{1,21} = 60.12$ $P < 0.001$] mRNA expression in the hypothalamus (Figure 2). URB597 did not significantly alter the expression of immune mediators in the absence of LPS. However, URB597 did attenuate the LPS-induced increase in IL-1 β expression [interaction effect: $F_{1,22} = 6.86$ $P = 0.016$] and concurrently augmented LPS-induced SOCS-3 expression [interaction: $F_{1,20} = 8.30$ $P = 0.009$]. Although URB597 enhanced and reduced the LPS-induced increase in IL-6 and IL-10 expression, respectively, ANOVA revealed that these effects failed to reach statistical significance. There was no significant effect of URB597 on LPS-induced expression in TNF- α or I κ B α mRNA in the hypothalamus.

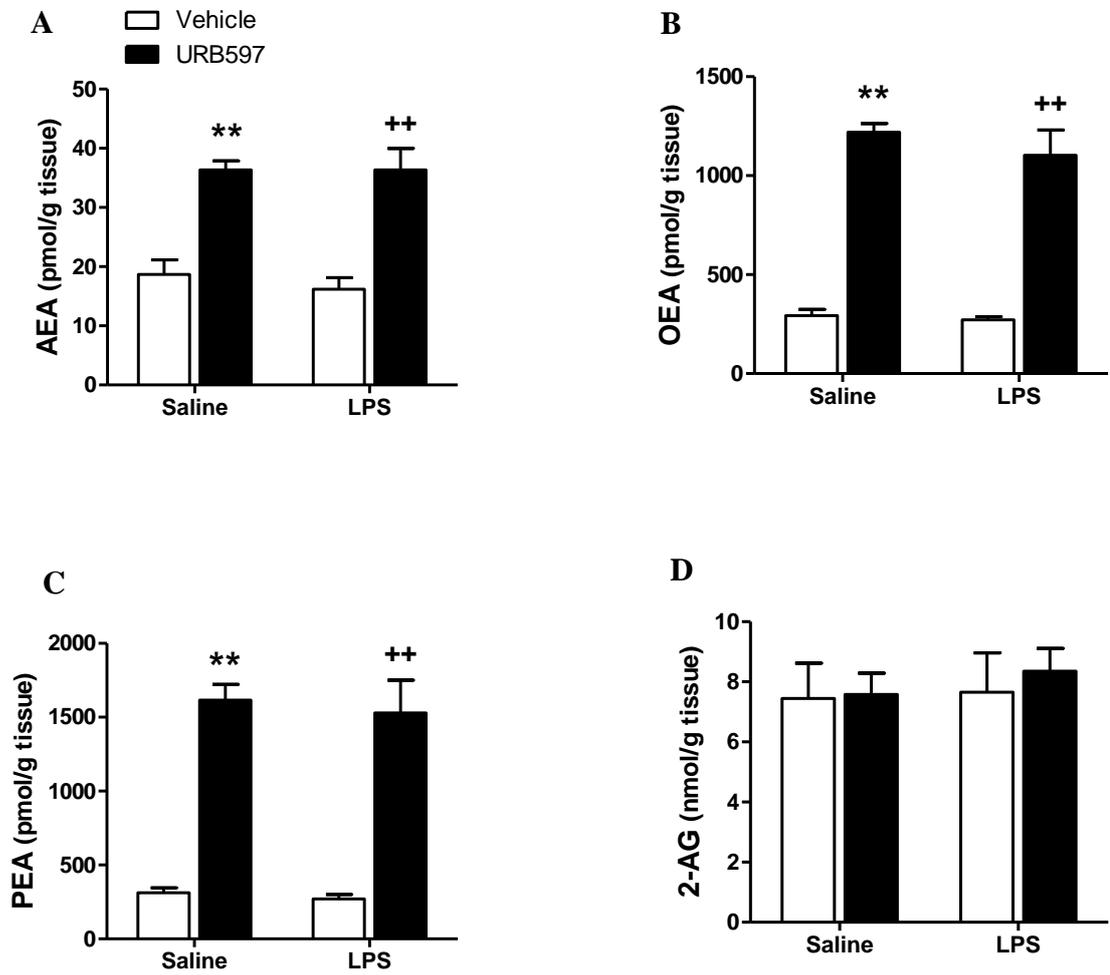


Figure 1. Systemic administration of URB597 enhances the levels of (A) AEA, (C) OEA and (D) PEA, but not (B) 2-AG, in the hypothalamus. Data expressed as mean + SEM (n = 4-8 per group). ** $P < 0.01$ vs. vehicle-saline. ++ $P < 0.01$ vs. vehicle-LPS.

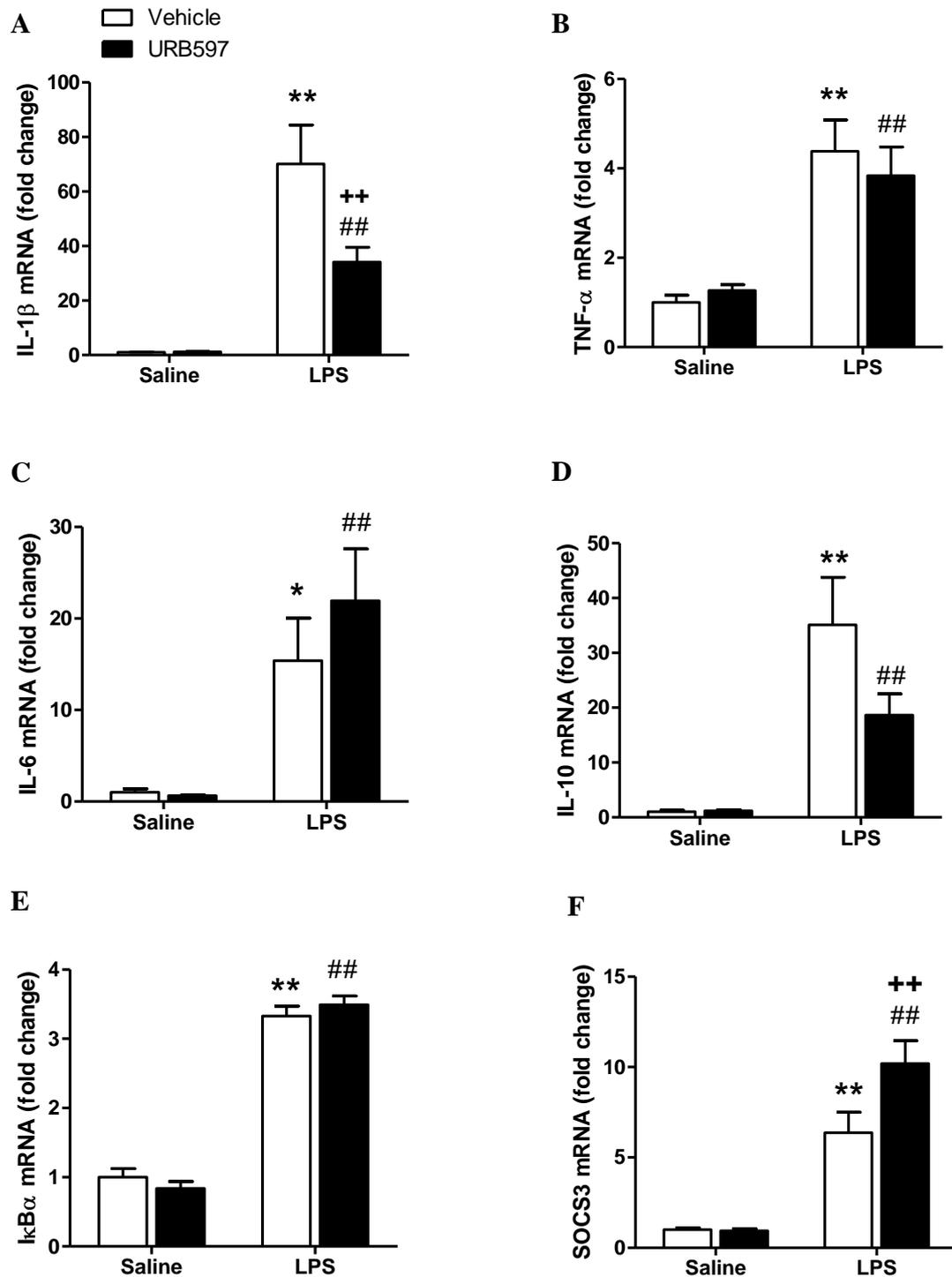


Figure 2. URB597 modulates LPS-induced changes in inflammatory gene expression in the hypothalamus. URB597 attenuates LPS-induced increase in (A) IL-1 β and augments the LPS-induced increase in (F) SOCS3 expression. Although URB597 appeared to modulate the LPS-induced increase in (C) IL-6 and (D) IL-10 expression, analysis revealed that this failed to reach statistical significance. There was no effect of URB597 on (B) TNF- α or (E) I κ B α expression, in the presence or absence of LPS. Data expressed as mean + SEM (n = 4-8 per group). mRNA data is expressed as fold-change vs. vehicle-saline. **P<0.01 *P<0.05 vs. vehicle-saline. ##P<0.01 #P<0.05 vs. URB597-saline. ++P<0.01 vs. vehicle-LPS.

Inhibition of FAAH by URB597 increases levels of AEA, OEA and PEA in the spleen without altering LPS-induced increases in cytokine levels

Similar to effects observed in the hypothalamus, URB597 increased AEA [$F_{1,22} = 44.51$ $P < 0.001$], OEA [$F_{1,22} = 97.74$ $P < 0.001$] and PEA [$F_{1,22} = 62.36$ $P < 0.001$], but not 2-AG, levels in the spleen, an effect not altered in the presence of LPS (Figure 3A-D). LPS significantly increased the levels of the pro-inflammatory cytokines, IL-1 β [$F_{1,17} = 264.23$ $P < 0.001$], TNF- α [$F_{1,21} = 30.79$ $P < 0.001$] and IL-6 [$F_{1,18} = 138.39$ $P < 0.001$] and the anti-inflammatory cytokine IL-10 [$F_{1,23} = 13.43$ $P = 0.001$] in the spleen (Figure 3E-H). URB597 did not alter the levels of pro- or anti-inflammatory cytokines in the spleen in the presence or absence of LPS.

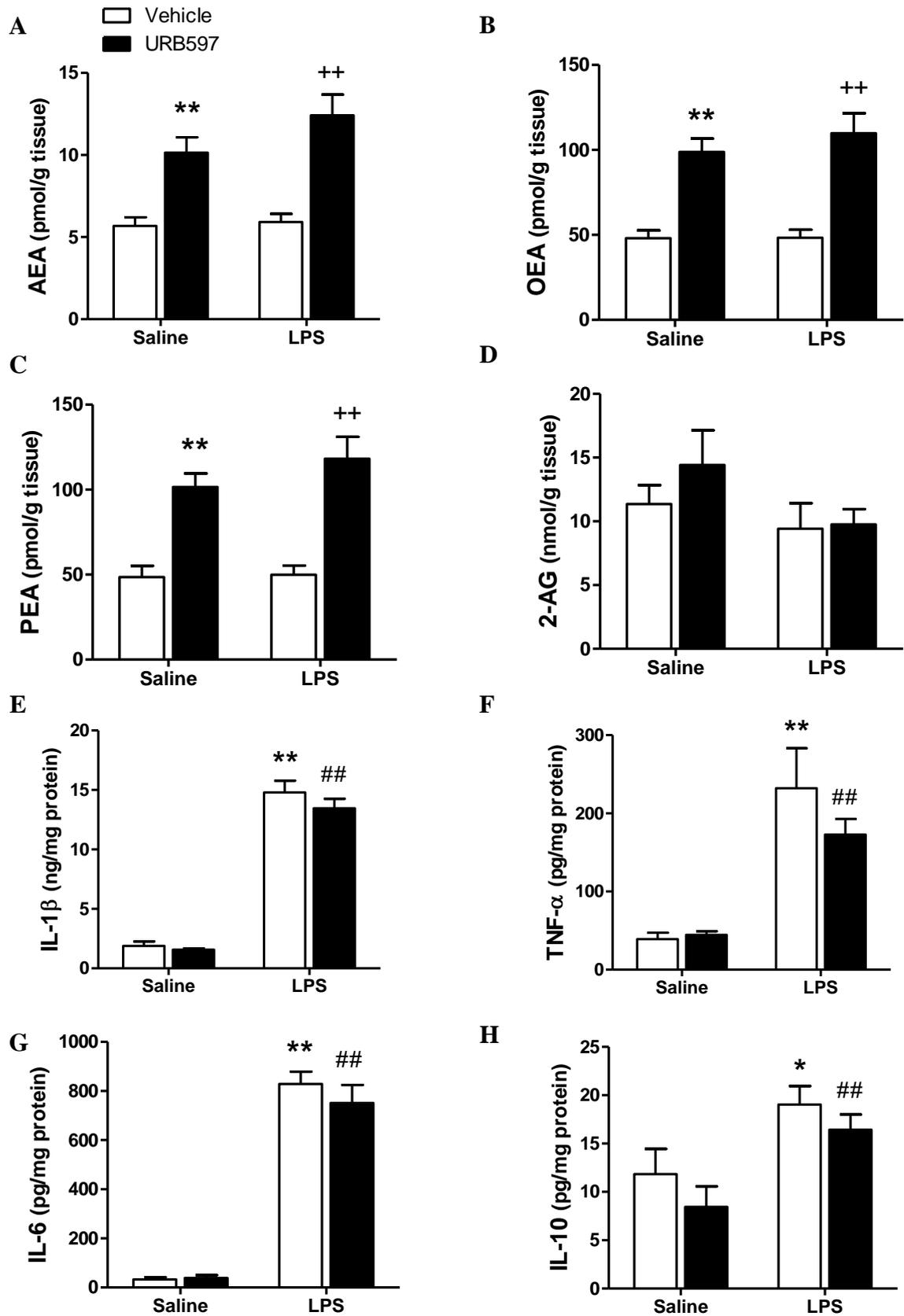


Figure 3. URB597 increases the concentration of (A) AEA, (C) OEA and (D) PEA, but not (D) 2-AG in the spleen. LPS induced an increase in (E) IL-1 β , (F) TNF- α and (G) IL-6 or (H) IL-10, an effect not altered by URB597. Data expressed as mean + SEM (n = 4-8 per group). ***P* < 0.01 vs. vehicle-saline. ##*P* < 0.01 vs. URB597-saline. ++*P* < 0.01 vs. vehicle-LPS.

URB597 does not significantly alter LPS-induced increases in pro-inflammatory cytokines or corticosterone levels in the plasma

LPS induced a significant increase in the levels of pro-inflammatory cytokine levels [IL-1 β : $F_{1,17} = 158.15$ $P < 0.001$; TNF α : $F_{1,18} = 10.10$ $P = 0.005$ and IL-6: $F_{1,18} = 97.52$ $P < 0.001$] and corticosterone [$F_{1,18} = 171.95$ $P < 0.001$] in the plasma (Figure 4 and 5). URB597 did not significantly alter plasma pro-inflammatory cytokine levels, in the presence or absence of LPS. LPS-induced corticosterone levels were slightly lower in URB597-treated animals than in vehicle-treated rats; however, this effect was not statistically significant.

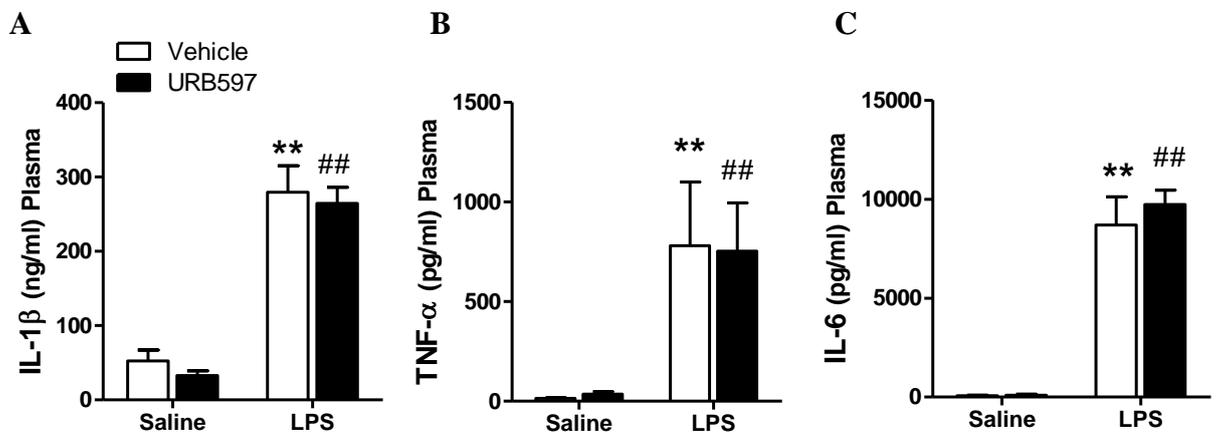


Figure 4. URB597 does not significantly alter LPS-induced cytokine levels in the plasma. LPS induced an increase in (A) IL-1 β , (B) TNF- α and (C) IL-6. There was no significant effect of URB597 on cytokine levels in the presence or absence of LPS. Data expressed as mean + SEM (n = 4-7 per group). ** $P < 0.01$ vs. vehicle-saline. ## $P < 0.01$ vs. URB597-saline.

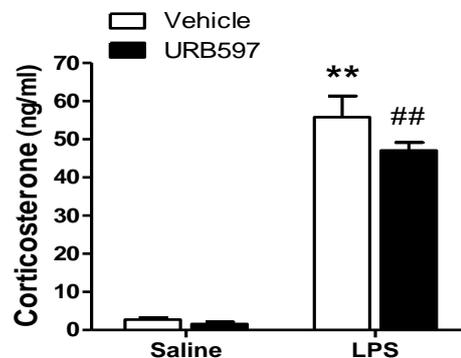


Figure 5. URB597 does not significantly alter LPS-induced corticosterone levels in the plasma. Data expressed as mean + SEM (n = 4-7 per group). ** $P < 0.01$ vs. vehicle-saline. ## $P < 0.01$ vs. URB597-saline.

Discussion

The present study demonstrated that systemic administration of the FAAH inhibitor URB597 increased AEA, OEA and PEA levels centrally, in the hypothalamus, and peripherally, in the spleen. URB597 attenuated the LPS-induced increase in IL-1 β expression while concurrently enhancing LPS-induced SOCS3 expression in the hypothalamus. There was no effect of FAAH inhibition on cytokine levels in the periphery (plasma or spleen) or on plasma corticosterone levels. The current study demonstrates an important role for FAAH in the modulation of central neuroimmune processes associated with acute inflammation.

Correlating with earlier studies (Felder et al., 1996), basal levels of the fatty acid amides, AEA, OEA and PEA were lower in the spleen than in the brain. Despite this, systemic administration of the FAAH inhibitor URB597, reliably and robustly increased levels of AEA and the related *N*-acyletholamines, OEA and PEA, in both the spleen and hypothalamus. These findings corroborate previous studies demonstrating an increase in these fatty acid amides in whole brain extracts and liver following administration of URB597 (Kathuria et al., 2003; Fegley et al., 2005). FAAH is also known to metabolise 2-AG (Di Marzo et al., 1998), although not to the same extent as MAGL (Dinh et al., 2002), however URB597 did not alter 2-AG levels in either the hypothalamus or spleen. This confirms that the dose of URB597 used in the present study selectively enhanced fatty acid amide levels. URB597-induced increases in the fatty acid amides were not altered in the presence of LPS. This may be somewhat surprising in light of *in vitro* evidence indicating that LPS increases AEA and 2-AG levels, an effect mediated by inhibition of FAAH and MAGL activity and/or enhancement of AEA biosynthesis (Varga et al., 1998; Di Marzo et al., 1999; Maccarrone et al., 2001; Liu et al., 2003). *In vivo*, LPS has been demonstrated to increase AEA synthesis (Fernandez-Solari et al., 2006) and reduce 2-AG levels (Borges et al., 2011) in the rat hypothalamus. Although the dose of LPS (100 μ g/kg) and time post injection (2 h) used in both the present and former (Borges et al., 2011) studies are identical, methodological differences such as strain of rat (Sprague Dawley vs. Wistar), strain of LPS (0111:B4 vs. 026:B6) or time of day of the injections (light phase vs. just prior to dark phase) may account for the

discrepancy between studies. In addition, in the study demonstrating that LPS increased AEA synthesis (Fernandez-Solari et al., 2006), LPS was administered at a dose 50 times greater than that given in the present study. To our knowledge, the effects of LPS on levels of OEA or PEA have not been reported, however, should LPS inhibit FAAH activity, it would have been expected that all three fatty acid amides would be enhanced. The results of this study argue against an LPS-induced increase in endocannabinoid synthesis or FAAH or MAGL inhibition at the dose used in the current study.

It is well established that endotoxin administration is associated with the induction of inflammatory cytokines in the brain and periphery (Breder et al., 1994; Pitossi et al., 1997; Konsman et al., 1999; Turrin et al., 2001; Roche et al., 2006; 2008), findings confirmed in the present study. Increased circulating pro-inflammatory cytokines communicate with the brain via many routes (diffusion into brain across the blood brain barrier deficient areas, sensory signals and vagus nerve stimulation) and induce cytokine synthesis within the CNS, which leads to a state of acute neuroinflammation and sickness behaviour. Thus, modulation of peripheral cytokines can impact on inflammatory signals sent to the brain. Although URB597 enhanced the levels of AEA, OEA and PEA in both the spleen and hypothalamus, it failed to alter cytokine levels in the periphery. URB597, administered both systemically and i.c.v., has previously been shown to augment LPS-induced increases in TNF α plasma levels (Roche et al., 2008; De Laurentiis et al., 2010). LPS-induced TNF α release in the plasma is primarily controlled by the CNS (Mastronardi et al., 2001) and recent evidence indicates that AEA induced augmentation of LPS-stimulated increases in TNF α is mediated by hypothalamic CB₁ receptors (De Laurentiis et al., 2010). However, a higher dose of URB597 (1mg/kg) was used in the current study in comparison to our earlier study (0.6mg/kg) (Roche et al., 2008) which may have resulted in increased fatty acid amide levels, in particular OEA and PEA. Indeed, it is possible that the effects of OEA and/or PEA may have counteracted the effects of AEA on LPS-induced TNF α levels in the periphery. For example, PEA is known to elicit potent anti-inflammatory effects, including inhibition of LPS-induced increases in plasma TNF α levels (Berdyshev et al., 1998; Hoareau et al., 2009). Although the present studies indicate that the effects of URB597 on LPS-induced cytokine expression in the hypothalamus are mediated

directly at the level of the CNS, the possibility exists that alterations in peripheral cytokine levels may have occurred at time points other than that examined in the present study. Further studies are required in order to more thoroughly evaluate the temporal profile of this response, both centrally and peripherally. Cytokines within the hypothalamus have been shown to be responsible for many aspects of the sickness response following endotoxin administration (for reviews see (Beishuizen and Thijs, 2003; Conti et al., 2004; Dantzer, 2009)). For example, 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). The present study demonstrates that enhanced levels of AEA, OEA and PEA in the hypothalamus following URB597 were associated with an attenuation of LPS-induced IL-1 β , but not TNF α or IL-6 expression. Thus, AEA-induced inhibition of IL-1 β expression may prevent/attenuate the fever and hypophagia associated with acute inflammation. However, further studies are required in order to determine if the reduction in LPS-induced IL-1 β expression is due to direct inhibition or a shift in the response curve over time, effects which would have very different functional consequences.

Pro-inflammatory cytokines in the hypothalamus, such as IL-1 β and TNF α , are also responsible for activation of the hypothalamic-pituitary-adrenal axis (HPA) axis and consequently glucocorticoid release following LPS (Beishuizen and Thijs, 2003). In addition, endocannabinoid modulation of neuroendocrine activity has been the topic of several studies (for reviews see (Cota, 2008; Steiner and Wotjak, 2008; Gorzalka and Hill, 2009)), with increasing evidence that endocannabinoids act to inhibit stress-induced HPA axis activation (Patel et al., 2004; Di et al., 2005; Evanson et al., 2010). The present study demonstrated that enhanced AEA tone following URB597 failed to alter LPS-induced increases in plasma corticosterone levels or the expression of TNF α in the hypothalamus. Therefore, hypothalamic TNF α may underlie the LPS-induced increase in plasma corticosterone, an effect not altered by URB597. Physiological stressors (such as immune challenge) and psychological stressors (such as restraint) activate the HPA axis via distinct mechanisms, which also may account for the lack of effect of URB597 on corticosterone levels in the present study. Based on this, it is possible that endocannabinoid signalling may regulate HPA axis activity evoked by psychological (Patel et al., 2004; Hill et al.,

2011), but not physiological, stressors. However, it should also be noted that URB597 may have elicited an effect on HPA axis activation and corticosterone levels at an earlier time point than that examined in the present study. It has been previously shown that URB597 (0.1-1mg/kg) attenuates restraint-stress induced increase in corticosterone levels 1 h post administration (Patel et al., 2004) and that blockade of the CB₁ receptor enhances stress-induced increases in corticosterone up to 90 minutes post stress (Patel et al., 2004; Hill et al., 2011). Thus, by examining corticosterone levels 2h post LPS, the effects of URB597 on LPS-induced HPA axis activation may have been missed.

