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**Interferon alpha-induced changes in emotional and
nociceptive processing: involvement of the endocannabinoid
system**

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Abstract

Interferon- α (IFN- α) is a pro-inflammatory cytokine used for the treatment of hepatitis B and C as well as various malignancies. However, therapy is often associated with neuropsychiatric side effects, including depression and anxiety (Schaefer et al., 2002, Raison et al., 2005), as well as the development of painful symptoms (Shakoor et al., 2010, Nogueira et al., 2012, Capuron et al., 2002). Several preclinical studies which have employed IFN- α for the investigation of inflammation-induced depression have yielded varying results and to date, no study has evaluated the effect of repeated IFN- α treatment on nociceptive behaviour. Therefore, this thesis sought to devise an experimental protocol that would mimic the clinical situation and provide a robust and reproducible model of IFN- α -induced depression and hyperalgesia. The results demonstrated that repeated subcutaneous administration of a high, but not low, dose of IFN- α resulted in depressive-like behaviour and hyperalgesia to a noxious inflammatory stimulus in C57Bl/6 mice. Establishment of this model of IFN- α -induced depression and pain subsequently allowed for the evaluation of possible neurobiological substrates underpinning concurrent changes in emotional and nociceptive responding. Given the recognised role for the endocannabinoid system in emotional and nociceptive responding, we chose to examine if changes existed in this system following repeated administration of IFN- α . The data revealed no change in the levels of the endocannabinoids or expression of the receptors or enzymes responsible for metabolism of the endocannabinoids between saline and IFN- α -treated mice. However, in the presence of a noxious stimulus, levels of the endocannabinoids, AEA and 2-AG, were increased in the PAG and RVM, key components of the descending pain pathway in IFN- α -treated animals. In comparison, formalin administration increased levels of 2-AG, AEA and related *N*-acylethanolamines at the site of injury in saline-, but not IFN- α -treated animals. Thus, IFN- α -treated mice may have an inability to mobilise endocannabinoids at the site of injury in response to a noxious stimulus, an effect which may underlie the hyperalgesia observed. As such, the remaining studies evaluated the effect of enhancing endocannabinoid tone either at the site of injury (paw) or globally, on formalin-evoked nociceptive behaviour. Inhibition of FAAH, the primary enzyme responsible for the metabolism of AEA and *N*-

acylethanolamines, or inhibition of MAGL, the 2-AG-metabolising enzyme, at the level of the paw, attenuated IFN- α -induced hyperalgesia, without altering formalin-evoked nociceptive behaviour in saline-treated animals. These data provide further support for an impaired ability of IFN- α -treated mice to engage the peripheral endocannabinoid system in response to a noxious stimulus, an effect which underlies the hyperalgesia observed in these animals. In comparison, systemic MAGL, but not FAAH, inhibition attenuated formalin-evoked nociceptive behaviour in both saline- and IFN- α -treated animals, indicating that enhancing 2-AG tone can elicit analgesic effects in the presence and absence of a depressive-like state. Taken together, this thesis has demonstrated for the first time that it is possible to establish a preclinical mouse model of IFN- α -induced depression and pain behaviour, and highlight a role for the endocannabinoid system in mediating and modulating IFN- α -related hyperalgesia.

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Declaration

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

Signed:..... Date:.....

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List of commonly used abbreviations

ABDH6, Serine hydrolase α/β -hydrolase domain containing protein 6;
ABDH12, Serine hydrolase α/β -hydrolase domain Containing Protein 12;
AEA, Anadamide;
AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;
BDNF, Brain-derived neurotrophic;
AMP, adenosine monophosphate;
CB₁, Cannabinoid receptor subtype 1;
CB₁^{-/-}, Cannabinoid receptor subtype 1 knockout;
CB₂, Cannabinoid receptor subtype 2;
CCI, Chronic constriction injury;
CCL2, Chemokine (C-C motif) ligand 2;
CCL3, Chemokine (C-C motif) ligand 3;
CCR2, C-C chemokine receptor type 2;
CFA, Complete Freud's adjuvant;
CNS, Central nervous system;
CRF, Corticotrophin-releasing factor;
CRF1, Corticotrophin-releasing factor receptor subtype 1;
CUS, Chronic unpredictable stress;
DAGL, Diacylglycerol lipase;
FAAH, Fatty acid amide hydrolase;
FST, Forced swim test;
GABA, γ -Aminobutyric acid;
GABA_A, γ -Aminobutyric acid receptor subtype A;
HIV, Human Immunodeficiency Virus;
HPA, Hypothalamo-pituitary-adrenal;
ICSS, Intracranial self-stimulation;
MAGL, monoacylglycerol lipase;
MAO, Monamine oxidase;
MAPK, Mitogen-activated protein kinase;
mRNA, Messenger ribonucleic acid;
NADA, N-arachidonoyl-dopamine;

NAPE, N-arachidonoylphosphatidylethanolamine;
NAPE-PLD, N-arachidonoylphosphatidylethanolamine phospholipase D;
NGF, Nerve growth factor;
NMDA, N-methyl-D-aspartate;
OB, Olfactory bulbectomised;
PNI, Peripheral nerve injury;
PNL, Partial sciatic nerve ligation;
PPARS, Peroxisome proliferator-activated receptors;
RVM, Rostral ventromedial medulla;
SD, Sprague Dawley;
SNI, Spared nerve injury;
TRPV1, Transient receptor potential vanilloid 1;
WKY, Wistar-Kyoto;
 Δ^9 -THC, Delta-9-tetrahydrocannabinol;
2-AG, 2-arachidonylglycerol;
4-MC, 4-methylcatechol;
5-HT_{1A}, 5-hydroxytryptamine receptor subtype 1A;
5-HT_{2A}, 5-hydroxytryptamine receptor subtype 2A;
5-HT_{2C}, 5-hydroxytryptamine receptor subtype 2C.

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

Publications and Proceedings

Peer Reviewed Published Manuscripts

1. **M. Fitzgibbon**, D.P. Finn, M. Roche (2015) High times for painful blues: the endocannabinoid system in pain-depression comorbidity. *Int J Neuropsychopharmacol.* Sep 5. doi: 10.1093/ijnp/pyv095. [Epub ahead of print].

Peer Reviewed Published Abstracts

- 1. M. Fitzgibbon, R.J. Henry, D.M. Kerr, D.P. Finn, M. Roche (2015)** Characterization of the endocannabinoid system in the descending pain pathway in a mouse model of IFN- α -induced hyperalgesia. *Int J Neuropsychopharmacol Suppl.* In press
- 2. M. Fitzgibbon, M. Liu, J. Bannon, S. Shen, M. Roche (2014)** Unc-51-like kinase 4 heterozygous mice display an anxiety-like behaviour profile and alterations in the GABAergic system within the amygdale. *Front. Neurosci. Conference Abstract: Neuroscience Ireland Young Neuroscientists Symposium 2014.* doi: 10.3389/conf.fnins.2014.87.00027 and *European Neuropsychopharmacology Suppl.* doi:10.1016/S0924-977X(14)70931-9
- 3. M. Fitzgibbon, N. O'Reilly, D.M. Kerr, D.P. Finn, M. Roche (2014)** Transient depressive-like behaviour in response to repeated interferon-alpha administration in mice. *European Neuropsychopharmacology Suppl.* doi:10.1016/S0924-977X(14)70042-2
- 4. M. Fitzgibbon, N.N. Burke, D.P. Finn, M. Roche (2013)** Chronic interferon alpha administration induces thermal hyperalgesia in mice. *International Behavioural Neuroscience Society Meeting abstracts P105* (<http://c.ymcdn.com/sites/ibns.siteym.com/resource/resmgr/IBNSProgram2013FINA L.pdf>)

Other Research Dissemination

International Conference Presentations

- 1. M. Fitzgibbon, R.J. Henry, D.M. Kerr, D.P. Finn, M. Roche (2015)** Peripheral inhibition of FAAH attenuates formalin-evoked nociceptive responding in a mouse model of IFN- α -induced hyperalgesia. Presented at 7th European Cannabinoid Workshop, Genoa, Italy, September 2015 (P).
- 2. M. Fitzgibbon, N O'Reilly, D.M. Kerr, D.P. Finn, M. Roche.** Enhanced inflammatory pain behaviour in the interferon-alpha induced mouse model of depression. Presented at International Association for the Study of Pain 15th World Congress on Pain, Buenos Aires, Argentina, October 2014 (P).
- 3. M. Fitzgibbon, N O'Reilly, D.M. Kerr, D.P. Finn, M. Roche.** Enhanced formalin-evoked nociceptive responding in the interferon-alpha induced mouse model of depression. Presented at European Behavioural Pharmacology Society Annual Workshop, Brighton, UK, June 2014 (P).
- 4. M. Fitzgibbon, A.A. Iglesias, L.G. Glynn, A.W. Murphy, F.M. Finucane.** The Influence of Body Mass Index on All-Cause Mortality in Patients with Established Cardiovascular Disease. Presented at 48th European Diabetes Epidemiology Group Meeting, Potsdam, Germany, April 2013 (P).

Irish Conference Presentations:

- 1. M. Fitzgibbon, R.J. Henry, D.M. Kerr, D.P. Finn, M. Roche (2015)** Peripheral inhibition of FAAH attenuates formalin-evoked nociceptive responding in a mouse model of IFN- α -induced hyperalgesia. Presented at Irish Pain Society Annual Scientific Meeting, Dublin, September 2015 (P).
- 2. M. Fitzgibbon, R.J. Henry, D.M. Kerr, D.P. Finn, M. Roche (2015)** Peripheral inhibition of FAAH attenuates formalin-evoked nociceptive responding in a mouse model of IFN- α -induced hyperalgesia. Presented at 9th neuroscience Ireland Conference, Dublin, September 2015 (P).
- 3. M. Fitzgibbon, R.J. Henry, D.M. Kerr, D.P. Finn, M. Roche.** Characterization of the endocannabinoid system in the descending pain pathway in a mouse model of IFN- α -induced hyperalgesia. Presented at College of Medicine, Nursing and Health Science Postgraduate Research Day, Galway, May 2015 (P).
- 4. M. Fitzgibbon, R.J. Henry, D.M. Kerr, D.P. Finn, M. Roche.** Characterization of the endocannabinoid system in the descending pain pathway in a mouse model of IFN- α -induced hyperalgesia. Presented at Molecular Medicine Ireland Annual Scientific Meeting, Dublin, March 2015 (P).
- 5. M. Fitzgibbon, M. Liu, J. Bannon, S. Shen, M. Roche.** Unc-51-like kinase 4 heterozygous mice display an anxiety-like behaviour profile and alterations in the GABAergic system within the amygdale. Presented at Careers in Neuroscience Symposium, NUI Galway, November 2014 (P).
- 6. M. Fitzgibbon, N O'Reilly, D.M. Kerr, D.P. Finn, M. Roche.** Enhanced formalin-evoked nociceptive responding in the interferon-alpha induced mouse model of depression. Presented at Research Showcase, NUI Galway, July 2014 (P).
- 7. M. Fitzgibbon, N O'Reilly, D.M. Kerr, D.P. Finn, M. Roche.** Enhanced formalin-evoked nociceptive responding in the interferon-alpha induced mouse

model of depression. Presented at College of Medicine Postgraduate Research Day, NUI Galway, May 2014 (P).

8. M. Fitzgibbon, N O'Reilly, D.M. Kerr, D.P. Finn, M. Roche. Transient depressive-like behaviour in response to repeated interferon-alpha administration in mice. Presented at Molecular Medicine Ireland Annual Scientific Meeting, Trinity College, Dublin, March 2014 (P).

9. M. Fitzgibbon, N.N. Burke, D.P. Finn, M. Roche. The effects of repeated interferon-alpha administration on depressive-like and nociceptive responding in two different mouse strains. Presented at Faculty of Pain Medicine Ireland Annual Scientific Meeting, College of Anaesthetists, Dublin, January 2014 (P).

10. M. Fitzgibbon, N.N. Burke, D.P. Finn, M. Roche. The effects of repeated interferon-alpha administration on depressive-like and nociceptive responding in two different mouse strains. Presented at 8th Neuroscience Ireland Conference, University College Cork, November 2013 (P).

11. M. Fitzgibbon, N.N. Burke, D.P. Finn, M. Roche. Chronic interferon alpha administration induces thermal hyperalgesia in mice. Presented at College of Medicine, Nursing and Health Science Postgraduate Research Day, NUI Galway, May 2013 (P).

12. M. Liu, M. Roche, **M. Fitzgibbon**, T. O'Brien, S. Shen. Roles of a novel kinase ULK4 in brain development and function. Presented at College of Medicine, Nursing and Health Science Postgraduate Research Day, NUI Galway, May 2013 (P).

13. M. Fitzgibbon, N.N. Burke, D.P. Finn, M. Roche. Chronic interferon alpha administration induces thermal hyperalgesia in mice. Presented at Molecular Medicine Ireland Annual Scientific Meeting, Dublin, March 2013 (O).

14. M. Fitzgibbon, A.A. Iglesias, L.G. Glynn, A.W. Murphy, F.M. Finucane. The Influence of Body Mass Index on All-Cause Mortality in Patients with Established Cardiovascular Disease. Presented at Irish Endocrine Society Annual Meeting, Dublin, October 2012 (P).

15. M. Fitzgibbon, A.A. Iglesias, L.G. Glynn, A.W. Murphy, F.M. Finucane. The Influence of Body Mass Index on Patterns of Cardiovascular Disease in an At-Risk Cohort. Presented at the first meeting of the Association of the Study of Obesity on the Island of Ireland, Dublin, September 2012 (P).

Chapter 1

General Introduction

1.1 Depression-Pain Co-morbidity

Depression is a state of low mood and aversion to activity that can affect a person's thoughts, behaviour, feelings and sense of well-being and is characterized as a constellation of psychological (depressed mood, anhedonia, feelings of worthlessness or guilt, altered concentration, and suicidality) and physical (altered appetite and weight, disturbed sleep, psychomotor agitation or retardation, and fatigue) symptoms (DSM-5, 2013). It is one of the most prevalent forms of psychiatric illness, affecting up to 120 million people worldwide (Lepine and Briley, 2011) with a varying lifetime prevalence ranging from 12.8% in Europe (Alonso et al., 2004) to 16.2% in the U.S. (Kessler et al., 2005). In particular, prevalence and incidence tends to be higher in women than in men whereby women are nearly twice as likely to develop the condition, especially in younger age groups, or where there exists a family history of the disorder or concurrent illness (Patten et al., 2006, Wang et al., 2010). According to World Health Organization predictions, depression is estimated to be second most important contributor to the global burden of disease by 2020, and the principal cause in developed countries by 2030 (WHO, 2011, Lepine and Briley, 2011). Depressive disorder is also associated with loss of physical and cognitive function and as such can be devastating to the health, daily lives, and occupational and social functioning of the individual afflicted as well as their family, co-workers, employers and others (Lepine and Briley, 2011, Birnbaum et al., 2010). Furthermore, the degree of disability correlates with the severity of depression (Lepine et al., 1997). Another devastating aspect of depression is the associated elevated risk of suicide in patients, which can be 20-fold higher than in the general population in the case of patients with major depressive disorder (MDD) (Lepine and Briley, 2011). While considered a debilitating disorder in its own right, depression is frequently associated with other disorders including neurological conditions, cancer, cardiovascular disease and chronic pain. Chronic pain and depression frequently co-exist, and it has been reported that this association may be as high as 80% of patients (Poole et al., 2009). For example, major depressive and bipolar disorder can associate with painful symptoms in up to 95% of patients (Grover et al., 2012, Maneeton et al., 2013, Nicholl et al., 2014). Specific pain-related symptoms of depression include headache as well as abdominal, joint, chest and back pain (Mathew et al., 1981, Kroenke et al., 1994, Hauser et al., 2014). However, depressed

patients have also been shown to have increased, unchanged or reduced sensitivity to painful stimuli such as cold, heat and pressure (Bar et al., 2005, Ben-Tovim and Schwartz, 1981, Boettger et al., 2013, Lautenbacher et al., 1999, Gormsen et al., 2004), effects which depend on the modality and intensity of the stimulus. However, despite varying responses to painful stimuli, data indicate that severity of depression correlates directly with increased intensity of pain symptomatology (Khongsaengdao et al., 2000). Conversely, patients suffering from inflammatory conditions such as rheumatoid arthritis, osteoarthritis or inflammatory bowel disease (Hawker et al., 2011, Khongsaengdao et al., 2000, Kvien, 2004, Kappelman et al., 2014) and neuropathic pain (Emery et al., 2014, Lin et al., 2015) are up to 4.9 times more likely to develop depression or anxiety disorder than the general population. In addition, co-morbid depression-pain has also shown to be more disabling and expensive to both patients and society than either condition alone (Gameroff and Olsson, 2006, Emptage et al., 2005). Thus, taken together, greater understanding of the pathophysiological mechanisms that underlie depression and pain and their interaction, is of utmost importance and may inform on the development of more effective therapeutic strategies for these debilitating disorders.

1.2 Preclinical evidence of depression and pain co-morbidity

Animal models provide a valuable means of investigating potential neurobiological substrates which may underlie the association between depression and pain.

1.2.1 Animal models of depression exhibit altered nociceptive responding

Several animal models of depression including that based on stress, genetics, lesion and pharmacological manipulation have been shown to exhibit alterations in nociceptive responding [for review see (Li, 2015)], supporting the clinical finding of an association between depression and pain. For instance, animals which have been exposed to chronic mild stress display reduced nociceptive thresholds to cold (Bardin et al., 2009, Bravo et al., 2012, Bravo et al., 2014) and mechanical (Bardin et al., 2009, Imbe et al., 2012) stimuli and an increased threshold to noxious thermal stimuli (Shi et al., 2010). Additionally, inflammatory (Gameiro et al., 2005, Rivat et al., 2010, Wang et al., 2013) and neuropathic (Bravo et al., 2012) pain behaviours

have shown to be enhanced in the same. Similarly, the Wistar-Kyoto (WKY) rat, a stress hyperresponsive strain, exhibits thermal hyperalgesia (Burke et al., 2010), visceral hyperalgesia to colorectal distension (Gibney et al., 2010, Gosselin et al., 2010, O'Malley et al., 2010), enhanced formalin-evoked inflammatory pain behaviour (Burke et al., 2010, Rea et al., 2014) and enhanced mechanical allodynia following peripheral nerve injury (neuropathic pain) (Zeng et al., 2008, del Rey et al., 2011). Pharmacological depletion of monoamines following reserpine administration results in depressive-like behaviour, and recent evidence has demonstrated accompanying thermal allodynia (Liu et al., 2014b), pronounced and long-lasting mechanical hyperalgesia and allodynia, and cold allodynia (Nagakura et al., 2009, Arora et al., 2011). Thus, this model has been proposed as a possible rodent model of fibromyalgia (Nagakura et al., 2009). Various lesion models of depression also associate with altered nociceptive responses. An example of this is the olfactory bulbectomised (OB) rat and recent work from our group has demonstrated that this lesion model of depression exhibits increased sensitivity to thermal and mechanical stimuli in the acetone drop, hot plate, tail flick and von Frey tests (Burke et al., 2010, Burke et al., 2013a) as well as increased inflammatory pain responding in the formalin test (Burke et al., 2010) and enhanced neuropathic pain responding following spinal nerve ligation (Burke et al., 2013a, Burke et al., 2014b). Collectively, these animal models of depression have been shown to exhibit altered nociceptive thresholds and enhanced inflammatory and neuropathic pain behaviour, mimicking effects observed clinically.

1.2.2 Animal models of chronic pain exhibit depressive-like behaviour

Several studies have identified depressive- and anxiety-related behaviour, as assessed in multiple behavioural paradigms, in a wide variety of preclinical models of chronic pain [for review see (Li, 2015, Yalcin et al., 2014)]. For example, peripheral nerve injury (PNI) or spared nerve injury (SNI) in mice, which induces a pronounced mechanical allodynia, is also accompanied by the development of depressive-like behaviour as evident through enhanced immobility in the forced swim test (FST) (Norman et al., 2010, Goncalves et al., 2008, Wang et al., 2011). An alternative model of neuropathic pain follows chronic constriction injury (CCI) and rodents which have been subjected to this procedure exhibit reduced sucrose

preference (Dellarole et al., 2014) and increased immobility in the FST (Hu et al., 2009, Jesse et al., 2010, Zhao et al., 2014, Fukuhara et al., 2012), indicating the development of anhedonia and behavioural despair respectively, which each represent depressive-like behaviour. In the complete Freund's adjuvant (CFA) model of inflammatory pain behaviour, both rats and mice exhibit depressive-like behaviour in the FST (Maciel et al., 2013, Borges et al., 2014) and tail suspension test (Maciel et al., 2013); as well as anxiety-related behaviour as seen in the elevated plus maze, open field test and social interaction test (Borges et al., 2014, Parent et al., 2012). Furthermore, altered emotional responding in chronic pain models would appear to occur subsequent to the development of enhanced somatosensory perception, as has been observed in neuropathic pain models, whereby altered emotional behaviour occurs 4-8 weeks post nerve injury (Yalcin et al., 2011, Suzuki et al., 2007), and not earlier (2-4weeks) when mechanical allodynia/hypersensitivity is observed (Kontinen et al., 1999, Hasnie et al., 2007). These studies highlight the development of depressive- and anxiety-like behaviour in models of neuropathic or inflammatory pain, and suggest that pathological alterations induced by persistent nociceptive input to brain regions that process both pain and affect may account, at least in part, for co-morbid depressive-like behavioural changes.

Animal models that replicate the clinical scenario are important for in-depth investigation of the possible neurobiological substrates that may mediate the association between depression and chronic pain.

1. 3 Shared substrates in depression and pain

Although the cause of the association between depression and pain remains somewhat elusive, a role for common anatomical substrates and neurobiological mediators including neurotransmitters, neurotrophins, neuroendocrine alterations and inflammatory mediators have been suggested. Any or all of the aforementioned may alter neural signalling and function in brain centres involved in the regulation of emotional and nociceptive processing and thus underlie the association between these disorders [for recent reviews see (Goesling et al., 2013, Doan et al., 2015, Walker et al., 2014, Meerwijk et al., 2013, Blackburn-Munro, 2004, Anderson et al., 2012, Maletic and Raison, 2009, Jennings et al., 2014)]. A comprehensive review of

the role of each of these substrates and mediators in depression, pain and their interaction is beyond the scope of this thesis and thus presented herein is an overview of the evidence supporting a role for specific neuroanatomical substrates and mediators with particular emphasis on the role of inflammatory mediators, namely pro-inflammatory cytokines, as a key mediator in depression-pain interactions.

1.3.1 Neuroanatomical Substrates

Aberrant regulation of emotional processing is associated with anatomical abnormalities in various brain regions including the thalamus, cortical areas such as the prefrontal cortex, anterior cingulate cortex and insular cortex as well as limbic regions, namely the hippocampus and amygdala [for review see (Rive et al., 2013, Dutta et al., 2014, Delvecchio et al., 2012)]. For example, depressed patients exhibit alterations in size, activity and/or connectivity in thalamic, cortical and limbic areas. More specifically, the thalamus, described as the relay centre of the brain, displays a reduction in grey matter volume in depressed patients, an effect which indirectly correlates with depressive symptoms, when compared with healthy controls (Hagan et al., 2015, Kempton et al., 2011), while imaging studies have also identified increased thalamic activity in subjects in response to psychological pain such as that observed in grief and recalled sadness (George et al., 1996, Lane et al., 1997, Kersting et al., 2009). Additionally, frontal cortical volume is reduced in patients diagnosed with disorders such as post-traumatic stress disorder (Tavanti et al., 2012), depression (Lai and Wu, 2014, Han et al., 2014) and in suicidal subjects (Monkul et al., 2007), while limbic regions such as the amygdala associate with increased activity in patients with MDD (Jaworska et al., 2014, Zhang et al., 2014b). The hippocampus also plays a prominent role in affective processes and studies have reported hippocampal atrophy in patients with such disorders including major depressive disorder (MDD) (Stratmann et al., 2014). While the exact mechanism(s) through which the above-described changes may occur remains somewhat elusive, many studies have identified a causative role for enhanced inflammatory states in the development of such events, in particular, central atrophy. For example, the gram negative bacterial endotoxin, lipopolysaccharide (LPS), an immune stimulant known to induce depressive-like behaviour in rodents, has been shown to enhance

hippocampal levels of pro-inflammatory cytokines, namely IL-6, TNF- α and IL-1 β (Ho et al., 2015). Importantly such inflammatory events also promote a reduction in local brain-derived neurotrophic factor (BDNF) levels (Guan and Fang, 2006) and overall hippocampal volume (Johansson et al., 2005), changes which may underlie the behavioural effects observed.

In addition to the well characterized ascending and descending pain pathways (see below for full description and figure 1.3.1), these same supraspinal regions are also involved in the processing of nociceptive information, and changes in these have been reported in chronic pain patients. For example, reductions in thalamic grey matter density and cerebral blood flow have been identified in patients with neuropathic pain (Iadarola et al., 1995, Hsieh et al., 1995, Apkarian et al., 2004). Similarly, alterations in size, activity and/or connectivity have also been reported in various cortical regions in chronic pain patients. For instance, reduced gray matter density is evident in discrete components of the prefrontal cortex (PFC) such as the bilateral dorsolateral region in patients suffering from chronic back pain (Apkarian et al., 2004), while painful conditions such as migraine associate with reduced gray matter volume in the cingulate cortex (Schmidt-Wilcke et al., 2008). Additionally, reduced cerebral blood flow has been identified in the PFC (Casey and Tran, 2006) and anterior cingulated cortex (ACC) (Honda et al., 2007) in clinical pain populations. An alternative cortical region, the insular cortex, is frequently activated in response to painful stimuli, as demonstrated in several imaging studies (Frot and Mauguiere, 2003, Casey and Tran, 2006, Baliki et al., 2006, Seifert and Maihofner, 2007) and patients with fibromyalgia, a chronic pain state, display increased connectivity of this region (Ichesco et al., 2014) such that the degree of connectivity correlates directly with the severity of painful symptoms in these populations (Schmidt-Wilcke et al., 2014, Ichesco et al., 2014). Similarly, changes in size, activity and/or connectivity have also been reported in the amygdala (Giesecke et al., 2005) and hippocampus (Berna et al., 2010, McCrae et al., 2015).

Changes in these thalamic, cortical and limbic regions are thought to modulate nociceptive responding through modulation of ascending and descending pain pathways (see figure 1.3.1). In brief, the perception of pain involves activation of

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peripheral nociceptors located on primary afferent neurons, A δ - and C-fibres. Activation of these nociceptors occurs following exposure to noxious mechanical, thermal or chemical stimuli and thresholds for activation can in turn be influenced by a variety of inflammatory mediators such as bradykinin, prostaglandins, thromboxanes, leukotrienes, platelet-activating factor, and nitric oxide [for review see (Petho and Reeh, 2012)]. Sensory information is then relayed to the dorsal horn of the spinal cord and subsequent conduction to the brain via second order neurons through various ascending pain pathways including spinothalamic pathways, the spinoparabrachial pathway, the spinomesencephalic, the spinohypothalamic and the spinoreticular pathways which innervate higher brain regions including the aforementioned thalamic, cortical and limbic structures [for review see (Millan, 1999)]. Thus, ascending pathways allow the perception of the somatosensory and affective appreciation of pain. The descending pain pathway consists of neuronal connections from the periaqueductal gray (PAG) to the rostral ventromedial medulla (RVM) and the dorsal horn of the spinal cord, which act to inhibit or facilitate pain transmission at the level of the spinal cord. The PAG can be modulated by input from some of the aforementioned cortical and limbic areas including the amygdala (Hopkins and Holstege, 1978, Oka et al., 2008) and the prefrontal cortex (PFC) (An et al., 1998, Neugebauer et al., 2009), and activation of the PAG has been shown to have a potent inhibitory action on pain transmission. The RVM then receives input from the PAG which acts to modulate the firing of ON- and OFF-cells which in turn facilitate and suppress pain transmission respectively and thus, are involved in the mechanisms modulating descending inhibition or facilitation (Heinricher et al., 2009). Thus, higher brain centers which are associated with affective responding can potentially modulate the descending mediation of pain. An example of such modulation includes the inhibition of pain by fear (fear-induced analgesia) while long-term low grade stress has been shown to enhance pain sensation and perception (stress-induced hyperalgesia). Higher brain centers such as the amygdala and prefrontal cortex have been shown to be important in mediating both of these effects of fear/stress on pain processing [for review see (Jennings et al., 2014, Butler et al., 2008)].

Figure 1.3.1. Ascending and descending pain pathways. The ventral spinothalamic pathway innervates thalamic nuclei and synapses with third-order neurons that terminate in the cortex and encodes the sensory-discriminative aspects of pain. Neurons in the spinoparabrachial pathway synapse with third-order neurons that terminate in the hypothalamus and the amygdala. The descending pain pathways originates in higher brain regions such as the cortex, hypothalamus, and amygdala and project to the periaqueductal grey (PAG) and the rostroventral medulla (RVM) and finally to the dorsal horn of the spinal cord, where they modulate pain signals. Pink arrows represent sensory-discriminative ascending pathway or content of pain, purple arrows represent the sensory-affective ascending pathways or emotional aspect of pain and green arrows represent descending pain pathways. DRG, dorsal root ganglia. Reproduced and adapted from (Olango, 2012).

Several insightful reviews have covered the plethora of evidence for alterations in neuroanatomical sites and their significance in depression-pain interactions [for review see (Meerwijk et al., 2013, Robinson et al., 2009)]. For example, similar to that mentioned above, changes in parameters such as size, activity and connectivity have been reported in thalamic, cortical and limbic brain regions in patients with co-existent mood and pain disorder. An example of such changes has been reported in the insular cortex of patients with fibromyalgia, an autoimmune condition highly

associative with symptoms of depression and pain. The insular cortex is a limbic-related cortex which plays a critical role in emotive and pain processing (Phan et al., 2002), through relay of information from afferent sensory pathways to a number of other limbic-related structures, including the amygdala and orbitofrontal cortex (Craig, 2003). Altered connectivity has been identified within the insular cortex of fibromyalgia patients (Ichesco et al., 2014), while the degree of connectivity in this region has been shown to positively correlate with the severity of pain symptoms (Schmidt-Wilcke et al., 2014, Ichesco et al., 2014). Similarly, activation of another cortical region, the PFC, correlates directly with clinical pain scores in fibromyalgia patients with co-morbid depression (Giesecke et al., 2005). Such alterations in size, activity and/or connectivity has also been reported in the thalamus (Hagan et al., 2015, Kempton et al., 2011, George et al., 1996, Lane et al., 1997, Kersting et al., 2009), alternative cortical areas namely the anterior insula, anterior midcingulate cortex and pregenual anterior cingulate cortex (Giesecke et al., 2005, Elsenbruch et al., 2010) and limbic regions including the amygdala (Giesecke et al., 2005) and hippocampus (Berna et al., 2010, McCrae et al., 2015). Given the susceptibility of descending nociceptive transmission to modulatory input from these higher centres, such anatomical changes as observed in depression and/or pain may thus influence descending facilitation and/or inhibition of pain responding.

In agreement with the clinical scenario, animal models of co-existent depressive and pain behaviour have also identified thalamic, cortical and limbic regions specifically implicated in depression-pain interactions. Such investigations have included direct stimulation of the nucleus submedius (Sm) (of the medial thalamus) which has been shown to inhibit visceral hypersensitivity, a pain state associated with altered emotional experience, in rodents (Hanamori et al., 1998). In addition, various cortical structures have also been identified as possible mediators in preclinical behavioural paradigms of this co-morbidity including the rostral (r)ACC, the lesion of which increases the duration of immobility in the FST, indicative of an enhanced depressive-like state (Bissiere et al., 2006), while stimulation of this region attenuates avoidance behaviour in the place escape/avoidance paradigm, a test for altered affective responding associated with chronic pain, following spinal nerve ligation in rodents (LaBuda and Fuchs, 2005). Furthermore, early-life stress models

of depression which exhibit changes in nociceptive thresholds have been shown display reductions in hippocampal volume (Aksic et al., 2013) and function (Jin et al., 2013). Thus taken together, both clinical and preclinical data suggest that alterations in anatomical changes in connectivity, activity or volume common in cortical and limbic areas may underlie the association between depression and pain.

1.3.2 Monoamines and the HPA axis

Mood and pain processes are influenced by common neurotransmitters such as GABA, glutamate and monoamines [for review see (Goesling et al., 2013, Walker et al., 2014)] and the neuroendocrine system, in particular the hypothalamic-pituitary adrenal (HPA) axis [for review see (Blackburn-Munro, 2004)]. A full overview of the role of the various neurotransmitter systems in emotional and nociceptive processing and the possible interaction between depression and pain is beyond the scope of this thesis but has been covered in detail by several excellent reviews (Robinson et al., 2009, Maletic and Raison, 2009). As such, the section will highlight the evidence supporting the role of the monoamine system and HPA axis, the most widely studied neurobiological mediators, as possible substrates in depression-pain interactions.

1.3.2.1 Monoamines

The role of monoaminergic transmission, namely that of serotonin, noradrenaline and dopamine remains the most investigated mode of neurotransmission implicated in both depressive and painful states. A vast body of experimental data demonstrates the importance of functionally competent monoaminergic pathways for combating depressive states, and most currently available treatments of depression aim to restore the compromised activity of corticolimbic monoaminergic pathways in this regard [for review see (Hamon and Blier, 2013)]. The importance of monoamine availability is further exemplified by evidence that several non-direct monoaminergic based approaches for the treatment of depression, such as electroconvulsive therapy, also influence monoaminergic transmission within corticolimbic structures (Tamminga et al., 2002). It is now generally accepted that depression does not simply reflect dysfunction of any one monoamine; but rather a

complex disruption of the overall operation of monoaminergic networks which is covered in the aforementioned reviews. Monoamine-based antidepressant therapies are now also considered first line therapy for the treatment of various chronic pain conditions including fibromyalgia and neuropathic pain (Marangell et al., 2011, Wernicke et al., 2006). There is also an array of preclinical studies depicting a role for serotonergic and/or noradrenergic-mediated analgesia [for review see (Hauser et al., 2013, Aziz et al., 2014)]. However, the variable capacity of 5-HT to differentially inhibit or facilitate nociception within the CNS is dependent on the type of 5-HT receptor subtype activated (Bannister et al., 2009). Serotonergic and noradrenergic neurons project into the limbic and prefrontal regions of the brain, areas most frequently associated with emotional processing and responding, as depicted in figure 1.3.2.1. However, neurons from these regions also project to brain stem areas such as the RVM and directly to the spinal cord, and regulate nociceptive processing (Fields et al., 1991, Bymaster et al., 2005). As described earlier, the PAG relays information from higher corticolimbic regions to the brainstem, in particular the RVM, where monoaminergic input associates with the activation of ON and OFF cells, thus engaging facilitatory and inhibitory neurons to respectively increase and decrease dorsal horn activity (Bannister et al., 2009). Therefore, serotonergic and noradrenergic neurons in the raphe nuclei and the locus coeruleus, respectively, can directly modulate pain and emotional responding via regulation of cortico-limbic and descending pain pathways and dysregulation of this system may underlie the pathophysiology of depression, pain and/or their interaction (Stahl and Briley, 2004).

Figure 1.3.2.1. Monoaminergic innervation of brain regions. A, Noradrenergic neurons of the locus ceruleus project to the limbic and cortical regions, as well as the thalamus, cerebellum, and spinal cord. (Note: Noradrenergic neurons of cell groups A1, A2, A5, and A7 project to more restricted regions and associate with autonomic function). B, Dopaminergic neurons of the substantia nigra and the adjacent ventral tegmental area (VTA) project to the striatum and various regions in the neocortex, while a dopaminergic cell group in the hypothalamus regulates neuroendocrine processes. C, Serotonergic neurons located in the raphe nuclei project to almost all parts of the brain and are involved in many functions including the regulation of emotional and nociceptive processes. Adapted from (Fuchs and Flugge, 2004).

A role for monoaminergic system as a common neurobiological substrate for depression and pain has also been evaluated in animal models. As described earlier, pharmacological depletion of monoamines (serotonin, noradrenaline and dopamine) following reserpine administration results in depressive-like behaviour as well as concurrent thermal allodynia (Liu et al., 2014b), pronounced and long-lasting mechanical hyperalgesia and allodynia, and cold allodynia (Nagakura et al., 2009,

Arora et al., 2011). Research from our group has recently shown that chronic treatment with the tricyclic antidepressant amitriptyline, which acts primarily as a serotonin-noradrenaline reuptake inhibitor, elicits an antidepressant-like effect and potently attenuates nerve-injury induced mechanical and cold allodynia, in the rat olfactory bulbectomy model of depression (Burke et al., 2015). Increases in post-operative pain have been reported in young animals following exposure to gestational stress, an effect which is significantly normalised upon developmental administration of fluoxetine, a selective serotonin reuptake inhibitor (Knaepen et al., 2013). In addition to the evaluation of monoaminergic tone in models of depression with altered nociceptive responding, a role for the monoaminergic system has also been demonstrated in animal models of pain which exhibit altered affect. For example, in the CCI model of neuropathic pain, the tricyclic antidepressant, imipramine, significantly attenuated the associated despair-like behaviour observed in the forced swim test (Yasuda et al., 2014). Thus, taken together, several clinical and preclinical studies have demonstrated altered monoaminergic tone in animal models of depression-pain comorbidity. However, it should be noted that monoamine-based antidepressants are only effective in approximately 60% of depressed patients and older tricyclic antidepressants which are associated with adverse side effects have been found to be more effective in treating neuropathic pain. Thus, other neurobiological substrates may also be involved in mediating the association between these conditions.