Enhancing anti-inflammatory cytokine levels such as IL-10 (systemically and centrally) has also been shown to attenuate LPS-induced hypophagia, fever, reduced motor activity and energy expenditure (Ledeboer et al., 2002; Hollis et al., 2010). The present study demonstrated that URB597 reduced LPS-induced expression of IL-10, in the hypothalamus. These findings are in contrast with *in vitro* data showing that AEA increases LPS/IFN γ -induced increases in IL-10 levels in microglia (Correa et al., 2010). It is possible that the effects of URB597 on IL-10 observed in the present study may be mediated by OEA and/or PEA and not AEA. In addition, as cytokine expression has only been examined at a single time point in the present study, we cannot exclude the possibility that the effects of URB597 are due to a delay in the response curve rather than a decrease in cytokine expression *per se*. However, whether the effects on IL-10 are due to direct inhibition or a delay in the response, the present findings suggest that IL-10 is not responsible for the reduction in LPS-induced IL-1 β expression observed following URB597 administration.

In an effort to examine possible molecular mechanisms by which enhanced endocannabinoid tone may modulate cytokine expression within the hypothalamus, this study also investigated the effect of URB597 on the expression of I κ B α , an indirect measure of NF- κ B signalling (Read et al., 1994), and SOCS3. Although AEA has previously been demonstrated to modulate NF- κ B activation (Sancho et al., 2003; Nakajima et al., 2006; Correa et al., 2010), LPS-induced I κ B α expression was not altered by URB597 in the present study. Therefore it is unlikely that NF- κ B signalling system is involved in the attenuation of cytokine expression in the hypothalamus following URB597 administration. In comparison, the present study

demonstrated that FAAH inhibition results in an augmentation of LPS-induced SOCS3 expression. SOCS proteins represent a rapid self-regulating mechanism to modulate cytokine signalling. Previous studies have demonstrated that cannabinoid ligands enhance SOCS3 expression in the periphery (Lavon et al., 2003; Caraceni et al., 2009) and inhibit SOCS3 activation in cultured microglial cells (Kozela et al., 2010). However, this is the first study to demonstrate endocannabinoid-induced changes in SOCS3 expression following an immune challenge *in vivo*. The expression of SOCS3 is primarily regulated by activation of signal transducer and activation of transcription (STAT)-3 by IL-6 GP130 and IL-10 cytokines, although other signalling cascades such as NF- κ B and MAPK are also known to be involved (Qin et al., 2007; Baker et al., 2009). Our data demonstrated that LPS-induced IL-6 expression in the hypothalamus was enhanced, although not significantly, following URB597. Thus the augmentation of LPS-induced SOCS3 expression may be the result of enhanced IL-6 signalling following the inhibition of FAAH. The primary function of SOCS3 is to inhibit signalling by IL-6 via inhibition of the JAK/STAT3 pathway, thus enhanced SOCS3 expression following URB597 may limit the pro-inflammatory action of this cytokine. In addition, IL-1 β -induced transcription and activation of NF- κ B and the MAPKs is inhibited by SOCS3 (Karlsen et al., 2004; Frobose et al., 2006). SOCS3 also mediates some of the anti-inflammatory effects of IL-10 (Berlato et al., 2002; Qin et al., 2007), however, as URB597 attenuated rather than enhanced LPS-induced IL-10, it seems unlikely that the enhanced SOCS3 expression in the present study was induced following IL-10 signalling. It should also be noted that LPS induces increases in other cytokine-like molecules such as leptin which are also capable of inducing STAT-3 phosphorylation and SOCS3 expression in the hypothalamus (Hubschle et al., 2001), an effect proposed to underlie, at least in part, LPS-induced hypophagia (Borges et al., 2011). Further studies are required to assess the contribution of the individual fatty acid amides to the activation of SOCS3 and the downstream consequences of this activation.

In summary, the present study demonstrates an important role for FAAH in the modulation of neuroinflammatory responses. As the hypothalamus is a critical site in the regulation of anorexia, fever, neuroendocrine and sympathetic activity in response to an acute inflammatory stress, the present findings may have important

implications in targeting the endocannabinoid system for the treatment of stress-related neuroinflammatory and psychiatric disorders.

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Chapter 4

***The monoacylglycerol lipase inhibitor JZL184
attenuates LPS-induced increases in cytokine
expression in the rat frontal cortex and plasma:
differential mechanisms of action***

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ABSTRACT

JZL184 is a selective inhibitor of monoacylglycerol lipase (MAGL), the enzyme that preferentially catabolizes the endocannabinoid 2-arachidonoyl glycerol (2-AG). Here, we have studied the effects of JZL184 on inflammatory cytokines in the brain and plasma following an acute immune challenge and the underlying receptor and molecular mechanisms involved.

JZL184 and/or the CB₁ receptor antagonist, AM251 or the CB₂ receptor antagonist, AM630 were administered to rats 30 min before lipopolysaccharide (LPS). 2 h later cytokine expression, MAGL activity, 2-AG, arachidonic acid and prostaglandin levels were measured in the frontal cortex, plasma and spleen.

JZL184 attenuated LPS-induced increases in IL-1 β , IL-6, TNF α and IL-10 but not the expression of the inhibitor of NF- κ B (I κ B α) in rat frontal cortex. AM251 attenuated JZL184-induced decreases in frontal cortical IL-1 β expression. Although arachidonic acid levels in the frontal cortex were reduced in JZL184-treated rats, MAGL activity, 2-AG, PGE₂ and PGD₂ were unchanged. In comparison, MAGL activity was inhibited and 2-AG levels enhanced in the spleen following JZL184. In plasma, LPS-induced increases in TNF α and IL-10 levels were attenuated by JZL184, an effect partially blocked by AM251. In addition, AM630 blocked LPS-induced increases in plasma IL-1 β in the presence, but not absence, of JZL184.

In conclusion inhibition of peripheral MAGL in rats by JZL184 suppressed LPS-induced circulating cytokines that in turn may modulate central cytokine expression. The data provide further evidence for the endocannabinoid system as a therapeutic target in the treatment of central and peripheral inflammatory disorders.

Introduction

Neuroinflammation is a key component of various neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis and psychiatric disorders such as depression. As such, the need to develop a greater understanding of the neurobiological mechanisms mediating neuroinflammation is critical at a fundamental physiological level and for the development of novel, more efficacious treatments. Accumulating evidence indicates that the endogenous cannabinoid (endocannabinoid) system plays a significant role in modulating the immune system, representing an important therapeutic target in the treatment of both central and peripheral inflammatory disorders [for reviews see (Ullrich et al., 2007; Nagarkatti et al., 2009; Stella, 2009; Finn, 2010; Jean-Gilles et al., 2010; Molina-Holgado and Molina-Holgado, 2010)].

The endocannabinoid system is comprised of the two cannabinoid G protein-coupled receptors CB₁ and CB₂; receptor nomenclature follows Alexander *et al.*, (2011), the endocannabinoids anandamide (N-arachidonoyl ethanolamide) and 2-arachidonoyl glycerol (2-AG), and the enzymes responsible for their synthesis and degradation. 2-AG, the most abundant endocannabinoid in the CNS and full agonist at both CB₁ and CB₂ receptors, is synthesised primarily via hydrolysis of cell membrane phospholipid precursors by diacylglycerol lipase- α (Mechoulam et al., 1995; Gao et al., 2010). Monoacylglycerol lipase (MAGL) is the enzyme primarily responsible for the metabolism of 2-AG to arachidonic acid and glycerol (Dinh *et al.*, 2002), accounting for up to 85% of 2-AG hydrolysis in the brain (Blankman *et al.*, 2007). Other enzymes involved in 2-AG hydrolysis include the serine hydrolases ABHD6 and ABHD12, fatty acid amide hydrolase (FAAH), cyclooxygenase-2 (COX-2) and carboxylesterases (Di Marzo et al., 1998; Blankman et al., 2007; Xie et al., 2010; Savinainen et al., 2012). 2-AG levels are enhanced in animal models of ischemia, Alzheimer's disease, Parkinson's disease and multiple sclerosis (Baker et al., 2001; Panikashvili et al., 2001; Ferrer et al., 2003; van der Stelt et al., 2005; van der Stelt et al., 2006), and it is possible that this endocannabinoid may play a protective role in these conditions, all of which have a neuroinflammatory/neuroimmune component. Indeed, evidence from *in vitro* studies indicates that 2-AG suppresses immune

function by reducing inflammatory cytokines such as IL-6, IL-2 and TNF- α and mediators such as nitric oxide and prostaglandins (Gallily et al., 2000; Chang et al., 2001; Facchinetti et al., 2003; Rockwell et al., 2006; Raman et al., 2011). In addition to being a substrate for COX-2, 2-AG also inhibited the increase in COX-2 expression in neurons and T cells, but not endothelial cells or macrophages, in response to inflammatory stimuli (Chang et al., 2001; Mestre et al., 2006; Zhang and Chen, 2008; Raman et al., 2011). Recent studies have demonstrated that enhancing 2-AG tone protects hippocampal neurons in culture from excitotoxic- and inflammation-induced degeneration and apoptosis, an effect mediated by CB₁ receptor-induced suppression of extracellular signal-regulated kinases, nuclear factor- κ B (NF- κ B) phosphorylation and COX-2 expression (Zhang and Chen, 2008; Chen et al., 2011). Although, *in vitro* data suggest potent anti-inflammatory and neuroprotective effects of 2-AG, there has been a paucity of studies *in vivo*, primarily due to the lack of pharmacological agents that selectively target 2-AG metabolism. Panikashvili and colleagues demonstrated that 2-AG activation of CB₁ receptors reduced infarct volume following closed head injury via inhibition of NF- κ B signalling (Panikashvili et al., 2001; Panikashvili et al., 2005). Subsequent studies from these researchers revealed that 2-AG reduced the expression of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6, reactive oxygen species and blood-brain-barrier permeability in this model of traumatic brain injury (McCarron *et al.*, 2003; Panikashvili *et al.*, 2006). Recent studies have also shown a neuroprotective effect of 2-AG in acute and chronic mouse models of multiple sclerosis, an effect accompanied by modulation of macrophage activity (Lourbopoulos *et al.*, 2011). URB602, a MAGL inhibitor, has been shown to elicit anti-inflammatory effects in several animal models (Comelli *et al.*, 2007; Guindon *et al.*, 2011). However, the low potency (Hohmann *et al.*, 2005) and lack of selectivity of URB602 for MAGL over FAAH (Vandevoorde *et al.*, 2007) has limited the usefulness of this compound. The recent development of a novel potent MAGL inhibitor JZL184 (Long et al., 2009b; Long et al., 2009a), has been a major advance, enabling detailed studies on the role of 2-AG in a number of physiological and pathophysiological processes including tumour cell growth, anxiety, nausea and pain (Kinsey et al., 2009; Kinsey et al., 2010; Sciolino et al., 2011; Sticht et al., 2011; Ye et al., 2011). In terms of inflammatory processes, JZL184 selectively increased gastric levels of 2-AG in mice and inhibited nonsteroidal anti-inflammatory-induced gastric haemorrhage and

associated increases in expression of the pro-inflammatory cytokines IL-1, IL-6 and TNF- α (Kinsey *et al.*, 2011). Similarly, in a mouse model of colitis, JZL184-induced increases in 2-AG were associated with reduced macroscopic and histological colon alterations and pro-inflammatory cytokine expression, effects mediated by CB₁ and CB₂ receptors (Alhouayek *et al.*, 2011). In the brain, increases in pro-inflammatory cytokine expression and PGE₂ levels following an immune challenge were inhibited following genetic or pharmacological MAGL inhibition, an effect not mediated by cannabinoid receptors but possibly via modulation of the arachidonic acid cascade (Nomura *et al.*, 2011). Similar anti-inflammatory and neuroprotective effects were observed following MAGL inhibition in a mouse model of Parkinson's disease (Nomura *et al.*, 2011).

To date, *in vivo* studies examining 2-AG effects on inflammation have been primarily carried out in mice. A large proportion of animal models are developed in the rat, and therefore investigation of effects across rodent species is paramount. Although JZL184 has reduced affinity for rat MAGL compared with the murine enzyme (Long *et al.*, 2009a), systemic administration of JZL184 exhibited anxiolytic-like effects at relatively low doses in rats (Sciolino *et al.*, 2011). However, only one study to date has reported that systemic administration of JZL184 increased 2-AG levels in the rat brain (Oleson *et al.*, 2012). The present study demonstrates that JZL184 reduced lipopolysaccharide (LPS)-induced cytokine expression/levels both in the rat frontal cortex and plasma, effects partially attenuated by CB₁ receptor antagonism. MAGL activity was inhibited, and 2-AG levels enhanced in the spleen, but not in frontal cortex, following JZL184 administration, indicating that different mechanisms may underlie the central and peripheral anti-inflammatory effects of JZL184.

EXPERIMENTAL PROCEDURES

Animals

All animal care and experimental protocols were 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.

Experiments were carried out on male Sprague Dawley rats (weight 220-260g; Harlan, 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 08:00 to 20:00 h). All experiments were carried out during the light phase between 08:30 h and 15:00 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 before experimentation in order to minimise the influence of the injection procedure on biological endpoints.

Experimental Design

Experiment 1: Effects of JZL184 on LPS-induced cytokine expression, 2-AG and arachidonic acid levels in the rat frontal cortex and plasma, and receptor mechanisms mediating these effects

Rats were randomly assigned to one of seven groups. Vehicle-Vehicle-Saline, Vehicle –Vehicle-LPS, AM251-Vehicle-LPS, AM630-Vehicle-LPS, Vehicle-JZL184-LPS, AM251-JZL184-LPS, AM630-JZL184-LPS (n = 6-10 per group). The CB₁ receptor antagonist AM251 (1 mg kg⁻¹, Cayman Chemicals, Estonia), CB₂ receptor antagonist AM630 (1 mg kg⁻¹, Cayman Chemicals, Estonia) or vehicle (ethanol: cremophor: saline; 1:1:18) were administered i.p. in an injection volume of 1 ml kg⁻¹. The doses of antagonists were chosen based on previous studies demonstrating their ability to block the effects of cannabinoid agonists *in vivo* (Jayamanne et al., 2006; Gonzalez et al., 2011). Immediately following the administration of antagonists, rats received either JZL184 (10 mg kg⁻¹ in an injection

volume of 2 ml kg⁻¹, generously received from Prof Benjamin Cravatt, Scripps Institute, La Jolla, CA, USA) or vehicle (ethanol: cremophor: saline; 1:1:18) followed 30 min later by an i.p. injection of LPS (100 µg kg⁻¹ B0111:B4 Sigma Aldrich, Dublin, Ireland) or saline vehicle (sterile 0.89% NaCl). The dose and time of JZL184 were determined from previous studies demonstrating that a minimum of 8 mg kg⁻¹ is required to induce behavioural effects in rats (Sciolino *et al.*, 2011), that 10 mg kg⁻¹ enhanced 2-AG levels in the ventral tegmental area of the rat brain (Oleson *et al.*, 2012) and that levels of endogenous 2-AG are enhanced 30 min post-administration (Long *et al.*, 2009b; Long *et al.*, 2009a). The dose and time of LPS administration were chosen on the basis of previous work within our laboratory demonstrating enhanced cytokine levels in the periphery and brain 2 h post-LPS administration (Roche *et al.*, 2006; Roche *et al.*, 2008; Kerr *et al.*, 2012). Animals were killed by decapitation 2 h post-LPS/saline, trunk blood collected into chilled lithium heparin collection tubes, centrifuged at 4°C for 15 min at 4 000× g, plasma was then removed and stored at -80°C until cytokine determination. Spleen and frontal cortex were excised, snap frozen on dry ice and stored at -80°C until assayed for MAGL activity, 2-AG, arachidonic acid, prostaglandin levels and cytokine expression.

Experiment 2: Effect of JZL184 on 2-AG levels in the rat frontal cortex over time

Rats were randomly assigned to one of nine treatment groups: Vehicle-Saline, Vehicle-LPS (10 min), JZL184-LPS (10 min), Vehicle-LPS (30 min), JZL184-LPS (30 min), Vehicle-LPS (60 min), JZL184-LPS (60 min), Vehicle-LPS (90 min) and JZL184-LPS (90 min) (*n* = 8-12 per group). Rats were administered JZL184 (10 mg kg⁻¹ i.p. Cayman Chemicals, Tallin, Estonia) or vehicle (ethanol: cremophor: saline; 1:1:18) followed 30 min later by an i.p. injection of LPS (100 µg kg⁻¹) or saline vehicle. Rats were killed 10, 30, 60 or 90 min after LPS (or saline), the brain excised, the frontal cortex dissected out and stored at -80°C until assayed for 2-AG concentration.

Analysis of inflammatory mediators using quantitative real-time PCR

RNA was extracted from cortical tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany). Genomic DNA contamination was removed with the addition of DNase to the samples according to the manufacturer's instructions. 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 (Kerr *et al.*, 2012). Assay IDs for the genes examined were as follows: IL-1 β (Rn00580432_m1), TNF- α (Rn99999017_m1), IL-6 (Rn00561420_m1) and IL-10 (Rn00563409_m1). In order to determine if the effects of JZL184 on cytokine expression were mediated by modulation of the NF- κ B pathway, the expression of the inhibitor of NF- κ B, I κ B α (Rn01473658_g1), an indirect measure of NF κ B activity (Read *et al.*, 1994), was also assessed. 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.

Determination of plasma cytokine protein levels

Plasma TNF- α , IL-1 β , IL-6 and IL-10 concentrations were determined using specific rat enzyme-linked immunosorbent assays (ELISAs) performed using antibodies and standards obtained from R&D Systems, UK (TNF- α and IL-10) or Peprotech, UK (IL-1 β and IL-6) as previously described (Roche *et al.*, 2006; 2008; Kerr *et al.*, 2012). ELISAs were carried out according to manufacturer's instructions and cytokine levels were expressed as pg ml⁻¹ plasma.

MAGL Activity Assay

MAGL activity assay was conducted as previously described (Cable *et al.*, 2011). In brief, frontal cortical or spleen tissue was weighed (~20 mg), homogenised in 1 ml of TE buffer (50 mM Tris, 1 mM EDTA, pH 7.4) and centrifuged at 14 000 \times g for 15 min. The pellet was resuspended in 1 mL of TE buffer, centrifuged and resuspended

in a final volume of TE buffer so as to give a 1 in 5000 or 1 in 500 dilution of the initial wet cortical or spleen tissue weights respectively. 90 μl of sample aliquots or blanks were pre-incubated with 5 μl of Hanks/HEPES buffer (116 mM NaCl, 5.4 mM KCl, 1.8 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25 mM HEPES, 0.8 mM MgSO_4 , 1 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) pH 7.4, containing 1 mg ml^{-1} defatted albumin for 30 min at 37°C. After pre-incubation, 5 μl of substrate (500 μl 2 mM 2-OG containing 3.75 μCi 2-oleoyl- $[\text{}^3\text{H}]$ -glycerol; American Radiolabelled Chemicals) was added with mixing to give a final $[\text{}^3\text{H}]$ -2-OG concentration of 100 μM . The reaction was allowed to proceed for 15 min at 37°C, following which 300 μl of stop solution (8% charcoal in 0.5 M HCl) was added with mixing. Samples were allowed to stand for a further 20 min and then centrifuged at 14 000 $\times g$ for 5 min to pellet the charcoal before removal of a 200 μl aliquot of the clear supernatant containing liberated $[\text{}^3\text{H}]$ glycerol for liquid scintillation counting. Homogenates were assayed in triplicate. Data were expressed as $\text{nmol min}^{-1} \text{g}^{-1}$ tissue.

Quantitation of endocannabinoids and N-acylethanolamine concentrations using liquid chromatography - tandem mass spectrometry (LC-MS/MS)

Quantitation of endocannabinoids and N-acylethanolamines was essentially as described previously (Ford et al., 2011; Olango et al., 2011; Kerr et al., 2012). In brief, samples were homogenised in 400 μL 100% acetonitrile containing deuterated internal standards (0.014 nmol anandamide-d8, 0.48 nmol 2-AG-d8, 0.016 nmol PEA-d4, 0.015 nmol OEA-d2). Lyophilised samples were re-suspended in 40 μL 65% acetonitrile and separated on a Zorbax® C18 column (150 \times 0.5 mm 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 20 min. 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 pmol g^{-1} of

tissue. The limit of quantification was 1.3 pmol g⁻¹, 12.1 pmol g⁻¹, 1.5 pmol g⁻¹, 1.4 pmol g⁻¹ for anandamide, 2-AG, PEA and OEA respectively.

Qualitative detection of JZL184 using LC-MS/MS

The system and protocol employed was similar to that described for the detection of endocannabinoid and N-acylethanolamine levels with the following modifications. Briefly, samples were prepared as for endocannabinoid determination and resuspended in 100% acetonitrile. Separation occurred by reversed-phase gradient elution initially with a mobile phase of 25% acetonitrile and 0.1% formic acid that was ramped linearly up to 100% acetonitrile and 0.1% formic acid over 10 min and held at this for a further 10min before being returned to 25% acetonitrile. Under these conditions JZL184 eluted at 14 min.

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 25 V.

Quantitation of PGE₂ and PGD₂ using LC-MS/MS

Spleen or cortical samples were homogenised in 400 µl of acetonitrile containing tetra deuterated labelled internal standards (1.7pmoles PGE₂ and PGD₂). Lyophilised samples were resuspended in 40 µl 25% acetonitrile and 4 µl injected onto a Zorbax® SB C18 column (150 × 0.5 mm internal diameter). The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) maintained at a flow rate of 12 µl per min. Reversed-phase gradient elution began initially at 25% B and over 20 min was ramped linearly up to 50% B and at 25 min was ramped to 100% B and held at this for a further 5 min. Under these conditions, PGE₂ and PGD₂ eluted at 19.8 and 21 min respectively. Analyte detection was carried out in electrospray-negative ionisation and multiple reaction monitoring (MRM) mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Ireland). Quantification of each analyte was performed by ratiometric analysis and expressed as pmol g⁻¹ tissue.

HPLC-UV determination of arachidonic acid concentration

Determination of arachidonic acid was carried out by HPLC as described (Lang *et al.*, 1996). In brief, cortical or spleen samples (60-100 mg) were homogenised in 1 ml of mobile phase (8.5% phosphoric acid/acetonitrile (10:90 v/v)) containing 50 ng biphenyl per 20 μ l as internal standard. Samples were centrifuged at 14 000 \times *g* for 15 min at 4°C and 20 μ l of the supernatant or standard (200 ng arachidonic acid (Sigma, Ireland) per 20 μ l) was then injected onto the Shimadzu HPLC system (Mason Technology, Dublin, Ireland). Separation was carried out at 30°C on a Synergie 4 μ m reverse phase column (Phenomenex, UK) at a flow rate of 1 ml per min and detected on a SPD-10A UV-vis detector at 204 nm. Quantification of arachidonic acid levels was based on the ratiometric analysis of sample and standard peak heights at 204 nm and expressed as nmol g⁻¹ tissue.

Statistical Analysis

SPSS was used to analyse all data. Results are expressed as group means + standard error of the mean (SEM). Data were analysed using unpaired *t-test* or two-way ANOVA (following log transformation) with the factors of antagonist/ vehicle and JZL184/vehicle treatment. *Post hoc* analysis was performed using Duncan's test when appropriate. Data were considered significant when $P < 0.05$.