1.3.2.2 HPA axis activity

The hypothalamic–pituitary–adrenal axis or HPA axis is a complex set of direct influences and feedback interactions among three endocrine glands; the hypothalamus, pituitary and adrenal glands. The interactions among these organs constitute the HPA axis, a major part of the neuroendocrine system that controls reactions to stress and regulates many body processes, including the immune system, metabolism as well as mood and emotions among other functions. Importantly, the HPA axis has a physiological role in stress response and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis has been long been implicated in the pathophysiology of depression [for review see (Martinac et al., 2014, Maric and Adzic, 2013, Zunszain et al., 2011, Pariante and Lightman, 2008)]

and pain disorders [for review see (Bomholt et al., 2004)]. In this way, the HPA axis has thus also been proposed as a possible mediator in the depression-pain dyad [for review see (Blackburn-Munro, 2004)]. The HPA axis is responsible for the neuroendocrine response to stress, an adaptive function that is critical for survival (Herman and Cullinan, 1997).

Figure 1.3.2.2. This hypothalamic-pituitary-adrenal (HPA) axis response pathway. Hormonal response begins in the hypothalamus which secretes corticotropin-releasing hormone (CRH) into the bloodstream. CRH then travels via blood vessels to the pituitary gland where it stimulates the release of adrenocorticotrophic hormone (ACTH). ACTH, in turn, stimulates the release of other hormones, such as glucocorticoids from the adrenal glands. Cortisol, the most potent glucocorticoid in humans, provides a "negative feedback system." If the stressor is mild, when cortisol reaches the hypothalamus it inhibits further excess release of CRH, restoring ACTH and cortisol to normal levels. During intense stress, signals in the brain for more CRF release override inhibitory mechanisms and the stress reaction continues. Reproduced from (Sweis, 2012).

Stress-induced activation of cortico-limbic structures results in enhanced activation of the paraventricular nucleus (PVN) of the hypothalamus and the release of corticotrophin-releasing Factor/hormone (CRF/CRH) which in turn acts on CRF-1 receptors of the anterior pituitary, thus stimulating the release of adrenocorticotrophic hormone (ACTH) into the systemic circulation. This hormone then acts upon the adrenal cortex to initiate synthesis and release of the glucocorticoids

(cortisol/corticosterone) that bind to glucocorticoid receptors (GR) in the hypothalamus and pituitary, resulting in negative feedback or reduced release of CRF and ACTH, thus preventing hyperactivity of the axis (Herman and Cullinan, 1997) as depicted in figure 1.3.2.2. Impairments of neuroendocrine regulation have been reported in mood disorders in the clinic, and correlation between increased secretion of the cortisol and depression is one of the oldest observations in biological psychiatry, whereby up to approximately 50% of patients with depression display elevated cortisol levels, attributed to impaired negative feedback regulation (Arana et al., 1985). In addition, clinical depression associates with altered diurnal release of ACTH and adrenal hypertrophy (Holsboer, 2000, Nemeroff et al., 1992, Plotsky et al., 1998) while successful antidepressant treatment and normalisation of HPA activity has been shown to preclude alleviation of these depressive symptoms (Himmerich et al., 2007, Holsboer et al., 1982). Arrays of preclinical studies also demonstrate altered HPA signalling and function in animal models of depression [for reviews see (Muller and Holsboer, 2006, Kehne and Cain, 2010, Neigh et al., 2013)].

Alterations in HPA activity have also been directly linked with painful conditions. For instance, as for depressed patients, increased cortisol levels are also observed in patients with severe non-malignant chronic pain (Van Uum et al., 2008), rheumatoid arthritis (Catley et al., 2000), chronic back pain (Vachon-Preseu et al., 2013) and fibromyalgia (Fatima et al., 2013), among others. Thus, given enhanced cortisol levels and dysfunction of the HPA axis has been shown in both pain and depression separately, it is not surprising that several clinical studies have also identified HPA dysfunction in patients exhibiting co-morbid depression and pain. In one such study, a cross-sectional analysis of patients with advanced breast cancer revealed increasing plasma cortisol levels which positively correlated with symptoms of both depression and pain (Thornton et al., 2010). However, in contrast, a separate study carried out in patients with fibromyalgia has reported enhanced cortisol release and dysregulation of HPA function associated with depressive, but not pain, symptoms, implying possible diverging mechanisms for both affective and nociceptive processing in this condition (Wingenfeld et al., 2010). Preclinical data are also available to demonstrate this reciprocity such as that observed in an experimental model of gastritis whereby gastrointestinal inflammation, pain, anxiety- and depressive-like behaviours in rats

are accompanied by dysregulation of the HPA axis, characterized by increased expression of CRF mRNA and reduced expression of glucocorticoid receptor in the hypothalamus and increased plasma levels of corticosterone (Luo et al., 2013). Increased expression of CRF has also been reported in the paraventricular nucleus of the hypothalamus and dorsal raphe nucleus of WKY rats and animals pre-exposed to neonatal maternal separation respectively (Bravo et al., 2011), two models of depression and associated hyperalgesia. Furthermore, the hyperalgesic visceromotor response to phasic colorectal distension following repeated water avoidance stress has been shown to be attenuated by CRF1 antagonism (Larauche et al., 2008). In addition, systemic or intra-amygdalar injection of the CRF1 receptor antagonist NBI27914, blocks anxiety-related and nocifensive behaviour in a rat model of arthritis (Ji et al., 2007), thus emphasising the key role of the CRF-HPA stress axis in affective and/or nociceptive processing.

1.4 Immune System

Over the past two decades a wealth of data has arisen supporting a role for inflammatory processes in both depression and pain individually and in light of the high prevalence of co-existence, it has also been proposed that inflammation and inflammatory processes play a causative role in their co-morbidity. A particular focus of this thesis is the evaluation of inflammation-induced changes in emotional and nociceptive responding and thus, this section will provide an overview of the wealth of evidence supporting this association.

1.4.1 Immune System in Depression

Inflammation is a physiological protective response to injury which is characterised by heat, redness, swelling and pain. Inflammatory processes which respond to noxious stimuli incorporate the activation of host immune cells, blood vessels and proteins, namely pro-inflammatory cytokines, in order to eliminate the cause of injury and promote the removal of necrotic cells and tissue while initiating repair processes. Such processes are important in fighting various diseases and infections, however, chronic inflammation or prolonged enhancement of such inflammatory

mediators has now emerged as a major contributing factor in the pathogenesis of psychiatric disorders including depression, as has been thoroughly described in several excellent reviews (Leonard, 2001, Vidrih et al., 2012, Lichtblau et al., 2013, Liu et al., 2014a, Slavich and Irwin, 2014). Initial evidence depicting this relationship emerged in the clinic, where it was observed that patients presenting with various inflammatory conditions such as heart disease, type-2 diabetes, autoimmune disease, arthritis and cancer often exhibit symptoms of depression (Waheed et al., 2006, Maneeton et al., 2014, Xiong et al., 2015, Semenkovich et al., 2015). Additionally, it was shown that pro-inflammatory cytokines including interleukin (IL)-1 β , IL-6 and tumour necrosis factor-alpha (TNF- α) are increased in the plasma and CSF of similar patient cohorts (Lichtblau et al., 2013, Young et al., 2014). Such increases, which may arise due to direct stimulation of the immune system and/or activation of specific glial cells within central compartments are broadly described in figure 1.4.1., and more specifically depicted in figure 1.4.1.2. Additionally, levels of anti-inflammatory cytokines such as IL-10 tend to be reduced in depressed patients (Dhabhar et al., 2009). Further evidence for an inflammatory role of in the pathogenesis of depression is provided in the finding that a reduction in the activity of cyclo-oxygenase 2 (COX-2) by celecoxib, a nonsteroidal anti-inflammatory drug, exhibits mood elevating effects in depressed patients (Muller et al., 2006, Abbasi et al., 2012) while aspirin was also reported to have similar effects to celecoxib in a pilot study in depression (Mendlewicz et al., 2006). These clinical findings are supported by preclinical studies demonstrating that several animal models of depression also exhibit changes in immune parameters (Stepanichev et al., 2014, Kubera et al., 2011, Song and Wang, 2011). Furthermore, direct stimulation of the innate and adaptive immune system is associated with changes in emotional responding and depressive-like symptomatology (anhedonia, lethargy, stress-induced immobility and altered appetite) [for review see (Dantzer and Walker, 2014)]. Pattern recognition receptors (PPRs) which are located on various immune cells function in the mediation and orchestration of the innate immune response. The most widely studied PRRs are a class of type-1 transmembrane glycoproteins known as toll-like receptors (TLRs). In particular, attention has focussed on TLR3 and TLR4 receptors, the activation of which induces a peripheral and central immune response. As depicted in figure 1.4.1, such processes can lead to increased levels of the

tryptophan-degrading enzyme, indoleamine 2, 3, dioxygenase (IDO) (Gibney et al., 2013, O'Connor et al., 2009), thus limiting tryptophan availability peripherally and provoking the excessive formation of kynurenine (Comai et al., 2011), itself a precursor of other toxins such as quinolinic acid (QA), an NMDA receptor agonist which upon transport to the CNS promote glutamate-mediated excitotoxicity and neurodegeneration (Mazarei et al., 2013, Walker et al., 2013, Wichers and Maes, 2004, McNally et al., 2008). Such limited availability of tryptophan for transport into the CNS also diminishes central levels of serotonin (Heyes et al., 1992). Alternatively peripheral immune activation can potentiate the entry of pro-inflammatory cytokines and other chemokines, prostenoids, glucocorticoids as well as other immune components into the brain, through a leaky blood-brain-barrier (BBB), activation of endothelial cells that line the cerebral vasculature and production of inflammatory mediators inside the BBB or by binding to cytokine receptors associated with the vagus nerve, signalling inflammatory changes in the brain (Dantzer et al., 2008). Such communication between peripheral and central compartments is depicted below in figure 1.4.1.

Figure 1.4.1. Effects of the inflammatory cascade on neural plasticity, cell survival and neurotransmission. Microglia are the primary recipients of peripheral inflammatory signals that reach the brain. Activated microglia, in turn, initiate an inflammatory cascade whereby release of relevant cytokines, chemokines, inflammatory mediators, and reactive nitrogen (RNS) and oxygen (ROS) species induces mutual activation of astroglia, thereby amplifying inflammatory signals within the brain. Cytokines, including IL-1 β , IL-6, and TNF-alpha, IFN- α and IFN- γ (from T cells), induce the enzyme, IDO, which breaks down tryptophan (TRP), the primary precursor of 5-HT, into quinolinic acid (QUIN), a potent NMDA receptor agonist and stimulator of glutamate (GLU) release. Excessive exposure to cytokines, compromises astrocyte function, thus leading to downregulation of glutamate transporters, impaired glutamate reuptake, and increased glutamate release, as well as decreased production of neurotrophic factors including BDNF. Collectively these processes ultimately disrupt neural plasticity through excitotoxicity and apoptosis. Reproduced from (Miller et al., 2009).

Once in the brain, the pro-inflammatory cytokines activate both neuronal and non-neuronal cells such as astrocytes and microglia (Rajkowska and Miguel-Hidalgo, 2007), the primary source of pro-inflammatory cytokines in the brain, which in turn, alter neurotransmission (Wichers and Maes, 2004), HPA axis activity (Kim et al., 2015) and neurotrophin levels such as that of BDNF, reducing neuronal repair and attenuating neurogenesis (Rajkowska and Miguel-Hidalgo, 2007, Kubera et al.,

2011). These immune changes result in an acute sickness response which is resolved by 24 hours, however a longer term depressive-like behavioural response is subsequently observed. For example, polyinosinic:polycytidylic acid (Poly I:C) is a dsRNA and viral mimetic which directly activates TLR3 subsequently inducing a reduction in saccharin (sweet) preference, a behavioural effect indicative of anhedonic behaviour, which correlated with enhanced microglial activity and expression levels of IFN- γ , IL-1 β , IL-6 and TNF- α , reduced BDNF and increased IDO and kynurenine in the frontal cortex and hippocampus of rats (Gibney et al., 2013). TLR4 agonism such as that occurring following administration of LPS and subsequent induction of an innate inflammatory cascade, similarly is associated with the development of depressive-like symptoms in rodents as determined through a reduction in locomotor activity and saccharin preference (Pan et al., 2013, O'Connor et al., 2009) and increased immobility time in the FST (Painsipp et al., 2011). Furthermore, LPS administration results in enhanced induction of the kynurenine pathway (O'Connor et al., 2009), altered glial morphology and neuronal loss in the hippocampus (Corona et al., 2013, Johansson et al., 2005) and other such regions which are highly functional in affective processing. Thus, at least in certain cases, the depressive state may arise due to altered immune function which ultimately impacts on neuronal processing underlying emotional responding.

1.4.1.1 IFN- α -induced depression

Direct evidence for the concept of inflammation-induced depression emerged when therapeutic immunomodulators were found to provoke the development of this psychiatric disorder. One such therapeutic is interferon-alpha (IFN- α), a pro-inflammatory cytokine commonly used to treat various viral diseases such as hepatitis B (Papatheodoridis et al., 2008) and C (Deutsch and Hadziyannis, 2008). IFN- α exerts such effects through targeting various stages of viral replication including viral entry, envelop uncoating, genome replication, protein assembly and release of viral progeny. IFN- α therapy also prevents viral spread through the induction of cellular apoptosis, an attribute which also promotes its employment for the treatment of various malignancies (Asmana Ningrum, 2014, Jablonowska et al., 2012). Additionally, this pro-inflammatory cytokine induces alterations in cell cycle

and differentiation in susceptible malignant cells (Sangfelt et al., 2000, Caraglia et al., 2013).

Endogenous IFN- α is a critical effector of the innate immune response. It is produced by plasmacytoid dendritic cells (pDCs), macrophages, fibroblasts and endothelial cells in response to PRR activation by viruses and as described above, targets many various stages of viral replication. Additionally, IFN- α can also activate different cell types in the immune system to promote viral clearance and inhibit replication. Its receptor, IFNAR, is ubiquitously expressed on all cell lineages and such expression on any and all cell types ensures that an IFN response to a virus may be induced upon infection [for review see Wang et al., 2012]. As depicted in the diagram below (figure 1.4.1.1), IFN- α has been proposed to mediate its effects specifically by binding to the IFNAR1 receptor which then induces activation of the JAK-STAT pathway (Platanias, 2005), leading to downstream nuclear events including the subsequent transcriptional induction of various IFN-stimulated genes (ISGs) including that of anti-viral proteins as well as pro-inflammatory cytokines such as IL-6 and TNF- α and other interferons including IFN- γ [for review see (Katze et al., 2008)]. Such pro-inflammatory responses in turn can activate alternative immune cells and inflammatory pathways including dendrites, neutrophils, natural killer (NK) and T cells, eventually leading to the exacerbated induction of IDO, the tryptophan-metabolising enzyme.

Figure 1.4.1.1. Schematic diagram illustrates IFN- α -mediated activation of the JAK-STAT signalling pathway. IFN- α has been proposed to mediate its effect by binding to its receptor which then induces activation of JAK-STAT pathway leading to downstream transcriptional induction of various IFN-stimulated genes (ISGs) including pro-inflammatory cytokines such as IL-6 and TNF- α and other interferons including IFN- γ [for review see (Katze et al., 2008)]. Reproduced from (Masumi, 2013).

While this treatment strategy has been very effective in treating hepatitis infections as well as malignancies, mood alterations have been shown to appear in up to 60% of patients (Raison et al., 2005, Capuron et al., 2002) and despite its therapeutic efficacy, the development of the aforementioned depressive symptoms often prompts dose reductions and eventual withdrawal or discontinuation of therapy (Ogawa et al., 2012, van Zonneveld et al., 2005).

Preclinical studies have aimed to mimic the clinical scenario, characterising depressive-like behaviour in rodents following administration of IFN- α . Repeated administration of IFN- α to rats and/or mice induces alterations in affective responding in many behavioural paradigms. Specifically, peripheral administration of the drug (s.c. and i.p.) induces despair behaviour in the tail suspension test (TST) (Ping et al., 2012, Friebe et al., 2013, Zheng et al., 2014) which continuous treatment with IFN- α , s.c., similarly promotes despair behaviour in the forced swim test (FST)

(Fahey et al., 2007a, Siddegowda et al., 2011b). Repeated administration of the drug, both centrally (i.c.v.) and peripherally (s.c.) also precludes an anhedonic phenotype as evidenced in the sucrose preference test (Fahey et al., 2007a, Ping et al., 2012, Hayley et al., 2013), another common evaluation of depressive and hedonic behaviour (see figure 3.1). However, it should be noted that not all preclinical studies have demonstrated emotional changes as a result of IFN- α administration, with the inconsistency being reported likely due to methodological differences including strain of animal and type of IFN- α , amount and duration of administration and behavioural testing used to examine responses. As a consequence of enhancing immune system activity (illustrated in figure 1.4.1.2), IFN- α is believed to modulate emotional responding by the induction of indoleamine 2, 3- dioxygenase (IDO), and subsequent induction of the kynurenine pathway as detailed above, whereby excessive formation of kynurenine (Comai et al., 2011), and its metabolites quinolinic acid (QA), NMDA agonist induces glutamate-mediated excitotoxicity and neurodegeneration (Mazarei et al., 2013, Walker et al., 2013, Wichers and Maes, 2004, McNally et al., 2008). Alternatively pro-inflammatory cytokines, including IFN- α , once in the brain, activate astrocytes and microglia (Rajkowska and Miguel-Hidalgo, 2007), which as stated earlier, alter neurotransmission (Wichers and Maes, 2004), HPA axis activity (Kim et al., 2015) and neurotrophin levels such as that of BDNF (Rajkowska and Miguel-Hidalgo, 2007, Kubera et al., 2011). Supporting the immune mediated effects on neuronal functioning, a recent study by Zheng and colleagues has shown that repeated administration of IFN- α in mice over a period of five weeks provokes chronic activation of microglial activity evidenced through enhanced protein expression of Iba1 in the hippocampal tissue, an essential limbic region in affective processing (Zheng et al., 2015). Furthermore, depressive-like behaviour as observed following IFN- α treatment was effectively reversed upon minocycline treatment, an inhibitor of microglial activity (Zheng et al., 2015).

While the effects of repeated IFN- α administration on depressive symptoms are now widely recognised, increasing evidence indicates that this treatment regime may also result in chronic pain, evidenced through the emergence of somatic symptomatology such as body pain, myalgias, headache, joint pain and abdominal pain (Shakoor et al., 2010, Nogueira et al., 2012, Capuron et al., 2002). It has been estimated that pain

may occur in up to 72% of IFN- α -treated patients (Nogueira et al., 2012). It is possible that such effects are also attributable to the above-described immune mediated effects on neuronal and glial functioning in discrete brain regions underlying emotional and/or nociceptive processing. Indeed, this mechanism has recently been proposed as a common substrate in the manifestation of pain-depression interactions (Walker et al., 2014). However, it is also possible that repeated exposure to this therapeutic cytokine may instigate changes in alternative pathways or neurobiological mediators that may specifically govern nociceptive responding such effects at the peripheral nociceptive terminals and/or ascending or descending pain pathways. Although to our knowledge, no studies have characterised pain behaviour following long-term administration of the drug in preclinical models, acute effects of IFN α on pain behaviour have been evaluated. Peripheral administration of IFN- α has been shown to evoke analgesic effects which are reversed upon treatment with naloxone, a μ -opioid receptor antagonist (Blalock and Smith, 1981), while central administration dose-dependently induces acute analgesia assessed as a reduction in the tail-flick reflex in both mice (Lee et al., 2010) and rats (Jiang et al., 2000). These data suggest that IFN- α may act at peripheral and/or central sites to modulate nociceptive tone, although the effects of long-term treatment are unknown.

Figure 1.4.1.2. Schematic diagram illustrates the cascade of inflammatory events leading to eventual neurotoxicity as a consequence of IFN- α therapy. Reproduced from (McNally et al., 2008).

1.4.2 Immune System in Pain Processing

It has long been recognized that inflammatory processes impact on nociceptive signalling and are critical in the development and maintenance of pathological pain conditions [for review see (Vallejo et al., 2010, Calvo et al., 2012b, Ji et al., 2013)]. While a full evaluation of this area is beyond the scope of this thesis, presented herein is an overview of the common held consensus on how the immune system may impact on nociceptive processing. Increased levels of IL-1 β , IL-6, TNF- α , IFN- γ and IL-2 have been identified in the plasma of chronic pain patients and the extent of this inflammatory state has shown to correlate directly with pain intensity (Koch et al., 2007, Ludwig et al., 2008, Kadetoff et al., 2012) while anti-inflammatory drugs, namely COX inhibitors are currently among the main class of prescribed analgesics, further exemplifying the importance of inflammatory processes in pain pathophysiology. Inflammatory mediators may modulate nociceptive transmission at various levels from peripheral, spinal and supraspinal levels. At a peripheral level, inflammatory mediators including bradykinin, prostaglandins, thromboxanes, leukotrienes, platelet-activating factor and nitric oxide can sensitise nociceptors, thus

altering pain thresholds in response to mechanical, thermal or chemical stimuli [for review see (Petho and Reeh, 2012)]. For example, intraplantar administration of LPS, a TLR4 agonist, induces an innate local inflammatory response resulting in a state of mechanical allodynia (Naidu et al., 2010, Booker et al., 2012). At a spinal level, activation of the innate immune system also provokes alterations in pain thresholds and several lines of evidence demonstrate that cytokines can directly activate astrocytes and microglia in the spinal cord prompting the respective initiation and maintenance of pathological or chronic pain states (Del Valle et al., 2009, Shi et al., 2012, Ji et al., 2013). For example, intrathecal administration of the TLR3 agonist, poly (I:C), induces behavioural, morphological, and biochemical changes similar to that of neuropathic injury while genetic deletion of TLR3 inhibits spinal nerve injury as induced by pro-inflammatory cytokines (Obata et al., 2008). Additionally, local induction of pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α , have each shown to induce a state of central sensitization evidenced through the emergence of hypersensitivity or enhanced excitatory synaptic transmission in isolated spinal cord slices (Kawasaki et al., 2008) (see figure 1.4.2). At a supraspinal level, magnetic resonance spectroscopy (MRS) has identified reduced N-acetylaspartate levels, indicative of neuronal injury or loss, and increased glial activation, in regions such as the frontal cortex and thalamus in a number of chronic pain conditions (Chang et al., 2013).

Figure 1.4.2. Pro- and anti-inflammatory roles for glia in central sensitisation. A, pro-inflammatory glia promotes sustained central sensitization leading to transcriptional changes in dorsal horn neurons thus altering function. Astrocytes respond to this ongoing synaptic activity by releasing of glutamate (Glu), ATP, TNF- α , IL-1 β , IL-6, nitric oxide (NO) and prostaglandin E2 (PGE2). Activated microglia are also a source of all of these pro-inflammatory factors. B, activated glia can also be anti-inflammatory or neuroprotective, as they release anti-inflammatory cytokines such as IL-10 and IL-4 and express cannabinoid receptors (CB1 and CB2) that have been shown to exert anti-inflammatory functions. Reproduced from (Milligan and Watkins, 2009).

1.4.3 Immune system in Depression-Pain Interactions

While substantial evidence indicates a role for inflammatory mediators and processes in depression and pain individually, there is now a wealth of evidence supporting a prominent role for immune processes in the development of co-morbid depression and pain. Clinically, it has been reported that many painful inflammatory conditions such as osteo- and rheumatoid arthritis as well as IBD and fibromyalgia frequently

associate with a high prevalence of depressive symptoms in comparison with healthy populations (Hawker et al., 2011, Khongsaengdao et al., 2000, Kvien, 2004, Kappelman et al., 2014) while in such instances, the intensity of depressive symptomatology has also been shown to directly correlate with pain severity (Khongsaengdao et al., 2000). Furthermore such co-morbid symptomatology is evident upon initiation of cytokine-based therapies such as IFN- α , whereby, as discussed earlier, up to 60% of patients develop depressive disorder and/or develop painful symptoms such as body pain, myalgias, headache, joint pain and abdominal pain (Shakoor et al., 2010, Nogueira et al., 2012, Capuron et al., 2002). Importantly, such painful symptoms present more frequently in the case of patients with co-morbid major depression e.g. headache, 90% vs. 70%; joint pain 54% vs. 48% (Shakoor et al., 2010), when compared with treated patients which do not display symptoms of altered affect, thus potentiating the evidence for inflammatory processes in the aetiology of depression-pain co-morbidity.

Furthermore, several studies have identified altered nociceptive responding in animal models of depression as described earlier (section 1.2), many of which have identified altered immune signalling concurrent with reported behavioural effects. For example, recent data from our own laboratory have shown enhanced microglial and astrocyte activation within supraspinal centres including the frontal cortex and amygdala following SNL in the OB rat model of depression, an effect greater than that observed when compared to either OB or SNL alone (Burke et al., 2015, Burke et al., 2014b). Furthermore, allodynic behaviour following SNL in the OB rat positively correlates with pro-inflammatory cytokine expression, namely IL-6 mRNA, in the amygdala (Burke et al., 2013b), while expression of markers of microglia and astrocyte activation, anti-inflammatory cytokines and proinflammatory chemokines are also increased in the PFC, when compared to either OB or SNL alone (Burke et al., 2014c, Burke et al., 2014a). The WKY rat, alternative genetic model of depression, exhibits exacerbated allodynia following the induction of peripheral nerve injury, an effect correlated with increased mRNA levels of IL-1 β in the brainstem and PFC (Apkarian et al., 2006). Maternal deprivation, an early life stress model of depression, has been shown to exhibit mechanical and cold allodynic sensitivity following peripheral nerve injury, effects associated with increased

expressional levels of IL-6 and TNF- α in the hippocampus (Burke et al., 2013c). Similarly, pain behaviour and associated exhibition of altered affective responding frequently associate with altered immune signalling. For instance, anxiety-related behaviour following administration of the inflammatory painful stimulus CFA, is associated with increased mRNA levels of TNF- α in the amygdala, while local blockade of the cytokine reversed the observed anxiety-like effects in mice with persistent inflammatory pain (Chen et al., 2013).

Thus, there is much clinical and preclinical evidence to suggest a role for underlying immune alteration in depression-pain co-morbidity. However, it remains to be determined if the inflammatory state induces changes in neuronal function in the same or alternative regions are responsible to the effects on emotional and nociceptive processing.

1.5 Endocannabinoid System

The plant *Cannabis sativa* has been used as a medicine throughout the world for several thousand years, with reports of its use in treating painful symptoms appearing as early as 2600 B.C. The principal psychoactive ingredient of *Cannabis sativa*, delta-9-tetrahydrocannabinol (Δ^9 -THC), was first identified in 1964 (Gaoni and Mechoulam, 1964) and subsequent studies to understand its mechanism of action have led to the discovery of the endogenous cannabinoid (endocannabinoid) system. This endocannabinoid system consists of the cannabinoid receptors (CB₁ and CB₂) (Matsuda et al., 1990, Munro et al., 1993, Devane et al., 1988), their naturally occurring endogenous ligands, the best characterized of which are N-arachidonylethanolamine (or anandamide; AEA) (Devane et al., 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam et al., 1995), and the enzymes involved in their biosynthesis and degradation. Other endocannabinoid ligands have also been identified including oleamide (Leggett et al., 2004), O-arachidonoyl ethanolamine (virodhamine) (Porter et al., 2002), 2-arachidonoyl glycerol ether (noladin ether) (Hanus et al., 2001) and N-arachidonoyl-dopamine (NADA) (Huang et al., 2002), although their physiological role has not been examined in detail. Endocannabinoid biosynthesis occurs on demand via hydrolysis of cell membrane phospholipid precursors. AEA is formed from the precursor *N*-

arachidonoylphosphatidylethanolamine (NAPE), due to the hydrolytic activity of the phospholipase D enzyme NAPE-PLD (Di Marzo et al., 1994, Sugiura et al., 1996), while fatty acid amide hydrolase (FAAH) is the primary enzyme responsible for the metabolism of this endocannabinoid (Cravatt et al., 1996) as evident in figure 1.5. In comparison, the main biosynthetic pathway for 2-AG involves the hydrolysis of the membrane phospholipid phosphatidylinositol by phospholipase C, producing 1,2-diacylglycerol which is then converted to 2-AG by diacylglycerol lipase (DAGL) (Sugiura et al., 1995, Prescott and Majerus, 1983). 2-AG is primarily metabolized by monoacylglycerol lipase (MAGL) (85%) (Blankman et al., 2007) (figure 1.5) although other enzymes including cyclooxygenase-2 (Kozak et al., 2000, Yu et al., 1997), lipoxygenase (van der Stelt et al., 2002), ABDH6 (serine hydrolase α/β -hydrolase domain) and ABDH12 (Blankman et al., 2007) have also been shown to play a role.

Figure 1.5. Schematic representation of the endocannabinoid system in pre- and postsynaptic neurons. The presynaptic terminal is located in the top, whereas the postsynaptic neuron is located in the bottom. Abbreviations: EMT, endocannabinoid membrane transporter; MAGL, monoacylglyceride lipase; DAGL, DAG lipase; AEA, anandamide; NAPE, N-arachidonoyl phosphatidylethanolamine; NAT, N-acyltransferase. Reproduced from (Pacher et al., 2006).

Upon release, endocannabinoids bind to and activate the G-protein coupled receptors CB₁ and/or CB₂. CB₁ receptors are highly expressed on presynaptic neurons throughout the human and rodent brain (Herkenham, 1991, Tsou et al., 1998, Mackie, 2008), the activation of which results in inhibition of cyclic AMP, activation of mitogen-activated protein kinase (MAPK), inhibition of N- and P/Q-type voltage-activated Ca²⁺ channels and induction of inwardly rectifying K⁺ currents, with the resultant inhibition of neurotransmitter release (Demuth and Molleman, 2006). CB₁ receptors have also been shown to be expressed on glia and on a wide range of peripheral tissues, though at lower levels than observed on neurons (Galiegue et al., 1995, Carlisle et al., 2002, Osei-Hyiaman et al., 2005, Cavuoto et al., 2007, Cota, 2007). In contrast, CB₂ receptors are widely distributed in peripheral tissues and organs, with a particularly high density on immune cells and tissues (Munro et al., 1993, Berdyshev, 2000, Sugiura et al., 2000) including on glia within the brain, with enhanced expression observed under neuroinflammatory conditions (Carlisle et al., 2002, Nunez et al., 2004, Rock et al., 2007). Accumulating evidence has also indicated that the CB₂ receptor is also expressed on subsets of neurons within the brain (Van Sickle et al., 2005, Gong et al., 2006, Baek et al., 2008, Zhang et al., 2014a), and thus also modulates neurotransmission (Roche and Finn, 2010, Atwood et al., 2012, Kim and Li, 2015). Endocannabinoids also have affinity for and activity at other receptors, 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). Activity at these receptors has been proposed to account, at least partially, for some of the differential effects observed with potent selective cannabinoid agonists and pharmacological modulators of endocannabinoid tone.

Due to the abundant central distribution of the endocannabinoid system, it is in a prime position to regulate neurophysiological activities such as emotional and nociceptive processing, which individually, have been active areas of research over the past decade with a number of excellent reviews synthesising the data supporting a role for the endocannabinoid system in modulating mood and nociception [for reviews see (Ashton and Moore, 2011, Hillard and Liu, 2014, Gorzalka and Hill, 2011, Boychuk et al., 2015, Rani Sagar et al., 2012, Ulugol, 2014)]. However, no

review to date has examined the evidence and potential role for the endocannabinoid system as a link between co-existent depression and pain, and thus the remainder of this introduction aims to collate and synthesize these data.

1.5.1 The role of the endocannabinoid system in depression-pain interactions

1.5.1.1 Clinical Evidence

There is much evidence currently available to demonstrate alterations in the endocannabinoid system in chronic pain (Richardson et al., 2008, Kaufmann et al., 2009) and in psychiatric (Gobbi et al., 2005, Hill and Gorzalka, 2005, Koethe et al., 2007) patient populations. For example, various genetic polymorphisms of CB₁ and CB₂ receptors have been identified in patients with major depression and bipolar disorder (Mitjans et al., 2013, Monteleone et al., 2010, Mitjans et al., 2012, Minocci et al., 2011), with a single nucleotide polymorphism in the CB₁ receptor reported to enhance the risk of treatment resistance in depression (Domschke et al., 2008) and the development of anhedonic depression following early life trauma (Agrawal et al., 2012). Alterations in the CB₁ receptor and FAAH have also been identified in patients with pain associated with migraine, Parkinson's disease and irritable bowel syndrome (Juhász et al., 2009, Park et al., 2011, Greenbaum et al., 2012). In addition, peripheral (serum) levels of endocannabinoids are reportedly reduced in both depressed patients (Hill et al., 2008b, Hill et al., 2009b) and those with chronic pain (Fichna et al., 2013). A recent study has reported enhanced plasma 2-AG levels and increased CB₁ and CB₂ mRNA expression on blood lymphocytes in osteoarthritic patients (La Porta et al., 2015). A significant positive correlation was observed between 2-AG levels, pain and depression, and a negative correlation of 2-AG with quality of life and visual memory was observed (La Porta et al., 2015). In addition, CB₁ receptor expression was positively correlated with depression scores, while CB₂ receptor expression was positively correlated with pain scores. These data indicate that key components of the endocannabinoid system are up-regulated in human osteoarthritis with significant correlations with pain and emotional symptoms. In addition to visual loss and sensory deficits, neuromyelitis optica is associated with significant pain (altered threshold responding and symptoms of

neuropathic pain), depression and increased plasma levels of 2-AG and AEA (Pellkofer et al., 2013). This study evaluated if a correlation existed between pain threshold and levels of endocannabinoids, demonstrating a considerable negative correlation between the plasma levels of 2-AG and mechanical pain thresholds in these patients, although this study did not evaluate if an association also existed with depressed mood. While the data suggest a possible association between pain, depression and the endocannabinoid system in osteoarthritis and neuromyelitis optica patients, further clinical studies are required to determine if alterations in the genetics, levels and activity of the endocannabinoid system exist in other patient groups exhibiting depression-pain comorbidity.

Few, if any, clinical studies have directly investigated the role or activity of cannabinoids in depression-pain interactions; however enhanced mood and improved quality of life have been reported in studies investigating the analgesic effects of cannabinoid-based therapies (table 1.5.1.1.) For instance, cannabis intake has been reported to improve muscle and nerve pain, as well as depression and anxiety symptomatology, in a group of HIV patients (Woolridge et al., 2005). In patients with advanced cancer, improvements in anxiety and overall distress have also been reported, in those of whom pain symptoms were managed by daily adjunctive administration of Cesamet® (nabilone, a Δ^9 -THC analogue) for 30 days (Maida et al., 2008), while similarly, a randomized, double blind, placebo controlled trial, examining the therapeutic benefit of nabilone in terms of pain management and quality of life in patients with fibromyalgia, identified significant pain relief and alleviation of anxiety symptoms after 4 weeks of therapy (Skrabek et al., 2008). In addition, seriously mentally ill correctional populations displayed a significant amelioration of symptoms as related to post traumatic stress disorder, as well as symptoms of chronic pain as reported following a retrospective evaluation investigating the efficacy of nabilone for the management of such disorders (Cameron et al., 2014). Furthermore, a multicentre retrospective survey of patients with chronic central neuropathic pain or fibromyalgia who were prescribed oral Δ^9 -THC (dronabinol), supplemental to existing medication, reported improved symptoms of both anxiety and depression after 7 months of treatment as assessed by the Hospital Anxiety and Depression Scale (Weber et al., 2009). Sativex® (1:1 ratio

of Δ^9 -THC:cannabidiol), a recently launched oromucosal spray which is indicated for resistant spasticity and pain in multiple sclerosis, has not yet been directly associated with significant mood changes. However, improvements in patients overall quality of life has been reported in clinical populations following 16 weeks of treatment (Vermersch, 2011) while in a separate randomized control clinical trial evaluating the effect of Sativex® in patients with chronic painful diabetic neuropathy, it was found that patients with co-morbid depression displayed significant improvements in pain symptomatology in comparison with non-depressed counterparts (Selvarajah et al., 2010). Collectively, the above studies suggest that when co-existent, both depression/anxiety and pain respond to exogenously administered cannabinoids, although it remains to be determined if the effects are mediated by common or parallel mechanisms. Recent evidence has demonstrated enhanced amygdala activity and reduced functional connectivity between the amygdala and somatosensory cortex correlate with Δ^9 -THC mediated reductions in the unpleasantness to ongoing pain (Lee et al., 2013), suggesting that the amygdala may provide a common neural circuit that for the association between emotional responding and pain.

	<i>Drug</i>	<i>Pain Measurement</i>	<i>Depression/ Anxiety Measurement</i>	<i>Outcomes in Pain</i>	<i>Outcomes in Depression/ Anxiety</i>	<i>Reference</i>
HIV	Cannabis	Pilot Questionnaire	Pilot Questionnaire	↓ muscle, nerve pain, headaches	↓ anxiety, depression	Woolridge <i>et al.</i> 2005
Cancer Pain	Nabilone (Cesamet®)	ESAS MSE	ESAS	↓ pain score, MSE	↓ anxiety, overall stress	Maida <i>et al.</i> 2008
Fibromyalgia	Nabilone	VAS FIQ	Anxiety	↓ pain	↓ anxiety	Skrabek <i>et al.</i> 2008
Mentally ill offenders	Nabilone	Self-reported pain severity	PCL-C GAF	↓ pain	↓ PTSD symptoms	Cameron <i>et al.</i> 2014
Multiple Sclerosis-related resistant spasticity	Sativex® (Δ ⁹ -THC, cannabidiol)	NRS spasticity score	QOL	↓ spasticity	↑ QOL	Vermersch 2011
Chronic Central Neuropathic Pain, Fibromyalgia	Δ ⁹ -THC	VRS, NRS, PDI	SF-12, QLIP, HADS,	↓ pain, pain intensity	↑ QOL, depression, ↓ anxiety	Weber <i>et al.</i> 2009
Painful Diabetic Peripheral Neuropathy	Sativex® (Δ ⁹ -THC, cannabidiol)	VAS	HADS, QOL	↓ pain (only in patients with baseline depression)	↑ QOL	Selvarajah <i>et al.</i> 2010

Table 1.5.1.1. Clinical studies which have shown the effects of cannabinoid-based therapies on symptoms of co-morbid depression and pain. Abbreviations: HIV, human immunodeficiency virus; VRS, verbal rating scale; NRS, numerical rating scale; PDI, pain disability index; SF-12, short form-12; QLIP, quality of life; HADS, hospital anxiety and depression scale; QOL, quality of life; VAS, visual analog scale; FIQ, fibromyalgia impact questionnaire; ESAS, Edmonton symptom assessment system; MSE, morphine sulphate equivalent; PCL-C, Post-traumatic Checklist–Civilian version; GAF, Global Assessment of Functioning; PTST, Post-traumatic stress disorder.