Results

JZL184 attenuates LPS-induced increases in cytokine expression in the frontal cortex

LPS increased IL-1 β (23-fold), IL-6 (21-fold), TNF- α (3.5-fold), IL-10 (17-fold) and I κ B α (6.2-fold) expression when compared to saline-treated controls (Vehicle-Vehicle-Saline vs. Vehicle-Vehicle-LPS; Fig. 1A-D). Systemic administration of the MAGL inhibitor JZL184, significantly attenuated the LPS-induced increase in IL-1 β (JZL effect: $F_{1,36} = 42.962$, $P < 0.001$), IL-6 ($F_{1,35} = 4.124$, $P = 0.050$), TNF- α ($F_{1,37} = 46.070$, $P < 0.001$) and IL-10 ($F_{1,37} = 10.977$, $P = 0.002$), but not I κ B α , expression. Administration of the CB₁ receptor antagonist AM251 partially blocked the JZL184-induced attenuation of IL-1 β mRNA expression following LPS administration (Antagonist x JZL184 interaction effect: $F_{2,36} = 6.452$, $P = 0.004$) (Figure 1A). Although there was no main effect of antagonist treatment on IL-6 expression, a strong trend for AM251-induced blockade of the action of JZL184 on the expression of this cytokine was observed. AM251 alone significantly attenuated the LPS-induced increase in IL-1 β expression. Pharmacological blockade of the CB₂ receptor with AM630 did not alter LPS-induced cytokine expression alone, nor did it alter the JZL-induced attenuation of LPS-induced cytokine expression.

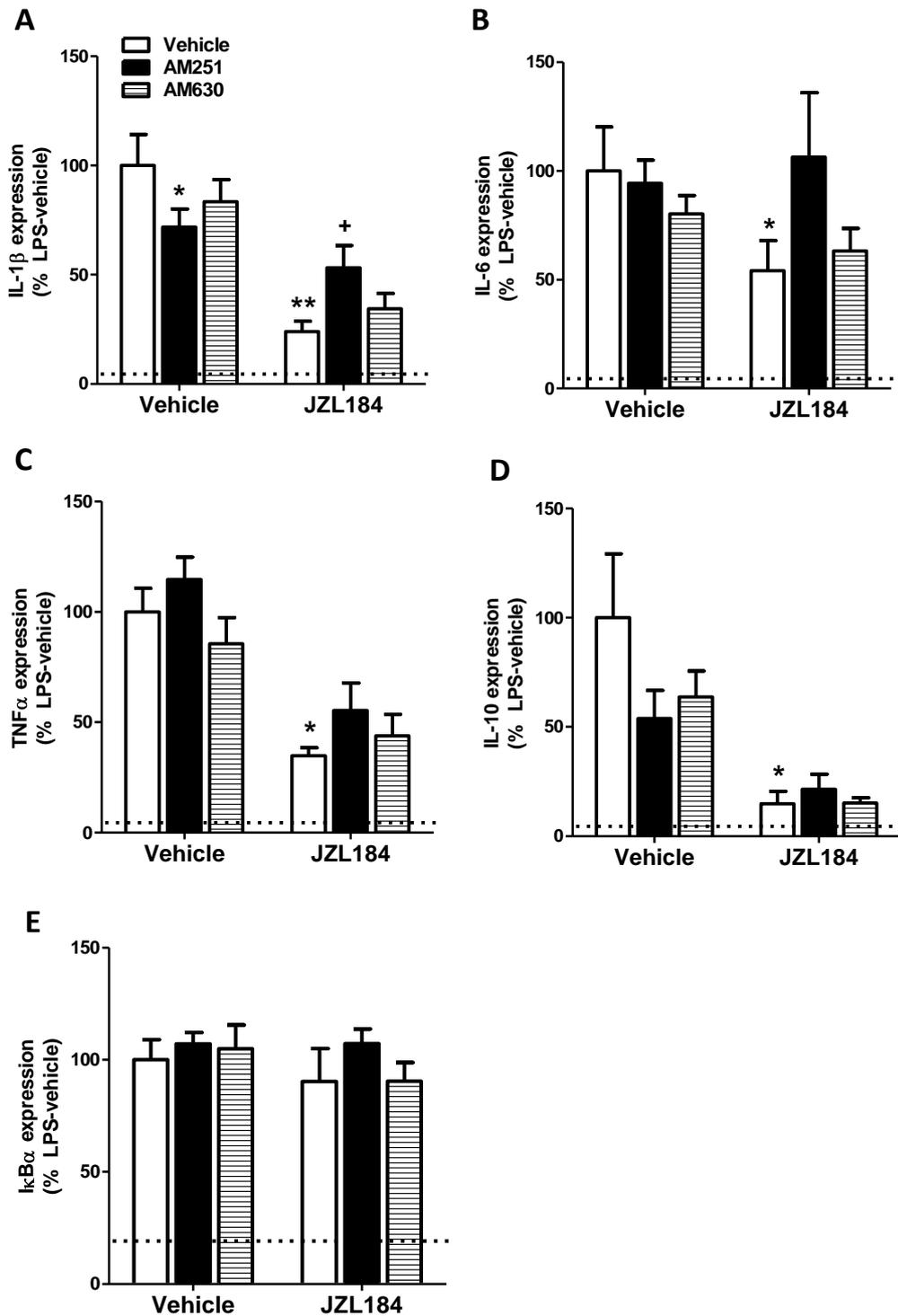


Figure 1. JZL184 attenuates LPS-induced increases in cytokine expression in the rat frontal cortex

JZL184 (10 mg kg⁻¹ i.p.) significantly attenuated LPS-induced increases in IL-1 β (A), IL-6 (B), TNF- α (C) IL-10 (D) mRNA expression in the rat frontal cortex. AM251 alone attenuated the LPS-induced increase in IL-1 β mRNA expression while also partially preventing the JZL184-induced attenuation of IL-1 β following LPS administration (A). Effect of LPS and JZL184 on I κ B α (E). Data expressed as means \pm SEM (n = 6-10 per group). Dotted line represents Vehicle-Vehicle-Saline. ** P < 0.01; * P < 0.05 vs. Vehicle-Vehicle-LPS. + P < 0.05 vs. Vehicle-JZL184-LPS.

JZL184 attenuates LPS-induced increases in TNF- α and IL-10 levels in plasma, an effect partially attenuated by CB₁ receptor antagonism

LPS increased IL-1 β (287-fold), IL-6 (5.9-fold), TNF- α (1300-fold) and IL-10 (169-fold) levels in the plasma when compared to saline-treated controls (Vehicle-Vehicle-Saline vs. Vehicle-Vehicle-LPS; Figure 2A-D). JZL184 significantly attenuated the LPS-induced increases in TNF- α (JZL effect: $F_{1,31} = 40.334$, $P < 0.001$) and IL-10 ($F_{1,30} = 7.337$, $P = 0.011$), but not IL-1 β or IL-6, levels (Figure 2C-D). AM251 and AM630 partially attenuated the JZL184-induced attenuation of TNF- α (Antagonist x JZL184 interaction effect: $F_{2,31} = 4.216$, $P = 0.024$) following LPS administration. Furthermore, the JZL184-induced attenuation of the increase in IL-10 levels following LPS was blocked by AM251 (Antagonist x JZL184 interaction effect: $F_{2,30} = 8.888$, $P = 0.001$; Figure 2D). AM251 alone attenuated the LPS-induced increase in IL-10 plasma levels (Figure 2D). AM630 significantly attenuated LPS-induced increase in IL-1 β cytokine levels in the presence of JZL184 (Antagonist x JZL184 interaction effect: $F_{2,32} = 4.614$, $P = 0.017$; Figure 2A).

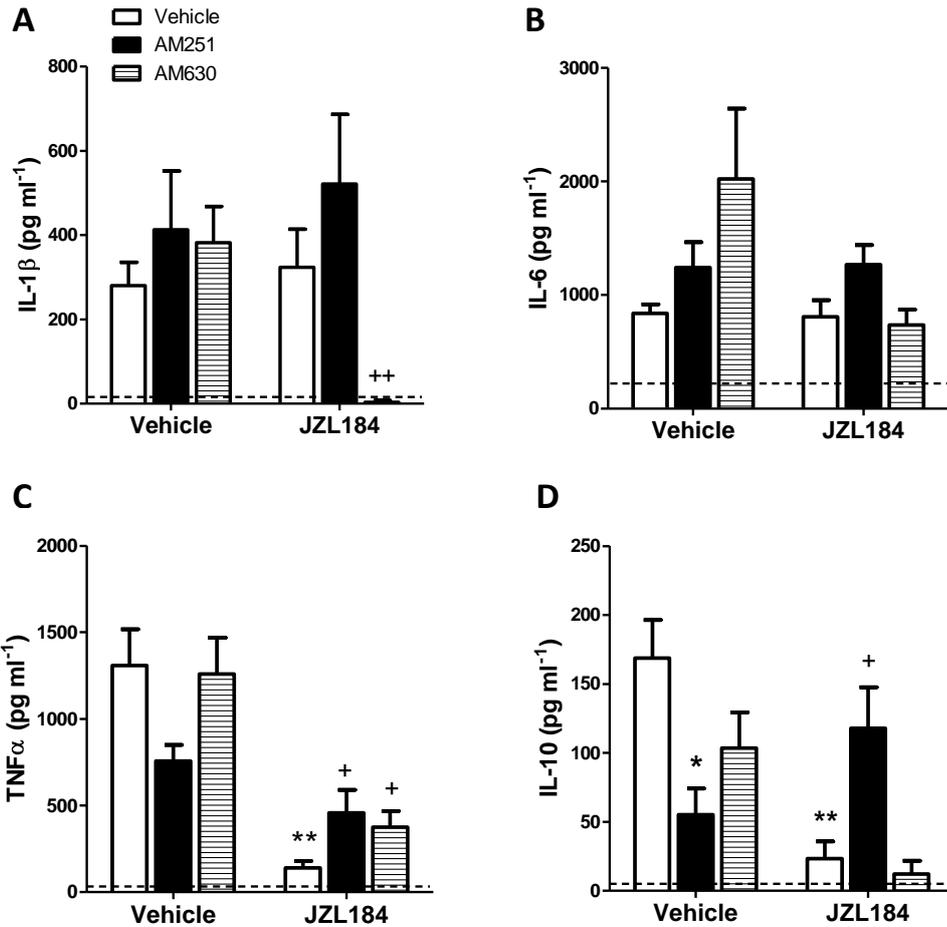


Figure 2. JZL184 attenuates LPS-induced increases in TNF- α and IL-10 levels in the plasma, an effect partially mediated by CB₁ receptors

LPS significantly increased IL-1 β (A), IL-6 (B), TNF- α (C) and IL-10 (D) plasma levels expression when compared to saline-treated controls (dotted line). JZL184 (10 mg kg⁻¹ i.p.) attenuated the LPS-induced increase in TNF- α (C) and IL-10 (D), effects partially attenuated by CB₁ receptor antagonism with AM251. AM630 also partially reversed the JZL184-induced attenuation of TNF- α levels following LPS administration (C). In the presence of JZL184, AM630 completely blocked the LPS-induced increase in IL-1 β (A). AM251 alone attenuated the LPS-induced increase in IL-10 levels (D). Data expressed as means \pm SEM (n = 6-10 per group). Dotted line represents Vehicle-Vehicle-Saline. * P < 0.05; ** P < 0.01 vs. Vehicle-Vehicle-LPS. + P < 0.05; ++ P < 0.01 vs. Vehicle-JZL184-LPS.

Systemic administration of JZL184 inhibits MAGL activity and increases 2-AG levels in the rat spleen, but not in the frontal cortex

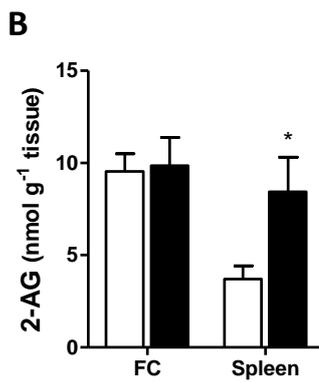
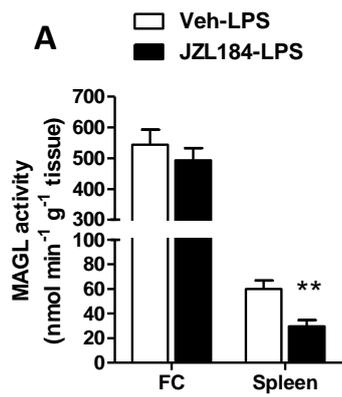
Systemic administration of JZL184 resulted in a significant inhibition of MAGL activity ($P = 0.002$) and an associated increase in 2-AG levels ($P = 0.023$) in the spleen, but not in the frontal cortex, of LPS-treated rats (Figure 3A-B). There was no effect of JZL184 on the levels of anandamide, OEA or PEA in either the frontal cortex or in the spleen (Figure 3C).

JZL184 reduces arachidonic acid levels but does not alter PGE₂ or PGD₂ levels in the frontal cortex of LPS-treated rats

Arachidonic acid levels were reduced in the frontal cortex ($P = 0.020$), but not in the spleen, of JZL184-LPS treated rats (Figure 3D). There was no effect of JZL184 on levels of the prostaglandins PGE₂ or PGD₂ in the frontal cortex or in the spleen (Figure 3E-F).

JZL184 is present in the rat spleen but not frontal cortex following systemic administration

As 2-AG levels were not enhanced in the frontal cortex at any of the time points examined, LC-MS/MS analysis was performed to qualitatively determine if JZL184 was present in the samples following systemic administration. Analysis revealed that although JZL184 was readily detectable in the rat spleen, the MAGL inhibitor could not be detected in the frontal cortex 2.5 h after administration (2 h after LPS) (Figure 3G-H).



C

		Veh	JZL184
FC	AEA	13.6 ± 0.8	14.9 ± 1.5
	OEA	94.0 ± 5.7	114.0 ± 14.4
	PEA	91.5 ± 7.5	123.8 ± 18.0
Spleen	AEA	1.36 ± 0.1	4.6 ± 2.9
	OEA	26.2 ± 2.6	54.2 ± 18.8
	PEA	29.5 ± 3.2	44.2 ± 9.8

Data expressed as mean (pmol g⁻¹) ± SEM

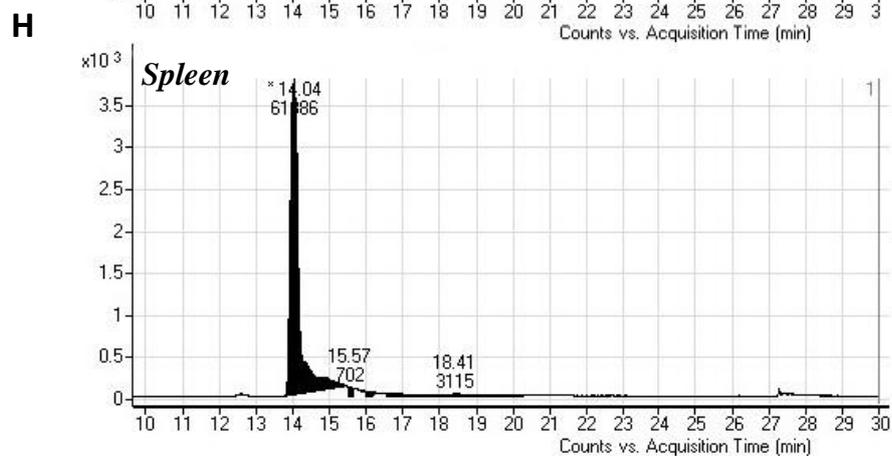
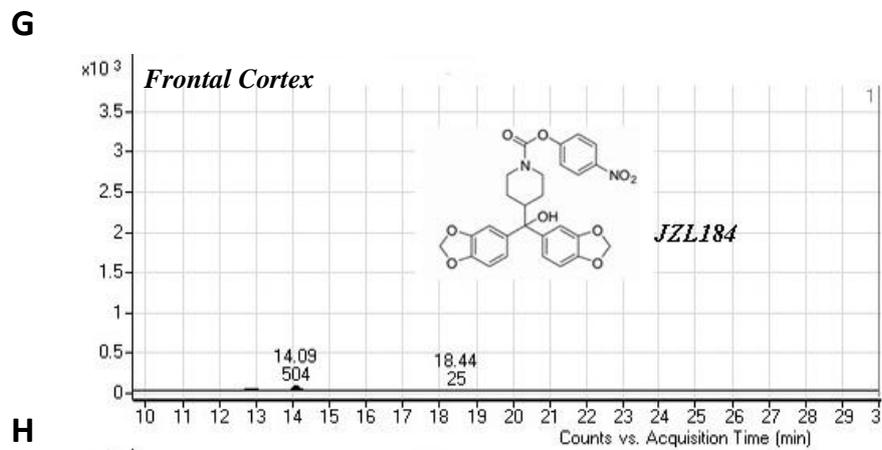
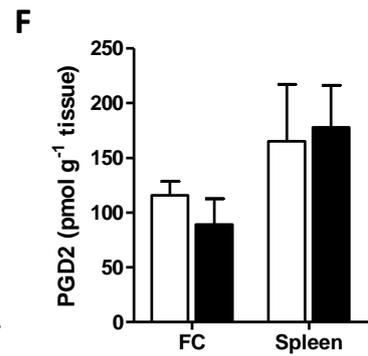
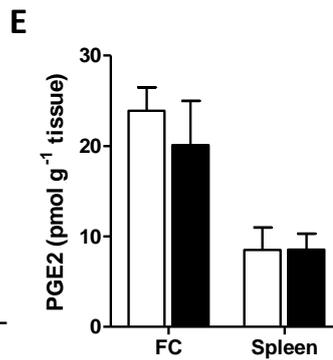
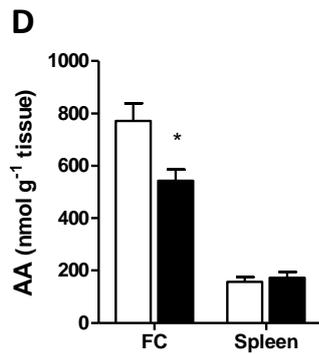


Figure 3. Effect of systemic administration JZL184 on MAGL activity, 2-AG, arachidonic acid and prostaglandin levels in the frontal cortex and spleen

Systemic administration of JZL184 (10 mg kg⁻¹, i.p.) inhibited MAGL activity (A) and increased 2-AG levels (B) in the spleen, but not frontal cortex. JZL184 did not alter concentrations of anandamide, OEA or PEA in either the frontal cortex or spleen (C). Arachidonic acid levels were increased in the frontal cortex, but not in the spleen of JZL184-treated rats (D). Levels of PGE₂ (E) and PGD₂ (F) in the frontal cortex or spleen of LPS-treated animals were not altered by prior administration of JZL184. JZL184 could be detected in the spleen (H) but not in the frontal cortex (G) following systemic administration. Structure of JZL184 presented as insert (F). Data expressed as means ± SEM (n = 6-10 per group). **P* < 0.05 vs. Vehicle-LPS.

The effect of JZL184 on 2-AG levels in the frontal cortex over time

As JZL184 did not alter 2-AG levels in the frontal cortex 2.5 h after administration (2 h following LPS), we sought to determine if the reduction in arachidonic acid might have resulted from increased 2-AG levels at an earlier time point than that examined in the studies described above. JZL184, administered 30 min before LPS, did not alter 2-AG levels in the frontal cortex measured at 10, 30, 60 and 90 min after LPS administration (Table 1). In addition, LPS alone did not alter 2-AG levels in the frontal cortex at any of the time points examined.

Table 1: Effect of systemic administration of JZL184 (10 mg kg⁻¹) on 2-AG levels in the frontal cortex over time

	Time (min)			
	10	30	60	90
Vehicle-LPS	6.80 ± 0.47	6.49 ± 0.55	7.64 ± 0.72	7.19 ± 0.81
JZL184-LPS	6.68 ± 0.40	7.13 ± 0.40	6.24 ± 0.78	7.70 ± 0.57

Vehicle-vehicle-saline: 7.00 ± 0.43 nmol g⁻¹ tissue. Data expressed as mean 2-AG concentration ± SEM in nmol g⁻¹ tissue. (n = 7-11 per group).

Discussion

The present study demonstrated that systemic administration of the MAGL inhibitor JZL184 robustly attenuated LPS-induced increases in cytokine expression in the rat frontal cortex. Although CB₁ receptor antagonism attenuated the JZL184-induced decrease in IL-1 β expression, this occurred in the absence of any JZL184-induced inhibition of MAGL activity or increase in 2-AG levels in the frontal cortex. Although arachidonic acid levels in this brain region were reduced in JZL184-LPS treated rats, this was not accompanied by changes in PGE₂ or PGD₂ levels. In comparison, JZL184 inhibited MAGL activity and increased 2-AG levels in the spleen, and attenuated the LPS-induced increases in plasma IL-10 and TNF- α levels, effects partially attenuated by CB₁ receptor antagonism. Together, these data demonstrate potent anti-inflammatory effects of JZL184 in the rat, both centrally and peripherally, although the mechanisms underlying these effects may differ.

Although JZL184 robustly and selectively enhanced 2-AG levels in the brain and peripheral organs of mice (Long et al., 2009b; Long et al., 2009a; Alhouayek et al., 2011; Kinsey et al., 2011; Nomura et al., 2011), to our knowledge only one study to date (Oleson *et al.*, 2012) has reported an increase in 2-AG levels in the rat brain (ventral tegmental area). In comparison to this latter study, the present study demonstrated that JZL184 inhibited MAGL activity and increased 2-AG levels in the rat spleen but not frontal cortex, 2.5 h after administration. Further analysis revealed that JZL184 could be detected in the spleen, but not frontal cortex, following systemic administration. Although the dose of JZL184 (10 mg kg⁻¹) was comparable between the present study and that of Oleson et al., (2012), the divergent results with respect to the ability of JZL184 to inhibit MAGL activity and increase 2-AG levels in the brain may result from differences between the two studies, such as the route of administration (i.p. vs. i.v.) or the brain regions under investigation. The lack of increase in 2-AG in the brain is also in contrast to that observed in mice at an equivalent time point (Long et al., 2009b; Long et al., 2009a). It should be noted that the affinity of JZL184 for rat MAGL is 10-fold lower than that for mouse or human MAGL (Long et al., 2009a) and, as such, higher doses of JZL184 in the present study may be required to inhibit MAGL and increase 2-AG in the rat brain. However, the present findings indicate that the dose of JZL184 used was capable of

inhibiting MAGL and increasing 2-AG levels in the spleen. These data, taken together with the data demonstrating detectable levels of JZL184 in the spleen but not in the frontal cortex, suggest that the lack of efficacy of the drug in the frontal cortex relates to insufficient brain permeability rather than dose.

While pharmacological blockade of MAGL with JZL184 reduced LPS-induced cytokines in both the frontal cortex and plasma, the profile and magnitude of the cytokine changes differed between these regions. JZL184 almost completely blocked LPS-induced expression of mRNA for cytokines (IL-1 β , TNF- α , IL-6 and IL-10) in the frontal cortex, similar to that previously reported in mouse brain (Alhouayek *et al.*, 2011; Nomura *et al.*, 2011). Further studies are required to determine if alterations in mRNA expression translate to changes in protein levels. Unlike these latter studies, this anti-inflammatory profile following JZL184 administration was not accompanied by an increase in 2-AG levels. It has been proposed that in the CNS, the inhibition of MAGL may shunt the hydrolysis of 2-AG onto other pathways such as COX-2, which would account for the lack of increase in 2-AG in the frontal cortex following JZL184. Such an effect would result in decreased arachidonic acid production via the MAGL hydrolysis of 2-AG, as observed in the current study. However, MAGL activity was not inhibited in frontal cortex following systemic administration of JZL184 and thus this is not a likely explanation. Nomura *et al.* have suggested that the anti-inflammatory effects of JZL184 in the brain are not directly mediated through cannabinoid receptors but rather due to reduced levels of arachidonic acid, with a consequent reduction in production of inflammatory mediators such as PGE₂ (Nomura *et al.*, 2011). Although arachidonic acid levels were reduced in the frontal cortex of JZL184-treated animals in the current study, this effect was not accompanied by alterations in PGE₂ or PGD₂ levels. In addition, CB₁ receptor antagonism with AM251 partially attenuated the JZL184-induced decrease in frontal cortical IL-1 β following LPS administration, indicating a potential role for the CB₁ receptor in mediating this response. 2-AG activation of CB₁ receptors has been shown to attenuate pro-inflammatory cytokine expression and protect against closed head injury via modulation of NF- κ B signalling (Panikashvili *et al.*, 2005; Panikashvili *et al.*, 2006). In addition, recent *in vitro* studies have demonstrated that JZL184-induced increases in 2-AG results in reduced

phosphorylation of NF- κ B and COX-2 expression in hippocampal neurons via activity at CB₁ receptors (Zhang and Chen, 2008; Du et al., 2011). However, the role of the CB₁ receptor in mediating the decrease in LPS-induced cytokine expression in the frontal cortex following JZL184 administration in the current study was not clear in light of the absence of an increase in 2-AG or changes in I κ B α expression, an indirect measure of NF- κ B signalling (Read *et al.*, 1994). Taken together, the data in the current study suggest that the anti-inflammatory effects of JZL184 in the rat frontal cortex, and their blockade by the CB₁ receptor antagonist, are most likely an indirect consequence of 2-AG-induced decreases in circulating cytokine levels following JZL184. Increased levels of circulating pro-inflammatory cytokines can communicate with the brain via many routes (diffusion into brain across the blood brain barrier deficient areas, sensory signals and vagus nerve stimulation) and induce cytokine synthesis within the CNS, which leads to a state of acute neuroinflammation. Thus, modulation of peripheral cytokines can profoundly affect brain neuroinflammatory processes. This has important implications as novel treatments targeting the levels of 2-AG in peripheral tissues or organs may indirectly modulate neuroimmune function.

In contrast to effects in the brain, JZL184 reduced LPS-induced increases in plasma levels of TNF- α and IL-10, but not IL-1 β or IL-6, effects accompanied by elevated 2-AG concentrations in the spleen, a major immune organ and source of circulating cytokines. To our knowledge, this is the first study to examine the effects of JZL184 on circulating cytokine levels following an acute immune challenge. The present findings correlate with recent studies demonstrating that a JZL184-induced increase in 2-AG was associated with reduced expression of several cytokines including TNF- α and IL-10 in models of gastric haemorrhage and colitis (Alhouayek *et al.*, 2011; Kinsey *et al.*, 2011). 2-AG-induced activation of CB₁ receptors was shown to prevent NSAID-induced gastric haemorrhage (Kinsey *et al.*, 2011). Both CB₁ and CB₂ receptors appear to be involved in the JZL184-induced amelioration of colon alterations in the mouse model of colitis; however, antagonism of these receptors only partially attenuated the JZL184-induced decrease in cytokine expression in the colon (Alhouayek *et al.*, 2011). In the current study, pharmacological antagonism of the CB₁ receptor with AM251 fully attenuated the JZL-induced decrease in plasma IL-10 levels, whereas antagonism of both CB₁ and CB₂ receptors partially blocked

the decrease in TNF- α levels. Therefore, JZL184 inhibition of rat MAGL increased peripheral 2-AG levels, which probably acted via CB_{1/2} receptors to attenuate LPS-induced increases in cytokines (IL-10 and TNF- α) levels. Although JZL184 did not alter levels of plasma IL-1 β or IL-6, effects on these cytokines at time points other than those examined in the present study cannot be ruled out.

It should be noted that a combination of CB₂ receptor antagonism and JZL184 resulted in complete inhibition of the LPS-induced increase in plasma IL-1 β , an effect not observed in the absence of MAGL inhibition. Although the significance of this finding is unclear, we propose that activation of other receptors by 2-AG, under circumstances where CB₂ receptors are blocked, may be responsible for this effect. For example, 2-AG suppressed IL-2 production via activation of PPAR γ (Rockwell *et al.*, 2006) and a similar mechanism may account for the reduction in IL-1 β levels observed here. PPAR γ activation has been repeatedly shown to elicit anti-inflammatory effects, including reductions in IL-1 β , and recent evidence indicates that this occurs by interfering with TLR4, the LPS receptor, and its downstream signalling components (Maggi *et al.*, 2000; Ji *et al.*, 2011). Thus, we propose that the tonic activity of 2-AG at PPAR γ is minimal; however, concomitant JZL184-induced MAGL inhibition and blockade of CB₂ receptors results in increased 2-AG availability, shunting its activity away from CB₂ receptors and onto PPAR γ , consequently inhibiting TLR4 signalling and LPS-induced IL-1 β production.

Immunosuppressive effects of cannabinoid receptor antagonists/inverse agonists have been previously reported, although the precise mechanisms by which these effects are mediated remain to be determined. Our data demonstrate that AM251 alone reduces LPS-induced IL-1 β expression in the frontal cortex and IL-10 levels in the plasma. Studies from our laboratory have previously reported that AM251 reduced plasma TNF- α levels and, to a lesser degree, IL-1 β and IL-6 (Roche *et al.*, 2008) and that another CB₁ receptor antagonist/inverse agonist, rimonabant, reduced IL-1 β levels in the brain and IL-1 β and TNF- α in the plasma (Roche *et al.*, 2006) under similar conditions. In addition, rimonabant has recently been shown to reduce the TNF- α , IL-6 and MCP-1 expression in a mouse model of colitis (Alhouayek *et al.*, 2011). Although in the current study, AM251 reduced IL-10 levels in rat plasma, rimonabant enhanced LPS-induced IL-10 levels in mice (Smith *et al.*, 2000), indicating potential, species-related, differences in the effects of the two compounds.

As highlighted earlier, the immunosuppressive effects of cannabinoid antagonists may be due to the unmasking of endocannabinoid actions at other receptors or direct activity at alternative targets such as GPR55 (Ryberg *et al.*, 2007). In addition, it has also been suggested that the cannabinoid receptor antagonists may act as partial agonists at CB₁ and CB₂ receptors when administered alone (Smith *et al.*, 2000; Croci *et al.*, 2003; Roche *et al.*, 2006; Roche *et al.*, 2008). Further studies are required in order to elucidate the mechanism underlying the immunomodulatory effects of these antagonists.

In conclusion, the current study demonstrated that JZL184 inhibited MAGL activity and increased 2-AG levels in a primary immune organ (the spleen) and attenuated LPS-induced increases in circulating cytokine levels in the rat, effects partially mediated by CB₁ receptors. In the frontal cortex, JZL184 robustly attenuated LPS-induced cytokine expression without elevating 2-AG levels or inhibiting MAGL activity, suggesting that the effects on central cytokine expression may be mediated indirectly via suppression of LPS-induced peripheral cytokine production. These results provide further evidence that MAGL inhibition may constitute a novel approach for the treatment of central and peripheral inflammatory disorders.

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Chapter 5

*Neuroinflammatory responses to TLR4 activation in
the valproic acid (VPA) rat model of autism*

Introduction

Autism is a neurodevelopmental disorder characterised by impairment in social interaction, deficits in verbal and non-verbal communications, and restrictive, repetitive stereotyped patterns of behaviours, interests and activities (DSM IV American Psychiatric Association 1994). Increasing evidence over the past 20 years has demonstrated altered immune function in autistic children and adults, an effect which has been proposed to underlie or modulate the behavioural deficits observed in autism [For review see (Onore et al., 2012)].

In brief, evaluation of peripheral cytokine levels has revealed increases in the levels of pro-inflammatory cytokines such as IL-1 β , IFN- α , IFN- γ , IL-1ra, TNF- α and IL-6 in autistic children when compared to normal controls (Singh, 1996; Croonenberghs et al., 2002; Ashwood et al., 2011a). Although the magnitude and specific cytokine changes differ slightly depending on the study, the general consensus is that autism is associated with increased levels of circulating pro-inflammatory cytokines. Furthermore, stimulation of monocyte cultures from autistic children with various TLR agonists has revealed an altered ability to mount an appropriate immune response, in particular, a blunted IL-10 and IL-6 response to TLR2/6 stimulation and enhanced IL-1 β response to TLR4 stimulation in the disorder (Jyonouchi et al., 2008; Enstrom et al., 2010). Evaluation of neuroinflammatory processes has revealed microglial and astrocyte activation in cortical and cerebellar regions in the brain of autistic patients (Vargas et al., 2005). In addition, levels of pro-inflammatory cytokines including IL-1, TNF- α and IL-6 in the brain and CSF have been shown to be increased (Vargas et al., 2005; Li et al., 2009). Thus, evidence suggests alterations in both peripheral and central inflammatory processing/function in autism.

In order to investigate the (neuro)biological mechanisms underpinning the development of autism spectrum disorders, numerous animal models have been developed. Several studies have examined immune responses in maternal immune activation models of autism [for review see (Patterson, 2009)]. Pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF α have been shown to be elevated in the foetus 2-24 hours following immunogen administration to the pregnant dam (Urakubo et al., 2001; Gayle et al., 2004; Ashdown et al., 2006; Meyer et al., 2006), effects which are sustained in adolescence and adulthood (Borrell et al., 2002; Basta-

Kaim et al., 2012). Apart from the maternal immune activation models there is a paucity of studies examining immune changes in animal models of autism.

Clinical evidence indicates that maternal exposure to the antiepileptic drug valproate during pregnancy has a tendency to increase the risk of autism in children (Rasalam et al., 2005; Meador et al., 2006) and, based on this evidence, the valproic acid (VPA) rodent model of autism was developed. Prenatal exposure of rodents to VPA at a critical time during gestation (G9 in mouse or G12.5 in the rat) impairs neural tube closure resulting in a host of neural abnormalities such as diminished number of cerebellar purkinje and cranial neurons (Rodier et al., 1997; Ingram et al., 2000), enhanced synaptic plasticity of the prefrontal cortex (Sui and Chen, 2012) and amygdala (Markram et al., 2008; Rinaldi et al., 2008), alterations in monoamine and amino acid neurotransmission (Schneider et al., 2007; Dufour-Rainfray et al., 2010; Kim et al., 2013). Assessment of behavioural alterations in VPA prenatally exposed offspring reveals that they exhibit reduced social behaviours, lower sensitivity to pain, increased tactile sensitivity, diminished acoustic prepulse inhibition, reduced locomotor and repetitive/stereotypic-like activity (Schneider and Przewlocki, 2005), increased anxiety-like behaviours (Markram et al., 2008) and altered circadian rhythms (Tsujino et al., 2007); behaviours which resemble those observed in autistic patients.

To date, few studies have examined immune alterations in the VPA rat model. Schneider and colleagues (2008) demonstrated that rats prenatally exposed to VPA exhibited decreased thymus weight, decreased proliferative capacity of splenocyte cultures to concanavalin A and increased production of NO by peritoneal macrophages, both basally and following LPS stimulation. In addition, while basal or Con A-induced IFN- γ and IL-10 production by splenic lymphocytes did not differ between controls and VPA-exposed animals, the Con A-induced IFN- γ /IL-10 ratio was decreased in VPA-exposed rats, possibly indicating an imbalanced Th1/Th2 response in the model (Schneider et al., 2008). These data lend further validity to the model and highlight altered immune processes in the prenatally exposed VPA rat in adulthood. However, the data are generated from *ex vivo* stimulation of immune cells (peritoneal macrophages and splenocytes) and thus the effects may not truly reflect the natural immune status of the model. A recent study in mice has demonstrated a trend for elevations of central immune mediators and markers (GFAP, CD11b, IL-1 β

and COX2) in the hippocampus of VPA-exposed offspring, although these were not statistically significant (de Theije et al., 2013). Similarly, a further recent study by Lucchina and co-workers demonstrated that central and peripheral basal levels of pro-inflammatory cytokines did not differ between VPA-exposed and control mice. However, VPA-exposed mice exhibited augmented LPS-induced expression of IL-6 in the spleen, microglia activation in the hippocampus and cerebellum and TNF- α and IL-6 in the cerebellum when compared to non-VPA-exposed counterparts (Lucchina and Depino, 2013). Thus, prenatal exposure to VPA may alter the ability of these animals to mount appropriate immune responses during adulthood. While this has been examined in the murine model, no study to date has examined if central immune processes are altered in the rat VPA model.

The objectives of the current study were

- To investigate if the expression of the pro-inflammatory cytokines IL-1 β , TNF α , IL-6, the anti-inflammatory cytokine IL-10, the chemokines CCL2 and CCL5 and markers of glial cell activation CD11b and GFAP were altered in the frontal cortex, hippocampus and cerebellum of VPA-exposed offspring, key brain regions implicated in autistic-like symptoms.
- To examine if VPA-exposed animals exhibit altered expression of the LPS-receptor TLR4 and its accessory binding protein MD-2 in the aforementioned brain regions.
- To determine if VPA-exposed animals exhibit altered LPS-induced changes in inflammatory mediators in key brain regions.
- To determine if adolescent animals prenatally exposed to VPA exhibit alterations in the peripheral expression of TLR4, MD-2 and immune mediators (IL-1 β , TNF α and IL-6). Furthermore, this study examined if LPS-induced increases in the expression of peripheral pro-inflammatory mediators were altered in the VPA rat model.

EXPERIMENTAL PROCEDURES

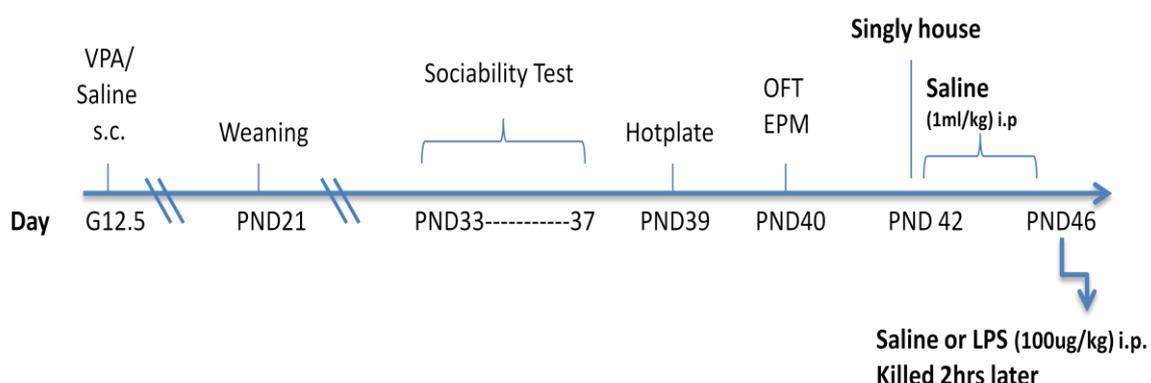
Animals

Male and female Sprague-Dawley rats (200-300g; Charles River Laboratories UK) were mated following determination of the oestrus phase of the reproductive cycle. The presence of spermatozoa in vaginal smears as determined by microscopy indicated the first day of gestation (G0.5). Following copulation, female rats were housed singly and maintained at constant temperature ($21 \pm 2^\circ\text{C}$) and humidity (30%-35%) under standard lighting conditions (12:12 h light–dark, lights on from 0700 to 1900 h). Food and water were available *ad libitum*. Experimental protocols were carried out under approval from the Animal Care and Research Ethics Committee at NUI Galway and under licence from the Irish Department of Health and Children, in compliance with the European Communities Council directive 86/609.

On gestational day 12.5 (G12.5), female rats received a single subcutaneous injection of sodium valproate (VPA) (Sigma, Dublin, Ireland) (600mg/kg) or saline vehicle. The dose and time of administration was chosen based on studies demonstrating that this regime elicits autistic-like behavioural changes in offspring (Schneider and Przewlocki, 2005). Females were allowed to raise their own litters and pups which were weaned on postnatal day (PND) 21. Following weaning, rats of either sex were housed separately in groups of 3-6 per cage.

Experimental Design

A schematic of the experimental design is presented in Fig 1.



Behavioural testing was carried out during adolescence between PND 33 and 40. The sequence of testing remained constant, and involved the sociability test followed by the hot plate test, followed by the open field and elevated plus maze test and was modelled on the study design described by Schneider and colleagues (Schneider and Przewlocki, 2005). All behavioural testing was carried out by an experimenter blinded to treatment (methodology and data presented in chapter 5). Two days following the final behavioural test (PND 40), animals were singly housed and administered i.p. injection of saline for a period of 3 days. On the 4th day animals were administered either sterile saline or the bacterial endotoxin LPS (100µg/kg) and 2hrs later were killed by decapitation, the spleen and brain removed, discrete brain regions including the frontal cortex, hippocampus and cerebellum dissected out, and all tissue was snap frozen on dry ice. Brain regions were stored at -80°C until assayed for mRNA expression of markers of glial activation (CD11b and GFAP), cytokines (IL-1β, TNFα, IL-6 and IL-10), chemokines (CCL2 and CCL5 (RANTES)), TLR4 and binding protein MD-2.

Analysis of inflammatory mediators using real-time PCR

RNA was extracted from cortical tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany). Genomic DNA contamination was removed with the addition of DNase to the samples according to the manufacturer's instructions. RNA was reverse transcribed to 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 (Kerr et al., 2012). Assay IDs for the genes examined were as follows: IL-1β (Rn00580432_m1), TNF-α (Rn99999017_m1), IL-6 (Rn00561420_m1), IL-10 (Rn00563409_m1), CD11b (Rn00709342-m1), GFAP (Rn00566603-m1), CCL2 (Rn00580555-m1), CCL5 (Rn00579590), TLR4 (Rn00569848-m1) and MD-2 (Rn01448830-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.

Statistical analysis

SPSS (Version 20) statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro-Wilks and Levene test, respectively. Parametric data were analysed using unpaired *t*-test and non-parametric data were analysed using an independent sample Mann-Whitney U test. Analysis was carried out to compare the effect of prenatal saline- vs. VPA exposure on cytokine expression. Data were considered significant when $p < 0.05$. Results expressed as group means + standard error of the mean (SEM).

Results

Expression of immune mediators in the frontal cortex, hippocampus and cerebellum in rats prenatally exposed to saline or VPA

The expression of IL-1 β , IL-10, CCL2 and CCL5 appeared to be increased in the frontal cortex of VPA-exposed animals, however statistical analysis revealed that only CCL5 expression ($U=0$, $p=.033$) was significantly increased when compared to saline controls (Fig 2a). In the hippocampus, the expression of GFAP ($t_{(12)}=2.236$, $p=.038$), a marker of astrocyte activation, was significantly reduced in VPA- vs. saline-exposed animals (Fig 2b). Although the expression of IL-1 β appeared to be elevated in the cerebellum of VPA-exposed animals when compared to saline-treated counterparts, this effect just failed to reach statistical significance (Fig 2c).

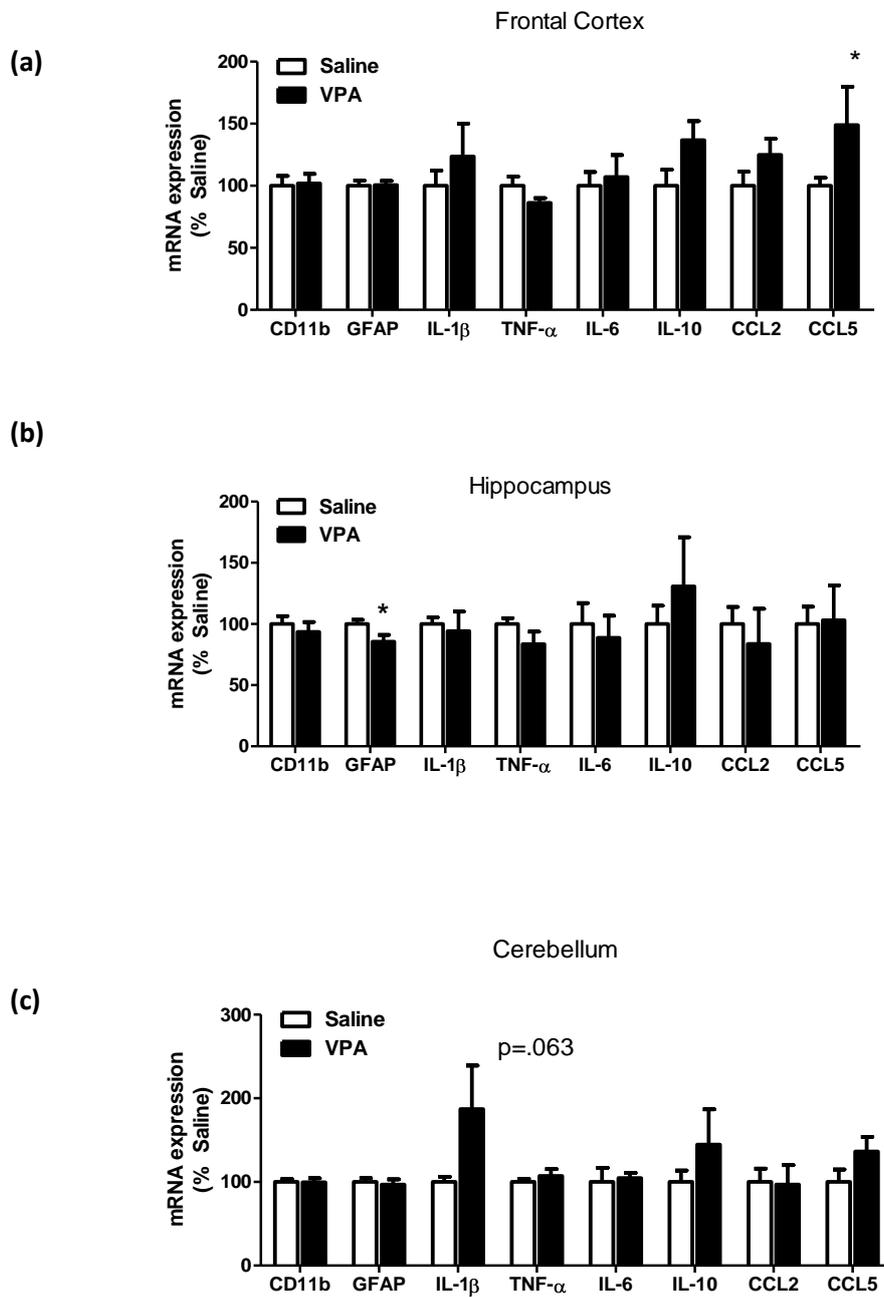


Fig 2. Expression of immune mediators and markers of glial activation in the (a) frontal cortex, (b) hippocampus and (c) cerebellum of animals exposed prenatally to saline or VPA. Data expressed as means +SEM (n = 6-8 per group). *P<0.05 vs saline-treated counterparts.

Expression of TLR4 and MD-2 in the frontal cortex, hippocampus and cerebellum in adolescent rats prenatally exposed to saline or VPA

The expression of TLR4 and MD-2 were unaltered in the frontal cortex and hippocampus of VPA exposed animals when compared to their saline counterparts (Fig 3a-b). However, analysis revealed a significant reduction in TLR4 ($t_{(12)}=3.655$, $p=.003$), but not MD-2, expression in the cerebellum of animals prenatally exposed to VPA when compared to saline controls (Fig 3c).

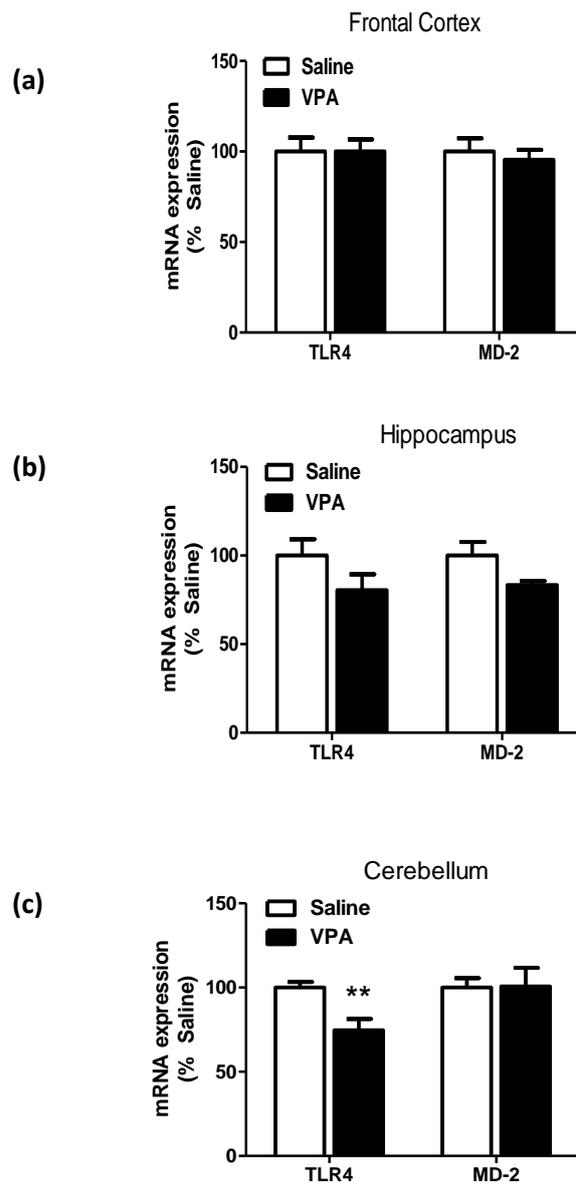


Fig 3. Expression TLR4 and MD-2 in the (a) frontal cortex, (b) hippocampus and (c) cerebellum of animals exposed prenatally to saline or VPA. Data expressed as means +SEM (n = 6-8 per group). * $P<0.05$ vs. saline treated counterparts.