1.5.1.2 Preclinical Evidence supporting a role for the endocannabinoid system in depression-pain interactions

Numerous studies have reported alterations in endocannabinoid signalling in several animal models of pain (Lim et al., 2003, Zhang et al., 2003, Walczak et al., 2005, Mitirattanakul et al., 2006) and mood-related behaviour (Marco et al., 2014, Navarria et al., 2014, Vinod et al., 2012). However, there is limited direct evidence available characterising alterations in endocannabinoid function in animal models of co-existent depressive- and pain behaviour (see table 1.5.1.2 and 1.5.1.3). One of the first studies examining the role of the endocannabinoid system in this interaction was conducted by Takahashi and colleagues (2003), whereby outbred Swiss-albino mice were stratified into groups of ‘anxious’ and ‘non-anxious’ animals as determined by behavioural responses in the elevated plus maze, before subsequent exposure to intraplantar formalin administration (Takahashi et al., 2003). Despite the current hypothesis that the degree of anxiety may contribute to the perception of and response to the noxious stimulus, both anxious and non-anxious animals displayed comparable formalin-evoked biphasic nociceptive response profiles. Systemic pretreatment with Δ^9 -THC elicited an analgesic effect in both groups of animals, which was effectively blocked by systemic pre-treatment with a CB₁ receptor antagonist, rimonabant (Takahashi et al., 2003), indicating therefore, that the endocannabinoid system, more specifically, the CB₁ receptor, represents a potential treatment strategy for inflammatory pain in the presence and/or absence of anxiety and possibly other neuropsychiatric disorders. However, given that direct activation of central CB₁ receptors is responsible for the psychoactive effects of potent cannabinoid-based therapies, direct agonism may not be therapeutically viable for such conditions and thus, modulation of endocannabinoid tone through inhibition of associated metabolising enzymes would confer improved efficacy and safety relative to direct cannabinoid agonists.

The Wistar-Kyoto (WKY) rat is a genetically stress-sensitive strain of rat that exhibits a depression- and anxiety-related phenotype (Pare and Redei, 1993) and heightened nociceptive responding (Burke et al., 2010, Zeng et al., 2008). Interestingly, WKY rats associate with higher levels of FAAH and CB₁ receptor

activity as well as lower levels of AEA in the frontal cortex and hippocampus, when compared to Wistar counterparts (Vinod et al., 2012), while enhancing AEA tone by pharmacologically inhibiting FAAH activity results in an attenuation of depressive-like behaviour (sucrose preference test and FST) in this stress sensitive strain (Vinod et al., 2012). We have recently identified alterations in the endocannabinoid system accompaniment to enhanced formalin-evoked nociceptive behaviour in the Wistar-Kyoto (WKY) rat (Rea et al., 2014) (table 1.5.1.2). More specifically, we found that in WKY rats, intraplantar administration of the noxious inflammatory pain stimulus formalin resulted in a significant reduction in AEA in the rostral ventromedial medulla (RVM), a component of the descending pain pathway and as described earlier, synonymous with pain facilitation and/or inhibition, an effect which was not observed in Sprague Dawley (SD) counterparts. While intraplantar administration of formalin increased levels of 2-AG in the RVM of SD rats, this effect was not observed in WKY animals. Furthermore, exposure to formalin induced significant increases in mRNA expression of NAPE-PLD and DAGL- α , precursors of AEA and 2-AG respectively, in the RVM of SD rats, an effect again, not observed in the WKY strain. Pharmacological studies were carried out in order to evaluate the functional significance of the alterations in the endocannabinoid system in WKY rats in response to formalin revealed that enhancing endogenous AEA tone following systemic administration of the FAAH inhibitor, URB597, attenuated formalin-evoked hyperalgesic responding in WKY rats, while in comparison, CB₁ receptor antagonism augmented nociceptive responding. Furthermore, local CB₁ receptor blockade within the RVM attenuated the reduction in nociceptive behaviour induced by URB597 in WKY rats, an anti-nociceptive effect which was not observed in SD rats (Rea et al., 2014). These findings indicate a causative role of endocannabinoid dysregulation in hyperalgesic behaviour associated with negative affect and moreover, identify a role for AEA-induced activation of CB₁ receptors in the descending pain pathway, more specifically, the RVM, as a mediator of pain suppression in animal subjects predisposed to anxiety and depression. Additionally, our lab has also recently shown that the endocannabinoid system may also play a role in the differential effects of repeated homotypic stress on inflammatory pain-related behaviour in WKY versus SD rats (Jennings et al., 2015). Specifically, repeated forced swim stress exposure prolonged and attenuated formalin-evoked

nociceptive behaviour in SD and WKY rats respectively, alterations accompanied by differential effects of stress on AEA levels in the amygdala and MAGL expression in the spinal cord between SD and WKY rats (Jennings et al., 2015). These data indicate that changes in the tone of the endocannabinoid system in the amygdala and spinal cord may underlie the differential effects of stress on inflammatory pain behaviour between SD and WKY rats.

The chronic unpredictable stress (CUS) model of depression has been shown to exhibit thermal hyperalgesia in the hotplate test (Lomazzo et al., 2015), cold allodynia (Bravo et al., 2012), exacerbated trigeminovascular nociception (Zhang et al., 2013), inflammatory hyperalgesia in response to formalin administration (Shi et al., 2010) and persistent mechanical hyperalgesia following nerve growth factor administration (NGF) (Lomazzo et al., 2015). As for the above described genetic models of depression, exposure to CUS has been shown to elicit site-specific alterations in the endocannabinoid system. Example of such changes include a significant a reduction in AEA levels in the hypothalamus, prefrontal cortex, hippocampus and striatum (Hill et al., 2008a), downregulation of CB₁ receptors, reduced 2-AG levels and increased FAAH levels in the hippocampus (Hill et al., 2005, Reich et al., 2009), a decrease in CB₁ receptor density in the hypothalamus and striatum and increased CB₁ receptor density in the PFC (Hill et al., 2008a, McLaughlin et al., 2013). Furthermore, pharmacological intervention has been shown to attenuate depressive-like behaviours in the CUS model including that observed following MAGL inhibition with JZL184 (Zhong et al., 2014, Zhang et al., 2015). However, to the best of our knowledge, only one study to date has evaluated endocannabinoid modulation on co-occurring affective and pain responding in CUS exposed animals. Pre-treatment with the FAAH inhibitor, URB597, or MAGL inhibitor, JZL184, which enhances endogenous levels of AEA and 2-AG respectively, was shown to significantly attenuate CUS-induced anxiety-related behaviour in the light-dark box along with concurrent thermal hyperalgesia as measured in the hot plate test (Lomazzo et al., 2015). Importantly, URB597, but not JZL184, significantly reduced chronic widespread mechanical hyperalgesia, associated with intramuscular administration of nerve growth NGF in rats pre-expose to CUS rats, an effect accompanied by significant increases in brain levels of AEA.

(Lomazzo et al., 2015). These findings would therefore indicate an important role for AEA signalling in anxiety- and pain-related behaviour, in stress-exposed mice.

Alterations in endocannabinoid signalling have also been observed in animal models of chronic pain with co-morbid alteration in affective responding (see table 1.5.1.3). A recent report by Racz and colleagues has revealed a prominent role of CB₁-mediated events in affective behaviour as induced by neuropathic pain (Racz et al., 2015). In this particular study, a behavioural paradigm representing neuropathic pain was achieved through partial ligation of the sciatic nerve (PNL), a procedure which wild type and CB₁^{-/-} mice were exposed to. Wildtype and CB₁^{-/-} mice exhibited mechanical allodynia following PNL, confirming neuropathological presence. However, evaluation of anxiety- (light-dark test and the elevated zero-maze) and depressive-like (sucrose preference test) behaviour 4-7 weeks following the PNL procedure revealed deficits in affective responding in CB₁^{-/-}, but not wildtype, mice (Racz et al., 2015), thus demonstrating that CB₁ receptor activity confers some form of resilience to pain-related anxiety/depression highlighting a protective role for CB₁ receptors against the emotional consequences of neuropathic pain. In a similar fashion, La Porta et al recently investigated the role of the endocannabinoid system in affective and cognitive manifestations in an animal model of osteoarthritis (La Porta et al., 2015). This study revealed that the anxiety-related behaviour of osteoarthritic mice, identified in the elevated plus maze, was enhanced in CB₁^{-/-} and absent in CB₂^{-/-} mice, indicating differential effects of CB₁ and CB₂ receptors in mediating the affective dimension of pain in the model. Similar to effects in a neuropathic model (Racz et al., 2015), the data would indicate that CB₁ receptors confer resilience, while CB₂ receptors confer susceptibility to the development of arthritis-related anxiety. The authors suggest and provide some support, that the differential effects of CB₁ and CB₂ receptors may be mediated by alterations in HPA axis functionality and responses (La Porta et al., 2015). In addition, this study also demonstrated that acute pharmacological blockade of CB₁ or CB₂ receptors ameliorated both the nociceptive and affective dimension of pain in the model (La Porta et al., 2015). Taken together, the data suggests that cortico-limbic endocannabinoid signalling is a key modulator of different osteoarthritis pain manifestations.

Two particular studies have investigated the potential role of CB₂ receptor functionality in the interaction between neuropathic or inflammatory pain and associated affective responding. The CCI model of neuropathic pain associates with mechanical hypersensitivity and depressive-like behaviour in mice (Hu et al., 2009). These associative effects following CCI are significantly attenuated by systemic administration of the CB₂ receptor agonist, GW405833, in the absence of effect in sham-operated animals. Furthermore, such behavioural effects were superior to administration of a tricyclic anti-depressant, first line treatment for depression and chronic pain (Hu et al., 2009). Valenzano and colleagues have also reported that the same CB₂ receptor agonist GW405833, dose-dependently attenuates mechanical allodynia in the complete Freund's adjuvant (CFA) model of inflammatory pain, without altering associated anxiety-related behaviour (Valenzano et al., 2005). In this way, it can be deduced that while CB₂ receptor agonism elicits analgesic effects in chronic neuropathic and inflammatory pain models, its effects on affective responding may depend on the emotional paradigm (depression vs. anxiety), or the particular pain model itself under investigation. The exact mechanism by which CB₂ receptor agonism elicits this potent analgesic and antidepressant-like effect has not been evaluated, however given the well recognized role for inflammatory processes in mediating chronic pain, it is possible that CB₂ receptor activation attenuates such responses, preventing the development of central sensitization and mechanical allodynia and the associated increase in neuronal input to affective supraspinal sites. However, this hypothesis requires further clarification with future studies in the area.

Hedonic behaviours (pain-depressed behaviour) have been observed following intraperitoneal administration of a dilute concentration of lactic or acetic acid, known to induce abdominal stretching/writhing (visceral pain behaviour). Evaluation of the role of the endocannabinoid system in mediating pain-stimulated and pain-depressed/suppressed responses in this model has revealed that genetic blockade of CB₁ receptors enhances acid-induced writhing (visceral pain stimulated behaviour) and while enhancing acid-induced reductions in feeding (Miller et al., 2011). On the other hand, CB₁ receptor agonism through treatment with Δ^9 -THC or CP55940, dose-dependently inhibits acid-stimulated stretching while eliciting either no effect (Miller et al., 2012) or exacerbating (Kwilasz and Negus, 2012) acid-induced

depression of feeding and scheduled controlled/intracranial self-stimulation (ICSS) or direct activation of reward circuitry in rats. Therefore, as demonstrated in the current situation, potent synthetic cannabinoids such as Δ^9 -THC and CP55940 potentially elicit differential effects on visceral pain and associated depressive-like or hedonic behaviour. Similar to direct CB₁ receptor agonism, the FAAH inhibitor URB597 exhibits a dose-related and CB₁ receptor-mediated decrease in acid-stimulated stretching while suppressing feeding behaviour (Kwilasz et al., 2014, Miller et al., 2012). Furthermore, URB597 also elicits a delayed but significant attenuation of acid-induced suppression of ICSS, an effect independent of CB₁ or CB₂ mediation (Kwilasz et al., 2014). Taken together, these data indicate a role for CB₁ receptors in mediating acid-induced visceral pain, with a possible common and/or alternative endocannabinoid mechanism mediating the associated anhedonic/depressive-like behaviour.

<i>Depression/Affective Model</i>	<i>Nociceptive Effects</i>	<i>Cannabinoid-based drugs</i>		<i>Endocannabinoid-related changes/effects</i>	<i>Reference</i>
Anxiety-stratified (EPM), mouse	↑ formalin-evoked nociceptive responding in anxious and non-anxious	Δ ⁹ -THC, Rimonabant	CB _{1/2} agonist CB ₁ antagonist	Δ ⁹ -THC ↓ nociception in both anxious and non-anxious mice, Rimonabant blocked effects of Δ ⁹ -THC	Takahashdi <i>et al.</i> 2003
WKY rat	↑ formalin-evoked nociceptive responding			Formalin-induced ↓ AEA in RVM, No formalin-induced ↑ 2-AG, NAPE-PLD or DAGL-α in RVM (compared with SD)	Rea <i>et al.</i> 2014
		URB597 AM251	FAAH inhibitor CB ₁ antagonist	Systemic URB597 ↓ nociception Systemic AM251 ↑ nociception AM251 within RVM blocked effect of URB597	
Repeated FST, WKY rat	↓ formalin-evoked nociceptive responding			No ↑ MAGL in spinal cord No ↓ AEA in amygdala	Jennings <i>et al.</i> 2015
CUS, mouse	↓ latency to respond in HPT	URB597 JZL184	FAAH inhibitor, MAGL inhibitor	URB597 ↓ anxiety (EPM, LD) JZL184 ↓ anxiety (LD) Both ↓ thermal hyperalgesia	Lomazzo <i>et al.</i> 2015
CUS, mouse	Chronic mechanical hyperalgesia following NGF	URB597 JZL184	FAAH inhibitor, MAGL inhibitor	URB597 ↓ hyperalgesia No change with JZL184	Lomazzo <i>et al.</i> 2015

Table 1.5.1.2. Endocannabinoid-mediated effects/changes on affective and nociceptive behaviour in animal models of depression. Abbreviations: EPM, elevated plus maze; WKY, wistar-kyoto; FAAH, fatty acid amino hydrolase; AEA, anandamide; RVM, rostral ventromedial medulla; 2-AG, 2-arachidonoylglycerol; NAPE-PLD, *N*-acyl phosphatidylethanolamine-specific phospholipase D; DAGL-α, diacylglycerol lipase-alpha; SD, sprague dawley; CUS, chronic unpredicted stress; HPT, hot plate test; LD, light-dark box; MAGL, monoacylglycerol lipase; NGF, nerve growth factor.

<i>Pain Model</i>	<i>Depressive Effects</i>	<i>Cannabinoid-based drugs</i>		<i>Endocannabinoid-related changes/effects</i>	<i>Reference</i>
PNL, mouse	↑ Anxiety in LD and Zero Maze ↓ Sucrose Preference in CB ₁ ^{-/-} mice only			Anxiety and depressive effects only in CB ₁ ^{-/-} mice	Racz <i>et al.</i> 2015
Monosodium iodoacetate, mouse	↑ Anxiety in EPM Memory impairment in object recognition memory task	ACEA JWH133	CB ₁ agonist CB ₂ agonist	↑ anxiety in CB ₁ ^{-/-} mice no anxiety in CB ₂ ^{-/-} mice ACEA and JWH133 ↓ mechanical allodynia and anxiety ACEA ↓ memory impairment	La Porta <i>et al.</i> , 2015
CCI, rat	↑ Immobility in FST	GW405833	CB ₂ agonist	GW405833 ↓ mechanical hyperalgesia GW405833 ↓ immobility	Hu <i>et al.</i> 2009
Acid-Stimulated Stretching, rat	↓ Food intake ↓ ICSS	Δ ⁹ -THC, CP55940	CB _{1/2} agonist CB _{1/2} agonist	Both blocked stretching Both exacerbated ↓ ICSS No effect on feeding	Kwilacz <i>et al.</i> 2012
Acid-Stimulated Stretching, rat	↓ ICSS	URB597 Rimonabant SR144528	FAAH inhibitor CB ₁ antagonist CB ₂ antagonist	URB597 ↓ stretching; blocked by rimonabant, URB597 induced delayed partial attenuation of ICSS; not attenuated by rimonabant or SR144528	Kwilacz <i>et al.</i> 2014

Table 1.5.1.3. Endocannabinoid-mediated effects/changes on affective and nociceptive behaviour in animal models of pain. Abbreviations: PNL, partial sciatic nerve ligation; LD, light-dark box; CCI, chronic constrictive injury; FST, forced swim test; CFA, complete Freud's adjuvant; MBT, marble burying test; FAAH, fatty acid amino hydrolase; ICSS, intracranial self-stimulation.

1.5.2 Mechanisms

While the exact mechanism(s) by which the endocannabinoid system may influence emotional and nociceptive processing remains undetermined, this system is known to elicit potent modulatory effects on neurotransmission, neuroendocrine and inflammatory processes, all known to be altered in both depression and chronic pain. Presented here is an overview of how the endocannabinoid system may modulate affective and pain processing via interacting with these systems.

1.5.2.1 Neurotransmitters

1.5.2.1.1 GABA and glutamate

As described earlier, GABA- and glutamatergic neurotransmission are well recognised as important mediators in affect and nociceptive processing, and alterations in these systems have been demonstrated in both depression and chronic pain [for reviews see (Kendell et al., 2005, Rea et al., 2007)]. Additionally, CB₁ receptors are highly expressed on presynaptic nerve terminals of GABAergic and glutamatergic synapses in cortical and limbic areas of the brain, important higher centres associated with emotional response and pain modulation (Herkenham et al., 1990, Wittmann et al., 2007, Katona et al., 2001, Domenici et al., 2006). Endocannabinoids have been shown to exert behavioural effects through CB₁ receptor agonism and subsequent presynaptic inhibition of GABAergic and glutamatergic transmission (Meng et al., 1998, Millan, 2002) in supraspinal and spinal regions (Ulugol, 2014). On the other hand, glutamatergic neurotransmission is also known to enhance endocannabinoid formation and subsequent CB₁ receptor activation (Galante and Diana, 2004) and therefore, in this way, bidirectional interaction exists between the endocannabinoid system and glutamatergic-GABAergic transmission. Several studies have also demonstrated that CB₁ modulation of GABAergic signalling is important in nociceptive (Pernia-Andrade et al., 2009, Manning et al., 2003, Naderi et al., 2005) and emotional (Reich et al., 2013, Rey et al., 2012, Haller et al., 2007, Rossi et al., 2010, Naderi et al., 2008) processing. Although it would appear intuitive, no specific study has investigated if endocannabinoid modulation of GABA and/or glutamate plays a direct role in depression-pain interactions. However, our lab has previously demonstrated that CB₁

receptors play an important role in mediating analgesia in response to acute stress (contextual fear conditioning) (Finn et al., 2004, Butler et al., 2008) and associative important role for GABAergic and glutamatergic signalling in the basolateral amygdala in mediating this effect (Rea et al., 2014). Thus, endocannabinoid modulation of GABAergic and glutamatergic tone can mediate stress-pain interactions, and therefore may play a prominent role in co-existent psychiatric and pain disorders.

1.5.2.1.2 Monoamines

In addition to the treatment of depression, and as mentioned earlier (section 1.3.3) monoamine-based antidepressants are now regarded as first-line therapy for fibromyalgia and neuropathic pain. In addition, endocannabinoid-mediated modulation of serotonergic, noradrenergic and dopaminergic transmission has been thoroughly investigated in several excellent reviews (Haj-Dahmane and Shen, 2011, Kirilly et al., 2013, Melis and Pistis, 2012). CB₁ receptors are highly expressed on serotonergic, noradrenergic and dopaminergic neurons, and thus have been shown to regulate monoaminergic activity. Local and systemic administration of exogenous CB₁ receptor agonists significantly increases serotonin (Bambico et al., 2007), noradrenaline (Jentsch et al., 1997, Oropeza et al., 2005, Page et al., 2008, Page et al., 2007) and dopamine (Solinas et al., 2006, Cheer et al., 2004) levels in discrete brain regions responsible for emotional and nociceptive processing as well as the interaction thereof. Increasing endogenous levels of AEA and 2-AG, through systemic administration of FAAH or MAGL inhibitors respectively, also enhances serotonergic and dopaminergic activity (Gobbi et al., 2005, Seif et al., 2011). Additionally, endocannabinoids can also inhibit the activity of monoamine oxidase (MAO) (Fisar, 2010), the enzyme responsible for serotonin, noradrenaline and dopamine metabolism, which would thus, increase the synaptic availability of these monoamines. CB₁ receptor activation by specific cannabinoids has also been shown to control the function and expression of specific serotonin receptors including 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C}, in discrete regions of the CNS (Aso et al., 2009, Moranta et al., 2009, Zavitsanou et al., 2010, Franklin et al., 2013). Furthermore, while CB₁ activity has been shown to attenuate pain behaviour in the formalin test, such effects have shown to be compromised following spinal noradrenergic depletion (Gutierrez

et al., 2003), demonstrating that cannabinoids induce nociception, in part through modulation of descending noradrenergic systems. Thus, endocannabinoid-induced enhancement of monoaminergic tone may modulate emotional and nociceptive processes and thus the interaction between depression and pain.

1.5.2.1.3 Opioids

Numerous studies have characterized the causative role and therapeutic potential of opioidergic signalling in affective and nociceptive processing [for review see (Lutz and Kieffer, 2013, Maletic and Raison, 2009)] and much evidence is now available to suggest that the endocannabinoid and opioidergic systems interact in therapeutically beneficial ways. CB₁ and μ -opioid receptors are highly co-localized on neurons in areas of the brain associated with emotional and pain processing such as the caudate putamen, PAG and spinal cord (Rodriguez et al., 2001, Wilson-Poe et al., 2012, Salio et al., 2001) and co-administration of opioids and cannabinoids results in synergistic and bi-directional anti-nociceptive effects in several animal models (Cichewicz et al., 1999, Cichewicz and McCarthy, 2003, Tham et al., 2005, Roberts et al., 2006, Smith et al., 2007, Wilson-Poe et al., 2013, Wilson et al., 2008). Furthermore, enhancement of endocannabinoid tone attenuates withdrawal symptoms in morphine-dependent animals (Shahidi and Hasanein, 2011, Smith et al., 2007, Wilson et al., 2008). Interestingly, evidence for endocannabinoid-opioidergic interactions is exemplified through the existence of cross tolerance between these neuromodulatory systems. For instance, decreases in the analgesic effects of Δ^9 -THC have been identified in morphine-tolerant animals and *vice versa* (Thorat and Bhargava, 1994). As regards the emotional consequences of such interactions, it has been shown that inhibition of opioid signalling (via κ -opioid receptors) attenuates the anti-depressant-like effect of rimonabant (CB₁ receptor antagonist/inverse agonist) in the FST (Lockie et al., 2011) while conversely the antidepressant-like effects of κ -opioid receptor antagonism is attenuated by the CB₁ receptor antagonist/inverse agonist AM251 (Braidia et al., 2009). Although further studies are required, collectively these findings suggest a regulatory role of the endocannabinoid system on opioid transmission, which may underlie the maintenance of co-existent depression-pain processes.

1.5.2.2 Neuroendocrine activity – Hypothalamic-pituitary adrenal (HPA) axis

Dysregulation of the HPA axis has been implicated in the pathophysiology of both depression and pain disorders for decades [for review see (Belvederi Murri et al., 2014, Maric and Adzic, 2013, Bomholt et al., 2004, Vierck, 2006)] and thus, has also been proposed as a possible mediator in the depression-pain dyad [for review see (Blackburn-Munro, 2004)]. Additionally, several lines of evidence now support an important role for the endocannabinoid system as a modulator of HPA axis function and vice versa [for review see (Finn, 2010, Riebe and Wotjak, 2011, Hill and Tasker, 2012)]. The majority of evidence collated to date suggests that basal HPA activity is subject to tonic inhibitory control by CB₁ receptors as has been shown in numerous reports whereby genetic deletion or pharmacological blockade of the CB₁ receptor *in vivo* enhances expression of CRF and reduces glucocorticoid receptor expression in the hypothalamus and pituitary gland respectively (Cota et al., 2007), and increases circulating levels of corticosterone and adrenocorticotrophic hormone (Steiner et al., 2008, Cota et al., 2007, Barna et al., 2004). Additionally, stress-induced increases in CRF expression in the paraventricular nucleus (PVN) of the hypothalamus and the basolateral amygdala, as well as corticosterone secretion, are effectively blocked by pharmacological enhancement of endocannabinoid levels (Hill et al., 2009a, Bedse et al., 2014, Roberts et al., 2014, Patel et al., 2004), indicating a role for endocannabinoid-CB₁ receptor signalling in diminishing such hyperactivity of the HPA axis. Furthermore, recent evidence has shown that CRF1 activation in the amygdala induces FAAH and reduces anandamide levels, an effect associated with anxiety-related behaviour (Gray et al., 2015). Given the important role of the amygdala in affective modulation of pain, it is possible that CRF mediated FAAH activation in this region may also modulate nociceptive processing and associated emotional alterations. While there have been a few studies examining endocannabinoid-HPA axis effects in mediating the effects of the stress response (Roberts et al., 2014, Hill et al., 2011), to date no study has investigated if cannabinoid mediated alterations of the HPA axis underlies alterations in nociceptive and/or affective behaviour observed in depression-pain comorbidity.

1.5.2.3 Neuroimmune processes

Increasing evidence indicates a potent and prominent interaction between inflammation, depression and pain [for review see (Walker et al., 2014)]. For instance, there is a high prevalence of depression among patients with inflammatory pain disorders such as fibromyalgia, arthritis and inflammatory bowel disease (Scheidt et al., 2014, Lin et al., 2015, Kappelman et al., 2014). In addition, patients receiving cytokine therapy for specific cancers and malignancies also develop depressive and/or painful symptomatology (Capuron et al., 2001, Capuron and Ravaud, 1999, Nogueira et al., 2012). Furthermore, increases in serum and cerebrospinal fluid levels of pro-inflammatory cytokines have been widely reported in both depression (Knuth et al., 2014, Tuglu et al., 2003, Bay-Richter et al., 2015) and pain conditions (Ludwig et al., 2008, Koch et al., 2007, Kadetoff et al., 2012). Inflammatory processes have also been shown to underlie the interaction between depression and pain in several animal models. For instance, increased expression of pro-inflammatory cytokines, concomitant with depressive-like behaviour has been identified in animal models of inflammatory (Kim et al., 2012a, Maciel et al., 2013) and neuropathic (Norman et al., 2010, Zhou et al., 2015, Dellarole et al., 2014, Burke et al., 2014b) pain. The innate inflammatory cascade has been shown to increase glutamate neurotransmission, central sensitization and excitotoxicity, reduce BDNF and neurogenesis and activate neurodegenerative cascades, events observed in both depression and pain conditions [for review (Dantzer et al., 2011, Maes et al., 2011, Song and Wang, 2011, Zunszain et al., 2013, Walker et al., 2014)].

Over the past decade, a wealth of data has demonstrated an important role for the endocannabinoid system in modulating innate immune function and inflammatory processes [for review, see (Henry et al., 2015, Zajkowska et al., 2014, Alhouayek and Muccioli, 2012)]. Interactions between the endocannabinoid system and inflammatory mediators has been shown to influence synaptic transmission and neuronal function (Rossi et al., 2014). Spinal cord injury has been shown to be associated with increased co-expression of CB₁ receptors with chemokines CCL2, CCL3, and/or CCR2 in the hippocampus, thalamus and periaqueductal grey; areas associated with affective pain responding (Knerlich-Lukoschus et al., 2011) and studies have also demonstrated that CB₁-chemokine interactions in the PAG can

modulate nociceptive responding (Benamar et al., 2008). Pharmacological enhancement of endocannabinoid tone also modulates inflammatory effects *in vivo*. For example, the FAAH inhibitor URB597 and the MAGL inhibitor JZL184 attenuates inflammation induced astrocyte and microglial activation (Katz et al., 2015) and neuroinflammatory processes (Henry et al., 2014, Kerr et al., 2013, Kerr et al., 2012). Furthermore, Zoppi and colleagues have demonstrated that pharmacological activation of CB₁ or CB₂ receptors attenuates, while genetic deletion of these receptors augments, repeated stress-induced pro-inflammatory responses in the frontal cortex (Zoppi et al., 2011, Zoppi et al., 2014). In addition, several studies have demonstrated that the analgesic effects of cannabinoids in chronic inflammatory and neuropathic pain are, at least, partially mediated by modulation of inflammatory responses (Lu et al., 2015, Burston et al., 2013, Burgos et al., 2012, Wilkerson et al., 2012). While there are no studies to date investigating if cannabinoids modulation of inflammatory processes underlies co-existent depressive-and pain behaviour, the above findings suggest a potential role for cannabinoid-mediated immunomodulation in the pathogenesis and treatment of co-occurring depression and pain.

As described above, there is much clinical and preclinical evidence available to support a role for the endocannabinoid system in mediating pain-depression interactions. While targeting the endocannabinoid system for therapeutic benefit in pain and psychiatric disorders has been explored, no study to date has specifically evaluated the effects of cannabinoids on depression-pain comorbidity, although some have evaluated mood and quality of life in patients receiving cannabinoid-based treatments for analgesic purposes (Weber et al., 2009, Skrabek et al., 2008, Maida et al., 2008, Cameron et al., 2014). Preclinical models that encapsulate the clinical scenario are particularly useful in gaining greater understanding on the neurobiology underlying depression-pain interactions and these have highlighted a particular role for AEA and CB₁ receptors in mediating and modulating the affective and nociceptive processes. However, there is limited data available examining the role of other components of the endocannabinoid system on depression-pain interactions (2-AG, CB₂, PPAR etc), whether the endocannabinoid modulation of affect and nociception occur through the same or parallel pathways, and the mechanism by

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which the endocannabinoid system may mediate its behavioural effects (neurotransmitters, HPA axis, inflammation or a combination). Such studies are essential towards a more comprehensive understanding of the neurobiology underlying the association between these pain and depression and fully explore the potential efficacy of targeting the endocannabinoid system for resolution of these co-morbid conditions.

1.6 Overall objectives and experimental approach

The main objective of the work presented herein was to improve our understanding of the role of the immune system in modulating emotional and nociceptive processing which may inform on the neurobiological mechanisms underlying co-existent depression and pain. Given the clinical evidence demonstrating the IFN- α treatment induces both depression and pain in patients, a particular aim of this thesis was to determine if this phenomenon could be replicated preclinically. The establishment of a preclinical animal model of IFN- α -induced depression and hyperalgesia would then enable the investigation of associated changes in inflammatory mediators, neurotrophins, HPA axis activity and neurotransmitters, changes which may underlie the behavioural phenotype. With the increasing interest in the endocannabinoid system as a potential target for depression and pain treatment, the role of this system in mediating the hyperalgesia observed following repeated IFN- α was evaluated.

Thus the hypothesis for this thesis was that repeated IFN- α administration induces depressive-like behaviour and alterations in nociceptive responding in mice, effects which are mediated by changes in inflammatory, neuroendocrine and/or neurotransmitter systems.

In order to appropriately investigate and evaluate this hypothesis, a series of aims were derived and are as follows:

1. Investigate the effects of repeated IFN- α exposure on depressive-like and nociceptive behaviour in two separate mouse strains. While many preclinical studies have characterized depressive-like behaviour following similar continuous administration of the drug to rodents (Siddegowda et al., 2011b, Ping et al., 2012), no study to date has investigated the potential of associative alteration in nociceptive responding in such models. Thus, thermal and inflammatory nociceptive responding was also assessed in these animals following repeated administration of hIFN- α (chapter 3 and 4).

2. Following the establishment of a treatment regime that resulted in both a depressive-like phenotype and hyperalgesia, associated changes in central and peripheral immune parameters were assessed in order to establish the presence of a persistent inflammatory state as result of chronic IFN- α treatment, while the neurotrophin BDNF, IDO and activity of the HPA axis were also evaluated at the time when behavioural changes were observed (chapter 4).

3. Given the proposed role of endocannabinoid signalling in mediating depression-pain interactions, the effect of repeated IFN- α administration on endocannabinoid and *N*-ethanolamine levels and associated enzyme and receptor expression in the supraspinal regions that regulate emotion and pain such as the prefrontal cortex, PAG and RVM, and in the spinal cord and the paw tissue were examined in the presence and absence of nociceptive tone (chapter 5). Subsequent studies examine the effect of enhancing AEA or 2-AG tone peripherally at the site of the noxious stimulus (chapter 6) and globally (chapter 7) on nociceptive responding in IFN- α -treated animals in comparison to controls.

Chapter 2

Materials and Methods

2.1 Animal Husbandry

Male C57Bl/6 mice (Charles River, UK) were used for the majority of studies (apart from studies described in Chapter 3, where CD1 mice were also used). Mice weighed 25-30g on arrival and were housed in groups of 3 or 4 in plastic bottomed ($14 \times 36 \times 20$ cm) containing wood shavings as bedding (changed twice weekly), in a temperature controlled room ($21 \pm 2^\circ\text{C}$), relative humidity of 40-60%, with a 12 : 12 hour light-dark cycle (lights on at 0700h). Mice were fed a standard laboratory diet of rat chow pellets (2014 14% rodent diet, Harlan Laboratories UK Ltd., Leics, UK); food and water were available *ad libitum*. The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

2.2. Pharmacological Treatments

Human interferon-alpha (hIFN- α -2a; 3M IU/0.5ml), Roferon-A®, was purchased from Roche Pharmaceuticals. The drug was prepared in saline from stock on a daily basis. IFN- α solution was administered subcutaneously in a volume of $3\mu\text{l/g}$ to give a final concentration of 400, 800 or 8,000 IU/g hIFN- α , to be injected subcutaneously on a daily basis. Note that 1IU hIFN- α is equivalent to 4pg of the same. Control animals were administered saline in an equivalent volume. Dosing occurred daily, between the hours of 10.00 and 12.00. For initial studies (chapter 3), the doses chosen were based on published research demonstrating depressive-like behaviour following repeated administration of hIFN- α (400-1,600IU/g) in mice (Siddegowda et al., 2011b). For later studies (chapter 4, 5, 6 and 7), the dose chosen was based on the minimum behavioural effects of lower doses observed in chapter 3 and published research demonstrating depressive-like behaviour following repeated administration of IFN- α (6,000IU/g) in mice (Ping et al., 2012). The lowest dose of IFN- α employed throughout, 400IU/g, is within that used clinically; IFN- α doses can range from, for example, 3 MIU, s.c., three times per week for chronic Hepatitis C virus to 36 MIU, s.c., daily, for AIDS-related Kaposi's sarcoma. The higher doses employed, 800IU/g and 8,000IU/g, are equivalent to that which are approximately

1.5 to 15 times higher than the maximum recommended daily dose of IFN- α for AIDS-related Kaposi's sarcoma.

2.3 Behavioural Testing

Behavioural testing was performed between the hours of 10.00 hr and 16.00 hr and occurred 20-24 hours after drug or vehicle administration to avoid acute effects of drug administration. Exceptions to this included the hot plate test which was carried out at one hour post IFN- α or saline administration (chapter 3) and the 24-hour sucrose preference test which began at 07.00, directly after IFN- α or saline administration (chapter 4). Behavioural testing occurred in a separate room with the exception of the open field, sucrose preference and hot plate tests which were carried out in the animal holding room. After each behavioural testing session, animals were returned to their cages. All tests were recorded by video camera onto DVD or hard drive and analysed at a later date, using EthoVision XT Version 8.5 video tracking software (Nodulus, Netherlands).

2.3.1. Behavioural measurements of locomotor activity

Many of the behavioural tests employed rely on motor responses as readout; therefore it was important to assess the effect of treatments on this parameter. Locomotor activity was assessed throughout each study at various time points using the open field test and was also assessed during the elevated plus maze (EPM) and formalin test.

2.3.1.1. Open field test

Locomotor activity was assessed using the open field test directly before initiation of treatment and on day 1, 3, 5, 7 and 10 of IFN- α or saline administration. The apparatus itself consisted of a wooden arena, divided into four equal quadrants forming four individual open fields (30 x 30 x 30 cm). Three/four mice, including at least one from each treatment group, were tested simultaneously for a five minute period and behaviour recorded onto DVD. Distance moved (cm) per minute and for the entire trial was assessed using Ethovision and this reflected locomotor activity. Animals were returned to their home cage following testing. The floors and walls of

the apparatus were cleaned and dried at the beginning of the day and between each trial with a mild disinfectant solution (Milton and warm water 1:10).