VPA-exposed animals exhibit a heightened neuroinflammatory response to TLR4 activation

Systemic administration of the TLR4 agonist LPS, increased the expression of the inflammatory cytokines IL-1 β , TNF α , IL-6, IL-10 and CCL2 in the cortex, hippocampus and cerebellum (Fig 4 a-c vs. dotted line) and CCL5 in the cerebellum of saline- and VPA-exposed animals (Fig 4 a-c vs. dotted line). In the cortex, LPS-induced IL-1 β (U=0, p=.025) and CCL2 (U=0, p=.025) expression were significantly augmented in VPA- vs. saline-exposed animals following systemic administration of LPS (Fig 4a). TNF- α (U=0, p=.025) expression was significantly enhanced in the hippocampus of VPA animals exposed to LPS when compared to saline-LPS treated counterparts (Fig 4b). In the cerebellum, the expression of GFAP (U=0, p=.025) and IL-1 β (U=0, p=.025) were increased in VPA-LPS treated animals when compared to their saline-LPS challenged counterparts (Fig 4c).

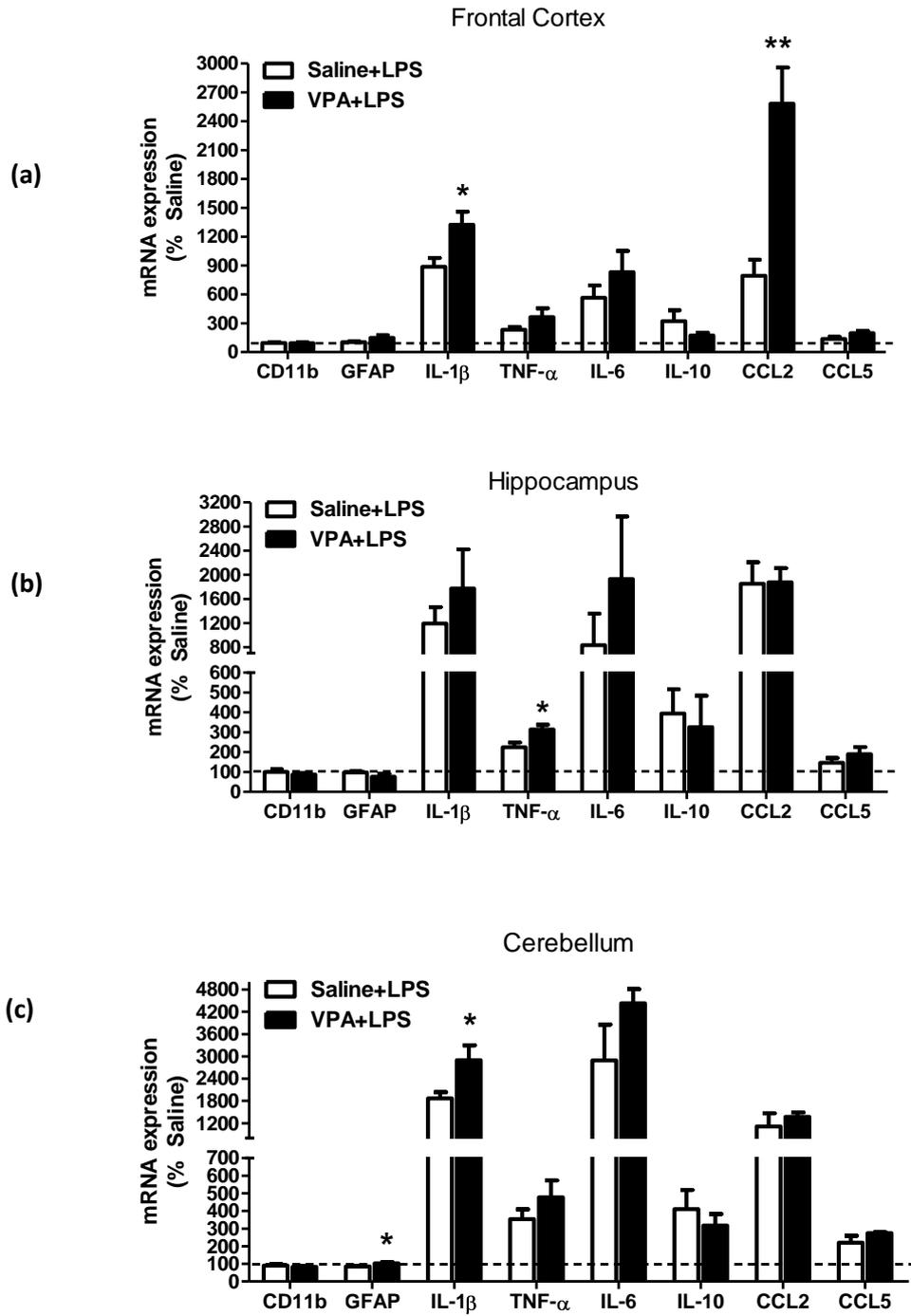


Fig 4. Expression of immune mediators and markers of glial activation following TLR4 activation in the (a) cortex, (b) hippocampus and (c) cerebellum of animals exposed prenatally to saline and VPA. Data expressed as mean + SEM (n = 3-5 per group). *P<0.05 vs. saline + LPS treated counterparts. Dotted line represents saline-vehicle treated controls

Prenatal VPA exposure does not alter the peripheral immune responses in the presence or absence of TLR4 activation

The expression of the pro-inflammatory cytokines IL-1 β , TNF- α , IL-6, TLR4 and MD-2, were unaltered in the spleen of animals prenatally exposed to VPA when compared to saline-treated controls (Fig 5 a-b). Systemic administration of the TLR4 agonist LPS induced an increase in the expression of splenic IL-1 β , TNF- α , IL-6, effects unaltered in animals exposed prenatally to VPA (Fig 5c).

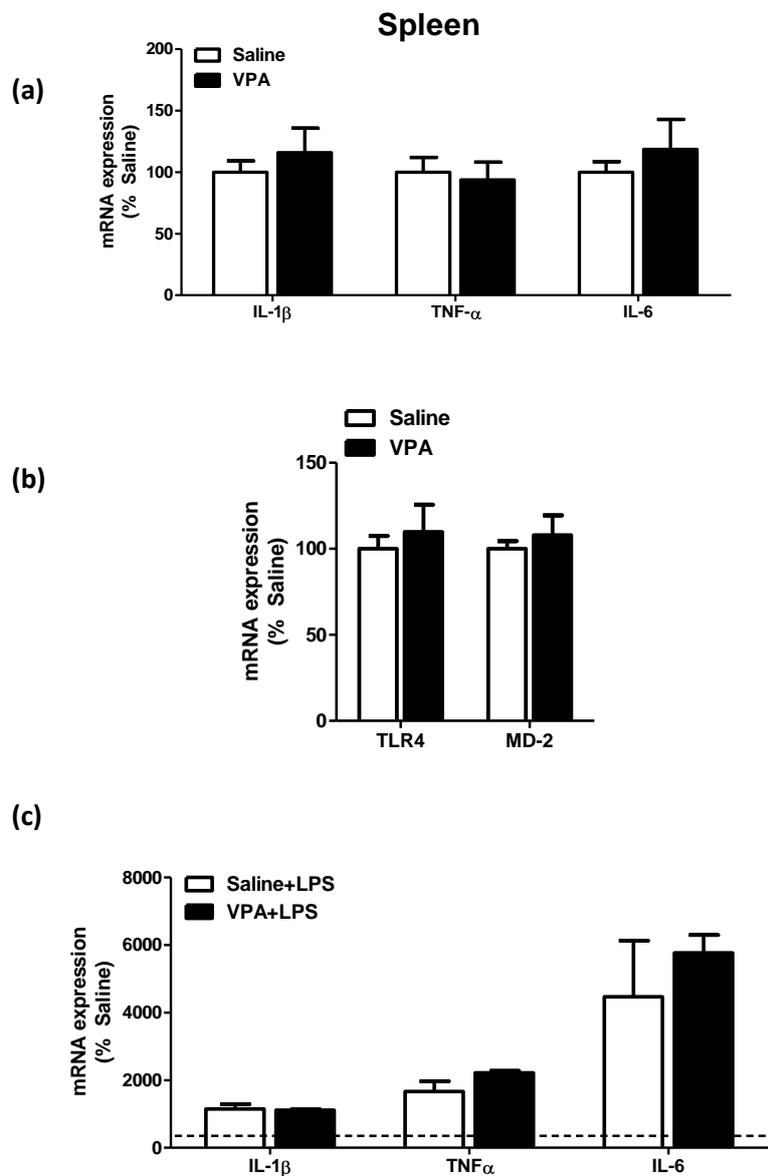


Fig 5. Expression of (a) IL-1 β , TNF- α , IL-6 and (b) TLR4 and MD-2 in the spleen of animals exposed prenatally to saline and VPA. (c) The expression of IL-1 β , TNF- α , IL-6 in the spleen of saline- and VPA-exposed animals following LPS administration. Data expressed as means + SEM (n = 3-8 per group). Dotted line represents saline-vehicle treated controls.

Discussion

Although alterations in cytokine expression have been demonstrated clinically and in other models of autism, the current study is the first to demonstrate that animals prenatally exposed to VPA exhibit increased expression of the chemokine CCL5 in the cortex, decreased astrocyte activation (GFAP expression) in the hippocampus and reduced TLR4 and slight but non-significant increases in IL-1 β and IL-10 expression in the cerebellum, when compared to saline-exposed counterparts. There was no significant difference in TLR4 or pro-inflammatory cytokine expression in the spleen between VPA- or Saline-treated animals. Thus, VPA-exposed animals appear to exhibit slight alterations in the expression of immune mediators in key brain regions that regulate emotional responding, but not in the periphery. Such alterations may modulate neuronal function within these brain regions, resulting in behavioural alterations observed in the model. In order to determine if VPA-exposed animals exhibit altered immune response to bacterial endotoxin exposure, the TLR4 agonist LPS was administered to both saline- and VPA-exposed animals. Systemic administration of LPS increased the expression of the inflammatory cytokines IL-1 β , TNF α , IL-6, IL-10 and CCL2 in the cortex, hippocampus and cerebellum of both saline- and VPA-prenatally exposed animals. The LPS-induced increases in IL-1 β and CCL2 mRNA expression in the cortex, TNF- α expression in the hippocampus and GFAP and IL-1 β expression in the cerebellum were significantly augmented in VPA-exposed animals when compared with saline-treated counterparts. While LPS increase pro-inflammatory cytokine expression in the spleen of both saline and VPA-exposed animals, this response did not differ between the two groups. These findings suggest animals prenatally exposed to VPA exhibit an exacerbated neuroimmunological response to TLR4 activation.

Given that it is now well recognised that immune alterations exist in, and may be responsible for some of the symptoms observed in autism, the current study is one of the few studies that has directly examined this in non-maternal immune activation models.

The current study has demonstrated that VPA-exposed animals do not exhibit altered expression of TLR4 or its adaptor protein MD-2 in a primary immune organ. Although we cannot rule out the possibility that alterations in the functionality or protein expression of these receptor molecules exist in the model, the data indicate that VPA-exposed animals express the same amount of TLR4 receptor (at least at a gene level) as control animals and are thus likely to be capable of mounting an immunological response to TLR4 agonists such as LPS. This is further confirmed due to the ability of systemic administration of LPS to increase levels of the pro-inflammatory cytokines IL-1 β , TNF α and IL-6 in the spleen in both saline- and VPA-exposed animals, an effect which did not differ between the groups. Thus, these rats prenatally exposed to VPA do not appear to exhibit an altered peripheral innate immunological profile. In comparison, Lucchina *et al* demonstrated that VPA-exposed mice, exhibit an augmentation of the LPS-induced increase in IL-6, possibly indicating increased innate immune activity in the murine VPA model (Lucchina and Depino, 2013).

In comparison to the lack of difference in the periphery, VPA-exposed animals exhibited increased expression of the chemotactic cytokine CCL5 in the frontal cortex and reduction in expression of TLR4 and GFAP in the cerebellum and hippocampus respectively, when compared to saline-treated counterparts. Although there were slight trends for an increased expression of IL-1 β and IL-10 in the cerebellum of VPA-exposed animals, expression of the pro- and anti-inflammatory cytokines did not significantly differ in any of the brain regions between saline- and VPA-exposed animals. Similarly, recently data has demonstrated that VPA-exposed mice do not exhibit altered central and peripheral (spleen) basal levels of pro-inflammatory cytokines (de Theije *et al.*, 2013; Lucchina and Depino, 2013). It is well recognised that astrocytes play a key role in brain development, neuronal function and glutamate metabolism. Several studies have reported increased astrocyte activation or GFAP expression in the brains from patients with autism (Laurence and Fatemi, 2005; Vargas *et al.*, 2005). In addition, mice prenatally exposed to the TLR-3 agonist poly I:C have been shown to exhibit increased GFAP (and microglial activation) in the hippocampus at PND1 and 100 (Ratnayake *et al.*, 2012). In contrast, the present study demonstrated a decreased GFAP expression, indicating a possible reduction in astrocyte activation/number, in the hippocampus of

VPA-exposed animals. In the resting state, astrocytes play a key role in neuronal homeostasis and glutamate metabolism. A recent study by Bristot Silvestrin and colleagues (2013) have examined glutamate metabolism in the hippocampus of VPA exposed animals, indicating altered astrocytic processing of glutamate in the model. In particular, this study demonstrated that young (PND15) VPA-exposed animals exhibit reduced expression of the glutamate transporter GLT1 and increased glutamine synthase (GS) activity while adult (PND120) animals exhibited increased glutamate uptake, GLT1 expression and glutathione activity and reduced GS activity in the hippocampus (Bristot Silvestrin et al., 2013). Further studies are required to determine if such alterations also exist in the model during adolescence as examined in the current study. However, taken together, these data suggest that astrocyte activation and functioning, at least in the hippocampus, are altered in the VPA model, effects which may underlie the spatial memory and affective impairments observed in the model (Schneider and Przewlocki, 2005; Moore et al., 2009).

Chemokines play a significant role in the recruitment of leukocytes into the CNS, regulate neuronal cell migration, proliferation and differentiation and are involved in communication between neurons and microglia. CCL5 plays an active role in recruiting leukocytes into sites of inflammation or injury and displays neurotropic (Bolin et al., 1998) and neuroprotective effects against various neurotoxins including glutamate, beta-amyloid, HIV and nitric oxide (Bruno et al., 2000; Kaul et al., 2007; Tripathy et al., 2010). CCL5 (RANTES) levels have been demonstrated to be enhanced in the plasma of autistic children (Enstrom et al., 2009; Ashwood et al., 2011b), effects correlated with greater communication and behavioural impairments (Ashwood et al., 2011b). However, changes in the expression/level of this chemokine have not been reported in the CSF or brain of autistic patients despite increases in other chemokines (CCL2 (MCP-1), MIP-1 β and IP-10) (Vargas et al., 2005; Garbett et al., 2008). To our knowledge, this is the first study to examine the expression of this chemokine in the brain in an animal model of autism, demonstrating enhanced expression in the frontal cortex, a key brain region regulating emotional and social processing. While the source and functional significance (inflammatory or neuroprotectant) of the increase in the chemokine remain to be determined, it should be noted that the CCL5 increase in VPA-exposed animals is not associated with a change in glial activation (as assessed by CD11b or

GFAP expression). This may indicate that the increase is not due to enhanced neuroinflammatory/glial activation in the model.

It should be noted that the expression of CCL5 was not altered in the frontal cortex or hippocampus following the administration of LPS to saline-exposed animals. In addition, cortical or hippocampal CCL5 expression in VPA-exposed animals did not differ from saline-exposed counterparts in response to TLR4 activation. However the expression of this chemokine was significantly elevated in the cerebellum of both saline- and VPA-exposed animals following LPS administration, effects associated with enhanced LPS-induced GFAP expression in VPA-exposed animals. It is unclear if CCL5 expression is elevated in VPA-exposed animals in response to enhanced astrocyte activation following LPS administration as saline-exposed counterparts exhibited a similar response. While the exact functional significance of the changes in CCL5 or CCL2 in the frontal cortex and cerebellum in this model is unknown, it is possible that enhanced chemokine expression may modulate neuronal function and account, at least in part, for the behavioural alterations observed in the VPA model.

It is well known that systemic administration of the bacterial endotoxin and TLR4 ligand LPS increases cytokine and chemokine expression in the brain. In accordance with this, our data demonstrate that LPS increased IL-1 β , TNF α , IL-6, IL-10 and CCL2 expression in both saline- and VPA-exposed animals. However, it should be noted that VPA-exposed animals exhibited augmented expression of IL-1 β and CCL2 in the cortex, IL-1 β and GFAP in the cerebellum and TNF α in the hippocampus following LPS. This is the first study to demonstrate enhanced neuroinflammatory response to TLR4 activation in a rat model of autism. A recent study by Lucchina and co-workers demonstrated augmented TNF- α and IL-6 in the cerebellum and heightened activation of microglia in the hippocampus and cerebellum of VPA-exposed mice following LPS administration. While the findings between these studies differ in terms of cytokine or glia activated, effects which may be species-dependent, taken overall this data further demonstrates altered immune responses in the VPA model of autism. CCL2 is released in response to acute brain injury, ischemia, and in neurodegenerative disease, binding primarily to the G-protein-coupled CCR2 to recruit inflammatory cells to sites of tissue damage and induce apoptosis/phagocytosis (Ransohoff et al., 1993; Kim et al., 1995; Hickman and El Khoury, 2010; Semple et al., 2010). In the current study, augmented increases

in CCL2 and pro-inflammatory cytokines, in VPA-exposed animals may indicate that these animals are at a greater risk of detrimental effects on neuronal survival and function in response to bacterial infection, as enhanced or continuous chemokine and cytokine signalling has been associated with neurodegeneration. (Gerard and Rollins, 2001; McGeer and McGeer, 2001, 2002; Semple et al., 2010). Thus, augmentation of cytokines and CCL2 in the aforementioned brain regions following LPS may ultimately alter neuronal function resulting in the exacerbation of behavioural changes observed in the VPA model. Several lines of evidence have demonstrated that immune activation is associated with heightened behavioural and social impairments in autistic patients and preclinical models (Opp et al., 1991; Zalcman, 2002; Moore et al., 2009; Enstrom et al., 2010; Ashwood et al., 2011a; Ashwood et al., 2011b). Further studies are required to determine if this is the case in the VPA model.

In conclusion, the present study indicates alterations in neuroinflammatory processes in the brain regions regulating social, emotional and motor responses in the VPA model, effects which may underlie the behavioural alterations observed. Thus, modulation of neuroimmune parameters may provide a novel therapeutic target for the treatment of some of the behavioural impairments seen in autistic patients.

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Chapter 6

Alterations in the endocannabinoid system in the rat valproic acid model of autism

Published Reference:

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ABSTRACT

The endocannabinoid system plays a crucial role in regulating emotionality and social behaviour, however it is unknown whether this system plays a role in symptoms associated with autism spectrum disorders. The current study evaluated if alterations in the endocannabinoid system accompany behavioural changes in the valproic acid (VPA) rat model of autism. Adolescent rats prenatally exposed to VPA exhibited impaired social investigatory behaviour, hypoalgesia and reduced locomotor activity on exposure to a novel aversive arena. Levels of the endocannabinoids, anandamide (AEA) and 2-arachidonylglycerol (2-AG) in the hippocampus, frontal cortex or cerebellum were not altered in VPA- versus saline-exposed animals. However, the expression of mRNA for diacylglycerol lipase α , the enzyme primarily responsible for the synthesis of 2-AG, was reduced in the cerebellum of VPA-exposed rats. Furthermore, while the expression of mRNA for the 2-AG-catabolising enzyme monoacylglycerol lipase was reduced, the activity of this enzyme was increased, in the hippocampus of VPA-exposed animals. CB1 or CB2 receptor expression was not altered in any of the regions examined, however VPA-exposed rats exhibited reduced PPAR α and GPR55 expression in the frontal cortex and PPAR γ and GPR55 expression in the hippocampus, additional receptor targets of the endocannabinoids. Furthermore, tissue levels of the fatty acid amide hydrolase substrates, AEA, oleoylethanolamide and palmitoylethanolamide, were higher in the hippocampus of VPA-exposed rats immediately following social exposure. These data indicate that prenatal VPA exposure is associated with alterations in the brain's endocannabinoid system and support the hypothesis that endocannabinoid dysfunction may underlie behavioural abnormalities observed in autism spectrum disorders.

Introduction

Autism is a neurodevelopmental disorder characterised by impaired social interaction, deficits in communication and restrictive, repetitive stereotyped patterns of behaviours. The aetiology of this disorder remains unknown, although several genetic and environmental factors have been identified which play a role in this spectrum of disorders. Prenatal exposure to teratogenic agents such as valproic acid (VPA) has been implicated in the pathogenesis of autism (Christianson et al., 1994, Williams and Hersh, 1997, Williams et al., 2001) and knowledge of this association has led to the development of a widely used and validated preclinical model of autism. Exposure of prenatal rats to VPA impairs neural tube closure and results in behavioural aberrations such as reduced social behaviour, lower sensitivity to pain and increased anxiety and fear in adolescent and adult rats (Schneider et al., 2001, Schneider and Przewlocki, 2005, Schneider et al., 2007, Markram et al., 2008), behaviours analogous to those observed clinically. Anatomical alterations such as diminished number of cerebellar purkinje and cranial neurons (Rodier et al., 1997, Ingram et al., 2000), enhanced synaptic plasticity of the prefrontal cortex (Sui and Chen, 2012) and amygdala (Markram et al., 2008, Rinaldi et al., 2008), alterations in monoamine and amino acid neurotransmission (Schneider et al., 2007, Dufour-Rainfray et al., 2010, Kim et al., 2013a) and immunological alterations (Schneider et al., 2008) have also been reported in the model.

Increasing evidence suggests a role for the endocannabinoid system in social and emotional processing (Viveros et al., 2007, Lutz, 2009), however there is a paucity of studies directly examining the role of this system in autism. Comprised of the G-protein coupled CB₁ and CB₂ receptors, the endogenous cannabinoid ligands (endocannabinoids) including anandamide (AEA) and 2-archidonylglycerol (2-AG) and the enzymes responsible for the synthesis and catabolism of the endocannabinoids, the neuroanatomical distribution of this system means that it is well positioned to modulate affective and social responding. A recent review has suggested metabolism of acetaminophen (paracetamol) to N-arachidonoylphenolamine (AM404) (Hogestatt et al., 2005), an AEA reuptake inhibitor, results in enhanced AEA tone which may alter neuronal development and

immunological function during critical neurodevelopmental phases possibly predisposing certain children to developing autism (Schultz, 2010). However, to date no detailed studies have been carried out investigating the link between acetaminophen, the endocannabinoid system and the development of autism. Polymorphisms in the gene encoding the CB₁ receptor, *CNRI*, have been shown to modulate striatal responses (Chakrabarti et al., 2006) and gaze duration (Chakrabarti and Baron-Cohen, 2011) to social reward cues, indicating that subtle changes in endocannabinoid affinity at the CB₁ receptors due to these polymorphisms may underlie deficits in social reward processing such as observed in autism. Preclinical studies have indicated that social play behaviour enhances AEA levels in several brain regions including the amygdala, nucleus accumbens (Trezza et al., 2012) and striatum (Marco et al., 2011) and that enhancing endogenous AEA tone following pharmacological inhibition of fatty acid amide hydrolyse (FAAH), the enzyme primarily responsible for the catabolism of this endocannabinoid (Di Marzo et al., 1998), or inhibition of AEA reuptake, and subsequent CB₁ receptor activation results in enhanced social play behaviour (Trezza and Vanderschuren, 2008, Umathe et al., 2009). In comparison, direct activation of CB₁ receptors with the potent agonist WIN55,212-2 reduces social behaviour (Trezza and Vanderschuren, 2008). The differential effects of global CB₁ receptor activation and enhancing AEA tone on social play behaviour have been proposed to be due to the selective activation of CB₁ receptors in brain regions involved in social and emotional responding following FAAH inhibition (Trezza and Vanderschuren, 2008, Trezza et al., 2012). However, it should be noted that in addition to increasing AEA levels, FAAH inhibition also increases *N*-acylethanolamines such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), although the role of these *N*-acylethanolamines on social and emotional behavioural responding remains to be investigated. Recent studies have demonstrated enhanced cortical levels of AEA, but not 2-AG, following social exposure in BTBR mice, (Gould et al., 2012), a mouse strain known to exhibit an autistic-like behavioural phenotype (Meyza et al., 2012). Agonist-induced GTP γ S binding of CB₁ receptors is enhanced in the BTBR mouse (Gould et al., 2012) and pharmacological activation of CB_{1/2} receptors has been shown to attenuate the hyperlocomotor activity displayed by these mice (Onaivi et al., 2011, Gould et al., 2012). Central activity of diacylglycerol lipase (DAGL) α and monoacylglycerol lipase (MAGL), the enzymes responsible for the synthesis and catabolism of 2-AG

respectively (Dinh et al., 2002, Gao et al., 2010), have been reported to be enhanced in the *fmr*^{-/-} mouse (Maccarrone et al., 2010, Jung et al., 2012), a model of fragile X syndrome, the most common genetic form of autism. In addition, pharmacological inhibition of MAGL and subsequent augmentation of endogenous 2-AG levels, results in the normalisation of locomotor and anxiety-related behavioural changes in *fmr*^{-/-} mice (Jung et al., 2012). As highlighted, several lines of evidence suggest a potential role for the endocannabinoid system in autism, however a detailed profile of the system in a validated preclinical model is lacking.