2.3.2 Behavioural measurements of affective behaviour

Affective behavioural responding was assessed in a variety of tests. Despair/depressive-like behaviour was evaluated in the tail suspension (TST) and forced swim (FST) tests, while hedonic behaviour was assessed in the sucrose preference test. Anxiety-related behaviour was analysed in the elevated plus maze (EPM)

2.3.2.1. Tail Suspension Test (TST)

The TST is widely used to assess depressive-like behaviour and anti-depressant activity in mice (Steru et al., 1985). This test involved mice being suspended by taping the distal part of the tail (figure 2.3.2.1) about 2cm from the end, to a 10cm long piece of tape which attached to a wooden surface approximately 30cm from the floor. Movements were observed and recorded for a total of six minutes. Behaviour during the final four minutes was then analysed with the aid of Ethovision XT software for the duration of immobility (seconds) over the trail, an increase in which has been proposed as an indicator of depression-like behaviour (Steru et al., 1985). Latency to immobility was also recorded.

Figure 2.3.2.1. Picture illustrates a mouse forming an immobile posture, indicative of despair behaviour, in the tail suspension test (TST), a common test employed for the assessment of despair behaviour. Adapted from (Berrocso et al., 2013)

2.3.2.2 Forced Swim Test

The FST is a commonly used method of assessing depressive-like behaviour and antidepressant-like activity in mice and rats. As previously described (Porsolt et al., 1977), mice were individually placed in a vertical glass cylinder (25 x 50 cm) filled with 25cm deep water at $25 \pm 2^{\circ}\text{C}$ (see figure 2.3.2.2) for six minutes and behaviour was recorded thereafter. Behaviour was recorded for six minutes, of which the last four minutes were analysed for the duration of immobility (seconds) while the initial two minutes allowed for habituation to the test. Mice were deemed immobile when they floated in an upright position or when only small movements were made to keep the head above water. Latency to immobility was also recorded. After testing, animals were removed from water and wrapped in paper towels for thorough drying before return to home cage.

Figure 2.3.2.2. Forced swim test (FST) for the assessment of despair behaviour. Reproduced from (Li et al., 2007).

2.3.2.3 Sucrose Preference Test

The sucrose preference test is commonly used to measure of depression-related anhedonia and was carried out similar to that previously described (Moreau et al., 2008, Painsipp et al., 2011, Ping et al., 2012). In this test rodents presented with the choice to drink from two bottles, one filled with tap water and the other with 1% sucrose solution (diluted in tap water) over a 24 hr period (see figure 2.3.2.3). After 12 hours, the relative position of the two bottles was changed to avoid side

preference. Animals were presented with the choice of solutions for a total of five days prior to IFN- α or saline administration, in order to establish baseline sucrose preference. After each 24hr period, bottles were weighed and the consumption of sucrose and water was calculated. Consumption of sucrose was expressed as (i) total sucrose intake, (ii) sucrose intake per 100g body weight (sucrose intake*100/body weight) and (iii) % sucrose preference relative to the entire quantity of fluid consumed (sucrose intake*100/total fluid intake). These methods were applied for the entire 24-hour test. Once baseline preference (>75%) established, the test was carried out at various stages following initiation of treatment.

Figure 2.3.2.3. Sucrose preference test (SPT) for the assessment of hedonic behaviour. Picture: Neurobehaviour Core, Lunenfeld-Tanenbaum Research Institute, 2015.

2.3.2.4 Elevated Plus Maze (EPM)

The elevated plus maze is a widely used behavioural assay for rodents and it has been validated to assess the anxiety-related behaviour. The test was carried out as previously described (Lister, 1987). Mice were subjected to the EPM after open field exposure. The arena consisted of a wooden apparatus, elevated to a height of 55 cm above the floor. The maze itself consisted of two open (50 x 10 cm; lux, 65) and two closed arms (50 x 10 x 30 cm; lux, 30) extending from a central platform (10 x 10 cm) (see figure 2.3.2.4). Mice were placed individually on the central platform facing an open arm and behaviour recorded for a period of five minutes. The maze was cleaned at the beginning of the day and between each trial with mild disinfectant solution (Milton and warm water, 1:10). Anxiety-related behaviour was analysed by

assessing the time spent in open (s) and closed arms, as well as the number of entries by the subjects into the open arms. Locomotor activity was also assessed as distance moved (cm) over the duration of the trial.

Figure 2.3.2.4. Elevated plus maze for the assessment of anxiety-related behaviour. Picture: Anonymous.

2.3.3 Behavioural measurements of nociceptive behaviour

2.3.3.1 Hot Plate Test

The hot plate test evaluates acute nociceptive responding to a noxious thermal stimulus observation of withdrawal behaviours including the licking of feet, jumping and rapidly stamping paws as previously demonstrated (Nishihara et al., 1995, Richardson et al., 1998). For the current series of studies, animals were exposed to the test 24 hours following the previous drug administration, unless the acute effect of the drug on thermal nociceptive responding was under evaluation (chapter 3). During the test period, mice were individually placed on a hot ceramic plate (temperature 54 – 55 °C) encased by a plastic transparent wall (Hot Plate Analgesia Meter, Harvard Apparatus, MA) similar to that in figure 2.3.3.1 and latency to respond was recorded. For consistency between subjects and studies, ‘response’ was specifically characterised as distinct licking of the front paw. Animal were removed from the testing apparatus immediately following the first observation of responding

with a time limit of 20 seconds allowed to respond. Animals which failed to respond to the stimulus by 20 seconds were removed from the arena. All animals were returned to their home cages following testing. The hot plate apparatus itself was cleaned and dried with mild disinfectant solution (Milton and warm water, 1:10) before testing and between each trial.

Figure 2.3.3.1. Hot plate test (HPT) for the evaluation of acute thermal nociceptive responding. Picture: Behavioural Testing Core Facility, University of California, Los Angeles, 2015.

2.3.3.2 Formalin Test

The formalin test was employed to examine nociceptive behavioural responding to a noxious inflammatory stimulus as previously described (Burke et al., 2010, Olango et al., 2012, Rea et al., 2014). The test itself is a well-established model of persistent pain characterized by a transient, biphasic pattern of pain behaviour. The initial early phase involves acute activation of C and A δ fibres (McNamara et al., 2007) while the late phase similarly represents primary afferent input as well as inflammatory processes in the injured tissue (Tjolsen et al., 1992) and the development of central sensitization (Coderre and Melzack, 1992).

For the test, 20 μ l of a 1% solution of formalin (Sigma Aldrich; Ireland) was injected into the left hind-paw of mice following which the animal was then placed in a plastic, transparent, square arena (30 x 30 x 30 cm) (see figure 2.3.3.2). Behaviour was recorded for one hour thereafter (or for 35 minutes, as is the case in chapter 5) and later rated with the aid of EthoVision XT software (Noldus Netherlands).

Formalin-evoked nociceptive behaviour was scored according to the weighted composite pain scoring technique (CPS-WST_{0,1,2}) (Watson et al., 1997). According to this method, pain behaviours are categorised as time spent raising the formalin-injected paw above the floor without contact with any other surface (C1), and holding, licking, biting, shaking, or flinching the injected paw (C2) to obtain a CPS [$CPS = (C1 + 2(C2)) / (\text{total duration of analysis period})$]. In early studies (chapter 3), general behaviours including locomotor activity as well as grooming (repetitive licking) and rearing (animal raising itself upright on its hind paws) behaviour was also evaluated during this 60-minute test. Formalin-induced oedema was assessed at the end of the test by measuring the change in diameter of the left hind paw immediately before and one hour after formalin administration, using Vernier callipers. Animals were sacrificed immediately following exposure to this test. The formalin arena itself was cleaned with mild disinfectant solution (Milton and warm water, 1:10) before testing and between each trial.

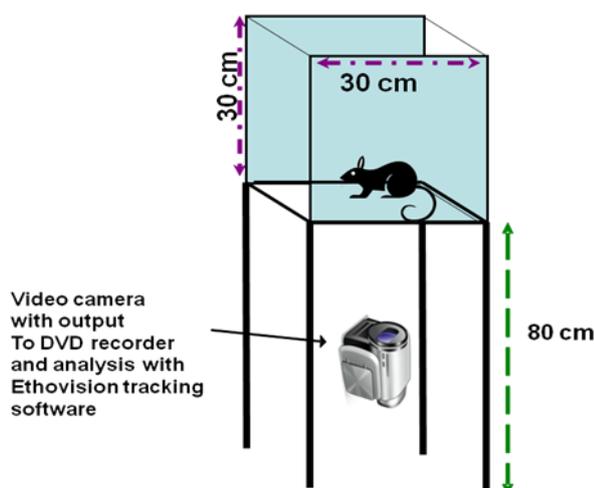


Figure 2.3.3.2. Formalin test arena for the evaluation of inflammatory pain behaviour.

2.4. Animal sacrifice and tissue collection

At the end of all testing, animals were sacrificed by deep isoflurane anesthesia. This was a rapid process to ensure humane experimental endpoint for all studies.

2.4.1. Blood Collection

Upon induction of deep anesthesia, blood was immediately removed through cardiac puncture with pre-coated heparin (1000IU) syringes. Blood was then collected in 15ml tubes, stored at 4 °C for one hour to allow for coagulation, before centrifugation at 5,000g for 15 minutes at the same temperature after which, plasma was removed and stored at -80 °C until analysis of corticosterone levels.

2.4.2. Spleen Collection

After blood collection, the spleen was removed with a forceps and stored at -80 °C until assessment of gene expression and protein analysis of inflammatory mediators.

2.4.3. Removal of brains and dissection of discrete brain regions

After sacrifice, the carcass was decapitated for brain removal. An incision was made using a scalpel along the top of the head and the skin pulled back to expose the skull. Using a scissors, a cut was made carefully along the midline of the skull from the back, while pressure was kept away from the brain surface, and the skull was removed. The dura mater was removed and using a curved forceps, the brain removed from the skull.

Discrete brain regions (prefrontal cortex (PFC), periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) were carefully gross dissected on an ice-cold plate which was sprayed with RNaseZap® (Invitrogen, Dublin, Ireland) to remove RNases. Figure 2.3.3 illustrates the approximate level of the dissections performed. The PFC was divided into right and left, while PAG and RVM were kept whole, placed into RNase free microfuge tubes, snap-frozen on dry ice and stored at -80°C for further analysis.

2.4.4. Removal of spinal cords

The spinal cord was removed using a scissors, an incision was made down the length of the back of the carcass and the muscle was removed until the spinal column was visible. Subsequently, two incisions were made either side of the column such that the dorsal part of the bone could be removed. The thoracic and lumbar vertebrae

were removed with scissors and the spinal cord was flushed from the column using a syringe filled with ice-cold sterile PBS. The lumbar enlargement containing the L4-L6 region was identified and isolated, the ipsi- and contralateral sides identified and removed, placed into RNase free microfuge tubes, snap-frozen on dry ice and stored at -80 °C for further analysis.

2.4.5 Removal of plantar paw tissue

The paw skin was dissected from the entire dorsal paw surface excluding the toes as illustrated in figure 2.3.5. This was achieved firstly through four incisions on the paw surface using a sterile scalpel blade and subsequent removal of the skin layer using a forceps and scissors. Once removed, the tissue was placed into RNase free microfuge tubes, snap-frozen on dry ice and stored at -80 °C for future analysis

Figure 2.4.5. Schematic diagram showing whole dorsal hind paw skin which was removed following animal sacrifice. Reproduced from (Guindon et al., 2011).

2.5 Gene expression analysis of inflammatory mediators, neurotrophin and components of the endocannabinoid system using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.5.1 RNA isolation

Total RNA was extracted from the PFC, PAG, RVM and paw tissue using Nucleospin® RNA II total isolation kit (Macherey-Nagel, Germany). Prior to the extraction process, due to increased tissue elasticity, paw tissue samples were ground into a fine powder using a mortar and pestle over dry ice. All samples were then homogenised with a polytron tissue disrupter, Ultra-Turrax (IKA®-Werke, Staufen, Germany) in 354µl of RA1 lysis buffer containing 1% β-mercaptoethanol (Sigma,

Dublin, Ireland). Homogenates were then filtered in a Nucleospin filter, centrifuged at 14,000g for one minute before addition of 350µl of 70% molecular grade ethanol (Sigma, Dublin, Ireland). This was then mixed by pipetting up and down 10 times. The samples were transferred to Nucleospin RNA II columns and centrifuged at 14,000g for 30 seconds to bind the RNA. 350µl of membrane desalting buffer (MDB, supplied with kit) was then added and columns were once again spun at 14,000g for 1 minute. DNA was removed using rDNase and DNase reaction buffer (supplied in kit). rDNase was diluted 1:10 in DNase reaction buffer and 95µl of this solution was pipetted directly onto the centre of each column. Columns were then left to stand for 15 minutes at room temperature. The rDNase was then inactivated by the addition of 200µl RA2 buffer, and the columns were then centrifuged at 14,000g for 30 seconds. 600µl of RA3 wash buffer was then added to the column which was then centrifuged again at 14,000g for a further 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 (1.5ml) and the RNA was eluted by the addition of 60µl of RNase-free water (Sigma, Dublin, Ireland) and centrifugation at 14,000g for 1 minute. The eluted RNA was then stored at -80 °C until quantification and reverse transcription.

2.5.2 RNA quantification and equalisation

The quantity and quality of RNA was assessed using a Nanodrop (ND-1000, Nanodrop, Labtech International, Ringmer, UK). Quantity of RNA was determined by measuring optical density (OD) at 260nm. Quality was concurrently determined by measuring the ratio OD260/OD280 and a ratio of approximately 1.8-2.1 was deemed indicative of pure RNA. Prior to cDNA synthesis, samples were equalised to the same concentration of RNA (average, 100ng/ul) with RNase free water and samples were then stored at -80 °C until reverse transcribed.

2.5.3 Reverse transcription of RNA

A high capacity complementary DNA (cDNA) kit (Applied Biosystems, Warrington, UK) was used to reverse transcribe RNA samples into cDNA. The kit master mix which provides the components required for first strand synthesis was prepared as follows (per sample): 2.0µl 10X RT buffer, 0.8µl 25X dNTP mix, 2.0µl 10X RT

random primers, 1.0µl Multiscribe Reverse Transcriptase, 4.2µl RNase free water. Once the required quantity of master mix was made up for the transcription of all samples, 10µl of this was added to an equal volume of equalised RNA sample in a PCR mini-tube. A negative control was included using the master mix with an equal volume of RNase free water. Samples were then placed in a Doppio thermal cycler (manufacturer and city) and incubated at 25 °C for 10 minutes, 37 °C for 2 hours, 85 °C for 5 minutes, before maintenance at 4 °C prior to collection. cDNA samples were then diluted 1:4 with RNases free water and stored at -80 °C until required for gene expression analysis

2.5.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Gene expression of target proteins was determined using commercially available TaqMan gene expression assays for the cytokines, immune modulators, neurotrophin and components of the endocannabinoid system (see table 2.4.4 for full list of expression assays used). All gene expression assays were purchased from Applied Biosystems (Warrington, UK) and contain specific forward and reverse target primers and FAM-labelled MGB probes. β -actin gene expression was used as an endogenous control to normalise gene expression between samples and was quantified using a β -actin endogenous control assay containing specific primers and a VIC-labelled MGB probe (product code, 1402019).

Target Gene	Assay ID
<i>Immune Modulators</i>	
Interleukin-6 (IL-6)	Mm99999064_m1
Interleukin-1 β (IL-1 β)	Mm00434288_m1
Glial fibrillary acidic protein (GFAP)	Mm01253033_m1
Integrin alpha M (CD11b)	Mm00434455_m1
Indoleamine 2, 3-dioxygenase (IDO)	Mm00492586_m1
Brain-derived neurotrophic factor (BDNF)	Mm04230607_m1
<i>Cannabinoid System</i>	
Fatty acid amide hydrolase (FAAH)	Mm00515684_m1
Monoacylglycerol lipase (MAGL)	Mm00449274_m1
Cannabinoid receptor subtype 1 (CB ₁)	Mm01212171_s1
Cannabinoid receptor subtype 2 (CB ₂)	Mm02620087_s1
peroxisome proliferator-activated receptor alpha (PPAR- α)	Mm00440939_m1
peroxisome proliferator-activated receptor gamma (PPAR- γ)	Mm01184322_m1

Table 2.5.4. Assay list for TaqMan probes.

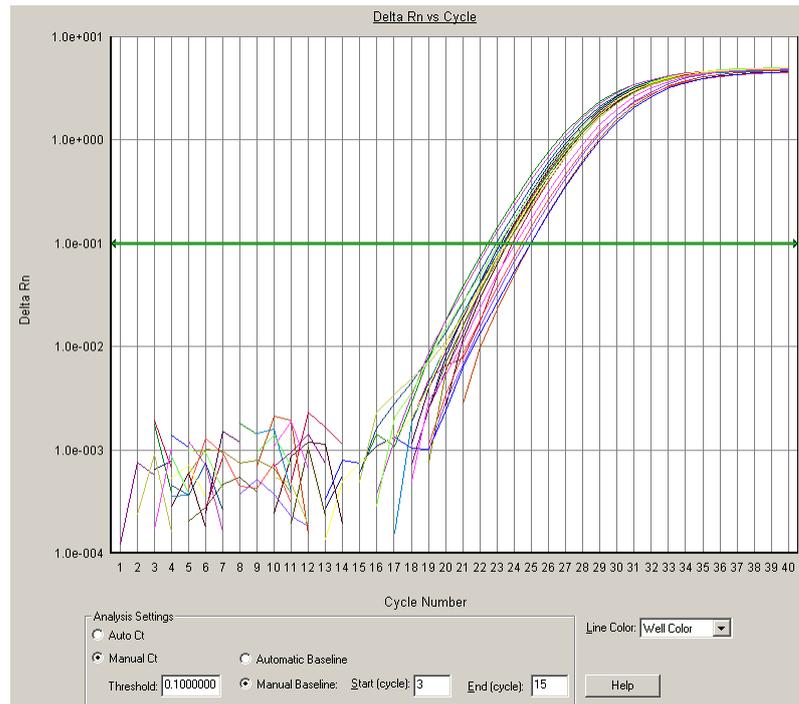
A reaction mixture was prepared and stored on ice for each target gene. This consisted of 0.67 μ l of the target primers, 0.67 μ l β -actin (multiplex version) and 6.67 μ l TaqMan master mix or SensiMix™ II Probe Low-ROX (Bioline, London, UK) per sample. Once the reaction mixture was prepared, it was kept on ice while 5 μ l of each diluted sample was pipetted onto a MicroAmp® optical 96 well plate. The above relevant reaction mixture (8 μ l) was then added to each well giving a total reaction volume of 13 μ l. Negative controls containing the master mix with RNase free water instead of cDNA sample were also included per probe. Plates were then covered with adhesive covers and spun at 1000g for 1 minute to ensure complete mixing. The plate was then placed in the real time PCR thermocycler (ABI Prism 7500 instrument, Applied Biosystems, Warrington, 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 program

2.5.5 Analysis of RT-PCR Results

Amplification plots and cycle threshold (Ct) values were examined using Applied Biosystems 7500 System SDS Software 1.3.1 as illustrated in figure 2.4.5. Ct values were then exported to Microsoft Excel for final analysis and relative quantitative measurement of target gene levels was performed using the $\Delta\Delta\text{Ct}$ method (Livak and Schmittgen, 2001). This method compares gene expression of experimental samples to control samples, allowing determination of the fold change in mRNA expression between experimental groups and involves 3 specific steps.

- Firstly, samples were normalised to endogenous control (β -actin) where ΔCt is determined: $\Delta\text{Ct} = \text{Ct Target gene} - \text{Ct Endogenous control}$.
- Then samples were normalised to control sample where $\Delta\Delta\text{Ct}$ is determined: $\Delta\Delta\text{Ct} = \Delta\text{Ct sample} - \text{average } \Delta\text{Ct of endogenous control group}$.
- Finally, the fold difference was calculated as $2^{-\Delta\Delta\text{Ct}}$. These values for each sample were then expressed as a percentage of the average of the $2^{-\Delta\Delta\text{Ct}}$ values for the control group. In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.

A.



B.

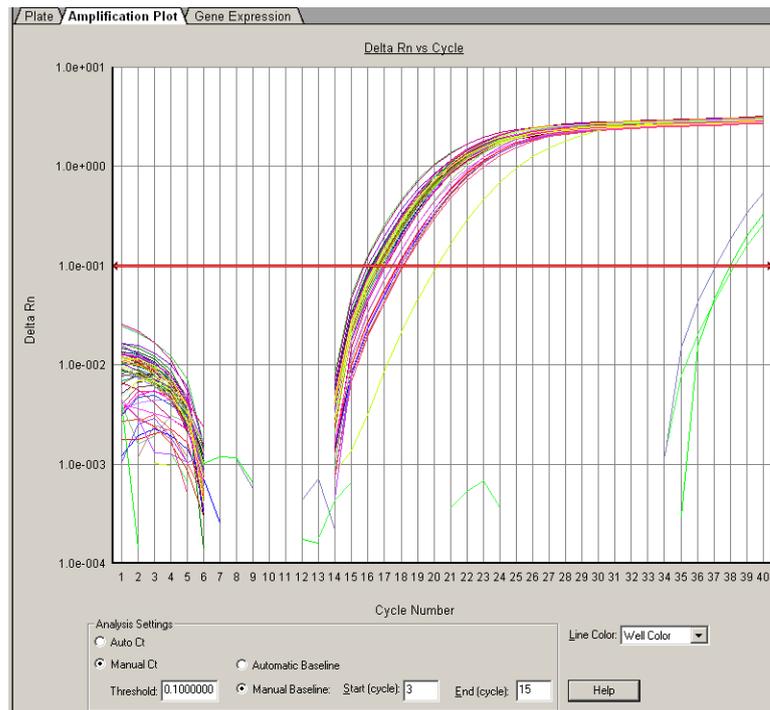


Figure 2.5.5. Amplification plots for A, BDNF and B, the endogenous control, β -actin in the mouse prefrontal cortex.

2.6 Analysis of pro- and anti-inflammatory cytokines using Enzyme-Linked Immunosorbent Assay (ELISA)

Each tissue (spleen) sample (approx 20mg) was homogenised in lysis buffer (1ml, see table 2.5 for details) and centrifuged at 14,000g for 15 minutes before supernatant was removed and stored at -80 °C for subsequent analysis.

Lysis Buffer (200 mls)		
Ingredient	Quantity	Directions
NaCl	1.60125g	
Trizma Base	0.48456g	Add NaCl and Trizma Base to 150mls DH2O. pH to 8.04 by adding a small amount of 1N HCl.
PMSF	0.034838g	Add PMSF and use dropper to rinse weigh boat to make sure all of PMSF gets into the solution (this will take a couple of hours to dissolve properly).
Sodium vanadate	0.183908g	Add sodium vanadate and wash down weigh boat with solution using dropper
Aprotinin	200µl (10µg/ml)	Add and leave to stir for a few minutes
Leupeptin	200µl (1µg/ml)	Add and leave to stir for a few minutes
Igepal	2ml	Add and wait for solution to go clear again
Glycerol	20ml	Add and wait for solution to go clear again. Bring solution up to 200ml mark by adding DH2O. Allow solution to stir for approximately 15 minutes to ensure that everything is completely dissolved. Pour into glass bottle, label and put into the fridge.

Table 2.6. Formulation for lysis buffer used for lysing spleen tissue.

2.6.1 Bradford Protein Assay

As cytokine protein expression was expressed as ng/mg of protein it was first necessary to determine protein concentration in each sample. Total protein in each sample was quantified by the Bradford assay.

Standard protein solutions (0-1500µg/ml) were prepared from a stock (2mg/ml) solution of bovine serum albumin (BSA: catalogue no: A8022: Sigma-Aldrich, Ireland). 5µl of samples (1:20 in distilled water) of samples or standards were pipetted into designated wells on a 96 well plate followed by 250µl of Bradford reagent (B6916: Sigma-Aldrich, Ireland). The plate was left at room temperature for 10 minutes to facilitate optimum colour development and absorbances were then read at 595nm. Protein content of samples could then be extrapolated from standard linear regression plots of protein concentration µg/ml versus optical density (OD) at 595nm (GraphPad Prism).

2.6.2 ELISA Assay Protocol

Protein levels of IFN-γ (catalogue no.: 430801), IL-1β (catalogue no.: 432601), TNF-α (catalogue no.: 430901), IL-12 (catalogue no.: 431601), IL-2 (catalogue no.: 431001), IL-4 (catalogue no.: 431101), and IL-10 (catalogue no.: 431401) in spleen tissue samples were determined using specific mouse enzyme-linked immunosorbent assays (ELISAs) performed using Biolegend ELISA MAX Standard Sets and in accordance with manufacturer instructions.

In brief, Maxisorb Nunc microtitre plates (Biosciences Ltd. Dublin) were coated with 100µl of anti-mouse cytokine antibodies (1:200 in coating buffer (8.4 g NaHCO₃, 3.56 g Na₂CO₃, add deionized water to 1.0 L, pH to 9.5) covered with an adhesive plate cover and left at room temperature overnight. Plates were then aspirated and washed four times with 300µL wash buffer (0.05% Tween 20 in PBS, pH 7.4) on an ELx50 BIO-TEK plate washer (Mason Technology, Ireland). 200 µl of assay diluent (1% BSA in PBS, pH 7.4) was then added to each well in order to block non-specific binding. Plates were then sealed and left to incubate for at least one hour at room temperature with shaking at 200rpm on a plate shaker. In the

meantime, standards for cytokines were prepared (1000-0 pg/ml) by 2 fold serial dilution in assay diluent. Following blocking, the plates were washed a further four times, following which 100µl aliquots of samples or standards were added to assigned well in duplicate and plates were sealed incubated as above at room temperature for a further two hours. After four washes, 100µl of specific diluted biotinylated anti-mouse cytokine detection antibody (1:200 in assay diluent) was added to each well and plates incubated for one hour as before. After four washes, 100µl of Avidin-HRP Conjugate (1:1,000) was added to each well, and plates were incubated at room temperature for 30 minutes. Following five washes, 100µl TMB substrate solution (catalogue no. 421101, Biolegend, CA) was added to each well and the plate was incubated at room temperature in the dark for approx. 20 to 30 minutes to allow for colour development. Reaction was then stopped upon colour development by adding 100µl of stop solution (2N H₂SO₄). Absorbance was then read at 450nm on a Tecan plate reader (Mason Technology, Ireland). Cytokine levels were then extrapolated from standard curves of cytokine concentration (pg/ml) versus optical density at 450nm (GraphPad Prism). The Bradford protein assay (described above) was used to determine protein concentration in spleen samples were then expressed as pg/mg protein.

2.7 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. 500655, Cayman Chemical Company, Cambridge Biosciences, UK). Plasma samples were defrosted on ice and spun at 5,000g, 4 °C for 15 minutes. To the 96-well plate provided, 100µl of EIA buffer (supplied with kit) was added to Non-Specific Binding (NSB) wells, and 50µl of EIA buffer was added to Maximum Binding (B0) wells. 50µl of corticosterone EIA standards (10,000, 4,000, 1,600, 640, 256, 102, 41, and 16 pg/ml) was added in duplicate into corresponding wells on the plate and 50µl of each sample (1: 2 with lysis buffer (table 2.5)) was added in duplicate into designated wells. 50µl of corticosterone acetylcholinesterase (AChE) tracer was added to each well except the Total Activity (TA) and the blank wells, and 50µl of Corticosterone EIA Antiserum was added to each well except the TA, blank and NSB wells. The plate was covered with an adhesive plate cover and

incubated for two hours at RT on an orbital shaker (gentle shaking), after which wells were washed five times with wash buffer. After washing, 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 B0 wells >0.3 units (blank subtracted). The plate was then read at a wavelength of 405nm after 60 minutes on a Tecan 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 B0 average readings to obtain corrected B0. Sample corticosterone levels were subsequently determined from a standard curve of $\ln(B/B0/(1-B/B0))$ versus log corticosterone concentration (pg/ml) using GraphPad Prism. The detection limit for the assay was 30pg/ml and all sample readings were above this limit of detection.

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

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) was employed for the quantification of lipids in various components of the descending pain pathway. The specific tissues which were assayed were the PFC, PAG, RVM and spinal cord as well as the paw tissue, which represented the site of injury in the formalin test. Prior to preparation of samples, paw tissue samples were ground into a fine powder using a mortar and pestle over dry ice. This was carried out due to the increased elasticity of the paw tissue which would otherwise make it more difficult to homogenise.

2.8.1 Preparation of Standards

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

Deuterated stock solutions of 2-AG (d8), AEA (d8), OEA (d2) and PEA (d4) (Cayman chemicals, Cambridge Biosciences, UK) were prepared in 100% acetonitrile, each at a concentration of 100µg/ml. From these stocks, a single deuterated homogenising buffer was prepared containing 2-AG (d8) at 100ng/400µl and AEA (d8), OEA (d2) and PEA (d4) at 5ng/400µl. The buffer was then dispensed into 20ml aliquots and stored with non-deuterated standards at -80 °C.

2.8.2 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 undeuterated standards to give a final concentration range of 187.5ng to 715pg for 2-AG and 18.75ng to 71.5pg for AEA, PEA and OEA. 400µl of the deuterated homogenising buffer was then added to each point of the standard curve. Quantification of endocannabinoids and *N*-acylethanolamines was carried out as previously described (Ford et al., 2011, Kerr et al., 2012, Kerr et al., 2013). Pre-weighed frozen tissue (~10 to 30mg) was first homogenised using a Branson sonicator in 400µl of the deuterated homogenising buffer. Homogenates were centrifuged at 14,000g, 4°C, for 15 minutes and the supernatant was subsequently collected and, along with standard curve preparations, evaporated to dryness in a centrifugal evaporator (Thermo SPD131DDA-230, Fischer Scientific, Ireland). Lyophilised samples and standards were then resuspended in 40µl acetonitrile (65%).

At this point, a technical expert in LC-MS/MS oversaw the injection of each sample (2µl) onto a Zorbax® C18 column (150 × 0.5 70 mm internal diameter) from a cooled autosampler maintained at 4°C. Mobile phases, which were prepared by the same technical expert, 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 2.7.2) and the collective run time was approx. 30 minutes.

Time (mins)	% 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 2.8.2. Gradient used to elute analytes.

Under these conditions, AEA, 2-AG, PEA and OEA were 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. As for the step before, such optimisation protocols were established and undertaken by a technical expert in mass spectrometry. Target endocannabinoids were then quantified by positive ion electrospray ionization and multiple reactions 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 2.7.3.)

Analyte	Parent---Daughter transition
AEA	348.3-----62.1
2-AG	379.3-----287.2
PEA	300.3-----62.1
OEA	326.0-----62.1
AEA (d8)	356.3-----63.1
2-AG (d8)	387.3-----294.2
PEA (d4)	304.3-----62.1
OEA (d2)	328.3-----62.1

Table 2.8.3. Parent---Daughter transitions (mass charge ratios (m/z)) for the deuterated (internal standard) and non deuterated forms of each analyte.

At this point, I was then able to subsequently quantify each analyte which was performed by determining the peak area response against its corresponding deuterated internal standard (see figure 2.8.2 and 2.8.3). 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 of analyte (undeuterated) / peak area analyte (deuterated)) vs (analyte concentration (undeuterated) / analyte concentration (deuterated)). The limit of quantification was 1.32 pmol/g, 12.1 pmol/g, 1.5 pmol/g, 1.41 pmol/g for AEA, 2-AG, PEA and OEA respectively.

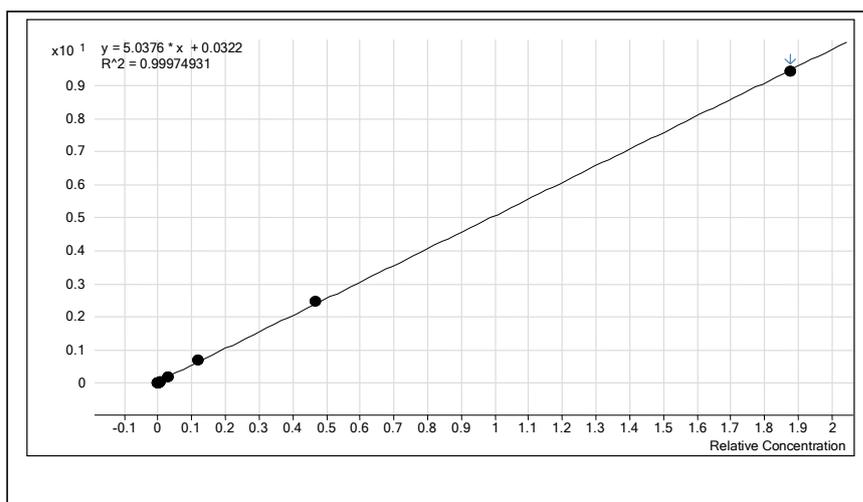


Figure 2.8.2. Plot of Relative Response vs. Relative Concentration for 2-AG.

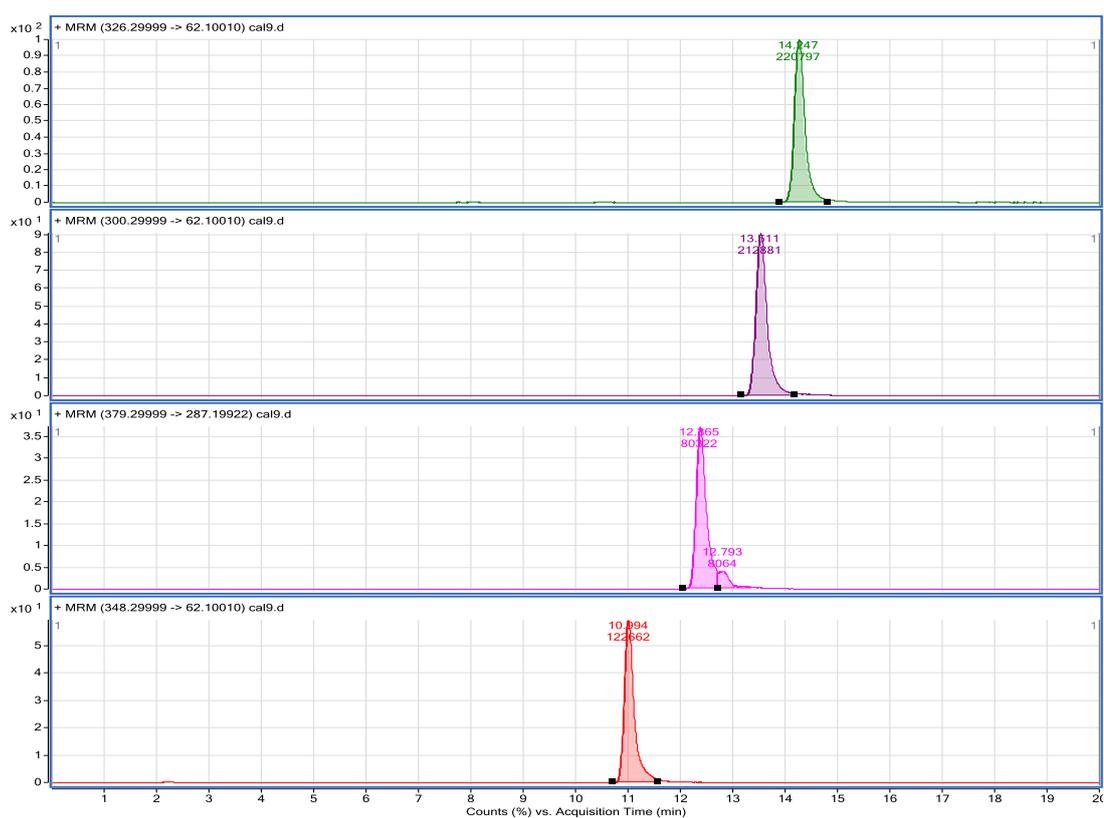


Figure 2.8.3. Chromatograms of 2-AG, AEA, OEA and PEA

2.9 Statistical Analysis

SPSS (IBM, New York, USA) statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro–Wilks and Levene test where $P > 0.05$, 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 while assessing one factor, whereas two-way ANOVA was used to compare the mean of more than two factors in more than two groups. One- or two-way repeated measures ANOVA was employed to compare the mean of two or more groups over time, while assessing one or two factors. Where data were non-parametric, and Kruskal-Wallis analysis by ranks was applied to compare the mean of more than two groups and Friedman's ANOVA was used to compare the mean of two or more groups over time. *Post-hoc* analysis was performed using paired *t*-tests, Fisher's LSD test and Kruskal-Wallis ANOVA 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

The effect of repeated administration of IFN- α on depressive- and anxiety-like behaviour and nociceptive responding to thermal and inflammatory stimuli in two strains of mice

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Interferon- α (IFN- α) is a pro-inflammatory cytokine used for the treatment of various viral diseases such as hepatitis B (Papatheodoridis et al., 2008) and C (Deutsch and Hadziyannis, 2008), as well as malignancy (Cirelli and Tyring, 1994), exerting its therapeutic effects through activation of the immune system, prompting viral suppression (Wang et al., 2002) as well as apoptosis (Asmana Ningrum, 2014) and alterations in cell cycle and differentiation (Sangfelt et al., 2000, Caraglia et al., 2013). However, IFN- α therapy is also associated with neuropsychiatric side effects, namely agitation, depression, anxiety and memory loss, in approximately 30-45% of patients (Schaefer et al., 2002, Raison et al., 2005). In addition, patients undergoing IFN- α treatment for chronic hepatitis C or other malignancy have reported increased incidence of somatic symptoms such as body pain, myalgia, headache, joint pain and abdominal pain (Shakoor et al., 2010, Nogueira et al., 2012, Capuron et al., 2002), effects occurring in up to 72% of some patient groups (Nogueira et al., 2012). Furthermore, such symptoms also presented more frequently in the case of patients with co-morbid major depression e.g. headache, 90% vs. 70%; joint pain 54% vs. 48% (Shakoor et al., 2010). Despite its therapeutic efficacy, the development of the aforementioned side effects following the initiation of IFN- α treatment often prompts dose reductions and eventual withdrawal or discontinuation of therapy (Ogawa et al., 2012, van Zonneveld et al., 2005).

Building on the clinical evidence of a high incidence of depression and anxiety in patients on IFN- α treatment and in order to improve our understanding of the relationship between the immune system and emotion, several studies have attempted to establish an animal model of inflammation-induced depression, employing IFN- α as a primary research tool (see table 3.1.1). These studies, evaluating behavioural modulation following IFN- α administration, have yielded varying results. In rats, acute administration of human recombinant IFN- α (hIFN- α) has been shown to increase immobility in the forced swim test (FST) and reduce sucrose consumption (Sammut et al., 2001, Makino et al., 2000), a finding that was not observed by De La Garza and colleagues in 2005 as illustrated in table 3.1.1. Repeated administration of hIFN- α also induced depressive-like effects in the forced swim and sucrose preference tests (Fahey et al., 2007b, Makino et al., 2000). However several other studies have failed to identify this phenotype at comparable

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doses (De La Garza et al., 2005, Loftis et al., 2006), although varying dosage regimes may account for such discrepancies. As with hIFN- α , acute administration of rat IFN- α (rIFN- α) has been shown to reduce sucrose consumption in rats (Sammut et al., 2001), without inducing depressive behaviour in the FST or alteration of brain stimulation reward thresholds (Makino et al., 2000, Kentner et al., 2007) (see table 3.1.1). To our knowledge, no studies to date have evaluated the behavioural effects of repeated rIFN- α administration. Importantly, it has been noted in several studies that neither human nor rat IFN- α administration associates with alterations in body weight or locomotor activity (Kentner et al., 2007, Loftis et al., 2006, Makino et al., 2000), symptoms which would otherwise confound the behavioural effects observed.

In mice, a number of studies have produced evidence for and against depressive- and anxiety-like behaviour following acute and repeated administration of IFN- α . Acute doses (50 IU/mouse (approx. 1.67IU/g) to 60IU/g) of hIFN- α have been shown to prolong immobility in the FST (Makino et al., 1998, Makino et al., 2000). Repeated administration of both low (6IU/g to 800IU/g) (Siddegowda et al., 2011a, Makino et al., 1998) and high (6,000IU/g) (Ping et al., 2012) doses of hIFN- α have also shown depressive-like behaviour in the FST, a finding that is not replicated when PEGylated hIFN- α is employed (Kosel et al., 2011) (see table 3.1.1). Additionally, continuous hIFN- α administration increased duration of immobility in the tail suspension test (TST) and reduced sucrose consumption (Ping et al., 2012), effects which were not observed by Kosel et al., although the latter employed an alternative treatment regime and route of administration when compared with the former. While acute administration of murine IFN- α did not induce depressive behaviour in either the FST and TST (Wang et al., 2009), repeated administration increased duration of immobility in the FST and TST, and reduced sucrose consumption in the preference test (Orsal et al., 2008, Friebe et al., 2013, Hayley et al., 2013, Zheng et al., 2014, Zheng et al., 2015) as evident in table 3.1.1. Furthermore, anxiety-related behaviours, as observed in the elevated plus maze (EPM) and light dark exploration test, were also observed following repeated, but not acute, administration of mIFN- α (Friebe et al., 2013, Wang et al., 2009). Discrepancies within and between studies may result from varying experimental design, type of IFN- α , drug formulation, dose,

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duration of treatment, housing conditions, choice of species and the strain employed. However, examination of data arising from mice studies reveals that several groups demonstrate depressive-like behaviour following repeated hIFN- α administration using a variety of tests and thus standardization of this protocol may provide a robust and reproducible model of IFN-induced depression. As stated above, clinical data indicate that IFN- α treatment is associated with enhanced painful symptoms; however there has been a paucity of studies examining the effects of IFN- α on nociceptive processing pre-clinically. Early investigations suggested analgesic-like effects of IFN- α following acute administration of the drug in rodents. More specifically, systemic administration of IFN- α evokes analgesic effects which are reversed upon treatment with naloxone, a μ -opioid receptor antagonist (Blalock and Smith, 1981). Additionally, central administration of IFN- α (i.c.v.) has been shown to dose-dependently induce acute analgesia through depression of the tail-flick reflex in both mice (Lee et al., 2010) and rats (Jiang et al., 2000). Furthermore, acute administration of IFN- α into the thalamus nucleus submedius, a brain region central to nociceptive modulation and descending inhibition/facilitation, produces an increase in paw withdrawal latency from a noxious heat stimulus (Wang et al., 2006), suggesting that IFN- α may act at various central sites to modulate nociceptive tone, possibly via opioid-receptor interaction. However, the effect of repeated IFN- α treatment on nociceptive responding has yet to be evaluated.

Thus, it was hypothesised that chronic treatment with IFN- α induces depressive-like behaviour and altered nociceptive responding in mice.

In order to address the above hypothesis, this project sought to examine the effect of repeated IFN- α administration on depressive-like, anxiety-related and nociceptive responding in two different mouse strains. The choice of protocol employed was based on the finding that 3 different groups demonstrated depressive like behaviour following repeated hIFN- α administration and thus this is currently the most consistent finding in the literature on this topic. However, these studies examined responses in different strains of mice (C57Bl/6J vs. CD1), over different time periods (5-15 days) and using different doses (6IU/g to 6,000IU/g). As such, this project examined two doses of IFN- α over 22 days in different tests of depressive-

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like and anxiety-related behaviour (TST, FST and EPM) in two different mouse strains (C57Bl/6J and CD1). Furthermore, nociceptive responding to both noxious thermal and inflammatory pain was also examined in the hot plate and formalin tests respectively.

Chapter 3: Effect of IFN- α on depressive, anxiety and nociceptive behaviour

3.1.1. Aims

1. Examine the effect of repeated administration of IFN- α on body weight and locomotor activity in two separate mouse strains
2. Investigate the effect of repeated administration of IFN- α on depressive-like and anxiety-related behaviour in various paradigms including the tail suspension, forced swim test and elevated plus maze in two separate mouse strains
3. Assess the effect of repeated IFN- α administration on thermal responding in the hot plate test and inflammatory nociceptive responding in the formalin test

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	Type of IFN- α		Behavioural Test	Result	References
Rat	Acute	hIFN- α	FST	↑ Immobility	Makino et al., 2000
			SPT	↓ Sucrose Consumption	Sammut et al., 2001
			SPR	-	De La Garza et al. 2005
		rIFN- α	FST	-	Makino et al., 2000
			SPT	↓ Sucrose Consumption	Sammut et al., 2001
			BSR	-	Kentner et al. 2007
	Chronic	PEG-hIFN- α	FST	-	Loftis et al., 2006
		hIFN- α	FST	↑ Immobility	Fahey et al., 2007, Makino et al., 2000, Fischer et al., 2015
		SPT	↓ Sucrose Consumption	De La Garza et al. 2005	
		EPM	↓ Time in OA	Fahey et al., 2007 Fischer et al., 2015 Fahey et al., 2007	
Mouse	Acute	hIFN- α	FST	↑ Immobility	Makino et al., 1998, 2000
		mIFN- α	FST TST EPM Light/Dark Test	↓ Immobility - - -	Wang et al. 2009
		PEG-rhIFN- α	FST SPT	- -	Kosel et al. 2011
		hIFN- α	FST TST SPT	↑ Immobility ↑ Immobility ↓ Sucrose Consumption	Siddegowda et al., 2011, Makino et al., 1998, Ping et al., 2012 Ping et al., 2012 Ping et al., 2012
		mIFN- α	FST TST SPT EPM OF Light/Dark Test	↑ Immobility - ↑ Immobility - ↓ Sucrose Consumption - ↓ Time in centre - ↓ Entries to centre - ↓ Time in light zone ↓ Entries to light zone	Orsal et al., 2008, Zheng et al., 2014 Zheng et al., 2015, Hoyo-Becerra et al., 2015 Friebe et al., 2013, Zheng et al., 2014, Zheng et al., 2015 Orsal et al., 2008, Hoyo-Becerra et al., 2015 Hayley et al., 2013 Orsal et al., 2008 Friebe et al., 2013 Hoyo-Becerra et al., 2015 Friebe et al., 2013 Hoyo-Becerra et al., 2015 Friebe et al., 2013

Table 3.1.1. Publications to date investigating the use of IFN- α in the attempt to develop an animal model of inflammatory-induced depression. Abbreviations: hIFN, human interferon; FST, forced swim test; SPT, sucrose preference test; SPR, sucrose pellet reward; rIFN, rat interferon; BSR, brain stimulation reward; PEG-IFN, PEGylated interferon; EPM, elevated plus maze; OA, open arms; mIFN- α , mouse interferon; TST, tail suspension test; OF, open field test.

3.2. Materials and Methods

3.2.1. Animals

Male C57BL/6 and CD1 mice (weight 25-30g; Charles River Laboratories, UK) were used in the parts 1 and 2 of the experiment respectively. Animals were housed in groups of three (with at least one mouse from each treatment group per cage), in plastic-bottomed cages containing wood shavings as bedding. Animals were maintained in a constant temperature (21 +/- 2°C) under standard lighting conditions (12:12 hr light-dark, lights on from 07.00 to 19.00 hr). All experiments were carried out during the light phase, between 10.00 and 14.00 hr. Mice were given free access to food and water.

The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

3.2.2. Pharmacological Treatments

Human interferon-alpha (hIFN- α -2a; 3M IU/0.5ml), Roferon-A®, was purchased from Roche Pharmaceuticals. The drug was prepared in saline from stock on a daily basis. IFN- α solution was administered subcutaneously in a volume of 3 μ l/g to give a final concentration of 400 or 800IU/g hIFN- α . Control animals were administered saline in an equivalent volume. Dosing occurred daily, between the hours of 12.00 and 14.00. The doses chosen were based on published research demonstrating depressive-like behaviour following repeated administration of hIFN- α (400-1,600IU/g) in mice (Siddegowda et al., 2011b). The lowest dose of IFN- α employed, 400IU/g, is within that used clinically; IFN- α doses can range from, for example, 3 MIU, s.c., three times per week for chronic Hepatitis C virus to 36 MIU, s.c., daily, for AIDS-related Kaposi's sarcoma, while the higher doses employed, 800IU/g is equivalent to that which is approximately 1.5 times the maximum recommended daily dose of IFN- α for AIDS-related Kaposi's sarcoma.

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3.2.3. Experimental Design

This study was conducted over 2 phases, experiment 1 which investigated the effect of hIFN- α (henceforth referred to as IFN- α) on behavioural responding in C57Bl/6J inbred mice and experiment 2 investigated the effects in CD1 outbred mice.

Following acclimatization to the animal unit (5 days), mice were tested in the open field test in order to determine baseline activity and then randomly assign to the different treatment groups. IFN- α administration began immediately following exposure to the open field and animals were retested 24hrs later. The sequence of behavioural testing over the course of the study is depicted in figure 3.2.3.

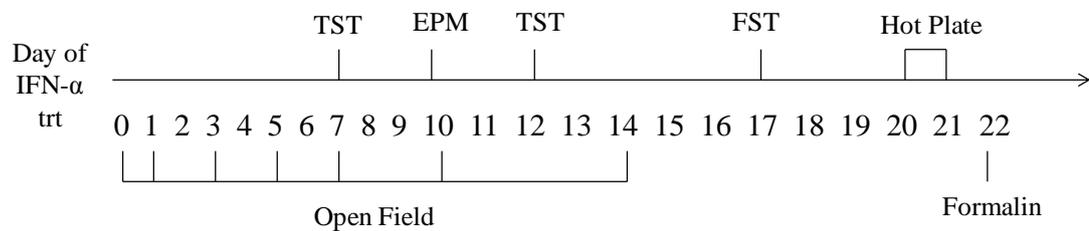


Figure 3.2.3. Timeline for Study 1, investigating the effect of chronic IFN- α on depressive-like behaviour, anxiety and nociception in C57BL/6 and CD1 mice (trt, treatment; TST, tail suspension test; EPM, elevated plus maze; FST, forced swim test).

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3.2.4 Behavioural tests

Behavioural testing was performed between the hours of 10.00 and 16.00, and occurred 20-24 hours after drug or vehicle administration to avoid acute effects of drug administration. All tests were carried out as described in chapter 2. A separate room was assigned for most behaviour analysis (apart from the open field and hot plate tests) and animals were tested immediately without time for habituation to the new environment. After each session, animals were returned to the cages in their own room. All tests including that assessing locomotor activity (open field), depressive-like behaviour (TST, FST), anxiety-related behaviour (EPM) and pain behaviour (hot plate and formalin tests) were recorded by video camera and analysed at a later date, using EthoVision video tracking software (Nodulus, Netherlands). In the case of the hot plate test, animals of both strains were tested one hour post IFN- α on day 20 of treatment and then 24 hours post the same dose, on experimental day 21.

3.2.5. Statistics

Data were analysed using SPSS statistical package, employing the Student's t-test, one- and two-way ANOVA, one-way repeated measures ANOVA followed by Fisher's LSD *post hoc* analysis where appropriate. Where data was not normally distributed, Friedman's ANOVA or Kruskal-Wallis analysis by ranks was applied. Data were considered significant when $P < 0.05$. Results are expressed as group means \pm standard error of mean (SEM).

3.3. Results

3.3.1. Experiment 1: The effect of repeated administration of IFN- α on depressive-, anxiety-related and nociceptive behaviour in C57BL/6 mice

3.3.1.1. Repeated IFN- α administration does not alter body weight or body weight gain of C57BL/6 mice

Throughout the experiment, mice were weighed daily directly prior to each injection of saline or IFN- α . This was in order to evaluate the influence of repeated IFN- α administration on body weight and body weight gain per week.

One-way repeated measures ANOVA revealed a significant effect of time [F (7, 175) = 25.83, P= 0.000], but no effect of drug [F (2, 25) = 1.066, P=0.360] or time x drug interaction [F (14, 175) = 0.43, P=0.965] on body weight. Fisher's LSD *post hoc* analysis revealed that body weight increased over the course of the study, an effect not altered by IFN- α administration (figure 3.3.1.1.A).

Similarly, two-way repeated measures ANOVA revealed a significant effect of time [F (2, 50) = 42.12, P=0.000] but not drug [F (2, 25) = 0.02, P=0.827] or drug x time interaction [F (4, 50) = 0.77, P=0.552] on body weight gain over 21 days. Fisher's LSD *post hoc* analysis revealed body weight gain increased throughout the study, an effect not modified by IFN- α administration (see figure 3.3.1.1.B).

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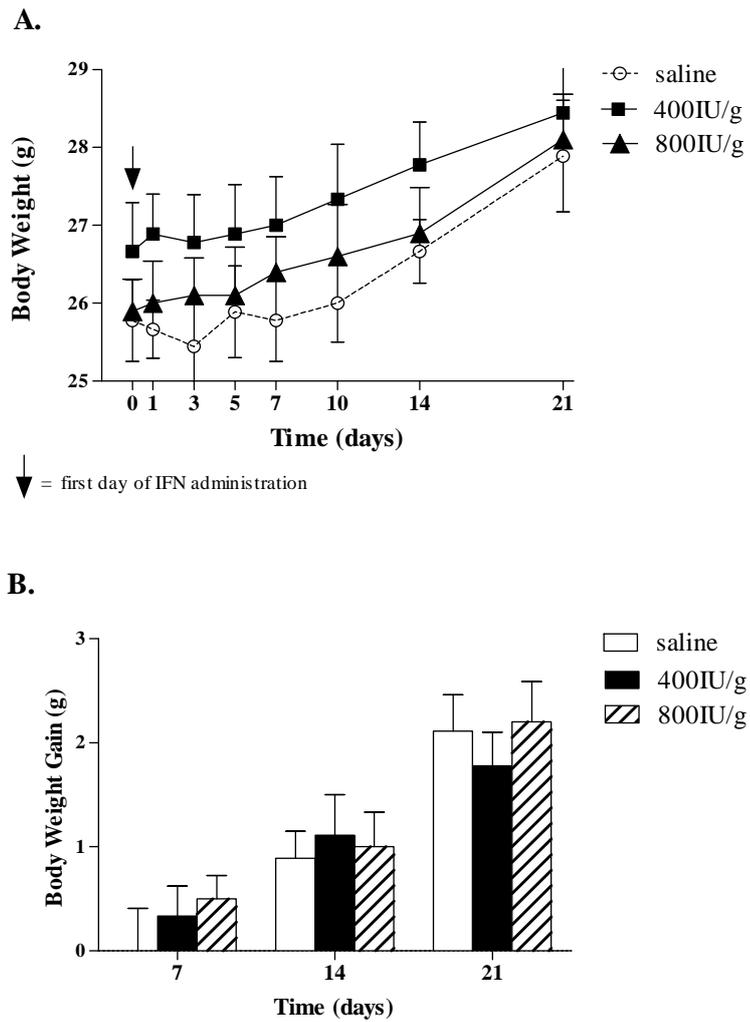


Figure 3.3.1.1. A, Body weight of C57BL/6 mice receiving saline, 400IU/g and 800IU/g/day IFN- α and B, body weight gain per week per week in mice receiving saline or IFN- α daily. Data are expressed as mean \pm SEM; n= 9-10 per group.

3.3.1.2. Repeated IFN- α administration does not alter locomotor activity of C57BL/6 mice in the open field test

In order to evaluate the influence of repeated IFN- α administration on locomotor activity, animals were exposed to the 5-minute open field test prior to and on day 1, 3, 5, 7, 10 and 14 of saline or IFN- α treatment. The distance moved over the 5 minutes was assessed using Ethovision XT software.

One-way repeated measures ANOVA revealed a significant effect of time [$F(6, 144) = 30.84, P=0.000$], but no effect of drug [$F(2, 24) = 0.48, P=0.626$] or drug x time interaction [$F(12, 144) = 0.92, P=0.533$] on the distance moved in the open field arena (figure 3.3.1.2). Fisher's LSD *post hoc* analysis revealed that while locomotor activity decreased significantly from day 0 at each time point throughout the study (habituation), this was unaffected by IFN- α administration.

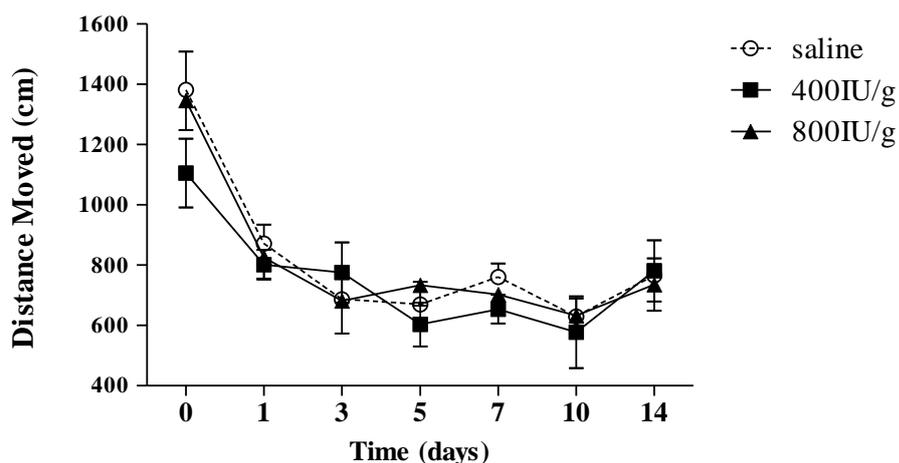


Figure 3.3.1.2. Locomotor activity (distance moved) of C57BL/6 mice receiving saline, 400IU/g or 800IU/g IFN- α per day. Data are expressed as mean \pm SEM; n= 9-10 per group.

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3.3.1.3. Repeated administration of IFN- α does not alter the duration of immobility of C57BL/6 mice in the TST

The TST was carried out following 7 and 14 days of repeated saline or IFN- α treatment. On both days, behaviour was recorded onto a DVD and later assessed for the duration of immobility throughout the 6-minute trial which is indicative of depressive-like behaviour.

On day 7, one-way repeated measures ANOVA, over the six minutes, revealed a significant effect of time [F (5, 125) = 26.10, P=0.000] but not drug [F (2, 25) = 1.204, P=0.317] or time x drug interaction [F (10, 125) = 1.14, P=0.336] on duration of immobility throughout the test (see Figure 3.3.1.3.A). Subsequent Fisher's LSD *post hoc* analysis revealed that immobility time increased for all animals after the initial minute of the test (figure 3.3.1.3.A).

On day 14, one-way repeated measures ANOVA, revealed no overall effect of drug [F (2, 25) = 1.45, P=253] but a significant effect of time [F (5, 125) = 30.84, P=0.000] and time x drug interaction [F (10, 125) = 2.67, P=0.005] on duration of immobility over the six- minute test, on day 14. Fisher's LSD *post hoc* analysis revealed increased duration of immobility throughout the test when compared with the initial minute in all groups (figure 3.3.1.3.B).

When the initial two minutes of the test were excluded to allow for habituation to the arena, two-way ANOVA revealed a significant effect of time [F (1, 25) = 24.39, p<0.000] but not drug [F (2, 25) = 1.23, p=0.3088] or time x drug interaction [F (2, 25) = 1.83, p=0.1820] on the duration of immobility in the TST (figure 3.3.1.3.C). Fisher's LSD *post hoc* analysis revealed that while IFN- α treatment did not alter immobility time on either of the days when compared to saline treated controls, paired t-tests revealed that time spent immobile was significantly increased in saline and 800IU/g treated mice on day 14 when compared to day 7, as seen in figure 3.3.1.3.C.

Wilcoxon signed rank test revealed a significant effect of time [P=0.000] and Kruskal-Wallis analysis by ranks analysis revealed no significant effect of IFN- α [P=0.862] on the latency to initiate immobility in the TST. Subsequent *post hoc* analysis revealed while IFN- α treatment did not alter latency to immobility on either of the days when compared to saline-treated controls, latency to immobility was

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reduced in the case saline-treated animals and group receiving 800IU/g/day on day 14 when compared with that on day 7 (see figure 3.3.1.3.D).

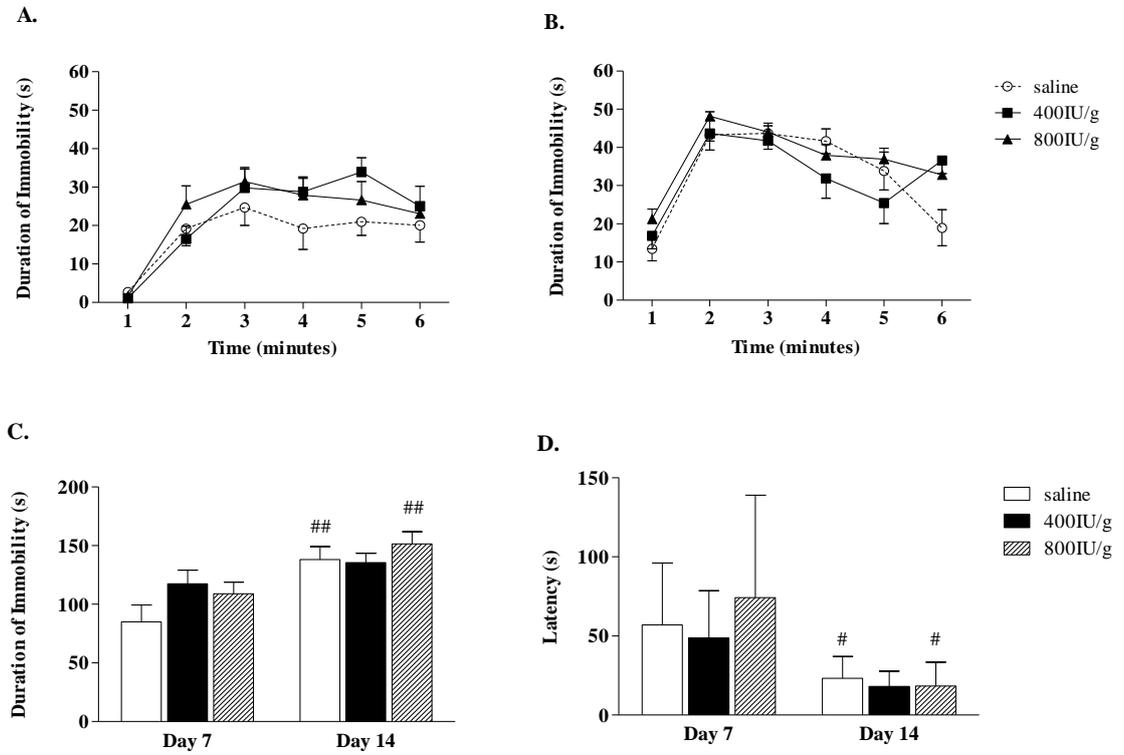


Figure 3.3.1.3. Effect of repeated IFN- α on immobility in the TST on day 7 and 14. A, time spent immobile within each minute of test on day 7 and B, day 14; C, total duration of immobility over the latter four minutes of test; D, latency of C57BL/6 mice to form immobile posture in TST on day 7 and 14. Data are expressed as mean \pm SEM; n= 9-10 per group. ^{##}P<0.01, [#]P<0.05 vs. day 7.

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3.3.1.4. Repeated administration of IFN- α does not alter the duration of immobility of C57BL/6 mice in the FST

The FST was carried out following 17 days of repeated saline or IFN- α treatment. Behaviour was recorded onto a DVD and later assessed for the duration of immobility throughout the 6-minute trial, a behaviour characteristic of a depressive phenotype.

One-way repeated measures ANOVA revealed an effect of time [$F(5, 125) = 54.58, P=0.000$] but not drug [$F(2, 25) = 0.18, P=0.837$] or time x drug interaction [$F(10, 125) = 0.42, P=0.936$] on the duration of immobility throughout the six-minute test. Fisher's LSD *post hoc* analysis revealed that time spent immobile increased throughout the test in the case of all groups, as evident in figure 3.3.1.4.A.

When the first two minutes of the test were excluded to allow for habituation in the arena, one way ANOVA revealed no significant effect of the drug on the total duration of immobility within the remaining four minutes [$F(2, 26) = 0.772, P=0.4732$] (figure 3.3.1.4.B).

One-way ANOVA also revealed no significant effect of IFN- α on latency to immobility in the FST [$F(2, 26) = 0.700, P=0.506$].

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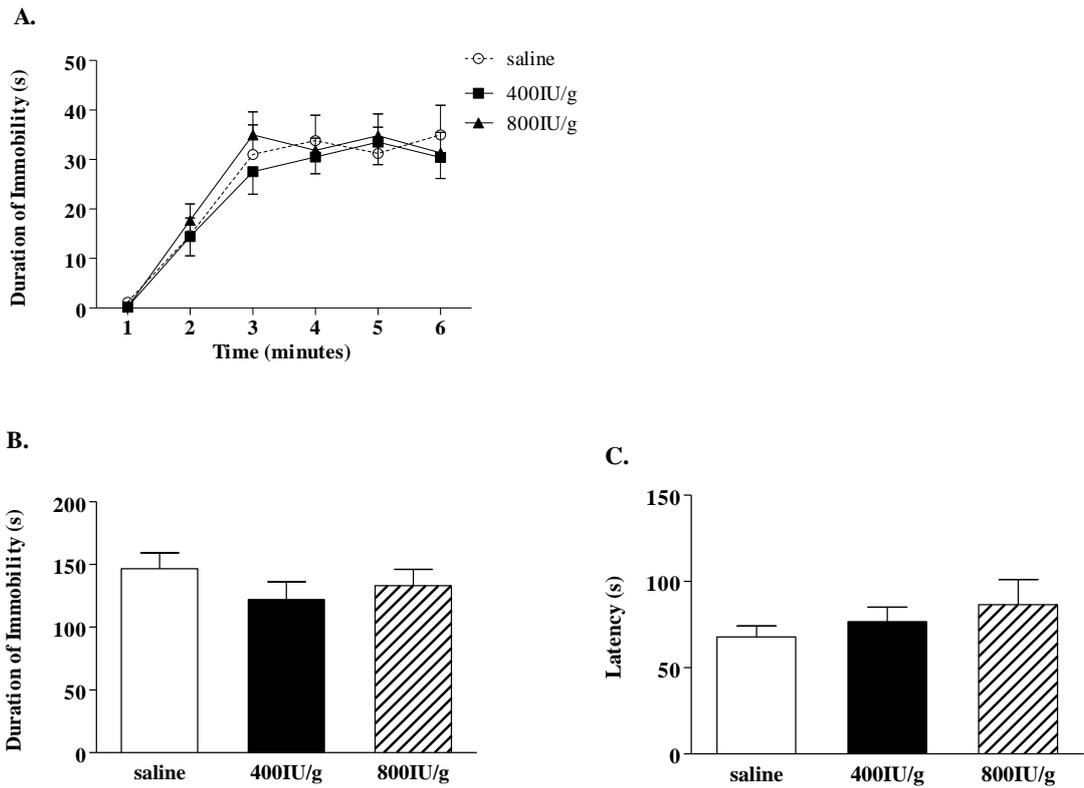


Figure 3.3.1.4. Effect of repeated IFN- α on immobility in the FST on day 17. A, time spent immobile within each minute of test; B, total duration of immobility over the latter four minutes of test; C, latency of C57BL/6 mice to form immobile posture in FST. Data are expressed as mean \pm SEM; n= 8-10 per group.

3.3.1.5. Repeated administration of IFN- α does not alter anxiety-related behaviour in the EPM in C57BL/6 mice

Animals were subjected to the EPM arena following 10 days of repeated saline or IFN- α treatment. Mice were placed individually on the central platform facing an open arm and behaviour recorded for the 5-minute trial. Assessment of the time spent in the open and closed arms, as well as the frequency of entry into the open arms was used to determine anxiety-related behaviour.

One-way ANOVA revealed no significant effect of IFN- α administration on distance moved [$F(2, 25) = 3.14, P=0.061$], time spent in the open arms [$F(2, 25) = 1.37, P=0.272$], entries into the open arms [$F(2, 25) = 3.18, P=0.059$] or time spent in the closed arms [$F(2, 25) = 1.46, P=0.252$] of the apparatus (figure 3.3.1.5.).

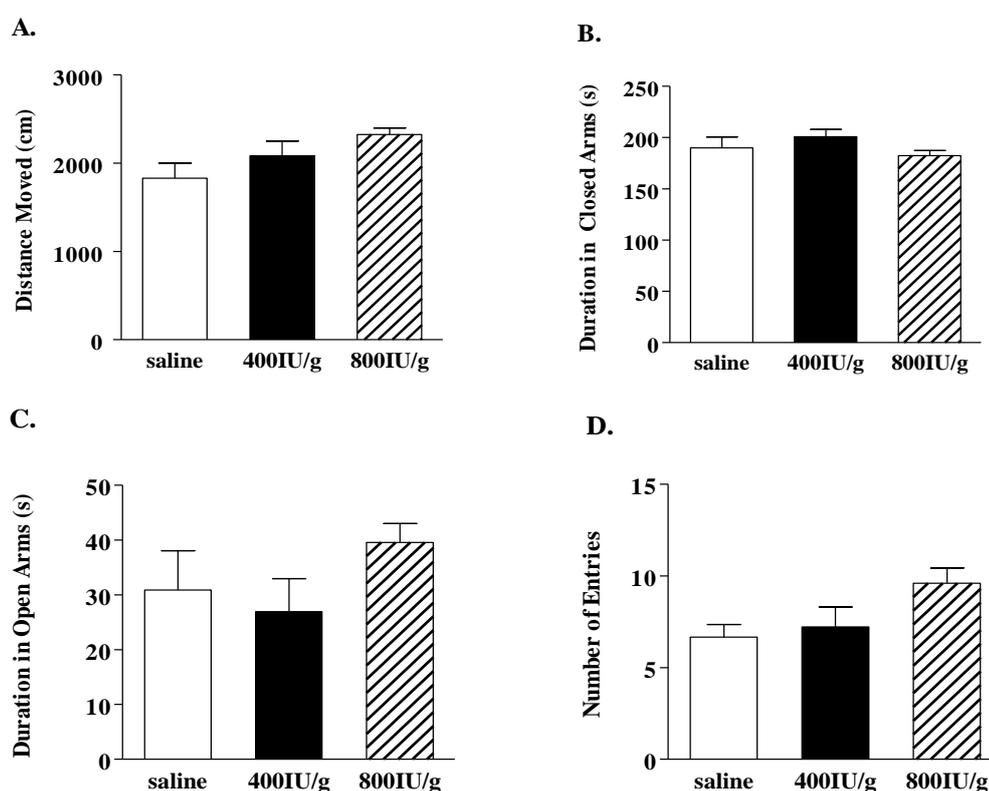


Figure 3.3.1.5. Effect of IFN- α on behaviour of C57BL/6 mice in an EPM apparatus: (a) Distance moved; (b) time spent in the closed arms; (c) time spent in open arms and (d) number of entries into open arms after 10 days of saline, 400IU or 800IU/g IFN- α administration. Data are expressed as mean \pm SEM; n= 9-10 per group.

3.3.1.6. Repeated IFN- α administration induces thermal hyperalgesia in the hot plate test in C57BL/6 mice

This test involved exposure of saline- and IFN- α -treated animals to a noxious thermal stimulus in the form of a ceramic plate, heated to a temperature of 54-55°C and the time taken or latency for animals to respond (elevate or lick hind paws) to the heat stimulus was recorded. This was carried out 1 hour post saline or IFN- α treatment on day 20 and 24 hours later (day 21).

Two-way ANOVA revealed a significant effect of time [F (1, 23) = 11.67, P=0.002] and drug [F (2, 23) = 6.59, P=0.006] on latency to lick the front paw in the hot plate test. Subsequent Fisher's LSD *post-hoc* analysis revealed a significant decrease in latency to respond in mice receiving 800IU/g when compared to the saline-treated control group of animals. Unpaired t-tests also revealed a reduction in latency 24 hours post treatment in comparison to just one hour, in mice receiving 800IU/g, an effect not observed in the 400IU/g group, as seen in figure 3.3.1.6.

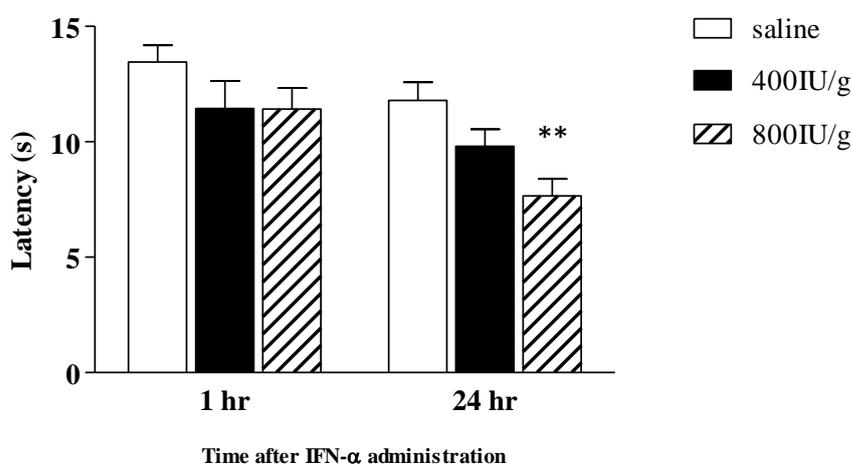


Figure 3.3.1.6. The effect of repeated IFN- α treatment on thermal nociceptive behaviour in C57BL/6 mice subjected to the hot plate test at one hour and 24 hours post drug, carried out on day 20 and 21 of the study, respectively. Data are expressed as mean \pm SEM; n= 8-10 per group; ** p<0.01 vs. saline at 24hrs.

3.3.1.7. Repeated administration of IFN- α does not alter nociceptive behaviour of C57BL/6 mice in response to a noxious inflammatory stimulus

In order to assess if repeated administration of IFN- α alters inflammatory nociceptive responding, mice received intraplantar administration of the chemical irritant formalin, on day 22, 24 hours following the last dose of IFN- α or saline vehicle treatment. Subsequent behavioural responding was assessed over the following 60 minutes.

One-way repeated measures ANOVA revealed a significant effect of time [F (11, 264) = 26.24, P=0.000] but not treatment [F (2, 24) = 0.05, P=0.954] or time x treatment interaction [F (22, 264) = 1.33, p=0.154] on elevation of the injected paw (pain '1' behaviour) in the formalin test (see figure 3.3.1.7.A). Furthermore, when assessed as a one-hour time block, one-way ANOVA also revealed no significant effect of IFN- α [P=0.954] on the total duration of this behaviour over 60 minutes, as seen in figure 3.3.1.7.B.

One-way repeated measures ANOVA revealed an effect of time [F (11, 264) = 18.23, p=0.000] but not treatment [F (2, 24) = 1.02, P=0.376] or time x treatment interaction [F (22, 264) = 0.75, P=0.781] on direct shaking, licking, biting, flinching of the affected paw (pain '2' behaviour) throughout the formalin test, as evident in figure 3.3.1.7.C. During assessment of pain 2 behaviour as a one-hour block, one-way ANOVA also revealed no significant effect of IFN- α [P=0.375] on the total duration of this behaviour over 60 minutes (see figure 3.3.1.7.D).

One-way repeated measures ANOVA revealed a significant effect of time [F (11, 264) = 21.50, P=0.000] not treatment [F (2, 24) = 0.81, P=0.457] or time x treatment interaction [F (22, 264) = 0.79, P=0.732] on composite pain score (CPS) in the formalin test (Figure 3.3.1.7.E). One-way ANOVA also revealed no significant effect of IFN- α [P=0.465] on the total duration of this behaviour over 60 minutes (figure 3.3.1.7.F).