The aim of the present study was to examine if the autistic-like behavioural changes exhibited by adolescent rats prenatally exposed to VPA are associated with endocannabinoid dysfunction in discrete brain regions known to modulate emotional and social behaviour. In addition to examining changes in endocannabinoid and *N*-acylethanolamine levels, and the expression of genes regulating the synthesis and catabolism of AEA and 2-AG, the expression of CB₁ and CB₂ receptors and other targets of the endocannabinoid system including peroxisome proliferator-activated receptor (PPAR) α , PPAR γ and GPR55 (Sun et al., 2006, Ryberg et al., 2007) were examined.

EXPERIMENTAL PROCEDURES

Animals

Male and female Sprague-Dawley rats (200-300g; Charles River Laboratories UK) were mated following determination of the oestrus phase of the reproductive cycle. The presence of spermatozoa in vaginal smears indicated the first day of gestation (G0.5). Following copulation, female rats were housed singly and maintained at constant temperature ($21 \pm 2^\circ\text{C}$) and humidity (30%-35%) under standard lighting conditions (12:12 h light–dark, lights on from 0700 to 1900 h). Food and water were available *ad libitum*. Experimental protocols were carried out under approval from the Animal Care and Research Ethics Committee at NUI Galway and under licence from the Irish Department of Health and Children, in compliance with the European Communities Council directive 86/609.

On gestational day 12.5 (G12.5), female rats received a single subcutaneous injection of sodium valproate (VPA) (Sigma, Dublin, Ireland) (600mg/kg) or saline vehicle. The dose and time of administration was chosen based on studies demonstrating that this regime elicits autistic-like behavioural changes in offspring (Schneider and Przewlocki, 2005). Females were allowed to raise their own litters and pups which were weaned on postnatal day (PND) 21. Following weaning, rats of either sex were housed separately in groups of 3-6 per cage.

Experimental Design

A schematic of the experimental design is presented in Figure 1.

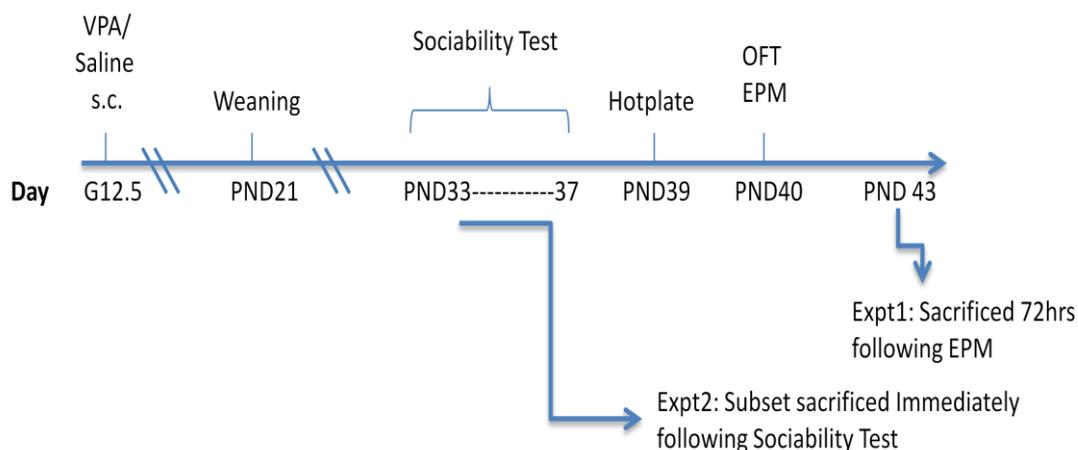


Fig 1. Schematic representation depicting the experimental design. Behavioural testing occurred during adolescence (PND 33–40). G12.5: gestational day 12.5, EPM elevated plus maze, OFT open field test, PND: postnatal day, s.c.: subcutaneous injection.

Experiment 1: Behavioural profile of the VPA model and associated changes in the endocannabinoid system

Behavioural testing was carried out during adolescence between PND 33 and 40. The sequence of testing remained constant, and involved the sociability test (saline-treated n = 16; VPA treated n = 14) followed by the hot plate test, followed by the open field and elevated plus maze test (saline-treated n = 10; VPA treated n = 8) and was modelled on the study design described by Schneider and colleagues [5]. All behavioural testing was carried out by an experimenter blinded to treatment. Seventy-two hours following the final behavioural test (PND 43) animals were killed by decapitation, the brain removed and discrete brain regions including the frontal cortex, hippocampus and cerebellum dissected out and snap frozen on dry ice. The frontal cortex was considered cortical tissue rostral to the central sulcus and included regions such as the prefrontal cortex, premotor cortex and motor cortex. All regions of the cerebellum (cerebro-, spino- and veribular) were included in the cerebellar tissue samples that were processed. The aforementioned regions have been implicated in autistic-like symptoms and alterations in these regions have previously been demonstrated in the VPA model of autism (Ingram et al., 2000, Dufour-Rainfray et al., 2010, Sandhya et al., 2012). Brain regions were stored at -80°C until assayed for endocannabinoid and *N*-acylethanolamine levels, and mRNA expression of endocannabinoid related genes.

Experiment 2: Endocannabinoid and N-acylethanolamine levels in discrete brain regions in VPA-exposed animals following exposure to the sociability test

Immediately following the sociability test, a subset of animals (saline-treated n = 6; VPA treated n = 6) were killed by decapitation, the frontal cortex, hippocampus and cerebellum excised, snap frozen on dry ice and stored at -80°C until assayed for endocannabinoid and *N*-acylethanolamine levels.

Behavioural Testing

Sociability test

The sociability test was conducted in a novel 3-chamber apparatus which allows for the measurement of social approach and social preference (Crawley, 2004, Nadler et al., 2004). In brief, animals were placed into a novel arena (80 cm x 31.5 cm) composed of three communicating chambers separated by Perspex walls with central openings allowing access to all chambers for 5 minutes. Distance moved (cm) and time spent (s) in the various compartments was assessed during this time to evaluate general locomotor activity and ensure that animals did not have a preference for a particular side of the arena. Following this acclimatization period, animals were briefly confined to the central chamber while an unfamiliar rat confined in a small wire cage was placed in one of the outer chambers. An identical empty wire cage was placed in the other chamber. The unfamiliar rat was randomly assigned to either the right or left chamber of the arena. The test animal was then allowed to explore the arena/chambers for a further 10 min. Distance moved in the arena, time spent engaging in investigatory behaviour with the novel rat and frequency of investigatory behaviour with the novel rat was evaluated with the aid of EthoVision XT software (Noldus Netherlands) in order to examine social approach and preference. All testing occurred during the dark phase (21.00-03.00 h) under red light illumination.

Hot plate test

The hot plate test was used to assess nociceptive responding to a noxious thermal stimulus. On the test day (10.00-12.00 hrs), the animal was taken from its home cage and placed directly onto a hot plate (IITC Life Science Inc, California, US) heated to $55 \pm 1^\circ\text{C}$. Thermal nociception was measured as the time elapsed (i.e. latency to respond (s)) between placement of the animal on the surface of the hot plate and when the animal first licked either of its hind paws, with a cut-off time of 40 sec to avoid tissue damage.

Open field test

On the experimental day, each animal was removed from the home cage during the light phase (between 1000h and 1500h) and placed singly into a brightly lit (lux 300-400) novel open field environment (diameter 75cm) where behaviour was assessed using a computerised video tracking system (EthoVision XT, Noldus Netherlands) for a 5 minute period. Behaviours assessed included locomotor activity (distance moved: cm) and duration of time spent (seconds; s) in the centre zone (45cm diameter), an indication of anxiety-related behaviour.

Elevated plus maze

Immediately following exposure to the open field, animals were placed directly onto the elevated plus maze. This 4-arm maze consisted of two open (lux 90) and two closed (30cm high wall, lux 30) arms (50 cm length × 12 cm wide) forming a plus shape, elevated approximately 50 cm from the floor. Each rat was placed in the centre of the maze facing an open arm and allowed to freely explore for 5 min. Time (s) in the open and closed arms was assessed over the trial with the aid of EthoVision XT video tracking system (Noldus Netherlands).

Quantitation of endocannabinoids and N-acylethanolamine levels using liquid chromatography - tandem mass spectrometry (LC-MS/MS)

Quantitation of endocannabinoids and *N*-acylethanolamines was essentially as described previously (Ford et al., 2011, Kerr et al., 2012a, Kerr et al., 2012b, Olango et al., 2012). In brief, samples were homogenised in 400µL 100% acetonitrile containing deuterated internal standards (0.014 nmol AEA-d8, 0.48nmol 2-AG-d8, 0.016nmol PEA-d4, 0.015nmol OEA-d2). Lyophilised samples were re-suspended in 40µL 65% acetonitrile and separated 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 10min and held at this for a further to 20min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 11.4min, 12.9min, 14.4min 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). Quantitation of each analyte was performed by ratiometric analysis and expressed as nmol or pmols per gram of tissue. The limit of quantification was 1.3pmol/g, 12.1pmol/g, 1.5pmol/g, 1.4pmol/g for AEA, 2-AG, PEA and OEA respectively.

Enzyme and receptor mRNA expression using quantitative real-time PCR

As previously described (Kerr et al., 2012a, Kerr et al., 2012b), RNA was extracted from cortical, hippocampal or cerebellar tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany) and 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). Assay IDs for the genes examined were as follows: NAPE-PLD (Rn01786262_m1), DAGL α (Rn01454304_m1), DAGL β (Rn01453775_m1), FAAH (Rn00577086_m1), MAGL (Rn00593297_m1), CB₁ (Rn00562880_m1), CB₂ (Rn03993699_s1), PPAR α (Rn00566193_m1), PPAR γ (Rn00440945_m1), GPR55 (Rn03037213_s1). 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.

FAAH and MAGL Enzyme Activity Assay

Enzyme activity assays were conducted essentially as previously described (Cable et al., 2011, Kerr et al., 2012b). In brief, hippocampal tissue was weighed (~20mg), homogenised in 1ml of TE buffer (50mM Tris, 1mM EDTA, pH7.4) and centrifuged at 14000 x g for 15 minutes. The pellet was resuspended in 1ml of TE buffer, centrifuged and resuspended in a final volume of TE buffer to give a 1:1000 dilution (FAAH determination) or 1:5000 dilution (MAGL determination) of the initial wet hippocampal tissue weight. 90µl of sample aliquots or blanks were pre-incubated with 5µl of Hanks/Hepes buffer (116mM NaCl, 5.4mM KCl, 1.8mM CaCl₂.2H₂O, 25mM HEPES, 0.8mM MgSO₄, 1mM NaH₂PO₄.2H₂O) pH 7.4, containing 1mg/ml defatted albumin for 30 min at 37°C. After pre-incubation, FAAH substrate (5µl: 40µM AEA containing 2µCi [³H]-AEA; American Radiolabelled Chemicals) or MAGL substrate (5µl: 2mM 2-OG containing 3.75µCi 2-oleoyl-[³H]-glycerol; American Radiolabelled Chemicals) was added to the samples to give a final [³H]-AEA concentration of 2 µM or [³H]-2-OG concentration of 100 µM. The reactions were allowed to proceed for 15 min at 37°C, following which 300µl of stop solution (8% w/v charcoal in 0.5M HCl) was added. Samples were allowed to stand for 20 min, centrifuged at 14000 x g for 5min and 200µl of the supernatant was used for liquid scintillation counting. Homogenates were assayed in triplicate. Data were expressed as pmol/min/g for FAAH activity or nmol/min/g for MAGL activity.

Statistical Analysis

SPSS statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro-Wilk and Levene test, respectively. All data were analysed using unpaired *t*-test to compare effect of prenatal saline- vs. VPA-exposure. Data were considered significant when *P* < 0.05. Results expressed as group means + standard error of the mean (SEM).

Results

Behavioural phenotyping of adolescent rats exposed prenatally to VPA

Analysis of behaviour during the acclimatization period of adolescent rats to the novel 3-chamber sociability area prior to the introduction of an unfamiliar conspecific rat revealed that prenatal exposure to VPA did not alter locomotor activity (Saline: 2482 ± 144 cm vs VPA: 2435 ± 77 cm) or time spent in either side of the arena (time in left side: Saline 103 ± 8 s vs VPA 112 ± 12 s; time in right side: Saline 99 ± 10 s vs VPA 85 ± 12 s). Following the introduction of the unfamiliar rat and novel object (empty wire container) into the 3-chamber test arena, analysis revealed that time in the chamber containing the unfamiliar rat and the time and frequency of investigatory behaviours towards the stimulus animal was significantly less in VPA-exposed rats when compared to controls ($P < 0.05$; Figure 2A, B, D, E, F). This decrease was accompanied by an increase in the time spent in the central chamber but (Figure 2F) was not related to alterations in locomotor activity as distance moved in the arena over the test period did not differ between the groups (Figure 2C).

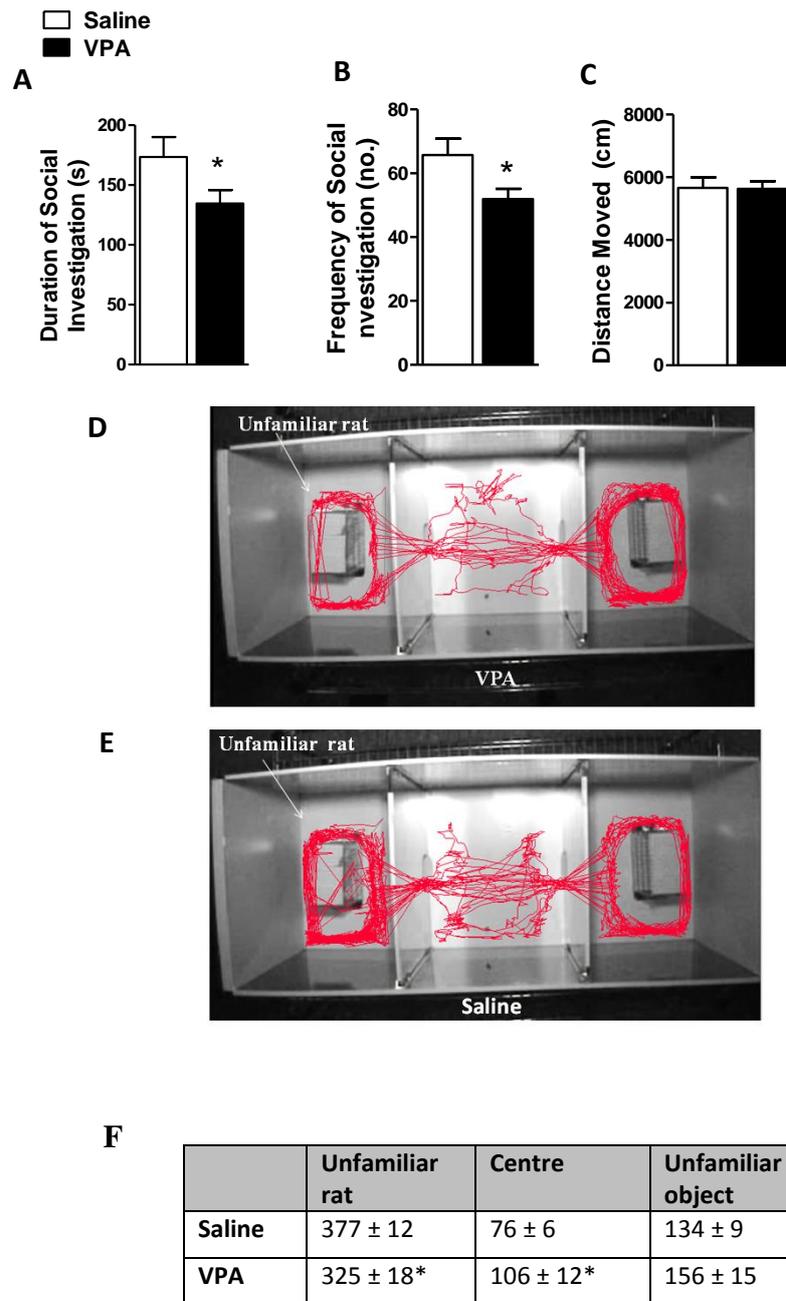


Fig 2. Rats prenatally exposed to VPA exhibit reduced time and frequency of investigative behaviour toward an unfamiliar con-specific rat. (A) Duration and (B) frequency of social investigatory behaviour of control and VPA-exposed rats in the sociability test (* $P < 0.05$ vs saline-treated counterpart). (C) Distance moved did not differ between the groups over the course of the test. Representative images demonstrating track tracing movements of (D) VPA- and (E) saline-exposed rats in the sociability test. Data expressed as mean + SEM ($n = 14-16$ per group). (F) Time spent (s) of saline and VPA exposed animals in each of the 3 chambers of the test area over the 10 min trial period. Data expressed as mean + SEM. $n = 14-16$ per group.

In the hotplate test, VPA-exposed animals exhibited a significant increase in latency to respond ($P < 0.01$) when compared to control animals (Figure 3A), indicating the development of heat hypoalgesia in the model.

On exposure to a novel brightly lit aversive open field arena, VPA-exposed rats exhibited reduced locomotor activity as demonstrated by a decrease in distance moved when compared to saline-treated counterparts ($P < 0.05$ Figure 3B). Furthermore, the duration of time spent in the central arena of the open field arena was also reduced ($P < 0.05$ Figure 3C), indicative of an anxiety-related phenotype. In order to further investigate possible anxiety-related behaviour, VPA-exposed rats were placed on the elevated plus maze. Time spent on the open (Figure 3D) and closed (Saline: 113 ± 11 s vs VPA: 101 ± 6 s) arms of the test arena did not differ between VPA- and saline-exposed rats.

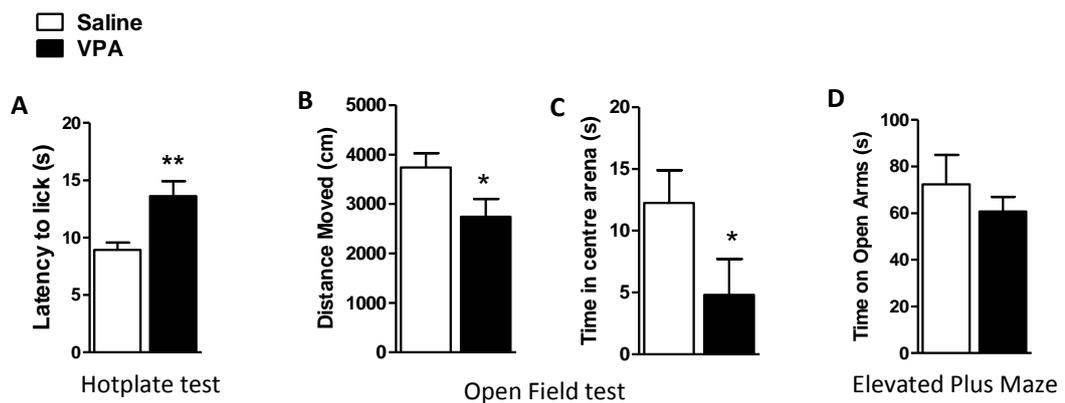


Fig 3. Rats prenatally exposed to VPA exhibit thermal hypoalgesia and reduced locomotor activity in open field test. (A) VPA-exposed rats exhibit an increased latency to lick the hindpaws in the hotplate test (** $P < 0.01$ vs saline). (B) Distance moved and (C) duration of time in the centre arena of a novel brightly lit open field is reduced in VPA-exposed animals when compared to saline-treated controls (* $P < 0.05$ vs saline). There was no significant difference between VPA- and saline- exposed rats in terms of % time on the open arms of the elevated plus maze. Data expressed as mean + SEM (n = 8-10 per group).

Endocannabinoid and N-acylethanolamine levels in discrete brain regions do not differ between animals prenatally exposed to VPA or saline

Although 2-AG levels in the frontal cortex were slightly reduced in VPA-exposed animals (Figure 4A), this effect failed to reach statistical significance ($P = 0.06$). Levels of the endocannabinoids, AEA and 2-AG, or the *N*-acylethanolamines PEA and OEA, in the frontal cortex (Figure 4A), hippocampus (Figure 4B) or cerebellum (Figure 4C) did not differ between VPA and saline-exposed rats.

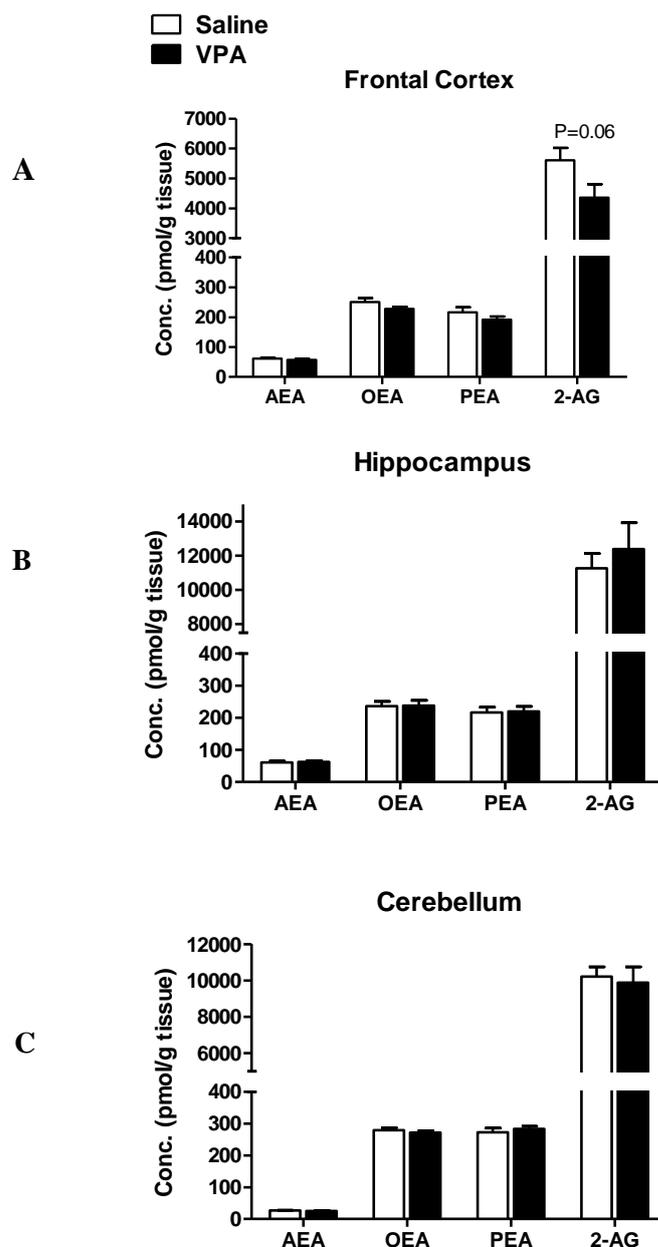


Fig 4. Endocannabinoid or *N*-acylethanolamine levels in the (A) frontal cortex, (B) hippocampus or (C) cerebellum did not differ between VPA or saline-exposed rats. AEA: anandamide, 2-AG: 2-arachidonyl glycerol, PEA: *N*-palmitoylethanolamide, OEA: *N*-oleoylethanolamide. Data expressed as mean + SEM ($n = 8$ per group).