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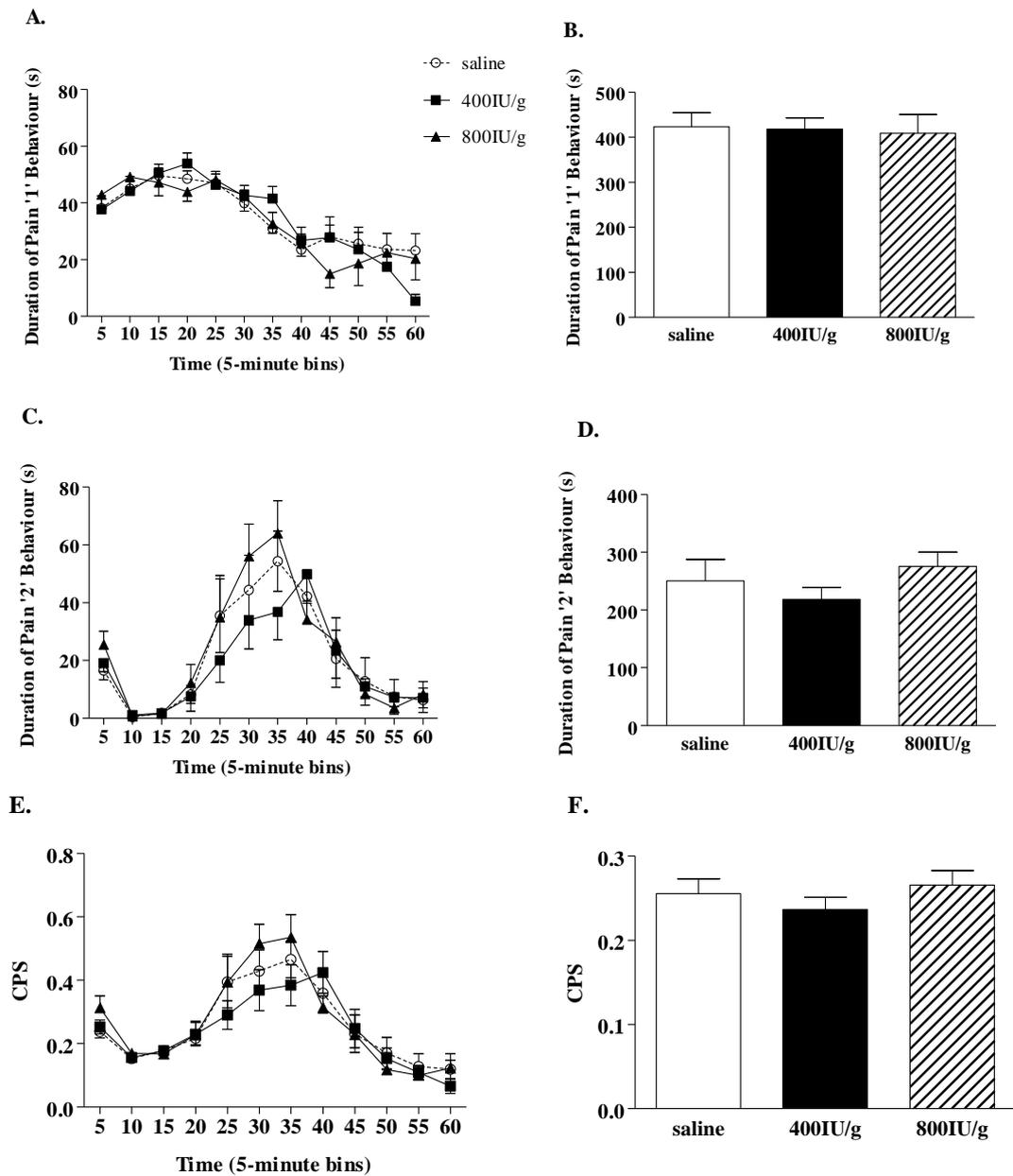


Figure 3.3.1.7. Nociceptive behaviour in the formalin test in C57Bl/6 mice: A, Profile of pain '1' behaviour over 60 minutes; B, Duration of pain '1' behaviour overall in the formalin test; C, Profile of pain '2' behaviour over 60 minutes; D, Duration of pain '2' behaviour overall in the formalin test; E, Profile of CPS over 60 minutes and F, Duration of CPS overall in the formalin test. Data are expressed as mean \pm SEM; n= 9 per group.

3.3.1.8. Chronic IFN- α does not alter locomotor activity or general behaviour of C57BL/6 mice in response to a noxious inflammatory stimulus

In order to assess if continuous administration of IFN- α alters pain behaviour, mice were exposed to the formalin test 24 hours following the final dose of IFN- α or saline vehicle. General behaviour i.e. locomotor activity, rearing and grooming, was also assessed throughout the 60-minute test.

One-way ANOVA revealed no significant effect of IFN- α treatment [$F(2, 25) = 2.345, P=0.117$] on distance moved, grooming [$F(2, 25) = 0.723, p=0.495$] or rearing behaviour [$F(2, 25) = 0.597, P=0.558$] during the formalin test (figure 3.3.1.8.).

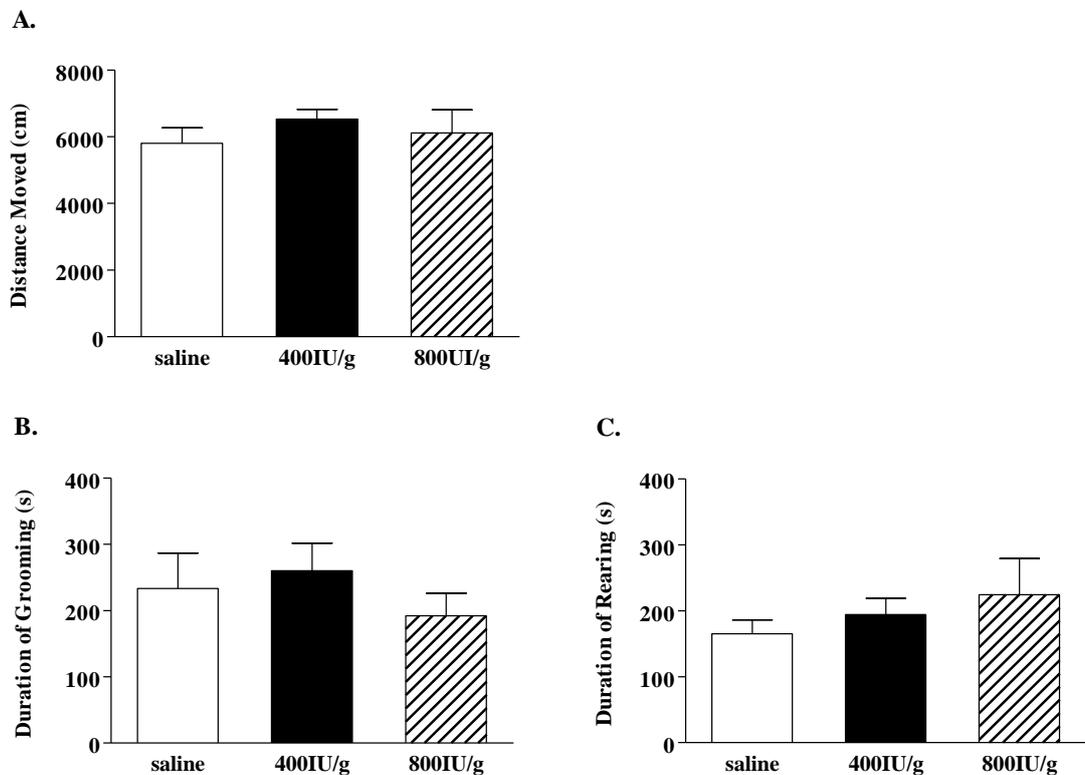


Figure 3.3.1.8. General behaviour in the formalin test in C57BL/6 mice: A, duration of locomotor activity, B, duration of grooming behaviour and C, duration of rearing behaviour overall in the formalin test. Data are expressed as mean \pm SEM; $n = 9$ per group.

3.3.1.9. Repeated IFN- α administration does not alter formalin-induced oedema of the left-hind paw in C57BL/6 mice

One-way ANOVA revealed no significant change in paw diameter across all treatment groups after formalin administration [$F(2, 27) = 0.947, P=0.402$] (Figure 3.3.1.9).

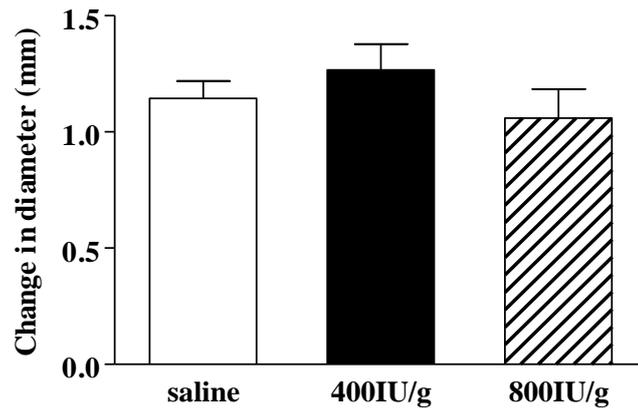


Figure 3.3.1.9. The effect of repeated IFN- α administration on Formalin-induced oedema of the left hind paw in C57BL/6 mice receiving daily injections of saline, 400IU/g or 800IU/g IFN- α . Data are expressed as mean \pm SEM; n= 9 per group.

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3.3.2. Experiment 2: The effect of repeated IFN- α administration on depressive, anxiety-related and nociceptive behaviour in CD1 mice

3.3.2.1. Repeated administration of IFN- α does not alter body weight or body weight gain in CD1 mice

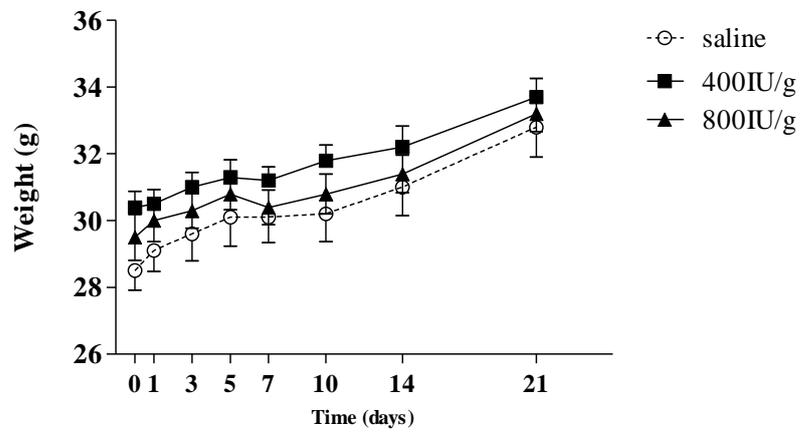
Throughout the experiment, CD1 mice were weighed daily directly prior to each injection of saline or IFN- α . This was in order to evaluate the influence of repeated IFN- α administration on body weight and body weight gain per week.

One-way repeated measures ANOVA revealed a significant effect of time [$F(7, 189) = 68.37, P=0.000$], but no effect of drug [$F(2, 27) = 1.31, P=0.287$] or time x drug interaction [$F(14, 189) = 0.56, P=0.895$] on body weight. Fisher's LSD *post hoc* analysis revealed that body weight increased in all groups by day 14 and 21, as seen in figure 3.3.2.1.A

Similarly, one-way repeated measures ANOVA revealed a significant effect of time [$F(2, 54) = 118.42, P=0.000$] but not drug [$F(2, 27) = 1.0, P=0.381$] or time x drug interaction [$F(4, 54) = 0.20, P=0.935$] on body weight gain over 21 days (see figure 3.3.2.1.B). Subsequent post hoc analysis revealed increased body weight gain over time in the case of mice treated with 800IU/g IFN- α .

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A.



B.

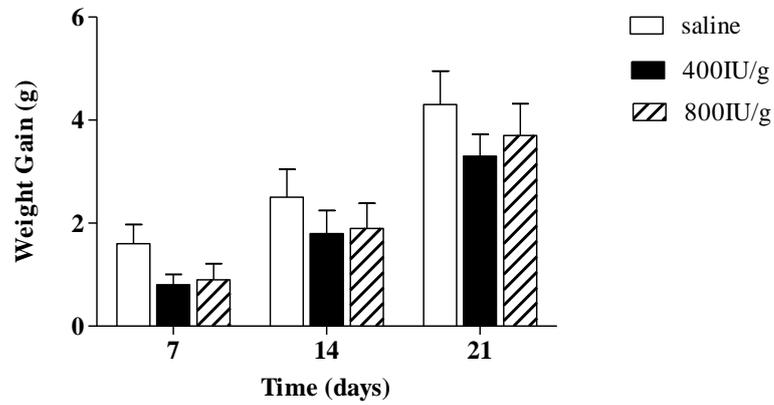


Figure 3.3.2.1. A, Body weight and B, body weight gain per week of CD1 mice receiving saline, 400IU/g or 800IU/g IFN- α daily for 21 days. Data are expressed as mean \pm SEM; n= 10 per group.

3.3.2.2. Repeated IFN- α does not alter locomotor activity of CD1 mice in the open field test

CD1 mice were exposed to the 5-minute open field test prior to and on day 1, 3, 5, 7, 10 and 14 of saline or IFN- α treatment. The distance moved over the 5 minutes was tracked and assessed using Ethovision XT software.

One-way repeated measures ANOVA revealed a significant effect of time [F (6, 144) = 9.89, P=0.000], but no effect of drug [F (2, 24) = 1.66, P=0.212] or time x drug interaction [F (12, 144) = 0.35, P=0.978] on the distance moved in the open field arena, as seen in figure 3.3.2.2. Fisher's LSD *post hoc* analysis revealed that while locomotor activity decreased significantly from day 0 at each time point throughout the study (habituation), this was unaffected by IFN- α administration.

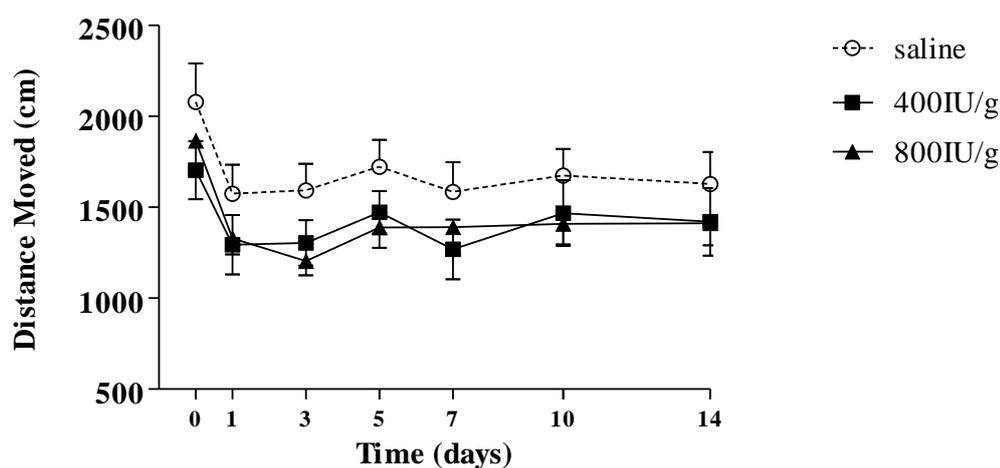


Figure 3.3.2.2. Locomotor activity (distance moved) in the open field arena of CD1 mice receiving saline, 400IU/g or 800IU/g IFN- α per day. Data are expressed as mean \pm SEM; n= 9-10 per group.

3.3.2.3. Repeated administration of IFN- α does not alter duration of immobility of CD1 mice in the TST

The TST was carried out following 7 and 14 days of repeated saline or IFN- α treatment. Behaviour was recorded and later assessed for the duration of immobility, a direct indicator of a depressive-like phenotype.

Specifically, on day 7, one-way repeated measures ANOVA revealed no significant effect of drug [$F(2, 23) = 0.33, P=0.719$] but a significant effect of time [$F(5, 115) = 17.29, P=0.000$] and time x drug interaction [$F(10, 115) = 2.23, P=0.020$] (see figure 3.3.2.3.A) on duration of immobility throughout the six-minute test. Fisher's LSD *post hoc* analysis revealed that while the duration of immobility increased throughout the course of test in the case of all treatment groups, there was no between group differences at any stage of the six-minute test.

On day 14, one-way repeated measures ANOVA, revealed a significant effect of time [$F(5, 115) = 13.83, p=0.000$] but not drug [$F(2, 23) = 0.18, P=0.838$] or time x drug interaction [$F(10, 115) = 0.56, P=0.845$] (see figure 3.3.2.3.B) on duration of immobility throughout the six- minute test. Fisher's LSD *post hoc* analysis revealed that immobility time increased significantly throughout the test in the case of all treatment groups, however there was no effect of drug treatment on this time at any stage of the six-minute test.

When the initial two minutes of the test were excluded to allow for habituation, two-way ANOVA revealed a significant effect of time [$F(1, 23) = 10.77, P=0.003$] but not drug [$F(2, 23) = 0.09, P=0.911$] or time x drug interaction [$F(2, 23) = 0.89, P=0.426$] on the duration of immobility in the TST on day 7 and 14. Paired t- tests revealed that time spent immobile increased significantly on day 14 in comparison to day 7 in the case of mice receiving 800IU/g IFN- α only, as seen in figure 3.3.2.3.C.

Two-way ANOVA revealed a significant effect of time [$F(1, 23) = 12.61, P=0.002$] but not drug [$F(2, 23) = 0.09, P=0.916$] or time x drug interaction [$F(2, 23) = 0.08, P=0.923$] on latency to immobility in the TST (figure 3.3.2.3.D). Subsequent paired t-tests revealed that latency to immobility reduced significantly by day 14 in the case of mice receiving 800IU/g IFN- α , an effect not observed in both alternative groups, as seen in figure 3.3.2.3.D.

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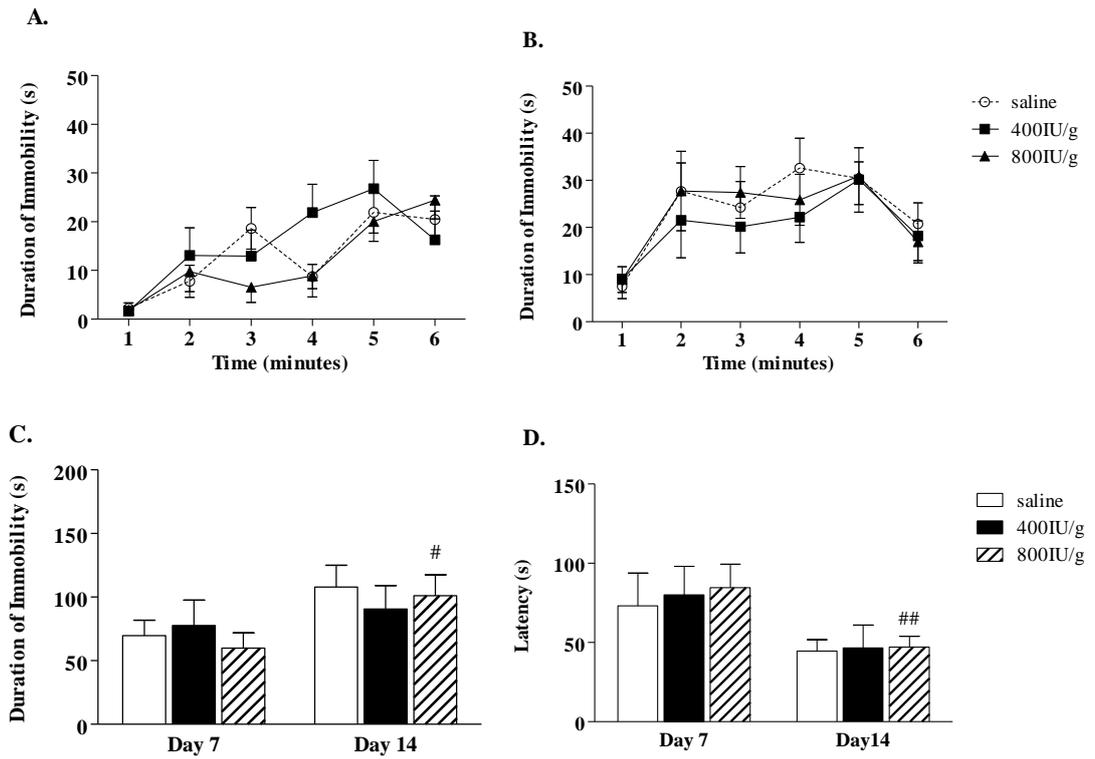


Figure 3.3.2.3. Effect of repeated IFN- α on immobility in the TST on day 7 and 14 in CD1 mice. A, time spent immobile within each minute of test on day 7 and B, day 14; C, total duration of immobility over the latter four minutes of test; D, latency of C57 mice to form immobile posture in TST on day 7 and 14. Data are expressed as mean \pm SEM; n= 8-10 per group. ##P<0.01, #P<0.05 vs. day 7.

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3.3.2.4. Repeated IFN- α administration does not alter time spent immobile of CD1 mice in the FST

The FST was carried out following 17 days of repeated saline or IFN- α treatment. Behaviour was recorded onto a DVD and later assessed for the duration of immobility throughout these 6 minutes.

Friedman's ANOVA revealed a significant effect of time [$X^2(5) = 70.197, P=0.000$] and Kruskal-Wallis ANOVA revealed a significant effect of IFN- α treatment [$X^2(2) = 7.647, P=0.022$] on time spent immobile throughout the six-minute test. *Post hoc* analysis employing Dunn's multiple comparisons revealed that the duration of immobility increased throughout the six-minute test in the case of all groups while further Kruskal-Wallis analysis by ranks revealed no significant effect of IFN- α treatment on immobility time at each time point throughout the test (see figure 3.3.2.4.A).

When the first two minutes of the test were excluded to allow for habituation in the arena, one way ANOVA revealed no significant effect of the drug on the total duration of immobility throughout the remaining four minutes [$F(2, 25) = 2.707, P=0.088$] (figure 3.3.2.4.B).

One-way ANOVA revealed no significant effect of IFN- α on latency to immobility in the FST [$F(2, 25) = 0.559, P=0.506$], as seen in figure 3.3.2.4.C.

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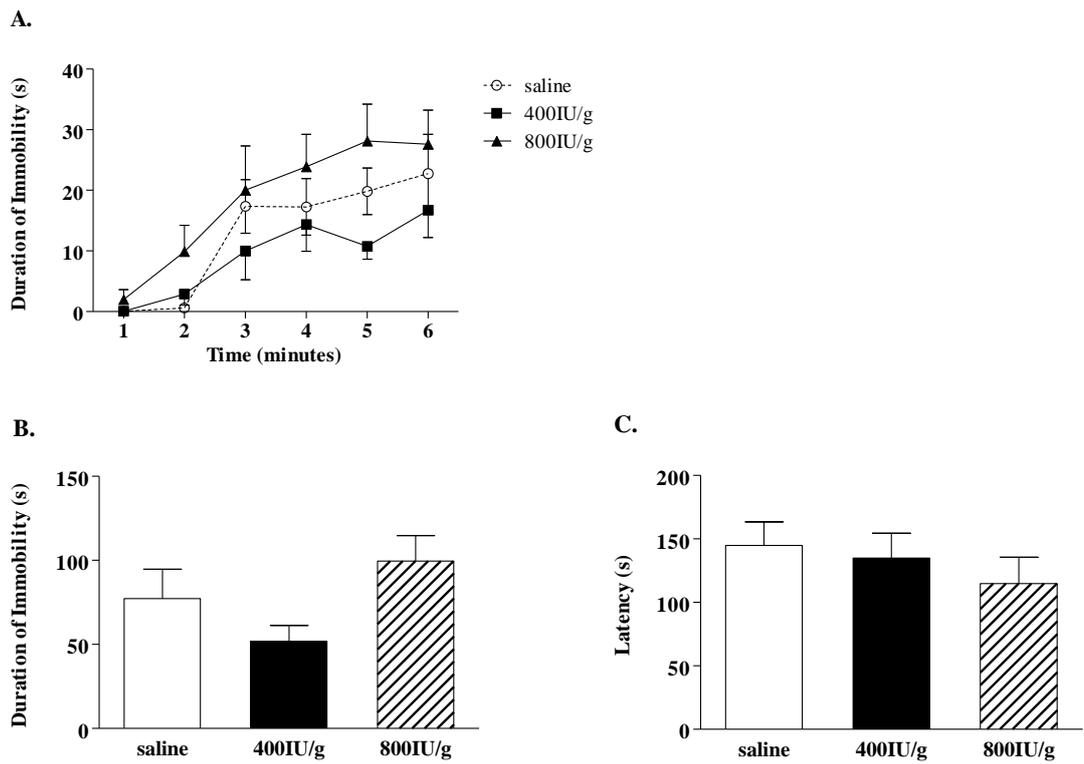


Figure 3.3.2.4. Forced Swim Test in CD1 mice receiving a chronic daily dose of saline, 400IU/g or 800IU/g IFN- α depicting A, time spent immobile within each minute of test; B, total duration of immobility over the latter four minutes of test; C, latency of C57 mice to form immobile posture in FST. Data are expressed as mean \pm SEM; n= 8-10 per group.

3.3.2.5. Repeated IFN- α does not alter anxiety-related behaviour in CD1 mice in the EPM

Animals were subjected to the EPM arena following 10 days of repeated saline or IFN- α treatment. Assessment of the time spent in the open and closed arms, as well as the frequency of entry into the open arms was used to determine the presence of anxiety-related behaviour.

One-way ANOVA revealed no significant effect of IFN- α treatment on distance moved [$F(2, 29) = 0.57, P=0.572$] in the EPM, as seen in figure 3.3.2.5.A. One-way ANOVA also revealed no significant difference between groups with regard to time spent in the open arms [$F(2, 27) = 0.347, P=0.710$], entries into open arms [$F(2, 29) = 1.86, P=0.176$] or time spent in the closed arms [$F(2, 29) = 1.22, P=0.310$] of the apparatus, also in figure 3.3.2.5.

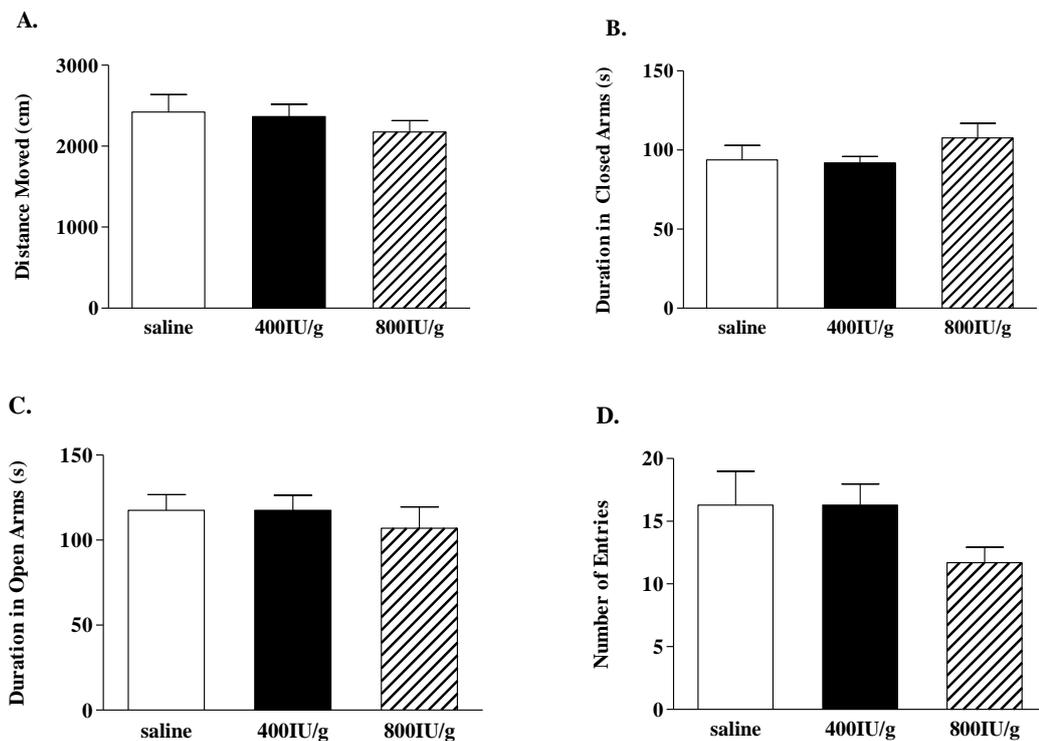


Figure 3.3.2.5. Effect of IFN- α on behaviour of CD1 mice in the EPM apparatus: A, Distance moved; B, time spent in the closed arms; C, time spent in open arms and D, number of entries into open arms after 10 days of saline, 400IU or 800IU/g IFN- α administration. Data are expressed as mean \pm SEM; n= 9-10 per group.

3.3.2.6. Repeated administration of IFN- α does not induce thermal hyperalgesia in CD1 mice the in the hot plate test

The hot plate test was carried out 1 hour post saline or IFN- α treatment on day 20 of the experiment and 24 hours following the same dose on the following day (day 21). Two-way repeated measures ANOVA revealed no significant effect of time [F (1, 20) = 0.08, P=0.784], drug [F (2, 20) = 0.44, P=0.652] or time x drug interaction [F (2, 20) = 0.44, P=0.961] on the latency to lick front paw (see figure 3.3.2.6).

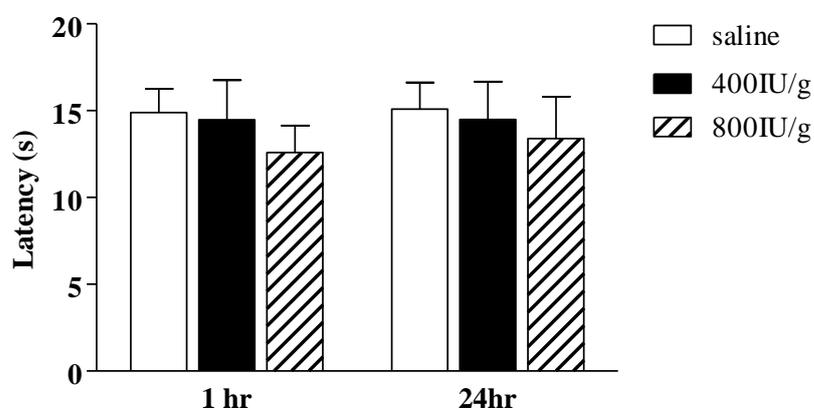


Figure 3.3.2.6. Effect of 400IU/g and 800IU/g on pain behaviour in CD1 mice subjected to the hot plate test at one hour and 24 hours post drug administration, carried out on day 20 and 21 of the study respectively. Data are expressed as mean \pm SEM; n= 7-10 per group.

3.3.2.7. Repeated IFN- α administration does not alter nociceptive behaviour of CD1 mice in response to formalin, a noxious inflammatory stimulus

One-way repeated measures ANOVA revealed a significant effect of time [F (11, 286) = 54.58, P=0.000] but not treatment [F (2, 26) = 0.02, P=0.975] or time x treatment interaction [F (22, 286) = 1.04, P=0.410] on pain '1' behaviour i.e. any elevation of the affected paw throughout the 60-minute test. Following assessment of pain 2 behaviour as a one hour block, one-way ANOVA also revealed no significant effect of IFN- α [P=0.975] on the total duration of this behaviour over 60 minutes (see figure 3.3.2.7.A,B).

Similarly, one-way repeated measures ANOVA revealed a significant effect of time [F (11, 286) = 12.19, P=0.000], but not drug [F (2, 26) = 0.358, P=0.709] or time x drug interaction [F (22, 286) = 0.51, P=0.969] on pain '2' behaviour i.e. direct shaking, licking, biting, flinching of the affected paw throughout the 60 minutes as observed in figure 3.3.2.7.C. Assessment of pain 2 behaviour as one hour block also revealed no significant effect of IFN- α [P=0.709] on the total duration of this behaviour over 60 minutes (figure 3.3.2.7.D).

One-way ANOVA also revealed a significant effect of time [F (11, 286) = 14.5, P=0.000] but not IFN- α treatment [F (2, 26) = 0.58, P=0.5665] or time x drug interaction [F (22, 286) = 0.46, P=0.983] on the overall CPS, subsequent to injection of formalin (figure 3.3.2.7.E). Assessment of pain 2 behaviour as a one hour block also revealed no significant effect of IFN- α [P=0.582] on the total duration of this behaviour over 60 minutes, as evident in figure 3.3.2.7.F.

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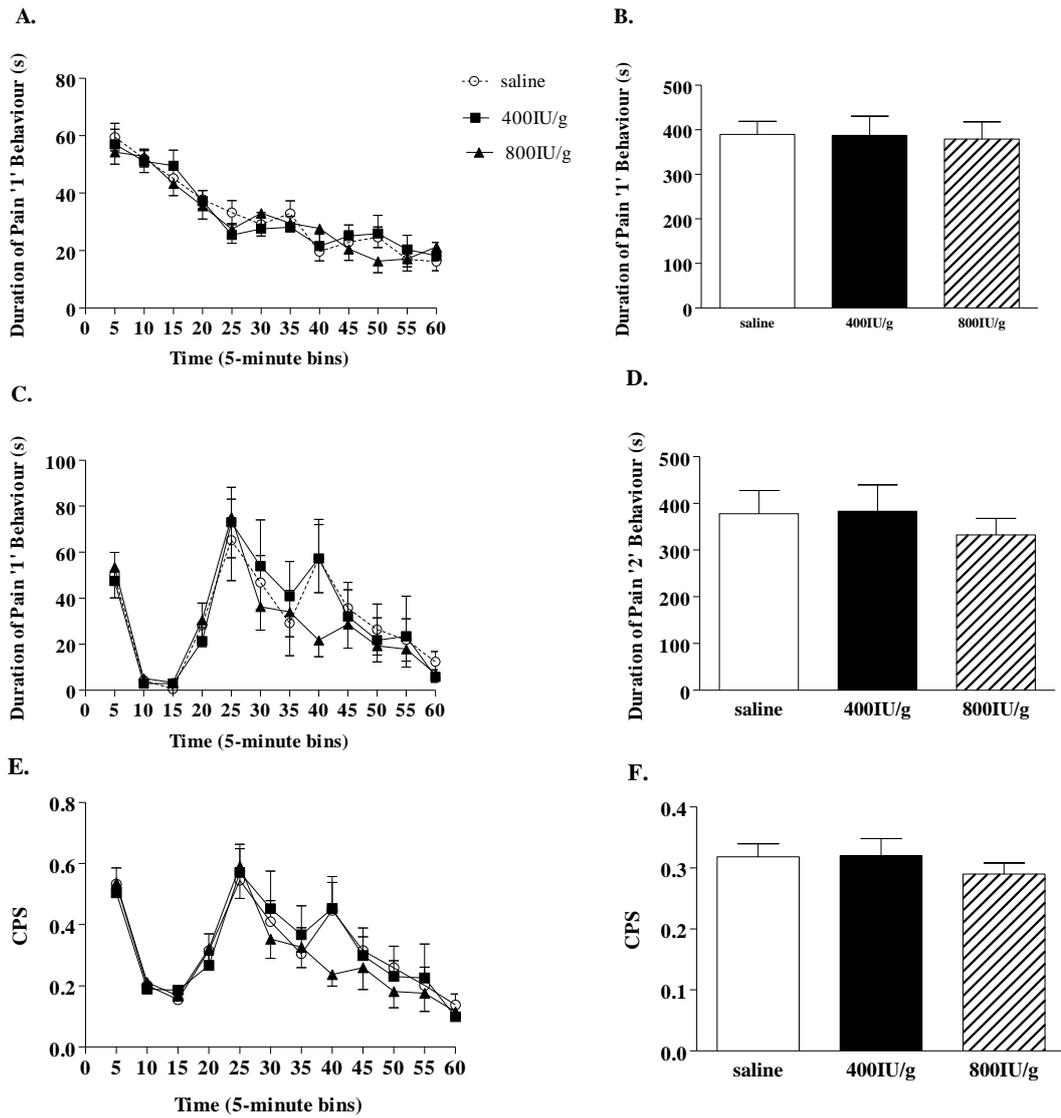


Figure 3.3.2.7. Nociceptive behaviour in the formalin test in CD1 mice: A, Profile of pain '1' behaviour over 60 minutes; B, Duration of pain '1' behaviour overall in the formalin test; C, Profile of pain '2' behaviour over 60 minutes; D, Duration of pain '2' behaviour overall in the formalin test; E, Profile of CPS over 60 minutes and F, Duration of CPS overall in the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group.

3.3.2.8. Repeated IFN- α administration does not alter general behaviour of CD1 mice in response to a noxious inflammatory stimulus

CD1 mice were exposed to the formalin test 24 hours following the final dose of IFN- α or saline vehicle. General behaviour i.e. locomotor activity, rearing and grooming was then assessed throughout the 60-minute test.

One-way repeated measures ANOVA revealed no significant effect of IFN- α treatment on distance moved [$F(2, 26) = 0.167, P=0.847$], grooming [$F(2, 26) = 1.037, P=0.369$] or rearing behaviour [$F(2, 26) = 0.422, P=0.660$] overall following formalin administration (figure 3.3.2.8).