Prenatal VPA exposure reduces expression and enhances activity of MAGL in the hippocampus

Evaluating the expression of genes which encode for the synthetic and catabolic enzymes of the endocannabinoid system in discrete brain regions revealed that VPA-exposed rats exhibit reduced MAGL ($P < 0.05$ Figure 5B) and DAGL α ($P < 0.01$ Figure 5C) mRNA in the hippocampus and cerebellum respectively, when compared to saline-treated counterparts. There was no significant difference in expression of synthetic or catabolic enzymes in the frontal cortex between VPA- and saline-exposed rats (Figure 5A). As VPA-exposed animals exhibit reduced MAGL mRNA expression in the hippocampus (Figure 5B), we investigated if altered MAGL activity may account for the lack of change in 2-AG levels observed in VPA exposed rats (Figure 4B). In accordance with this, MAGL (saline: 588 ± 36 vs VPA 786 ± 64 nmol/min/g, $P < 0.05$), but not FAAH (saline: 951 ± 42 vs VPA 951 ± 67 pmol/min/g), activity was enhanced in the hippocampus of VPA-exposed rats ($P < 0.01$ vs saline-treated counterparts).

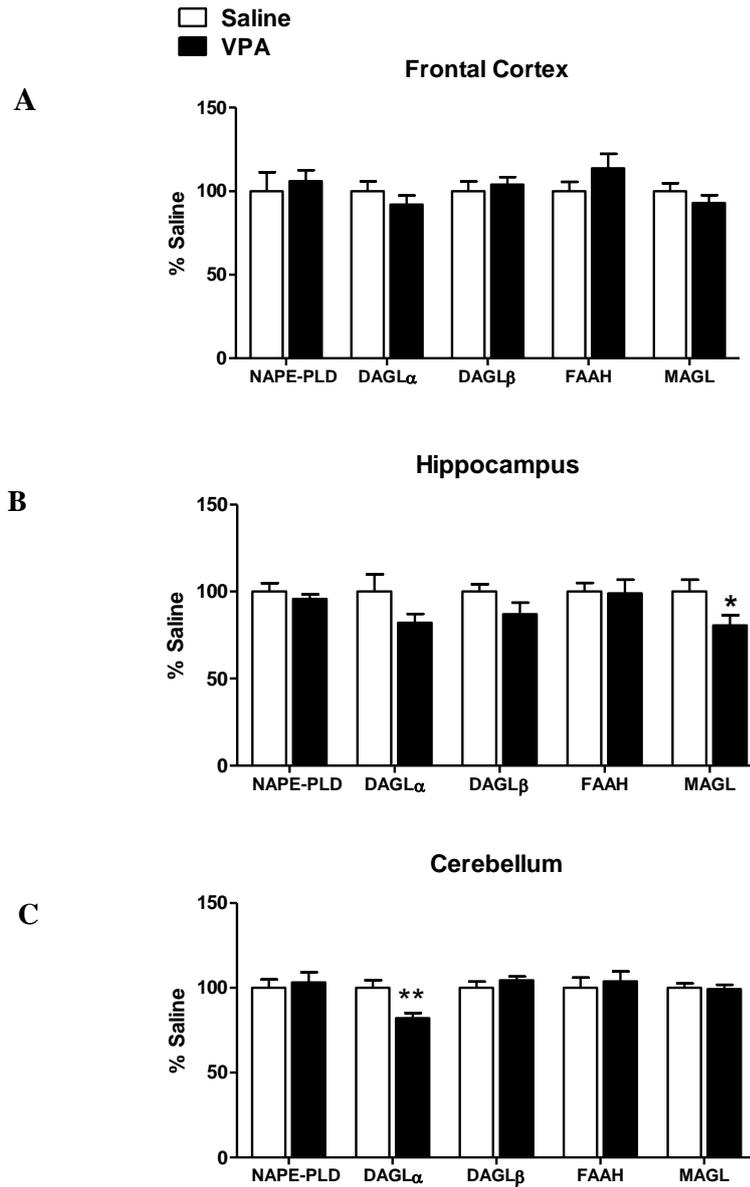


Fig 5. Expression of genes encoding for the enzymes involved in the synthesis and catabolism of endocannabinoids in discrete brain regions. VPA-exposed rats exhibit a decrease in the expression of (B) MAGL mRNA in the hippocampus and (C) DAGL α mRNA in the cerebellum, when compared to saline-treated controls ($*P < 0.05$). (A) No change was observed in gene expression in the frontal cortex between the groups. NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D; DAGL: diacylglycerol lipase, FAAH fatty acid amide hydrolyase, MAGL monoacylglycerol lipase. Data expressed as mean % change from saline-treated control + SEM (n = 8 per group).

PPAR and GPR55 expression is reduced in the frontal cortex and hippocampus of VPA-exposed rats

Neither CB₁ nor CB₂ receptor gene expression in the frontal cortex, hippocampus or cerebellum differed between VPA- or saline-exposed rats (Figure 6). As endocannabinoids are known to have affinity and activity at additional non-cannabinoid receptor targets, the effect of prenatal VPA exposure on PPAR α/γ and GPR55 was assessed. The expression of PPAR α ($P < 0.05$) and PPAR γ ($P < 0.01$) was reduced in the frontal cortex and hippocampus respectively, of VPA-exposed rats when compared to saline-treated counterparts (Figure 6A and B). In addition, GPR55 expression was reduced in the frontal cortex ($P < 0.01$) and hippocampus ($P < 0.05$), but not cerebellum, of VPA-exposed rats (Figure 6A and B).

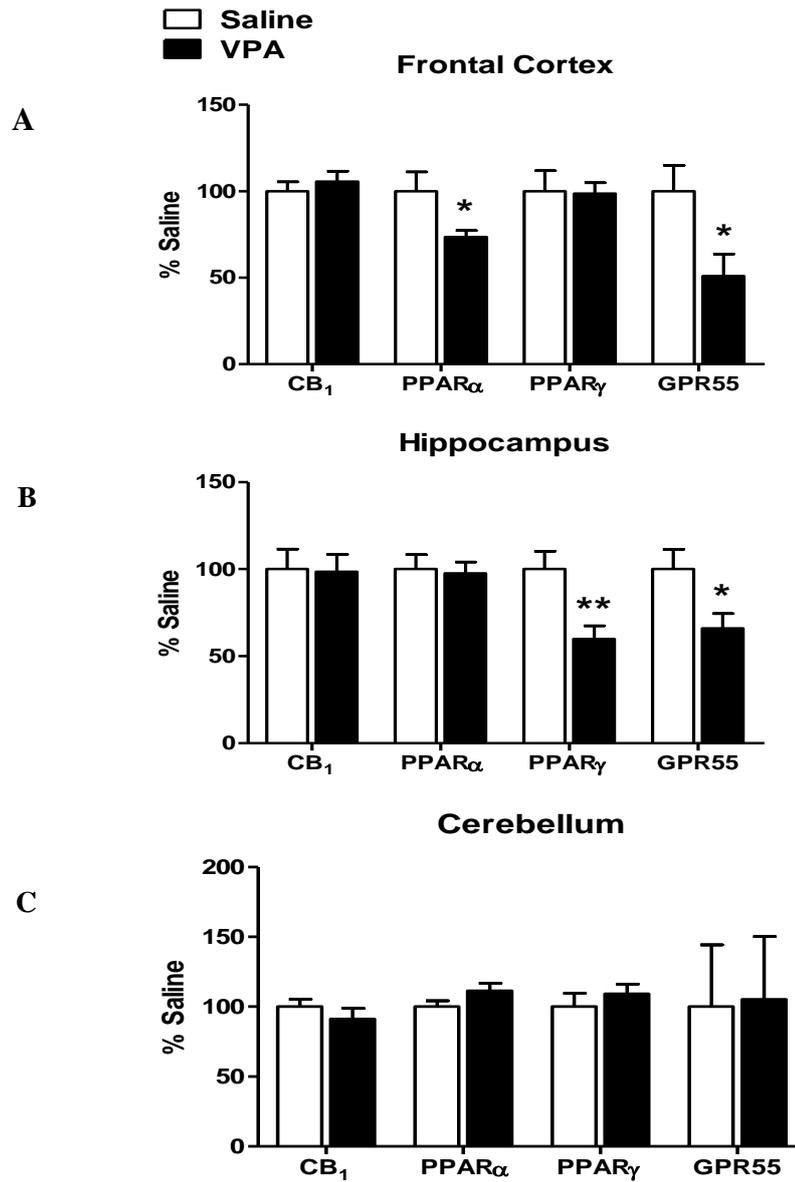


Fig 6. VPA-exposed rats exhibit a decrease in the expression of genes encoding for receptor targets of the endocannabinoid system in the (A) cortex and (B) hippocampus (** $P < 0.01$ * $P < 0.05$ vs saline-exposed rats). (C) No change was observed in gene expression in the cerebellum between the groups. CB₁ Cannabinoid receptor 1, PPAR peroxisome proliferator-activator receptor, GPR55 G-protein receptor 55. Data expressed as mean % change from saline-treated control + SEM (n = 8 per group).

Social exposure enhances FAAH substrates in the hippocampus of VPA exposed rats

Following the sociability test a subset of rats were sacrificed in order to determine if the social deficits observed in VPA-exposed animals are accompanied by alterations in endocannabinoid levels in discrete brain regions. While neither endocannabinoid nor *N*-acylethanolamine levels were altered in the frontal cortex (Figure 7A) or cerebellum (Figure 7C) of VPA-exposed animals following the sociability test, the FAAH substrates, AEA ($P < 0.05$), OEA ($P < 0.05$) and PEA ($P < 0.05$), were increased in the hippocampus (Figure 7B) when compared to saline-treated counterparts.

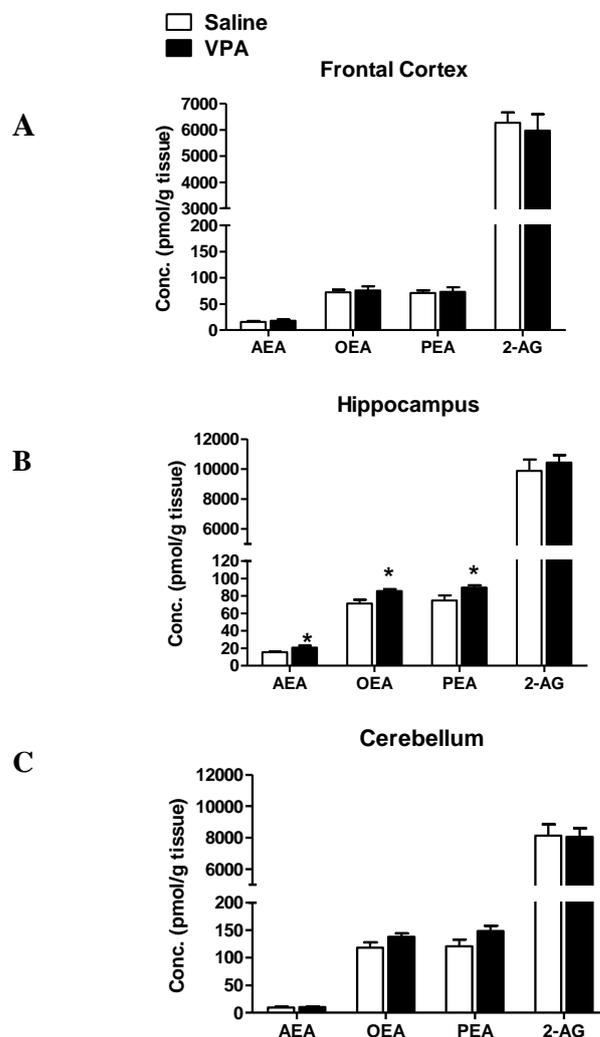


Fig 7. Endocannabinoid and *N*-acylethanolamine levels in the (A) frontal cortex, (B) hippocampus and (C) cerebellum immediately following exposure of saline- or VPA-exposed rats to the sociability test. AEA: anandamide, 2-AG: 2-arachidonyl glycerol, PEA: *N*-palmitoylethanolamide, OEA: *N*-oleoylethanolamide. Data expressed as mean + SEM. * $P < 0.05$ vs saline-exposed rats. $n = 6$ per group.

Discussion

The results of the present studies demonstrate that rats prenatally exposed to VPA exhibit autistic-like behavioural changes including reduced sociability, increased anxiety-related behaviour in an open field and reduced sensitivity to noxious stimuli, behavioural changes accompanied by alterations in various components of the endocannabinoid system. Specifically, VPA-exposed animals exhibited reduced expression of the 2-AG synthesising enzyme DAGL α in the cerebellum, reduced expression and enhanced activity of the 2-AG catabolising enzyme MAGL in the hippocampus, reduced expression of mRNA for PPAR α and GPR55, endocannabinoid receptor targets, in the frontal cortex, and reduced expression of PPAR γ and GPR55 mRNA in the hippocampus. In addition, the FAAH substrates, AEA, OEA and PEA were enhanced in the hippocampus of VPA-exposed animals following the sociability test. Thus, dysfunction in the endocannabinoid system may underlie some of the autistic-like behavioural changes observed in the VPA rat model.

Impaired social behaviour, a core symptom of autism spectrum disorders, has been repeatedly demonstrated both in adult and adolescent rats that have been exposed prenatally to VPA (Schneider and Przewlocki, 2005, Markram et al., 2008, Dufour-Rainfray et al., 2010, Kim et al., 2011). In accordance with these findings, the present study demonstrated that VPA-exposed animals exhibited reduced time and frequency interacting with an unfamiliar con-specific rat in the 3-chamber sociability test during adolescence. VPA-exposed animals did not exhibit altered locomotor activity during the acclimatization or testing phase of the sociability test, confirming that alterations in social behaviour are not related to motor impairments. However, it has been proposed that enhanced anxiety and fear processing may exacerbate an aversion to environmental interactions typical of social conditions (Markram et al., 2008), thus leading to impaired social behaviours as seen in the VPA model. Anxiety-related behaviour in this study were assessed in the open field and elevated plus maze and revealed that locomotor activity and time in the centre of the test

arena was reduced in VPA-exposed animals on exposure to a novel brightly lit open field environment. In comparison, previous studies have demonstrated that VPA-exposed animals exhibit increased locomotor activity in an open field test (Schneider et al., 2008, Kim et al., 2013a, Kim et al., 2013b), however, experimental conditions such as size of the test arena, lighting conditions and periods of testing differed significantly from those used in the current study. It appears that aversive stressful conditions, as employed in the open field test used in the current study, elicit anxiety or fear-related behaviour in VPA-exposed animals. Similarly, several studies have demonstrated that VPA-exposed animals exhibit reduced open arm entries and time on the open arms in the elevated plus maze, indicative of enhanced anxiety-related behaviour (Schneider et al., 2007, Markram et al., 2008, Schneider et al., 2008, Sandhya et al., 2012). Although we failed to observe such changes in the present study, it is possible that performing the elevated plus maze test immediately following exposure to the open field, where anxiety-related behaviour was evident, may have reduced the aversive, anxiety-provoking nature of this test. Autistic patients exhibit reduced sensitivity to painful stimuli (Militeri et al., 2000, Tordjman et al., 2009), a phenotype also observed in various pre-clinical models including prenatal exposure to VPA (Schneider et al., 2001, Schneider and Przewlocki, 2005, Markram et al., 2008, Schneider et al., 2008, Sandhya et al., 2012, Zhang et al., 2012). In accordance with these data, the present study demonstrated that adolescent rats prenatally exposed to VPA exhibited thermal hypoalgesia in the hotplate test. Together, the present study confirms that exposure to VPA during a critical stage in neo-natal development (G12.5) induces a behavioural phenotype during adolescence similar to that observed in autism, further highlighting the validity of this model.

In addition to behavioural alterations, morphological (Markram et al., 2008, Sandhya et al., 2012, Kim et al., 2013a), neurotransmitter/neuropeptide (Schneider et al., 2007, Dufour-Rainfray et al., 2010, Sandhya et al., 2012, Kim et al., 2013a) and immune changes (Schneider et al., 2008, Sandhya et al., 2012, Zhang et al., 2012) have been reported in VPA-exposed rats. The endocannabinoid system has been demonstrated to play a role in a wide variety of physiological processes including social and emotional behaviour, nociception and anxiety/fear (Viveros et al., 2007, Trezza and Vanderschuren, 2008, Marco et al., 2011). Enhanced DAGL activity in the prefrontal

cortex and striatum, enhanced MAGL activity in the striatum and unaltered 2-AG levels have been reported in the *fmr*^{-/-} model of fragile X syndrome (Maccarrone et al., 2010, Jung et al., 2012). However, to the best of our knowledge, the present study is the first to examine if post-mortem alterations in the endocannabinoid system are evident in a non-genetic model of autism. Our results demonstrate reduced expression of the 2-AG synthesising enzyme DAGL α in the cerebellum, reduced expression and enhanced activity of the 2-AG catabolising enzyme MAGL in the hippocampus, and unaltered central 2-AG concentrations, in VPA-exposed rats. Thus, under resting conditions, homeostasis in the endocannabinoid system may allow for the maintenance of steady state 2-AG levels in the brain. However, under certain conditions, changes in the ability to synthesise or metabolise 2-AG may lead to altered levels of 2-AG, modulation of neurotransmission and altered behavioural responding. Similar to that previously reported following social interaction (Marco et al., 2011), 2-AG levels were unaltered in any of the brain regions examined following the sociability test, and therefore alterations in the mobilisation or catabolism of this endocannabinoid may not underlie the social deficits observed in the VPA model. It is however possible that, 2-AG levels were altered during the test period and had returned to levels similar to controls by the end of the trial, or were altered in brain regions other than those investigated. Furthermore, it remains possible that alterations in 2-AG tone may play a role in one or more of the other behavioural changes observed in the model such as hypoalgesia, stereotypic and anxiety-related behaviour. Augmentation of 2-AG levels by pharmacological inhibition of MAGL results in the normalisation of enhanced locomotor and anxiety-related behavioural changes in *fmr*^{-/-} mice (Jung et al., 2012), although effects on social behaviour were not investigated. The authors indicate that the behavioural effects mediated by enhanced 2-AG are most likely via CB₁ receptor activation. In accordance with this, administration of the cannabinoid CB₁/CB₂ receptor agonists Δ^9 -THC and WIN55,212-2 reduced the hyperlocomotor activity of BTBR mice (Onaivi et al., 2011, Gould et al., 2012), a mouse strain also known to exhibit autistic-like behaviours. Further studies are required in order to decipher if enhancing central 2-AG tone and consequently CB₁ receptor activation may ameliorate some of the behavioural changes in VPA-exposed animals.

Although 2-AG levels were unaltered following exposure to the sociability test, the FAAH substrates AEA, OEA and PEA were increased in the hippocampus of VPA-exposed animals. BTBR mice have been reported to exhibit increased cortical AEA, but not 2-AG or OEA, levels following exposure to the sociability test (Gould et al., 2012), however it is unknown if alterations also exist in other brain regions. Social play behaviour enhances AEA levels in the amygdala, nucleus accumbens (Trezza et al., 2012) and striatum (Marco et al., 2011), but not in the prefrontal cortex or hippocampus. Pharmacological and genetic inhibition of FAAH (Trezza and Vanderschuren, 2008, Cassano et al., 2011), inhibition of AEA transport (Trezza and Vanderschuren, 2009) and central administration of AEA (Umathe et al., 2009) enhances social behaviour, indicating that enhanced endocannabinoid activity facilitates social play behaviour. Additional studies have revealed that enhanced AEA tone in the basolateral amygdala and nucleus accumbens (Trezza et al., 2012) but not piriform cortex (Zenko et al., 2011) mediates social interactive behaviour. In comparison, broad central activation of CB₁ receptors impairs social play behaviour (Trezza and Vanderschuren, 2008). The authors suggest that enhancing AEA levels and activating CB₁ receptors in brain circuits regulating social behaviour facilitates social play, however broad excitation of central CB₁ receptors interferes with the normal excitation of complex social acts (Trezza and Vanderschuren, 2008, Trezza et al., 2012), possibly by interfering with cognitive functions required for normal social interactions (Egerton et al., 2006). It should also be noted that the experimental conditions (social interactions vs sociability) and test subjects (naive rats vs VPA-exposed rats) used in the latter studies are significantly different to those used in the present study and alterations in endocannabinoid levels in brain regions such as the nucleus accumbens, amygdala or striatum cannot be ruled out. However, the role of the hippocampus in cognition is well recognised with a wealth of data demonstrating that CB₁ receptor activation reduces, while blockade enhances, cognitive performance (Egerton et al., 2006). As such, it is possible that AEA-induced activation of CB₁ receptors in the hippocampus of VPA-exposed animals during the sociability test results in impaired cognitive ability and subsequent deficits in social investigatory behaviour. Increased OEA and PEA levels as observed following the sociability test, may compete with AEA at the FAAH catalytic site leading to reduced catabolism of AEA, increased levels and subsequent enhanced activity at the CB₁ receptor. Alternatively, as neither OEA nor PEA exhibit

affinity for CB₁ receptors, it is possible that competition with the FAAH substrates for binding at PPARs, shunts AEA activity back onto the CB₁ receptor. Some of the behavioural changes may also be mediated by AEA activation of alternative receptor targets to CB₁ or direct activation of PPARs by OEA or PEA. The present study demonstrated a reduced expression of PPAR γ and GRP55 in the hippocampus of VPA-exposed animals. Although the role of PPARs or GPR55 on social behaviour is unknown, recent data indicate that activation of hippocampal PPAR γ enhances cognitive performance (Denner et al., 2012). Thus, downregulation of PPAR γ in the hippocampus of VPA-exposed rats may result in reduced cognitive performance and impaired behavioural responding to stressful situations. PPAR α activation by OEA or selective agonists facilitates memory consolidation via noradrenergic activation of the amygdala (Campolongo et al., 2009). PPAR α and GPR55 expression are reduced in the cortex of VPA-exposed animals, and although endocannabinoid levels were not altered in this region, altered activity at these receptors may account for some of the behavioural changes observed such as hypoalgesia or anxiety-related behaviour.

In conclusion, the present data demonstrates alterations in the endocannabinoid system in adolescent rats exposed prenatally to VPA, effects which may underlie some of the behavioural changes observed in the model. Thus, modulation of the endocannabinoid system may provide a novel pharmacological target for the treatment of behavioural traits associated with autism spectrum disorders.

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Chapter 7

General Discussion

Neuroinflammation is a key component of various neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis and chronic pain, and of psychiatric and neurodevelopmental disorders such as depression and autism. As such, the need to develop a greater understanding of the neurobiological mechanisms mediating neuroinflammation is critical at a fundamental physiological level and for the development of novel, more efficacious treatments. Furthermore, endocannabinoid modulation of neuroinflammation in these disorders, has received considerable attention over the past 10 years (McGeer and McGeer, 2001; Nagatsu and Sawada, 2005; Compston and Coles, 2008; Lucchina and Depino, 2013). Accumulating evidence indicates that the endocannabinoid system plays a significant role in modulating the immune system and endocannabinoids themselves represent a potential therapeutic target for the treatment of both central and peripheral inflammatory disorders without the concomitant side effects associated with potent exogenous cannabinoid treatment. However, there is a paucity of data relating to neuro-immuno-modulatory effects of endocannabinoids *in vivo*. Thus, one of the main objectives of the work presented herein was to enhance our understanding of the role of the endocannabinoid system in the modulation of peripheral and central inflammation *in vivo*.

Some of the key work presented herein has demonstrated that systemic administration of pharmacological agents that inhibit the enzymes responsible for the metabolism of the two most widely characterised endocannabinoids, anandamide and 2-AG, induces profound effects on TLR4-induced inflammatory responses in the brain. While both the FAAH inhibitor URB597 and the MAGL inhibitor JZL184 suppress TLR4-induced proinflammatory cytokines to varying degrees, the data presented in this thesis suggest that the mechanisms and sites by which these pharmacological agents elicit their effects are different. URB597 increases anandamide (and related N-acylethanolamines) in the brain (hypothalamus) an effect accompanied by suppression of TLR4-induced increases in IL-1 β . In contrast, systemic administration of JZL184 increases 2-AG levels peripherally, but not centrally, an effect associated with attenuation of TLR4-induced increases in proinflammatory mediators both peripherally and in the brain. These data have added to the body of knowledge on endocannabinoid modulation of (neuro)inflammation.