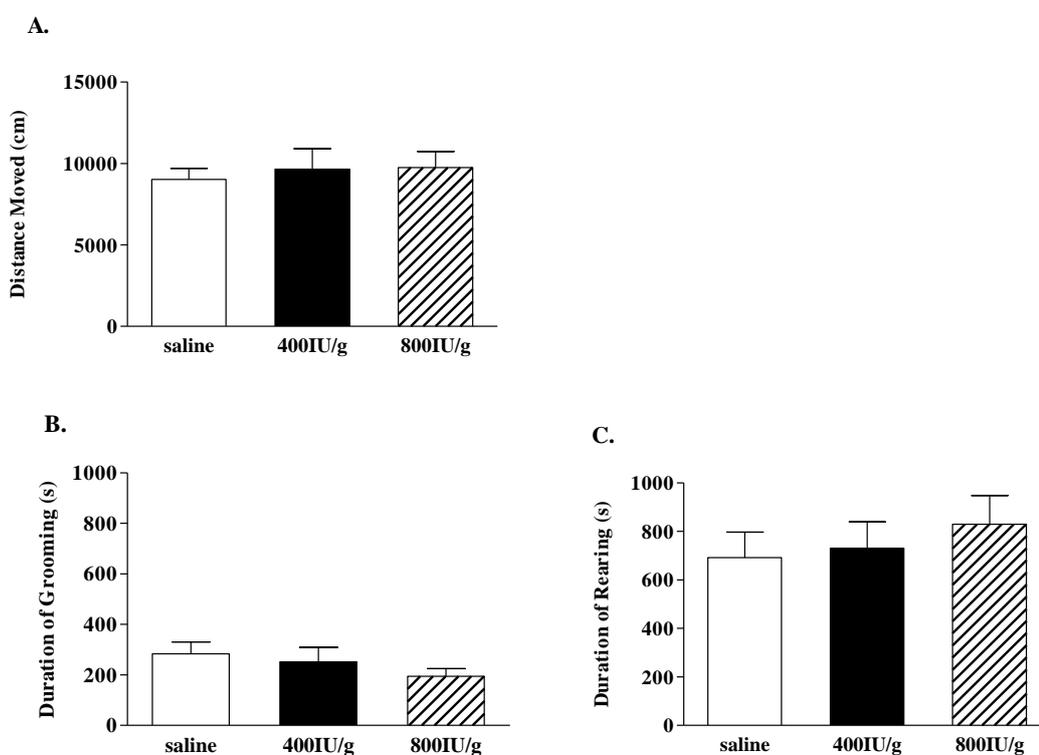


Figure 3.3.2.8. General behaviour in the formalin test in CD1 mice: A, duration of locomotor activity, B, duration of grooming behaviour and C, duration of rearing behaviour overall in the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group.

3.3.2.9. Repeated IFN- α administration does not alter formalin-induced oedema of the left- hind paw in CD1 mice

Formalin-induced oedema of the affected paw was assessed by measuring the change in diameter of the left hind paw immediately before and one hour after formalin administration.

One-way ANOVA revealed no significant change in paw diameter across all groups after formalin administration, [F (2, 28) = 1.29, P=0.292], as evident in figure 3.3.2.9.

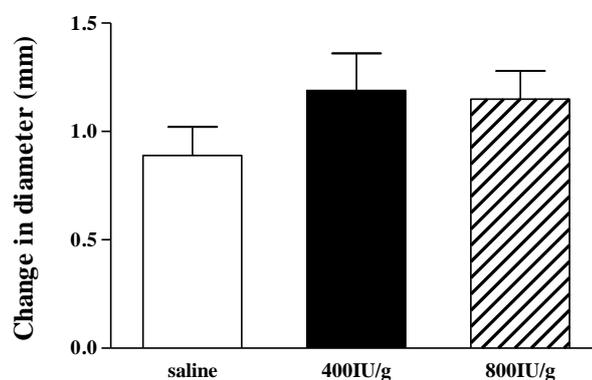


Figure 3.3.2.9. Formalin-induced oedema of the left hind paw in CD1 mice. Data are expressed as mean \pm SEM; n= 9-10 per group.

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3.4. Discussion

IFN- α is a pro-inflammatory cytokine, commonly used to treat infectious diseases such as hepatitis B and C as well as certain malignancies. However, this therapeutic strategy is associated with many unwanted side effects namely depression and anxiety, among other emotional and cognitive changes. Emergence of such depressive symptoms frequently prompts initiation of anti-depressant treatment or discontinuation of therapy. More recently, clinical studies have reported a high prevalence of pain disorders in patients undergoing this treatment regime (Shakoor et al., 2010, Nogueira et al., 2012). Thus, immune activation and subsequent alterations in neuronal functioning following repeated IFN- α treatment may underlie the high incidence of these co-morbidities.

In order to investigate the neurobiological mechanisms underpinning this response, this series of studies aimed to examine the effect of repeated IFN- α administration on depressive-, anxiety-like and nociceptive responding in two mice strains, which have each previously exhibited depressive-like behaviour in various paradigms of behavioural despair following continuous administration of IFN- α (Siddegowda et al., 2011b, Ping et al., 2012). Overall, the data presented above demonstrated that IFN- α (800IU/g) induced thermal hyperalgesia in C57, but not CD1 mice, following 21 days of treatment. This effect was in the absence of depressive- and anxiety-like behaviour or changes in locomotor activity in both strains.

Repeated administration of IFN- α in rodents has yielded conflicting data with respect to depressive-like behaviour. The present study demonstrated that 7 days of IFN- α administration to male C57 mice dose-dependently resulted in a slight, although non-significant, increase in immobility in the TST, an effect not observed in CD1 mice. Similar to these findings, Ping et al., (2012) also demonstrated that at comparable doses of subcutaneous IFN- α (60IU/g and 600IU/g) in C57 mice, immobility time in the TST was not altered (Ping et al., 2012). In addition, administration of murine IFN- α (mIFN- α), 60IU/g, i.p., also did not alter immobility time in the TST in BALB/C mice following 7 days of drug administration (Orsal et al., 2008). However, the duration of immobility in the TST was increased following treatment with 6,000IU/g/day IFN- α for 7 days. As such, it is possible that a longer treatment regime or a higher dose of IFN- α would be required to elicit a change in depressive-

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like behaviour as assessed in the TST. Re-examination of immobility in the TST on day 14 revealed that immobility was increased in all groups upon subsequent testing compared with immobility at day 7, while no effect of IFN- α (400 or 800IU/g/day) treatment on immobility observed at this time point in either C57 or CD1 mouse strain. It is likely that the repeated testing of the mice in the TST and increases of the immobility scores would mask any further increase in immobility that may be induced by IFN- α treatment.

In order to evaluate depressive-like behaviour in a further test, immobility in the FST was examined. Our data revealed that repeated administration (17 days) of IFN- α (400 and 800IU/g/day) did not alter immobility time of C57 or CD1 mice in the FST. This result is in contrast to a recent published finding whereby depressive-like behaviour was reported in the FST by day 5, 10 and 15 following daily administration of the drug at these doses (Siddegowda et al., 2011a). Similarly, earlier studies have also reported increased duration of immobility in the FST by day 7 following daily administration of IFN- α , (i.v. and i.p.), employing doses as low as 6IU/g (Makino et al., 1998, Makino et al., 2000, Ping et al., 2012). However, in general agreement with the current data presented, a recent study also failed to observe any notable alteration in despair-like behaviour throughout the forced swim test, albeit following central administration of mIFN- α (Hoyo-Becerra et al., 2015). However, based on this and other aforementioned studies, it can be deduced that the induction of despair behaviour as observed in the FST following IFN- α administration in rodents is dependent on a distinct combination of specific parameters which may include strain, dose, route, treatment regime, time of testing etc. Thus, future studies employing this behavioural paradigm should possibly incorporate an increase in dose, combined with a more appropriate time of testing in order unmask potential behavioural deficits following continuous treatment with IFN- α .

Previous publications have reported that acute and chronic administration of mIFN- α does not induce anxiety-related behaviour in the EPM and light/dark test (Wang et al., 2009, Orsal et al., 2008). However, repeated administration of hIFN- α (170 IU/g/day, 4 weeks s.c.) to rats results in anxiety-like behaviour in the EPM expressed as reduced time spent and entries into open arms (Fahey et al., 2007b).

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The data from our study demonstrated that time spent and entries into the open arms of the EPM, indicative anxiety-related behaviour, were unaffected by repeated (10 day) IFN- α administration (400 and 800 IU/g/day) in both C57BL/6 and CD1 mice. Thus, under the current conditions repeated administration of IFN- α does not alter anxiety-related behaviour as evidenced in the EPM. However, anxiety-like behaviour may be present and unmasked through other tests of such behaviour such as social interaction or conditioned fear which each have different mechanisms underlying them than the conflict associated with EPM.

As all of the aforementioned tests (TST, FST EPM) rely on motor responses as readout, it is important to assess the effect of treatments on this parameter. Locomotor activity was assessed throughout each study at various time points using the open field test and also assessed during the EPM and formalin test. Data from this revealed that repeated administration of IFN at any of the doses tested (400 and 800IU/g/day) did not alter locomotor activity at any timepoints examined. Thus, none of the data generated was impacted by effects on locomotor activity.

Although IFN- α therapy is frequently associated with pain complaints in the clinic, to our knowledge nociceptive responding has not been assessed in any preclinical studies evaluating the effects of repeated IFN administration to date. The present study demonstrated that 21 days of repeated IFN- α administration dose-dependently induced thermal hyperalgesia in male C57, but not CD1 mice, in the hot plate test. While there was a slight, non-significant reduction in latency to respond at the lowest dose of drug, a significant reduction in response latency was observed following repeated administration of 800IU/g/day IFN- α , an effect indicative of thermal hyperalgesia. As described in chapter 2, the hot plate test, originally characterized by Eddy and colleagues identifies licking and/or jumping behaviour as a direct indicator of nociceptive threshold (Eddy and Leimbach, 1953) and observed alterations in latency may reflect changes in nociceptive processing through peripheral (Kanaan et al., 1996, Menendez et al., 2002), spinal (Yamamoto et al., 2002, Huang et al., 2014) or supraspinal (Menendez et al., 2002, Piercey and Schroeder, 1981, Kubo et al., 2009) mechanisms. Furthermore, it is now known that affective predisposition influences responding to such stimuli (Burke et al., 2010). Thus, thermal hyperalgesia as observed in the case of C57 mice, but not CD1, may associate with

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the trend towards depressive-like behaviour as observed in the TST earlier in the study, an effect which was not evident in CD1 mice. Additionally, thermal hyperalgesia may also reflect a dependent-like syndrome in these animals as a result of the known morphine-like analgesic activity of IFN- α , an effect which may be thus evident in C57 but not CD1 mice. In this way, it would appear that C57 mice are more susceptible to the behavioural consequences of continuous IFN- α treatment than CD1 counterparts.

Interestingly, IFN- α did not alter inflammatory pain behaviour in response to formalin administration following 22 days of IFN- α administration. However, while repeated IFN- α administration did not alter inflammatory nociceptive responding in this model, it may do so in other models employing alternative noxious stimuli including carrageenan and complete Freund's adjuvant (CFA). Alternatively, IFN- α treatment may provoke alterations in neuropathic pain-related behaviour, which would be identified through alternative behavioural paradigms. Therefore, while this data indicates that formalin-evoked nociception is not altered in the current study, alterations in pain processing cannot be ruled out and future studies may include alternative approaches such as a higher dose IFN- α or alternative tests for pain behaviour.

Overall, although repeated administration of IFN- α induces thermal hyperalgesia after 21 days of treatment in the absence of a depressive phenotype, the present data highlight the complexities of investigating the effect of treatment on behaviour with issues such as strain, dose, housing conditions, behavioural tests used and time of testing all possible confounding factors.

Chapter 4

The effect of repeated IFN- α (8,000 IU/g) administration on depressive-like behaviour and nociceptive responding to thermal and inflammatory stimuli in mice

Chapter 4: Effect of IFN- α (8,000 IU/g) on depressive and nociceptive behaviour

IFN- α is a pro-inflammatory cytokine, commonly used to treat infectious diseases such as hepatitis B and C as well as certain malignancies. However, this therapeutic strategy is associated with many unwanted side effects namely depression, among other psychiatric disorders (Schaefer et al., 2002, Raison et al., 2005). More recently, clinical studies have reported a high prevalence of pain disorders in patients undergoing this treatment regime (Shakoor et al., 2010, Nogueira et al., 2012) and these were more frequently reported by patients with co-morbid major depression (Shakoor et al., 2010). Given the established role of inflammatory processes in the development of neuropsychiatric and neurological disorders, it is thought that immune activation following IFN- α treatment may underlie the high incidence of these co-morbidities.

Several studies, including that presented in the previous chapter, have examined depressive-like behaviour following acute and repeated IFN- α administration, with results often inconsistent between studies (see Table 3.1.1). In agreement with the clinical scenario, repeated administration of this pro-inflammatory cytokine prompts the induction of depressive-like effects in the forced swim (Orsal et al., 2008), tail suspension (Friebe et al., 2013) and sucrose preference (Hayley et al., 2013) tests in mice. In addition, chronic administration of IFN- α also exhibits a reduction in sucrose preference in the treated animals (Hayley et al., 2013), although others have failed to demonstrate such effects (Hoyo-Becerra et al., 2015). With specific regard to the administration of human (h)IFN- α , a number of studies have identified that repeated administration of both low (Siddegowda et al., 2011a, Makino et al., 1998) and high (Ping et al., 2012) doses of hIFN- α have also exhibited despair-like behaviour in the FST. Additionally, continuous hIFN- α infusion increased duration of immobility in the tail suspension test (TST) and reduced sucrose consumption (Ping et al., 2012). In comparison, Kosel and colleagues failed to observe such increases in immobility in the FST and hedonic behaviour in the sucrose preference test following repeated exposure of mice to pegylated hIFN- α (Kosel et al., 2011). Similarly, the data presented in chapter 3 demonstrate that relatively low doses of hIFN- α administered repeatedly over a 2-3 week period does not modulate depressive-like behaviour in the TST and FST tests in mice. However, it is possible that a higher dose of hIFN- α is required to induce such an effect. This would also

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seem plausible given that hIFN- α has been shown to exhibit differential affinity for murine IFNAR1 (Streuli et al., 1981) and thus, at the doses employed in chapter 3, may not induce a sufficient immune response with associated behavioural outcomes.

While the previous chapter depicts a trend towards an increase in immobility throughout the TST following 7 days of continuous IFN- α administration in C57Bl/6 mice, no change was observed following 14 days or indeed, in the FST following 17 days of IFN- α treatment (chapter 3). While this lack of effect is in agreement with some studies, possible discrepancies between this and studies depicting behavioural despair as a consequence of hIFN- α treatment include variances in study design, type of IFN- α , drug formulation, duration of treatment, choice of species and the strain employed. In particular, the dose employed in such studies may dictate reported disparities in behavioural outcomes. Given the lack of significant behavioural effects obtained employing doses as low as 400 and 800IU/g (chapter 3), it is possible that increasing the dose of drug employed may produce depressive-like effects as observed by Ping and colleagues (Ping et al., 2012). Furthermore, application of an alternative test for the assessment of depressive-like behaviour such as the sucrose preference test may also unmask behavioural sensitivities in response to the drug that were not previously detected in chapter 3. It is also possible that the multiple testing in the same animals as conducted in chapter 3 may have impacted on the data generated and possibly masked possible effects. Such an observation was noted in the TST, whereby upon suspension, test subjects somewhat anticipated the inescapable scenario and exhibited a reduction in latency to immobility as evident in figure 3.3.1.3, thus masking potential differences in duration of immobility. Repeated handling and exposure to novel environments may have elicited a stress response that impacted on behavioural responding of the animals and the effect of hIFN- α .

Recent reports from the clinic have revealed a high prevalence of pain disorders in patients undergoing IFN- α treatment (Shakoor et al., 2010, Nogueira et al., 2012). Some early investigations suggest an associative role with mechanical and thermal analgesia following acute administration of the drug in rodents (Lee et al., 2010, Jiang et al., 2000, Wang et al., 2006, Blalock and Smith, 1981). However, the effect

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of repeated administration of hIFN- α treatment on nociceptive responding has yet to be published on. In contrast to these early reports of analgesic properties following acute administration of the drug, our data (chapter 3) have identified a dose dependant thermal hypersensitive phenotype following 21 days of IFN- α treatment in C57Bl/6, but not CD1, mice. Thus, while acute hIFN- α may elicit analgesic effects in tests of thermal nociceptive responding, repeated administration appears to elicit hyperalgesia under these conditions. However, no such hyperalgesia was observed in the formalin test of inflammatory pain behaviour following 22 days of repeated administration. While these data may indicate effects on thermal but not inflammatory processing of pain, it is possible that the dose of hIFN- α employed was not sufficient to elicit changes in pain mechanisms that underlie inflammatory pain (as well as emotional) processes.

As referred to in chapter 3, IFN- α has been proposed to exert its behavioural effects through direct activation of the innate and adaptive immune system. More specifically, the pro-inflammatory cytokine binds to its receptor and activates various immune cells that highly express this receptor including macrophages and B lymphocyte cells, which in turn activates the JAK-STAT pathway (Platanias, 2005). The product of JAK-STAT activation then binds IRF9 before translocation to the nucleus (Stark et al., 1998, Darnell, 1997, Aaronson and Horvath, 2002), attachment to the IFN-stimulated response element (ISRE) and subsequent transcriptional induction of various IFN-stimulated genes (ISGs) including IL-6, TNF- α , IRFs such as IRF7, STATs and other interferons [for review see (Katze et al., 2008)]. Such responses in turn can activate alternative immune cells and inflammatory pathways including dendrites, neutrophils, natural killer (NK) and T cells. For example, IFN- α can modulate lymphocyte activity through upregulation of IFN- γ production (Marshall et al., 2006). Collectively, such responding eventually leads to the exacerbated induction of indoleamine 2, 3- dioxygenase (IDO), a tryptophan-metabolising enzyme. Induction of IDO limits tryptophan availability peripherally and provokes the excessive formation of kynurenine (Comai et al., 2011), itself a precursor of other toxins such as quinolinic acid (QA) and NMDA agonist which upon transport to the CNS promote glutamate-mediated excitotoxicity and

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neurodegeneration (Mazarei et al., 2013, Walker et al., 2013, Wichers and Maes, 2004). Additionally, cytokine-induced enhancement of HPA activity and associated increased glucocorticoid secretion can also induce tryptophan 2-3-dioxygenase (TDO) (Knox, 1951, Salter and Pogson, 1985), an alternative tryptophan-degrading enzyme. Collectively, such limited availability of tryptophan for transport into the CNS also diminishes central levels of serotonin (Heyes et al., 1992), a monoaminergic neurotransmitter central to the development and treatment of depression-pain interactions. Importantly, many of the above-mentioned pro-inflammatory cytokines have been implicated in the pathogenesis of depression and/or pain disorder as levels of the cytokines have been shown to be enhanced in the blood and cerebrospinal fluid of both depressed [for review see (Young et al., 2014, Lichtblau et al., 2013)] and chronic pain [for review see (Rodriguez-Pinto et al., 2014, Ji et al., 2013)] patients. More specifically, some of these cytokines, namely IL-6, IL-1 β and TNF- α are also implicated in the development of treatment-induced depression upon initiation of IFN- α therapy (Udina et al., 2013, Prather et al., 2009, Wichers et al., 2006, Loftis et al., 2013, Krueger et al., 2011, Raison et al., 2010). Altered levels of anti-inflammatory cytokines also associate with the development of depression and pain. For example, serum levels of IL-10 have shown to be reduced in depressed patients (Dhabhar et al., 2009). Additionally, in a clinical population of stroke patients, induction of major post-stroke depression is associated with a genetic polymorphism of IL-4 (Kim et al., 2012b), an anti-inflammatory cytokine known to inhibit the expression and associated activity of IDO in human monocytes (Musso et al., 1994).

IFN- α -induced production of pro-inflammatory cytokines such as IL-6, TNF- α and IFN- γ which, once in the brain, themselves activate glial cells and such pathological changes in the central nervous system (CNS) have also been reported in depressed subjects (Rajkowska and Miguel-Hidalgo, 2007) and in patients with chronic pain conditions (Loggia et al., 2015). Furthermore, such inflammatory processes have been shown to provoke central increases in IDO activity (Kwidzinski et al., 2005, O'Connor et al., 2009), thus reducing serotonin availability, enhancing glutamate excitability (Wichers and Maes, 2004) and altering neurotrophin levels such as

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BDNF, thus limiting neurogenesis and promoting neurodegeneration (Rajkowska and Miguel-Hidalgo, 2007, Kubera et al., 2011). These effects in emotional and pain centres may ultimately be responsible for depressive and/or pain disorder.

Previous published findings characterising the behavioural effects of IFN- α treatment (Siddegowda et al., 2011a, Ping et al., 2012) as well as the preliminary findings in chapter 3 collectively suggest that the C57Bl/6 mouse strain in particular may be a more appropriate strain to examine depressive-like symptoms and hyperalgesic behaviour following repeated administration of IFN- α rather than outbred CD1 mice. As such this strain of mice will be used in the current investigations.

Thus, the hypothesis for the current study was that repeated administration of a higher dose of IFN- α induces depressive-like behaviour and enhanced nociceptive responding in C57 mice, effects which are mediated by concurrent changes in peripheral and central immune signalling, neurotrophin levels and/or HPA activity.

Chapter 4: Effect of IFN- α (8,000 IU/g) on depressive and nociceptive behaviour

4.1.1. Aims

1. Examine the effect of repeated IFN- α administration (8,000 IU/g) on depressive-like behaviour in mice using the TST, FST and sucrose preference test
2. Assess nociceptive responding to a noxious thermal stimulus and persistent noxious inflammatory stimulus following repeated IFN- α administration using the hot plate and formalin tests
3. Determine if formalin-evoked nociceptive responding is altered following an acute single administration of IFN- α (8,000 IU/g)
4. Investigate if repeated IFN- α administration results in persistent chronic immune activation, either peripherally or centrally, altered neurophin expression or enhanced corticosterone levels, at the time when behavioural changes are observed.

4.2 Materials and Methods

4.2.1 Animals

As before, male C57Bl/6 mice (weight 25-30g; Charles River Laboratories, UK) were used. For experiment 1, part A, animals were housed in groups of three to 4 (with at least one mouse from each treatment group per cage) in plastic-bottomed cages containing wood shavings as bedding. For experiment 1, part B, and for experiment 2, animals were singly housed. All animals were maintained in a constant temperature (21 +/- 2°C) under standard lighting conditions (12 : 12 hr light-dark, lights on from 07.00 to 19.00 hr). All experimental procedures were carried out during the light phase, between 07.00 and 16.00 hr. Mice were given free access to food and water.

The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

4.2.2 Pharmacological Treatments

Human interferon-alpha (hIFN- α -2a; 3M IU/0.5ml), Roferon-A®, was purchased from Roche Pharmaceuticals. The drug was diluted in sterile saline from stock to the desired concentration on a daily basis. This drug/vehicle solution was administered subcutaneously in a volume of 3 μ l/g to give a final concentration of 8000IU/g/day IFN- α . For experiment 1, part A, experiment 2, 3 and 4, dosing occurred daily, between the hours of 10.00 and 12.00 whereas in experiment 1, part B, dosing occurred at 07.00 in order to avoid disturbance of the sucrose preference test, which began at 07.00 on each day of testing. The dose chosen was based on the minimum behavioural effects of lower doses observed in chapter 3 and published research demonstrating depressive-like behaviour following repeated administration of IFN- α (6,000IU/g) in mice (Ping et al., 2012) and is equivalent to that which is approximately 15 times higher than the maximum recommended daily dose of IFN- α for AIDS-related Kaposi's sarcoma.

4.2.3 Experimental Design

4.2.3.1 Experiment 1

This study evaluated the effect of IFN- α (8,000 IU/g/day) on depressive-like responding in C57Bl/6 mice. In brief, following acclimatization to the holding facilities, all mice were exposed to the open field arena for a five-minute period and distance moved assessed. Daily IFN- α or saline administration began between 10.00 hour and 12.00 hour, immediately following exposure to the open field and animals were then retested 24 hours later, and on days 3, 5, 7 and 10. On day 7 and day 10 of treatment, mice were also tested in the TST and FST respectively which took place directly after open field test. All behavioural testing occurred 24 hours post drug administration (sequence of behavioural testing is illustrated in figure 4.2.3.1.A) and was recorded onto DVD and assessed using EthoVision software as described in chapter 2.

A separate set of animals was used to examine the effect of repeated IFN- α administration on sucrose preference. On each day prior to the test, animals were administered a saline injection, s.c., in order to dissociate the injection procedure itself from possible hedonic-like effects upon induction of IFN- α treatment. Over the course of the following 24 hours, animals were offered the choice to drink from two bottles, one filled with tap water and the other with 1% sucrose solution. After 12 hours, the relative position of the two bottles was changed to avoid side preference. Animals were exposed to the choice of solutions for a total of five days prior to IFN- α administration, in order to establish baseline sucrose preference. In order to evaluate this, bottles were weighed and the amount of water and sucrose consumed was recorded after each 24-hour exposure. Consumption was expressed as (i) total sucrose intake, (ii) total fluid intake, (iii) sucrose intake per 100g body weight (sucrose intake*100/body weight) and (iv) % sucrose preference relative to the entire quantity of fluid consumed (sucrose intake*100/total fluid intake). Once sucrose preference greater than 75% was established, IFN- α or saline was administered once daily thereafter and mice were retested immediately after and on days 3-4, 6-7 and 10-11 of treatment, as seen in figure 4.2.3.1.B.

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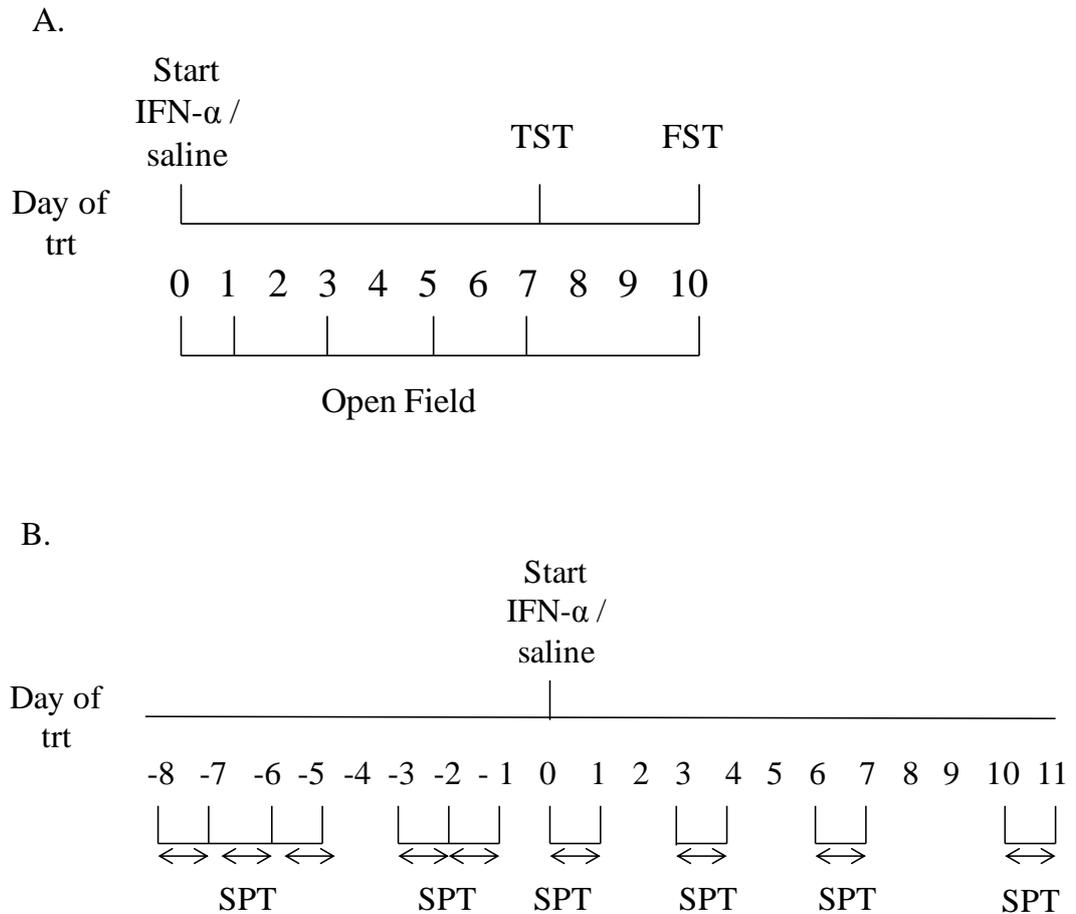


Figure 4.2.3.1. Timeline for Experiment 1, investigating A, the effect of repeated IFN- α (8,000 IU/g) on depressive-like behaviour in the TST and FST and B, investigating the effect of repeated IFN- α on depressive-like behaviour in the sucrose preference test in C57Bl/6 mice (trt, treatment; SPT, sucrose preference test).

4.2.3.2 Experiment 2

This experiment investigated the effect of IFN- α (8,000IU/g/day) on nociceptive behaviour in response to thermal and inflammatory noxious stimuli in C57Bl/6 mice. As in the case of the sucrose preference test described above, mice were administered saline for five days followed by eight days of saline or IFN- α administration, during which animals were exposed to the hot plate test on day 4 and 7 and the formalin test on day 8 (see figure 4.2.3.2). All behavioural testing occurred 24 hours following saline or IFN- α administration. Latency to lick the hind paw was

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recorded in the hot plate test and behaviour in the formalin test was recorded onto DVD and assessed using EthoVision software as previously described (chapter 2).

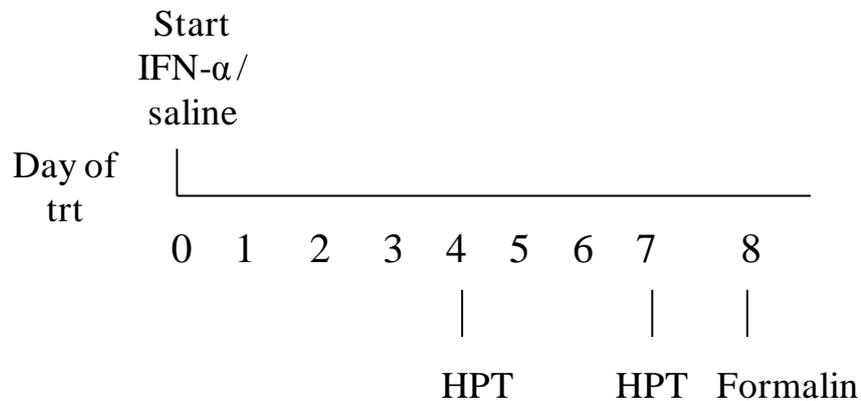


Figure 4.2.3.2. Timeline for experiment 2, investigating the effect of repeated IFN- α treatment (8,000 IU/g) on thermal and inflammatory nociceptive responding in C57Bl/6 mice.

4.2.3.3 Experiment 3

This experiment evaluated the effect of an acute single administration of IFN- α (8000IU/g/day) or saline on nociceptive responding in the formalin test 24 hours later. Mice were administered saline for five days to acclimatize to injection procedure. Animals then received either saline or IFN- α and 24 hours later, were exposed to the formalin test. Behaviour was recorded onto DVD and assessed using Ethovision software as previously described (chapter 2).

4.2.3.4 Experiment 4

This experiment investigated the effect of IFN- α (8,000 IU/g) on immune mediators and neurotrophin levels both peripherally and centrally as well as HPA axis activity following 8 days of treatment. In this way, a separate group of mice were administered IFN- α or saline, once every day for eight days and sacrificed 24 hours following the final injection. Blood was collected under anaesthesia by cardiac puncture and plasma isolated. The spleen was then removed, the PFC dissected out

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and all were stored at -80°C until analysis for gene expression, cytokine protein and corticosterone levels.

4.2.4 Behavioural tests

Behavioural testing was performed primarily between the hours of 10.00 and 16.00, and occurred 20-24 hours after drug or vehicle administration to avoid acute effects of drug administration. All experiments were carried out as described in chapter 2. As for previous experiments, some behavioural analysis took place in the animal holding room including the open field, hot plate and sucrose preference tests while the others were carried out in a separate testing room (TST, FST and formalin test) and animals were tested immediately without time for habituation to the new environment. As before, after each session, animals were returned to the cages in their own room. All tests were recorded by video camera onto DVD and analysed at a later date, using EthoVision XT video tracking software (Noldus, Netherlands).

4.2.5 RNA Extraction, Reverse Transcription and RT-qPCR

This was carried out as described in chapter 2 and performed using Taqman gene expression assays for mRNA expression of IL-1 β , IL-6, IDO, integrin alpha M (CD11b) and BDNF in the spleen and PFC tissue. The mRNA expression of glial fibrillary acidic protein (GFAP), a marker of astrocyte activation, was also assayed in the PFC tissue.

4.2.6 ELISA

ELISAs carried out on spleen lysate as described in chapter 2 and performed using ELISA MAXTM standard sets (Biolegend, UK) for pro- (IFN- γ , IL-1 β , TNF- α , IL-12, IL-2) and anti-inflammatory (IL-4, IL-10) cytokines.

The corticosterone assay was carried out on plasma samples, as described in chapter 2, and performed using a Corticosterone EIA kit (Cayman Chemical Company, MI).

4.2.7 Statistical Analysis

Data were analysed using SPSS statistical package, employing Student's unpaired t-test, one-way ANOVA and one-way repeated measures ANOVA followed by

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Fishers LSD *post hoc* analysis where appropriate. In the case of two treatment groups, where data were non-parametric, the Mann-Whitney U-test was employed. Data were considered significant when $P < 0.05$. Results are expressed as group means \pm standard error of mean (SEM).

4.3 Results

4.3.1 Experiment 1. The effect of repeated administration of IFN- α (8,000 IU/g) on depressive-like behaviour in C57Bl/6 mice

4.3.1.1 Repeated administration of IFN- α (8,000 IU/g) does not alter body weight or body weight gain in C57Bl/6 mice

In order to evaluate the influence of repeated IFN- α administration on body weight and body weight gain per week, mice were weighed directly prior to each injection of saline or IFN- α .

One-way repeated measures ANOVA revealed a significant effect of time [F (10, 180) = 0.48, P= 0.000], but no effect of IFN- α [F (1, 18) = 0.27, P=0.608] or time x IFN- α interaction [F (10, 180) = 0.48, P=0.903] on body weight throughout the study. Fisher's LSD *post hoc* analysis revealed that body weight increased over time, with no significant difference between the groups (figure 4.3.1.1).

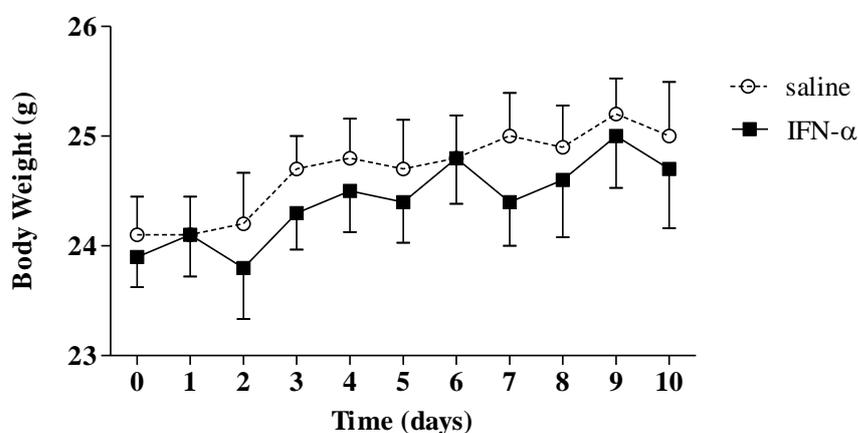


Figure 4.3.1.1. Body weight of C57Bl/6 mice receiving saline or 8,000IU/g IFN- α daily for 10 days. Data are expressed as mean \pm SEM; n= 10 per group.

4.3.1.2 Repeated administration of IFN- α (8,000 IU/g) does not alter locomotor activity of C57Bl/6 mice in the open field test

In order to evaluate the influence of repeated IFN- α administration on locomotor activity, animals were exposed to the 5-minute open field test prior to and on day 1, 3, 5, 7 and 10 of saline or IFN- α treatment. The distance moved over the 5 minutes was tracked and assessed using Ethovision XT software.

One-way repeated measures ANOVA revealed a significant effect of time [F (5, 70) = 24.20, P=0.000], but no effect of IFN- α [F (1, 14) = 0.30, P=0.593] or time x IFN- α interaction [F (5, 70) = 1.17, P=0.335] on the distance moved in the open field arena throughout the study (Figure 4.3.1.2). Fisher's LSD *post hoc* analysis revealed that locomotor activity decreased from the first exposure (day 0) likely due to habituation to the test, an effect not altered by IFN- α administration.

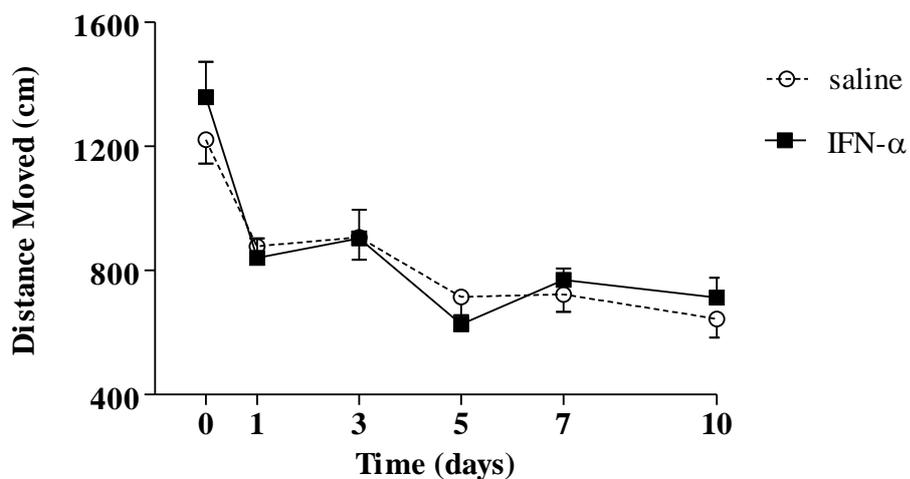


Figure 4.3.1.2. Locomotor activity (distance moved) in the open field arena of C57Bl/6 mice receiving saline or 8,000IU/g IFN- α per day. Data are expressed as mean \pm SEM; n= 10 per group.