Given that endocannabinoids elicit such profound effects on inflammatory processes, a further aim of this work was to characterise immune and endocannabinoid changes in a clinically relevant animal model of autism, the valproic acid (VPA) rat model. Data presented in this thesis demonstrate for the first time that rats prenatally exposed to VPA exhibit increased neuroinflammatory responses to TLR4 stimulation. Our findings are in accordance with mounting experimental evidence that associates immune and inflammatory alterations with the pathogenesis of autism (Croonenberghs et al., 2002; Vargas et al., 2005; Jyonouchi et al., 2008; Enstrom et al., 2010; Ashwood et al., 2011). In addition we have demonstrated for the first time that VPA-exposed rats also exhibit alterations in various components of the endocannabinoid system within several brain regions. Alterations in neuroinflammatory processes and/or the endocannabinoid system may underlie some of the behavioural abnormalities observed in the VPA model. Thus, endocannabinoid-neuro-immune interactions may provide a novel therapeutic target for some of the symptoms associated with autism.

This discussion will focus on appraising the most significant findings of this thesis and how they contribute to increasing our understanding of the endocannabinoid-(neuro)immune interactions and the implications for the treatment of disorders with an associated neuroinflammatory component such as autism. A schematic overview of the primary findings of this thesis is presented in Fig 7.1.

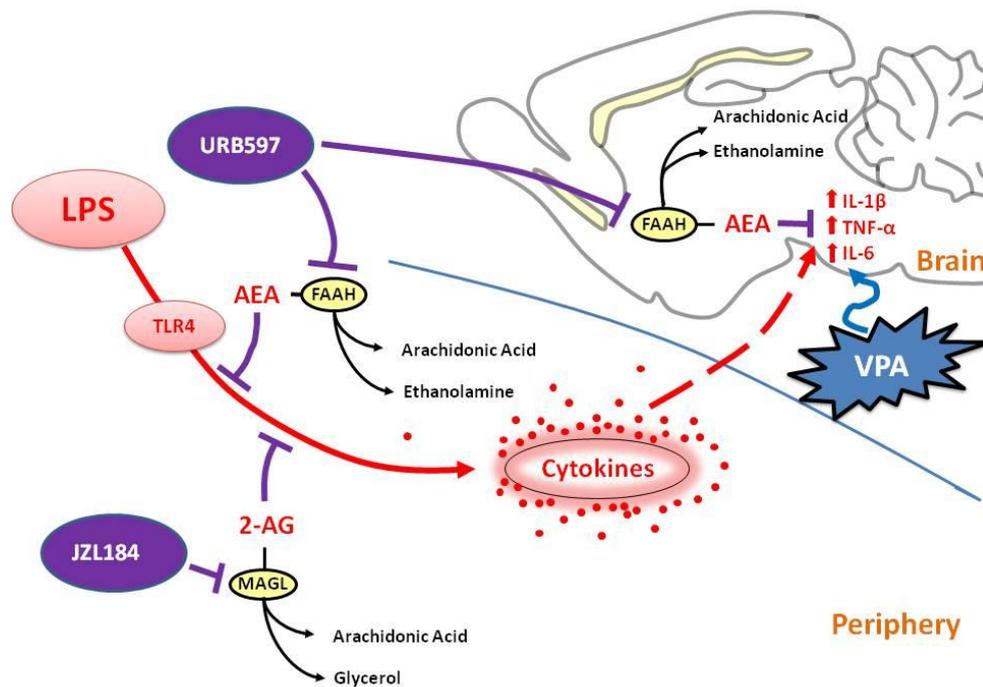


Fig 7.1 Schematic depicting primary findings of data presented in chapters 3-5.

TLR4 activation following peripheral administration of LPS results in activation of NF κ B and the production of pro-inflammatory cytokines. These communicate via various mechanisms with the central nervous system, ultimately resulting in enhanced expression of pro-inflammatory cytokines in the brain. Systemic administration of URB597 inhibits FAAH and increases anandamide (and related N-acylethanolamines, OEA and PEA) both periphery and in the brain. These FAAH substrates in turn slightly suppress the peripheral pro-inflammatory immune response to TLR4 activation while potentially inhibiting TLR4-induced IL-1 β in the brain. In comparison, systemic administration of JZL184 inhibits peripheral MAGL activity and increases 2-AG levels peripherally. This MAGL inhibitor does not cross the blood brain barrier and modulate central levels of 2-AG. JZL184 potentially attenuates TLR4-induced increases in proinflammatory mediators both peripherally and in the brain.

Prenatal exposure to VPA results in slight changes in central expression of inflammatory mediators. However, the inflammatory responses following TLR4 activation are exaggerated in the brain of VPA exposed animals when compared to control. Further studies are required to examine the effect of endocannabinoid modulators on TLR4-induced neuroinflammation in this model of autism.

Pharmacological inhibition of endocannabinoid degradation modulates the TLR4-induced increase in inflammatory mediators in key brain regions

It has long been known that systemic administration of the endotoxin and TLR4 agonist LPS activates NF- κ B signalling and induces increases in cytokine and chemokine expression both peripherally and centrally. Increased proinflammatory cytokines within the brain can then result in fever (via the production of prostaglandins), hypothalamic-pituitary-adrenal (HPA) axis activation (Rivest et al., 2000; Romanovsky, 2000) and behavioural alterations such as anorexia, anhedonia, hypolocomotion and depressed mood (Dantzer, 2001, 2004). Data presented in Chapters 3-5 confirm that systemic administration of a low dose of LPS (100 μ g/kg) increases expression of chemokines (CCL2, CCL5) and the pro- and anti-inflammatory cytokines, IL-1 β , IL-6, TNF- α and IL-10, both peripherally and centrally in conjunction with increased plasma corticosterone levels. Data presented also confirm that systemic LPS activates NF- κ B signalling as measured indirectly via I κ B α mRNA levels.

The data presented in chapters 3 and 4 are some of the first to examine and report on the effect of endocannabinoid modulators on acute neuroinflammatory processes in an *in vivo* model system. Prior data relating to AEA and 2-AG modulation of neuroinflammatory responses was limited primarily to *in vitro* studies demonstrating that modulation of the endocannabinoids attenuates the production of inflammatory mediators in stimulated cells (Chang et al., 2001; Facchinetti et al., 2003; Ortega-Gutierrez et al., 2005; Rockwell et al., 2006). *In vivo* studies demonstrated that direct endocannabinoid administration or enhancement following either FAAH or MAGL inhibition using URB597 or URB602 mediated anti-inflammatory and neuroprotective effects in various pre-clinical models including Parkinson's disease, multiple sclerosis, inflammatory pain and closed head injury (CHI) (Holt et al., 2005; Mestre et al., 2005; Costa et al., 2006; La Rana et al., 2006; Panikashvili et al., 2006; Comelli et al., 2007; Morgese et al., 2007). However, there was a paucity of studies investigating the effect of endocannabinoid modulation on acute neuroinflammatory responses *in vivo*, such as following TLR4 activation.

Chapter 3 of this thesis demonstrates that systemic administration of the FAAH inhibitor URB597 increased AEA, OEA, and PEA centrally, in the hypothalamus, an effect associated with attenuation of the LPS-induced increase in IL-1 β but not TNF- α or IL-6 expression. This is the first study to show endocannabinoid attenuation of TLR4-induced IL-1 β in the rat hypothalamus. As mentioned above, cytokines within the hypothalamus have been shown to be responsible for many aspects of the sickness response following endotoxin administration. For example, 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, central AEA has been shown to increase, while CB₁ receptor antagonism attenuates, LPS-induced hypothermia (Steiner et al., 2011), further demonstrating a role for AEA-CB₁ signalling in modulation of thermal responses to systemic inflammation. Thus, the AEA-induced inhibition of IL-1 β expression in the hypothalamus as demonstrated in the current study may ameliorate the fever and hypophagia associated with acute inflammation. These data add to the body of knowledge regarding AEA modulation of neuroinflammatory processes.

Although 2-AG is the preferred natural ligand for CB₁ receptors in the brain, our understanding of the role of this endocannabinoid in regulating physiological function has been limited due to a lack of pharmacological and genetic tools. The recent advent of selective pharmacological tools such as the potent MAGL inhibitor JZL184 and the development of MAGL^{-/-} mice, have enabled more detailed *in vivo* studies to be carried out in this area. The data presented in chapter 4 demonstrate that systemic administration of JZL184 to rats increases 2-AG levels peripherally but fails to inhibit MAGL or increase 2-AG in the frontal cortex. While JZL184 has been shown to increase central and peripheral 2-AG levels in mice (Long et al., 2009; Kinsey et al., 2010; Nomura et al., 2011), only one study to date has reported an increase in 2-AG levels in the rat brain (Oleson et al., 2012). Other recent studies report unaltered levels of rat 2-AG following JZL184 administration (Wiskerke et al., 2012; Woodhams et al., 2012). It is possible that JZL184 may enhance 2-AG levels in the rat CNS in situations where the BBB is compromised and where basal MAGL activity is already partially inhibited, such as during sepsis or in chronic disease models. Despite the lack of increase in 2-AG, the data presented in chapter 4 demonstrate that LPS-induced increases in IL-1 β , IL-6, TNF- α and IL-10 in the

frontal cortex were attenuated following systemic administration of JZL184. JZL184 effects are possibly mediated by modulation of peripheral cytokines which can communicate with the brain via various routes (diffusion into brain across the BBB-deficient areas, sensory signals and vagus nerve stimulation) resulting in altered cytokine profiles within the CNS. Similar anti-inflammatory effects were reported following systemic JZL184 administration in mice, although central 2-AG levels were increased in this case (Nomura et al., 2012). Nomura *et al* attributed the anti-inflammatory effects observed with JZL184 to a reduction in the arachidonic acid (AA) pool available for prostaglandin synthesis rather than 2-AG signalling at the CB₁ or CB₂ receptors. The data presented herein is the first to suggest that brain neuroinflammatory responses may be modulated by enhancing 2-AG peripherally. Thus, endocannabinoid modulators do not need to cross the BBB to induce potent anti-inflammatory effects centrally, thereby avoiding potential psychotropic side effects. In addition, it should be noted that systemic administration of JZL184 is capable of inducing anxiolytic-like effects in rats (Sciolino et al., 2011). Thus, 2-AG modulation of peripheral physiological processes is capable of modulating both neuroinflammatory and behavioural responding in the rat. The present study demonstrates a differential mode of action where increased peripheral 2-AG acting at cannabinoid receptors induce peripheral anti-inflammatory effects which in turn mediate similar central effects indirectly, possibly via cytokine signalling. Increased/chronic 2-AG signalling at central cannabinoid receptors and associated concomitant psychotropic effects are thus avoided.

Taken together, the data presented in this thesis have enhanced the body of knowledge regarding endocannabinoid modulation of acute neuroinflammatory responses *in vivo*, highlighting that enhancing anandamide tone centrally modulates the hypothalamic immune response to TLR4 activation, effects which may in turn attenuate at least some of the associated physiological responses. In comparison, enhancing 2-AG levels peripherally can induce profound anti-inflammatory responses in the brain (the present study; Nomura et al., 2011) and peripherally (present study; Kinsey et al., 2011, Alhouayek et al., 2011). The potent anti-inflammatory effects and potential lack of psychotropic side effects associated with MAGL inhibitors make modulation of the endocannabinoid system via this

mechanism a very interesting and exciting novel therapeutic target for a host of peripheral and central inflammatory conditions. See Fig 7.1

Neuroinflammatory responses to TLR4 activation in the valproic acid (VPA) rat model of autism.

While evaluating endocannabinoid modulation of acute neuroinflammatory responses has provided insight into the physiological mechanism underlying this association, evaluating such responses in a clinically relevant animal model would indicate if such associations are altered in the disease state. While several studies have examined this to various degrees in models of neurodegeneration, up to this point, few studies have evaluated/characterised inflammatory or endocannabinoid changes in an animal model of autism. Increasing evidence suggests neuroinflammatory processes may be involved in the pathophysiology of autism and that autistic patients may have altered response to immune stimulation (Croonenberghs et al., 2002; Vargas et al., 2005; Onore et al., 2012). Apart from maternal immune activation (MIA) models of autism, there have been a limited number of studies investigating immune changes in other pre-clinical models. *Ex vivo* stimulation of peritoneal macrophages and spleenocytes from rats prenatally exposed to VPA revealed a decreased IFN- γ /IL-10 ratio, indicating a possible imbalance in Th1/Th2 response in this model of autism (Schneider et al., 2008). Our data (chapter 5) have expanded on these findings and demonstrate that adolescent rats prenatally exposed to VPA exhibit slight changes in basal expression of inflammatory mediators, namely increased expression of the chemokine CCL5 in the cortex, decreased astrocyte activation (GFAP expression) in the hippocampus and reduced TLR4 expression in the cerebellum, when compared to control animals. However, following an immune challenge with the TLR4 agonist LPS, VPA exposed offspring exhibit augmented expression of IL-1 β and CCL2 in the cortex, TNF- α expression in the hippocampus and GFAP and IL-1 β expression in the cerebellum, when compared to LPS challenged control animals. Overall, these data demonstrate that VPA-exposed offspring exhibit an exacerbated neuroinflammatory response to TLR4 activation.

These results have been corroborated in a recent study by Lucchina and co-workers who investigated altered peripheral and central immune responses in a mouse VPA model of autism (Lucchina and Depino, 2013). Mice prenatally exposed to VPA exhibited unaltered central and peripheral basal levels of proinflammatory cytokines, but augmented LPS-induced expression of IL-6 in the spleen and TNF- α and IL-6 in the cerebellum when compared to LPS-challenged controls. Mice prenatally exposed to VPA also exhibited elevated basal levels of activated microglia in the hippocampus and cerebellum, and augmented LPS-induced microglial activation in these brain regions when compared to LPS challenged controls. The authors also demonstrated that direct administration of LPS into the cerebellum resulted in reduced social interaction (Lucchina and Depino, 2013). Thus, alterations in neuroinflammatory processes in VPA-exposed animals may underlie some of the behavioural changes observed in the model such as deficits in social exploration. In conclusion, the data presented herein (chapter 5) contribute to the face validity of the VPA model typifying the neuroinflammatory and immune alterations observed in autism (Croonenberghs et al., 2002; Vargas et al., 2005; Jyonouchi et al., 2008; Li et al., 2009).

Alterations in the endocannabinoid system in the rat valproic acid model of autism

The focus of this thesis was to examine the interaction between endocannabinoid and TLR4 responses and given that VPA-exposed animals exhibit altered neuroinflammatory responses to TLR4 activation, a further objective was to determine if these animals also exhibited alterations in the endocannabinoid system. The data presented herein (chapter 6) represent the first characterisation of the endocannabinoid system in a non-genetic model of autism. The results demonstrate that rats prenatally exposed to VPA exhibit autistic-like behavioural changes including reduced sociability, increased anxiety-related behaviour in an open field and reduced sensitivity to noxious stimuli. It has also been reported that VPA exposed offspring display delayed maturation (later eye opening), lower body weight, reduced brain mass, increased physical malformations, delayed motor development and poor integration of coordinated series of reflexes (swimming test) and delayed nest-seeking responses in the olfactory discrimination test (Schneider and Przewlocki, 2005; Favre et al., 2013). In accordance with this, we noted in our

studies that rats prenatally exposed to VPA exhibited tail deformations and slight delay in eye opening when compared to saline-exposed controls.

Furthermore the observed behavioural changes were accompanied by alterations in various components of the endocannabinoid system. Specifically, VPA-exposed animals exhibited reduced expression of the 2-AG synthesising enzyme DAGL α in the cerebellum, reduced expression and increased activity of MAGL in the hippocampus, reduced expression of PPAR α and GPR55, endocannabinoid receptor targets, in the frontal cortex, and reduced expression of PPAR γ and GPR55 in the hippocampus. Thus, VPA-exposed animals exhibit alterations in various aspects of the endocannabinoid system in key brain regions that modulate social and emotional processing. Comparable studies are limited to those involving genetic models of autism that also exhibit similar alterations in this system. Specifically, recent evidence has demonstrated that in a mouse model of fragile X syndrome (the *fmr*^{-/-} mouse), the most common genetic form of autism, central activity of DAGL α and MAGL, the enzymes responsible for the synthesis and catabolism of 2-AG respectively, are increased (Maccarrone et al., 2010; Jung et al., 2012). In addition, pharmacological inhibition of MAGL resulted in the normalisation of locomotor and anxiety-related behavioural changes in *fmr*^{-/-} mice (Jung et al., 2012). Furthermore, pharmacological or genetic blockade of the CB₁ receptor normalised cognitive impairment, nociceptive desensitization and susceptibility to audiogenic seizures, whereas blockade of the CB₂ receptor normalised anxiolytic-like behaviours in *fmr*^{-/-} mice (Busquets-Garcia et al., 2013). In addition, endocannabinoid signalling has been shown to be impaired in two mouse models of autism associated with different mutations of the neuroligin-3 gene. In essence, while the neuroligin-3 functionality is different, tonic endocannabinoid signalling at the CB₁ receptor and consequent suppression of GABA release from hippocampal cholecystinin (CCK) basket cells was found to be impaired in both models, effects which may account for the similar behavioural profile of these animals (Foldy et al., 2013). These latter studies indicate a possible role for the endocannabinoid system in the pathogenesis of autism, however, the present study is the first to demonstrate that alterations also exist in this system in a non-genetic animal model, lending further support for a role of the endocannabinoid system in the pathogenesis of autism and identifying a possible therapeutic target for this disorder.

In addition to the basal changes in the endocannabinoid system, it should be noted that following exposure to a novel conspecific animal, VPA-exposed animals exhibit increased levels of AEA, OEA and PEA in the hippocampus. While the functional significance of these changes is unknown, it is possible that the increases in N-acylethanolamines may be a compensatory mechanism to overcome the stress associated with social exposure. Elevation of AEA following administration URB597, has been shown to increase social play (Trezza and Vanderschuren, 2008) and evidence suggests that increased AEA in the basolateral amygdala and nucleus accumbens mediates social behaviour in rats (Trezza et al., 2012). Furthermore, interaction with a non familiar, but not familiar, social partner increases striatal levels of AEA in rats (Marco et al., 2011), an effect proposed as a management strategy to cope with novel social encounters.

Overall, the data presented herein (Chapters 5 & 6) are the first to demonstrate that VPA-exposed rats exhibit alterations in neuroinflammatory responses to TLR4 activation and various components of the endocannabinoid system. While further studies are required to determine if endocannabinoid modulation of neuroinflammatory processes may provide a novel therapeutic target for some of the behavioural symptoms associated with autism, the data presented have contributed significantly to the present knowledge base and have laid the foundation for subsequent studies in this area.

In conclusion, the work presented in this thesis has provided further evidence for a role of FAAH and MAGL in the regulation of TLR4-induced neuroinflammatory responses. Importantly, this data suggests that targeting 2-AG/MAGL may provide a means of modulating (neuro)inflammatory responses without possible psychotropic side effects associated with CB₁ receptor activation. Combining this with data from other groups indicates that the endocannabinoid system is a viable novel therapeutic target for the treatment of a host of central and peripheral inflammatory disorders. While neurodegenerative disorders such as Alzheimer's and Parkinson's disease would be the most obvious possible beneficiaries of neuro-immune modulatory therapies, increasing evidence indicates that inflammatory processes are also involved in the pathogenesis of neurodevelopmental disorders such as autism. The

data provided here has demonstrated for the first time that in a clinically relevant rat model of autism, alterations exist in various components of the endocannabinoid system and that neuroinflammatory responses to TLR4 activation are heightened in key brain regions that govern affective, social and emotional responding. While we recognise that there are certain limitations to this work and that further studies are required (see sections 7.1 and 7.2 below), the data provided in this thesis indicate that endocannabinoid-neuro-immune interactions may provide a novel therapeutic target for the treatment of some of the behavioural traits associated with autism. This is particularly relevant given that there is currently no defined psychopharmacologic treatment for the core symptoms of autism. Taken overall this thesis has enhanced our understanding of endocannabinoid-neuroimmune interactions, which has implications for identifying novel treatment targets for a host of neuroinflammatory disorders including autism.

7.1 Limitations of the studies

The data presented has demonstrate that for the first time that enhancement of endocannabinoid tone in vivo following inhibition of FAAH or MAGL potently modulates TLR4-induced inflammatory effects peripherally and centrally and that alterations exist in the endocannabinoid and immune system in the VPA model of autism. While this work has added significantly to the body of knowledge, a number of limitations are inherent in this work which may be the subject of studies in the future. These include:

- While our data demonstrate anti-inflammatory effects of endocannabinoid modulators following acute TLR4 activation; it should be noted that these effects were observed at a single timepoint and as such it cannot be ruled out that alterations may have occurred at timepoints other than those examined in the current study. For example we cannot conclusively rule out that prior administration of endocannabinoid modulators may have delayed the expression of TLR4 induced inflammatory responses, and such effects may occur to a similar potency at a later timepoint. However, combining our data with the published literature would indicate that this is unlikely and that endocannabinoids directly inhibit immune responses and elicit potent anti-inflammatory effects.
- Inhibition of FAAH resulted in increased levels of anandamide, OEA and PEA. As such the immunomodulatory effects following inhibition of FAAH with URB597 cannot be attributed solely to anandamide. Thus, further studies are required in order to determine the contribution of the individual FAAH substrates to the immunomodulatory effect of URB597 and the receptor mechanisms involved. Moreover it should also be noted that MAGL also catabolises other Monoacyl glycerides (MAG) to their free fatty acid and glycerol and their contribution has not been assessed in the current study.
- The current data is the first to demonstrate that VPA exposed offspring exhibit only slight alterations in basal levels/expression of immune mediators and exhibit an exacerbated response to TLR4 activation. However, it should be noted that these effects were only evaluated on one timepoint during

adolescence and we did not evaluate if sex differences existed. Furthermore, it is unknown if VPA exposed animals would have similar alterations in immune responding to other TLRs and the behavioural consequences of such an immune challenge. The investigation of alternative TLR activation, sexual dimorphism or adolescent vs adult responses was constrained by the requirement of a large number of animals available only via an 8 week in house breeding program but should be the focus of future studies.

- While every effort was made to evaluate mRNA and protein expression simultaneously or downstream gene expression that would result from and be thus indicative of changes in protein levels, we are cognizant of the fact mRNA is not always translated or indicative of protein levels. This is primarily due to methodological constraints including tissue amounts and number of animals required to evaluate over multiple timepoints. However, where possible we evaluated both mRNA and protein levels and combining our data with other published studies allows us to draw firm conclusions regarding endocannabinoid-immune interactions.

7.2 Future studies.

While the data presented in this thesis demonstrate that enhancement of endocannabinoid tone following inhibition of FAAH or MAGL has potent anti-inflammatory effects peripherally and centrally; and that the endocannabinoid and immune system are altered in the VPA model of autism, these data form the basis for several future studies. In particular, this data highlights the need to address the following questions in relation to the VPA model. .

- Do VPA exposed animals exhibit altered (neuro)immune responses to activation of other TLRs (e.g. TLR3)?
- What effect does immune activation have on social and anxiety-like behaviours in VPA exposed offspring? Would VPA animals exhibit altered TLR4-induced neuroimmune effects following enhancing endocannabinoid tone via FAAH or MAGL activation? What effect would enhancing endocannabinoid tone via FAAH or MAGL activation have on behavioural changes observed in the VPA model?

- Would reducing endocannabinoid tone by antagonism of CB₁/CB₂ receptors or other targets (TRPV1, PPARs, GPR55) modulate behaviour responding in VPA exposed offspring?
- Schneider *et al* found that male VPA exposed offspring exhibited a myriad of behaviour aberrations including decreased social interaction while female VPA exposed animals exhibited only increased repetitive/stereotypic-like activity when compared to control animals (Schneider et al., 2008). Similarly Kim et al demonstrated that VPA exposed male offspring displayed significantly impaired social interaction and hyperactivity while female offspring showed only marginal deficits (Kim et al., 2013). Further studies are required to determine if sexual dimorphic changes exist in the neuroimmune or endocannabinoid systems of VPA animals.

Thus future studies may investigate these questions in more detail providing further data for the possible role of endocannabinoid-neuroimmune interaction in autistic-like behaviours. Ultimately, data arising from these and other studies in the area may inform on the development of novel cannabinoid based therapies for the treatment of symptoms associated with ASD.

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