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4.3.1.3 Repeated administration of IFN- α (8,000 IU/g) does not alter the duration of immobility of C57Bl/6 mice in the TST

Mice were assessed for depressive-like behaviour in the TST following 7 days of saline or IFN- α treatment. The test itself was carried out 24 following the previous dose of saline or IFN- α . Behaviour throughout this six-minute test was recorded and then analysed for the duration of immobility.

One-way repeated measures ANOVA revealed a significant effect of time [F (5, 85) = 18.07, P=0.000] but not drug [F (1, 17) = 0.74, P=0.401] or time x drug interaction [F (5, 85) = 1.12, P=0.355] on time spent immobile throughout the six-minute test. Fisher's LSD *Post hoc* analysis revealed that time spent immobile increased significantly after the first minute of the test, an effect not significantly different between saline- and IFN- α -treated mice (Figure 4.3.1.3.A).

When the first two minutes of the test were excluded from analysis to allow for habituation to the arena, unpaired t-tests revealed no significant effect of IFN- α on the total time spent immobile in the latter four minutes of the test (P=0.281) (Figure 4.3.1.3.B). Unpaired t-test revealed no effect of IFN- α on latency to immobility (P=0.638) in the TST, as evident in figure 4.3.1.3.C.

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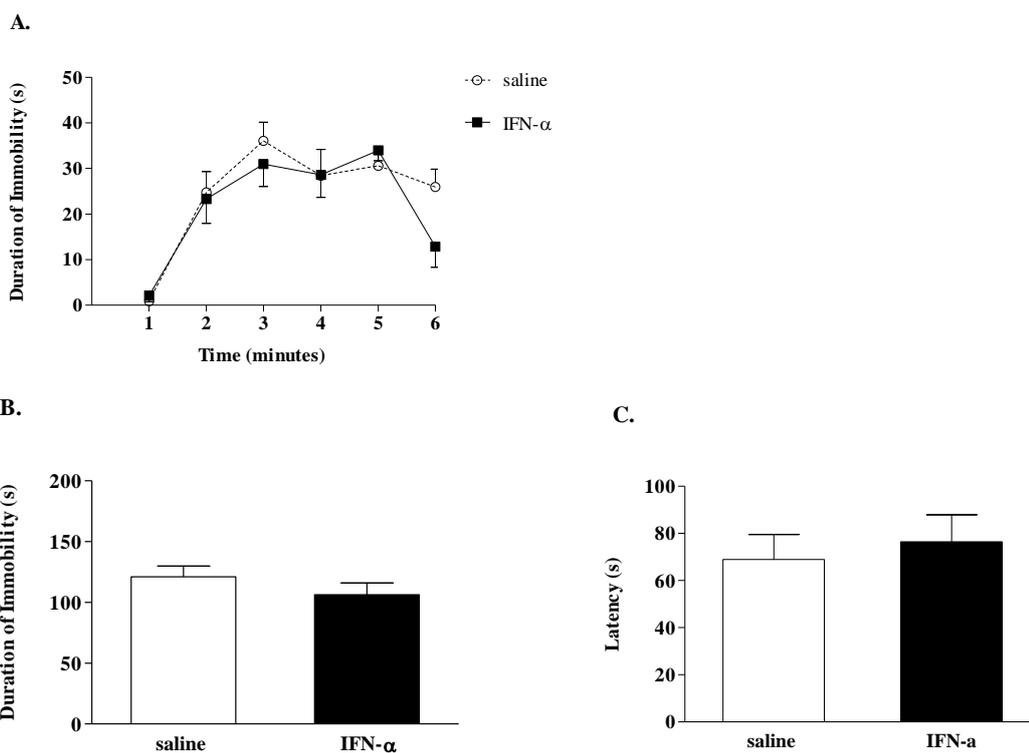


Figure 4.3.1.3. TST in C57Bl/6 mice receiving a chronic daily dose of saline or 8,000IU/g IFN- α : A, time spent immobile within each minute of test; B, total duration of immobility over the latter four minutes of test and C, latency to immobility. Data are expressed as mean \pm SEM; n= 9-10 per group.

4.3.1.4 Repeated administration of IFN- α (8,000 IU/g) for 10 days increased duration of immobility of C57Bl/6 mice in the FST

The FST was carried out following ten days of repeated saline or IFN- α treatment. Throughout the course of the 6-minute trial exposure, behaviour was recorded and the duration of immobility was later assessed.

One-way repeated measures ANOVA revealed a significant effect of drug [F (1, 18) = 15.45, P=0.001] and time [F (5, 90) = 79.60, P=0.000] but not drug x time interaction [F (5, 90) = 2.14, P=0.068] on the duration of immobility throughout the six-minute test. Fisher's LSD *post hoc* analysis revealed that time spent immobile increased after the first minute of the test in the case of both groups of animals. Time spent immobile was also significantly increased in the IFN- α -treated group from 3 to 6 minutes, when compared to saline-treated counterparts (figure 4.3.1.4.A).

When the first two minutes of the test were excluded for habituation, unpaired t-tests revealed a significant increase in time spent immobile in IFN- α treated mice throughout the latter four minutes of the test [P=0.000] when compared to saline-treated counterparts (figure 4.3.1.4.B). An unpaired t-test revealed no significant effect of repeated IFN- α administration on latency to immobility [P=0.814] in the FST.

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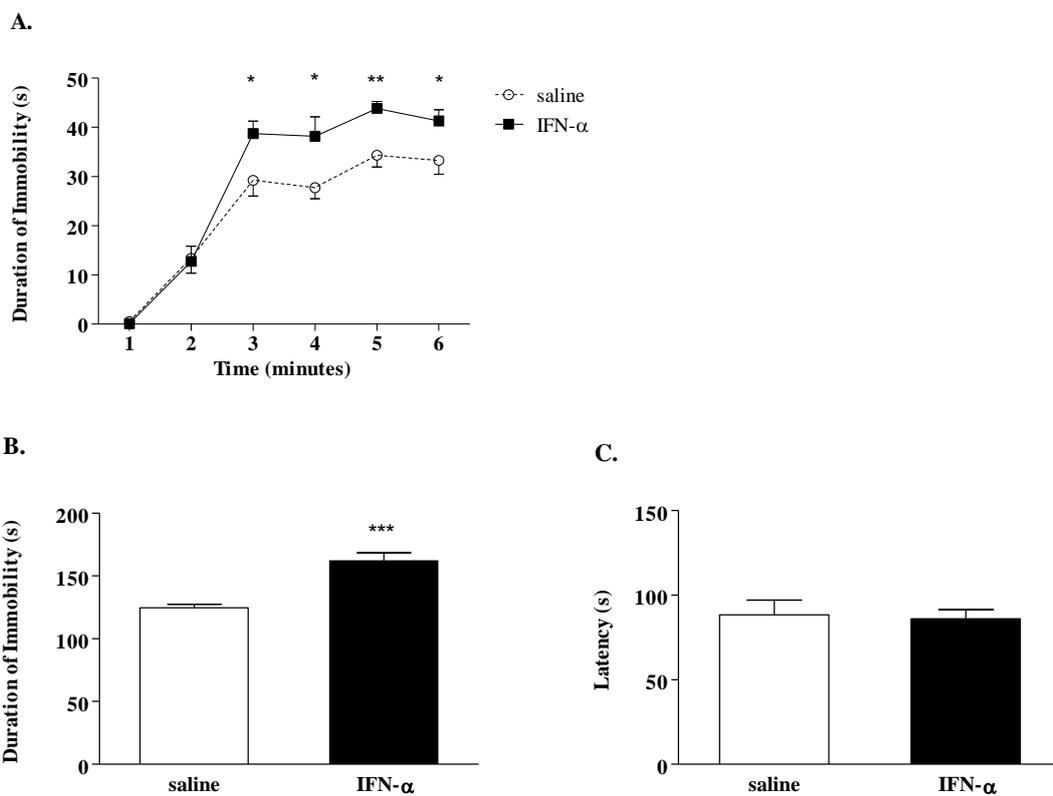


Figure 4.3.1.4. FST in C57Bl/6 mice receiving a chronic daily dose of saline or 8,000IU/g IFN- α : A, time spent immobile within each minute of test; B, total duration of immobility over the latter four minutes of test and c, latency to immobility. Data are expressed as mean \pm SEM; n= 10 per group, * p <0.05, ** p <0.01, *** p <0.001 vs. saline-treated animals.

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4.3.1.5 Repeated administration of IFN- α (8,000 IU/g) reduces sucrose preference

In order to evaluate the influence of repeated IFN- α administration on hedonic-like behaviour, animals were exposed to the 24-hour sucrose preference test prior to and during day 1, 4, 7 and 10 of saline or IFN- α treatment. After each day of testing, bottles were weighed and the consumption of sucrose and water was recorded for calculation of total sucrose intake, total fluid intake, sucrose intake per 100g body weight and % sucrose preference relative to the entire quantity of fluid consumed.

One-way repeated measures ANOVA revealed no significant effect of drug [F (1, 7) = 1.54, P=0.254], time [F (4, 28) = 2.02, P=0.084] or time x drug interaction [F (4, 28) = 0.82, P=0.526] on overall sucrose intake (figure 4.3.1.5.A). Similarly two-way repeated measures ANOVA revealed no significant effect of drug [F (1, 7) = 0.27, P=0.617], time [F (4, 28) = 0.97, P=0.438] or drug x time interaction [F (4, 28) = 0.36, P=0.833] on total fluid intake over the course of the study (figure 4.3.1.5.B).

One-way repeated measures ANOVA revealed a significant effect of drug [F (1, 8) = 6.409, P=0.035] and time [F (5, 40) = 2.538, P=0.044] but not time x drug interaction [F (5, 40) = 0.917, P=0.480] on sucrose preference. Subsequent Fisher's LSD *post hoc* analysis revealed a significant reduction in sucrose preference on day 4 in the IFN- α -treated group when compared with saline-treated counterparts. Sucrose preference was significantly reduced on day 4 and 7 in the IFN- α -treated group when compared with baseline values [Figure 4.3.1.5.C]. In comparison, there was no significant reduction in sucrose preference from baseline levels in saline-treated animals over the course of the study.

One-way repeated measures ANOVA revealed a significant effect of time [F (4, 28) = 3.05, P=0.033] but not drug [F (1, 7) = 0.96, P=0.360] or time x drug interaction [F (4, 28) = 0.93, P=0.460] on sucrose intake per 100g body weight throughout the study. Fisher's LSD *post hoc* analysis revealed that sucrose intake per 100g body weight was significantly reduced by day 7 in the IFN- α -treated mice, when compared with baseline values, as seen in figure 4.3.1.5.D. There was no effect of time on sucrose intake per 100g body weight in the saline-treated animals.

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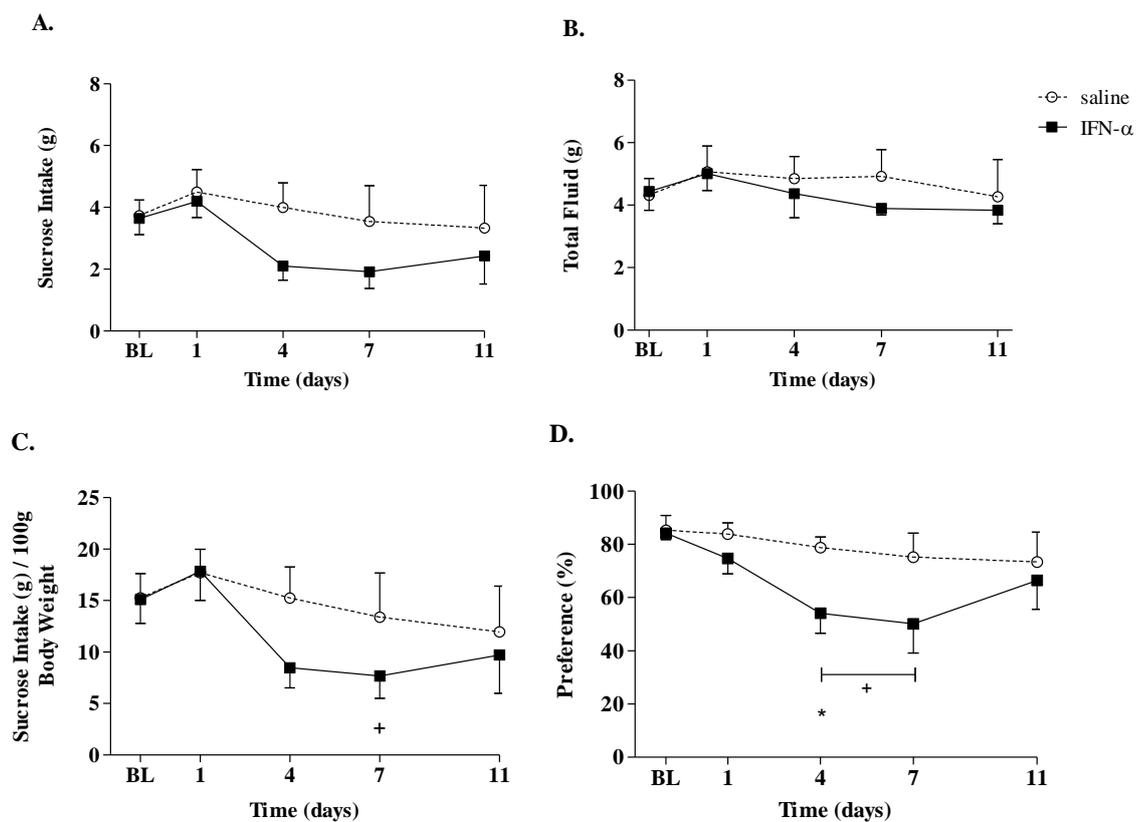


Figure 4.3.1.5. A, Sucrose intake; B, total fluid intake, C, sucrose intake per 100g body weight and D, sucrose preference in C57Bl/6 mice subjected to daily administration of 8,000IU/g IFN- α or saline. Data are expressed as mean \pm SEM; n= 4-5 per group; *p<0.05 vs. saline-treated mice; †p<0.05 vs. baseline (day-1) values (BL, baseline).

4.3.2. Experiment 2. The effect of repeated administration of IFN- α (8,000 IU/g) on thermal and inflammatory nociceptive behaviour in C57Bl/6 mice

4.3.2.1 Repeated IFN- α does not induce thermal hyperalgesia in the hot plate test in C57Bl/6 mice following 4 or 7 days of administration

Following four and seven days of saline or IFN- α treatment, animals were exposed to a noxious thermal stimulus in the form of a ceramic plate, heated to a temperature of 54-55°C (hot plate test) and the time taken or latency for animals to respond to the heat stimulus was recorded.

One-way repeated measures ANOVA revealed no significant effect of time [F (1, 18) = 3.684, P=0.071], IFN- α [F (1, 18) = 0.045, P=0.834] or time x IFN- α treatment [F (1, 18) = 0.093, P=0.764] on latency to respond in the hot plate test, as evident in figure 4.3.2.1.

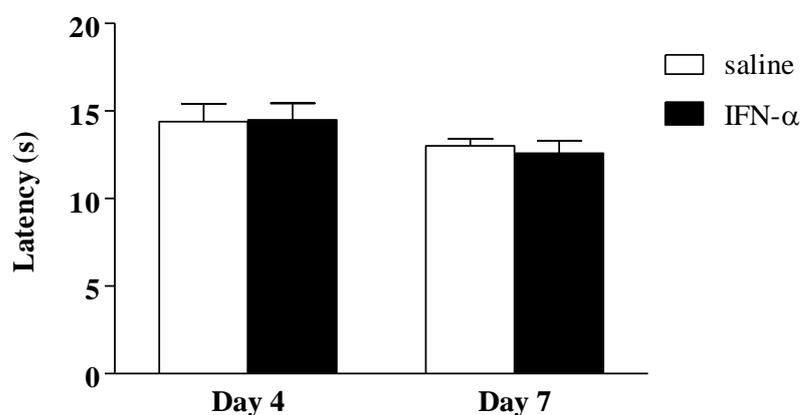


Figure 4.3.2.1. Latency to respond in the hot plate test following 8 days of saline or IFN- α treatment. Data expressed as mean \pm SEM; n = 10 per group.

4.3.2.2 Repeated administration of IFN- α (8,000 IU/g) induces inflammatory hyperalgesia in the formalin test following 8 days of administration

In order to assess if repeated administration of IFN- α alters inflammatory nociceptive responding, mice were exposed to the formalin test 24 hours following the last of 8 daily administrations of saline or IFN- α . This test involves the intraplantar injection of dilute formalin into the left hind paw and subsequent nociceptive responding was assessed over the following 60 minutes.

One-way repeated measures ANOVA revealed a significant effect of time ($F(11, 176) = 7.785, P=0.000$) but not drug [$F(1, 16) = 0.004, P=0.949$] or time x drug interaction [$F(11, 176) = 0.350, P=0.973$] on elevation of the injected paw (pain '1' behaviour), over the course of the 60-minute test (figure 4.3.2.2.). Fisher's LSD *post hoc* revealed no significant difference between saline- and IFN- α -treated animals in pain '1' behaviour during the early (0-5 min) or later (40-50 min) phase of nociceptive responding in the formalin test.

One-way repeated measures ANOVA revealed a significant effect of time [$F(11, 176) = 19.732, P=0.000$] and IFN- α [$F(1, 16) = 14.702, P=0.001$] but not time x treatment interaction [$F(11, 176) = 1.505, P=0.133$] on direct shaking, licking, biting, flinching of the injected paw (pain '2' behaviour) (figure 4.3.2.2.). Fisher's LSD *post hoc* analysis revealed that IFN- α -treated animals exhibit a significant increase in CPS during 0-5 and 40-50 minutes of the test when compared with saline-treated counterparts. Further temporal analysis revealed that IFN- α -treated animals exhibited enhanced pain '2' behaviour during the early (0-5 min) [$P=0.009$] and later (40-50 min) [$P=0.05$] phase of nociceptive responding in the formalin test when compared with saline-treated counterparts.

One-way repeated measures ANOVA revealed a significant effect of time [$F(11, 176) = 30.376, P=0.000$] and IFN- α treatment [$F(1, 16) = 13.310, P=0.002$] but not time x treatment interaction [$F(11, 176) = 1.720, P=0.072$] on composite pain score (CPS) in the formalin test (figure 4.3.2.2.). Fisher's LSD *post hoc* analysis also revealed that IFN- α -treated animals exhibit a significant increase in CPS at 0-5, 40-45, 45-50 minute time bins when compared with saline-treated counterparts. Further temporal analysis revealed that CPS was significantly increased in IFN- α -treated animals during both the early (0-5 min) [$P=0.004$] and late (40-50 min) [$P=0.020$]

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phase of nociceptive response in the formalin test when compared with saline-treated controls.

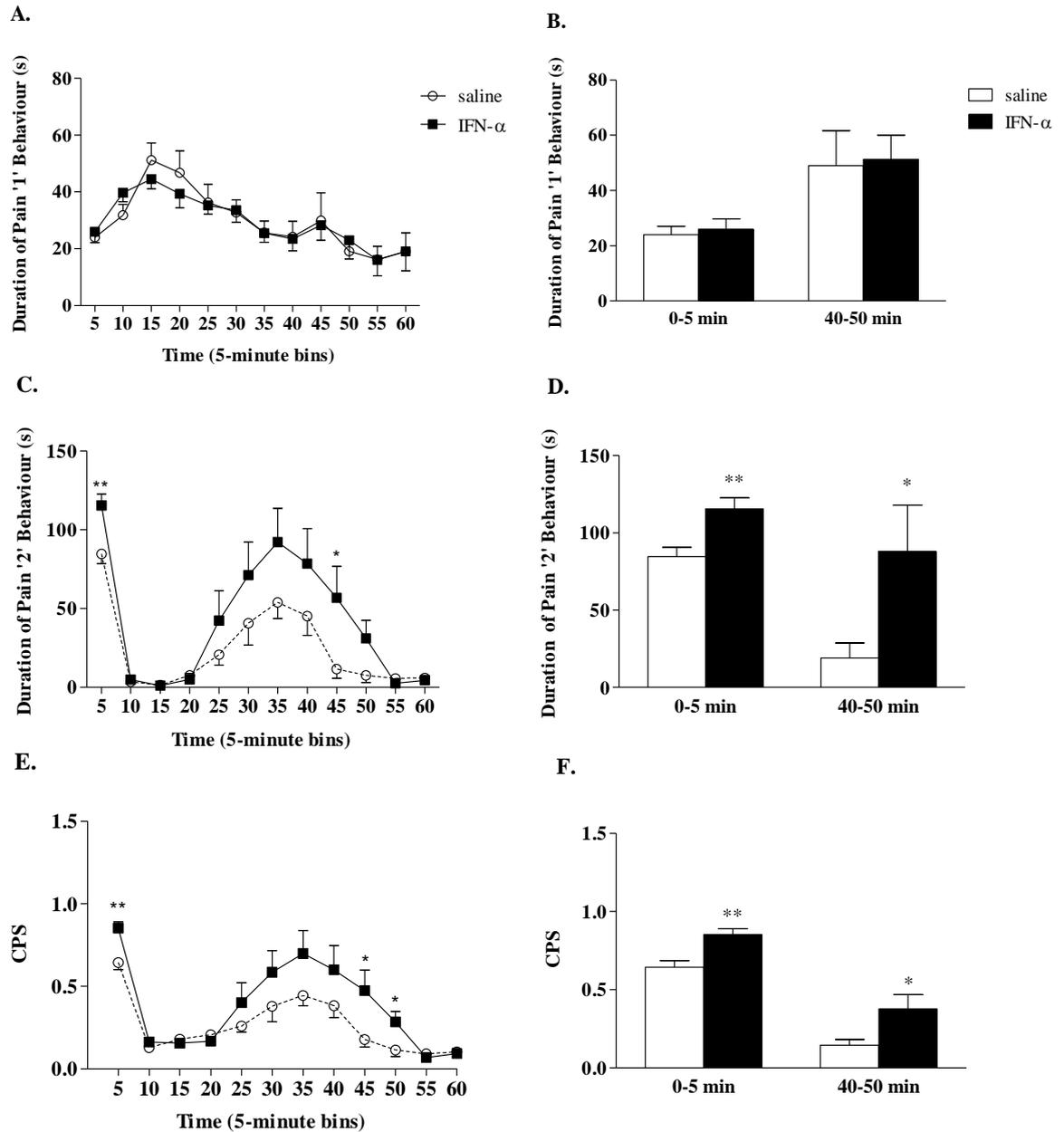


Figure 4.3.2.2. Nociceptive behaviour in the formalin test following 8 days of saline or IFN- α treatment: A, Profile of pain '1' behaviour over 60 minutes; B, Duration of pain '1' behaviour in the early (0-5min) and late (40-50min) phase of the formalin test; C, Profile of pain '2' behaviour over 60 minutes; D, Duration of pain '2' behaviour in the early (0-5min) and late (40-50min) phase of the formalin test; E, Profile of CPS over 60 minutes and F, Duration of CPS (composite pain score) in the early (0-5min) and late (40-50min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group; **p<0.01, *p<0.05 vs. saline-treated mice.

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4.3.3. Experiment 3. The effect of acute single administration of IFN- α (8,000 IU/g) on formalin-evoked inflammatory pain behaviour

The data in experiment 1 and 2 demonstrated a depressive-like phenotype and enhanced formalin-evoked nociceptive responding in repeated IFN- α treated mice. The data also revealed that a single acute administration of IFN- α does not alter locomotor activity or sucrose preference 24 hrs after administration (experiment 1). This experiment aimed to examine if inflammatory nociceptive responding is altered by a single acute administration of IFN- α

4.3.3.1 An acute single dose of IFN- α (8,000 IU/g) does not alter formalin-evoked inflammatory pain behaviour

One-way repeated measures ANOVA revealed a significant effect of time ($F(11, 187) = 25.033, P=0.000$) but not drug [$F(1, 17) = 0.368, P=0.552$] or time x drug interaction [$F(11, 187) = 1.396, P=0.177$] on elevation of the formalin injected paw (pain '1' behaviour), over the course of the 60-minute test.

One-way repeated measures ANOVA revealed a significant effect of time [$F(11, 187) = 41.099, P=0.000$] but not IFN- α [$F(1, 17) = 0.012, P=0.913$] or time x treatment interaction [$F(11, 187) = 0.498, P=0.903$] on direct shaking, licking, biting, flinching of the injected paw (pain '2' behaviour).

One-way repeated measures ANOVA revealed a significant effect of time [$F(11, 187) = 45.887, P=0.000$] but not IFN- α treatment [$F(1, 17) = 0.072, P=0.791$] or time x IFN- α interaction [$F(11, 187) = 0.426, P=0.943$] on CPS in the formalin test in C57Bl/6 mice.

Taken together, acute single administration of IFN- α does not alter inflammatory pain behaviour in the formalin test.

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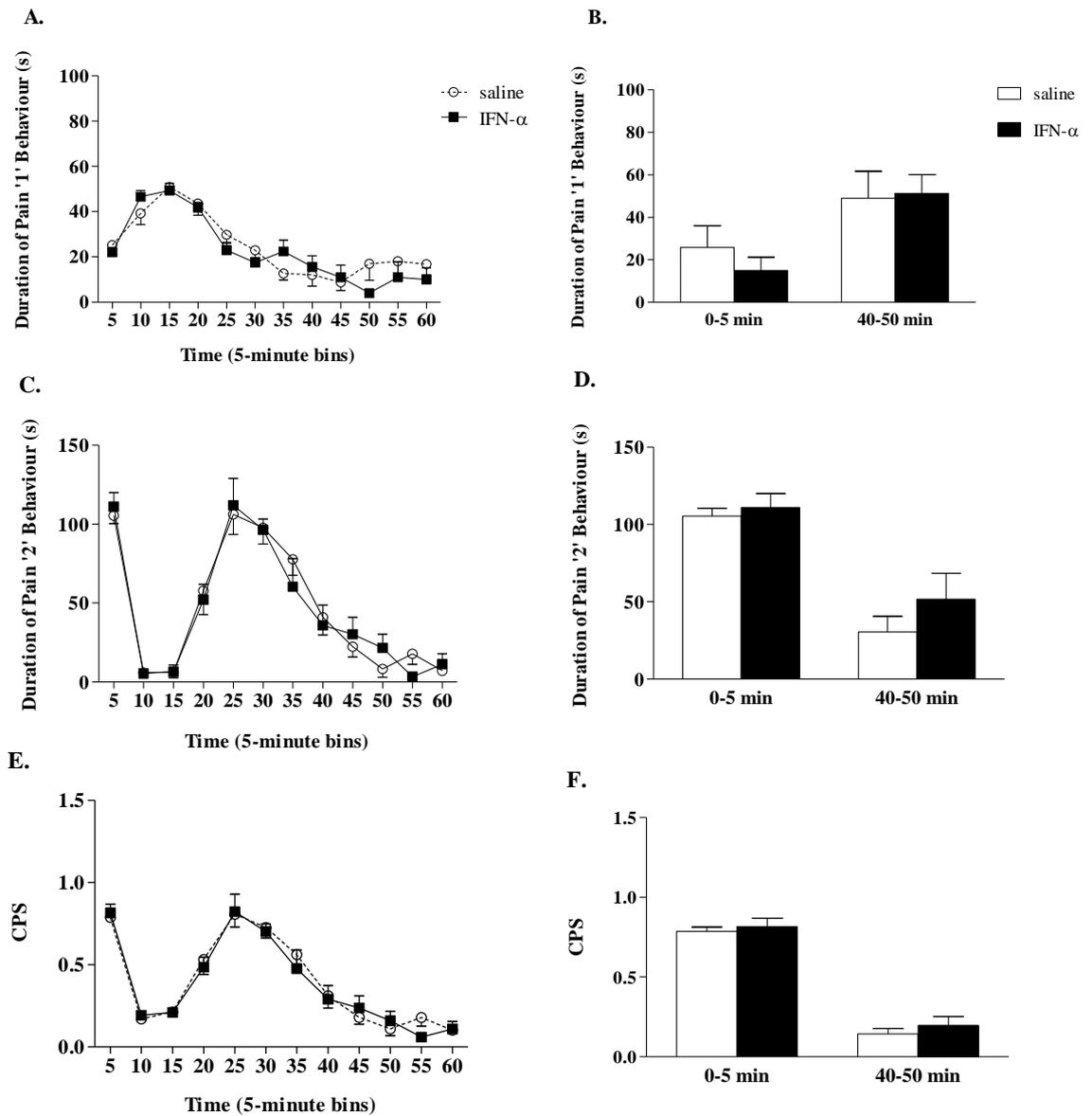


Figure 4.3.3.1. Nociceptive behaviour in the formalin test following one dose of IFN- α or saline treatment: A, Profile of pain '1' behaviour over 60 minutes; B, Duration of pain '1' behaviour in the early (0-5min) and late (40-50min) phase of the formalin test; C, Profile of pain '2' behaviour over 60 minutes; D, Duration of pain '2' behaviour in the early (0-5min) and late (40-50min) phase of the formalin test; E, Profile of CPS over 60 minutes and F, Duration of CPS in the early (0-5min) and late (40-50min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group.

4.3.4 Experiment 4. The effect of repeated IFN- α administration on peripheral and central inflammatory mediators and HPA axis activity

4.3.4.1 Repeated administration of IFN- α (8,000 IU/g) upregulates mRNA expression of CD11b in the spleen following 8 days of treatment

Following eight days of saline or IFN- α treatment, animals were sacrificed and spleen removed for subsequent quantitative RT-PCR analysis of the mRNA expression of the cytokines IL-1 β , IL-6, the monocyte/macrophage marker CD11b, the tryptophan-degrading enzyme, IDO, and neurotrophin, BDNF.

IFN- α treated mice exhibit a slight increase in IL-1 β , CD11b and IDO expression when compared to saline-treated counterparts, however unpaired t-tests revealed a significant effect of IFN- α treatment on mRNA expression of CD11b only in the spleen. Unpaired t-tests revealed no significant effect of IFN- α treatment on mRNA expression of IL-1 β [P=0.283], IL-6 [P=0.115], IDO [P=0.202] or BDNF [P=0.409] in the spleen (see figure 4.3.4.1.).

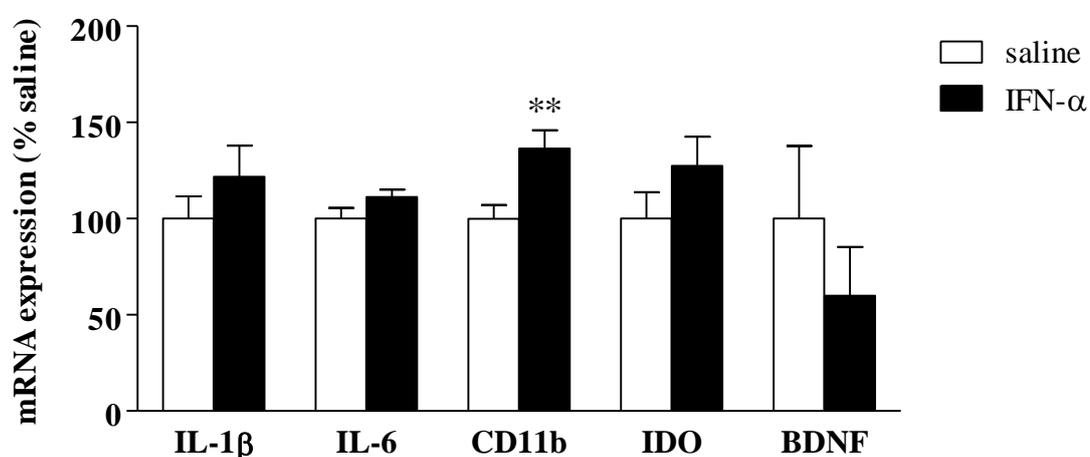


Figure 4.3.4.1. mRNA expression of pro-inflammatory cytokines, CD11b, IDO and BDNF following 8 days of IFN- α or saline treatment: Data are expressed as mean \pm SEM; n = 7-8 per group; **P<0.01 vs. saline-treated mice.

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4.3.4.2 Repeated administration of IFN- α (8,000 IU/g) does not alter cytokine protein levels in the spleen following 8 days of treatment

As there was an increase in mRNA expression of the macrophage marker CD11b in the spleen and a slight but not significant increase in IL-1 β expression, we then examined if perhaps protein levels of pro-inflammatory (IFN- γ , IL-1 β , TNF- α , IL-12 and IL-2) and anti-inflammatory (IL-4 and IL-10) cytokine would be altered by repeated IFN- α administration.

Unpaired t-tests revealed no significant effect of IFN- α treatment on protein levels of IFN- γ [P=0.856], IL-1 β [P=0.811], TNF- α [P=0.621], IL-12 [P=0.652], IL-2 [P=0.683], IL-4 [P=0.467] or IL-10 [P=0.503], as evident in figure 4.3.4.2.

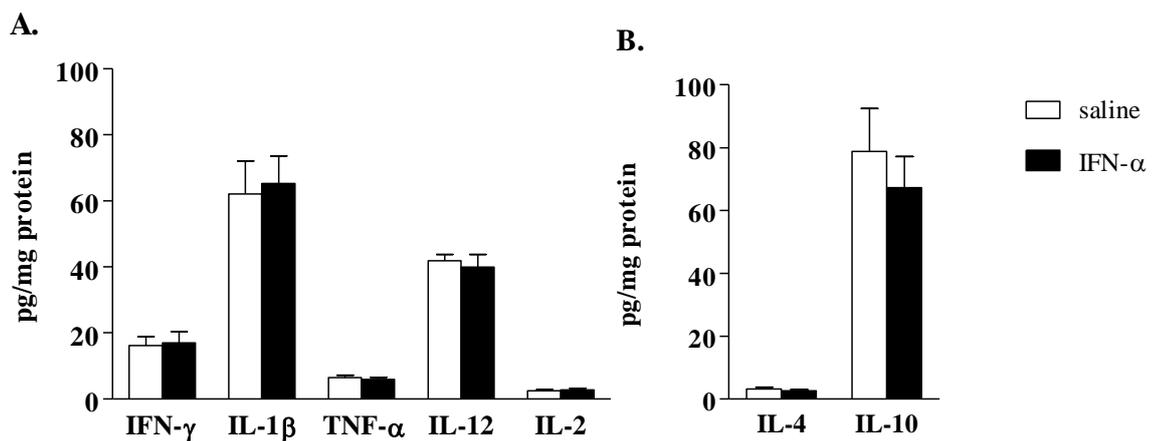


Figure 4.3.4.2. Protein expression of A, pro-inflammatory cytokines and B, anti-inflammatory cytokines in the spleen following 8 days of IFN- α or saline treatment: Data are expressed as mean \pm SEM; n = 8 per group.

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4.3.4.3 Repeated administration of IFN- α (8,000 IU/g) does not alter expression of immune mediators or BDNF in the PFC following 8 days of treatment

In order to determine if repeated IFN- α administration resulted in a persistent neuroinflammatory response with accompanying alterations in IDO and BDNF we examined the expression of the pro-inflammatory cytokines IL-1 β , IL-6, the macrophage/microglial marker CD11b, astrocyte activation marker GFAP, IDO and BDNF in the PFC of saline- and IFN- α treated mice.

Although IL-1 β mRNA expression tended to be increased in the PFC of IFN- α treated mice, unpaired t-tests revealed no significant effect of IFN- α treatment on mRNA expression of IL-1 β [P=0.390], IL-6 [P=0.559], CD11b [P=0.914], GFAP [P=0.839] or BDNF [P=0.577] in the PFC while IDO mRNA levels were undetectable (see figure 4.3.4.3.).

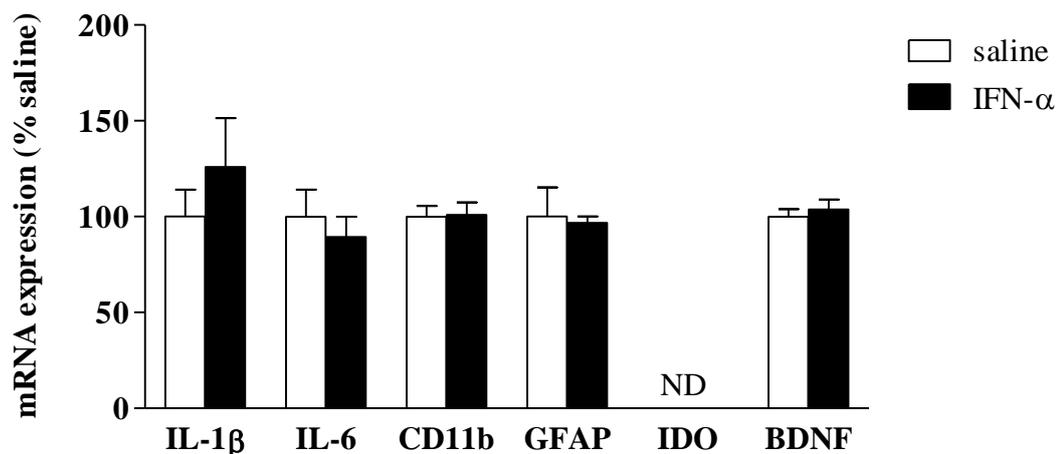


Figure 4.3.4.3. mRNA expression of pro-inflammatory cytokines, markers of glial activation and IDO and BDNF in the PFC following 8 days of IFN- α or saline treatment: Data are expressed as mean \pm SEM; n = 5-8 per group. ND: not detectable.

4.3.4.4 Repeated administration of IFN- α (8,000 IU/g) does not alter plasma corticosterone levels following 8 days of treatment

In order to determine if repeated IFN- α administration resulted in persistent alteration of HPA axis activity, we examined the corticosterone levels in the plasma of saline- and IFN- α -treated mice.

Although a trend towards an increase in corticosterone levels was observed in IFN- α treated mice, Mann-Whitney U-test revealed no significant effect of IFN- α treatment on plasma corticosterone levels [U=0.286] (see figure 4.3.4.4.).

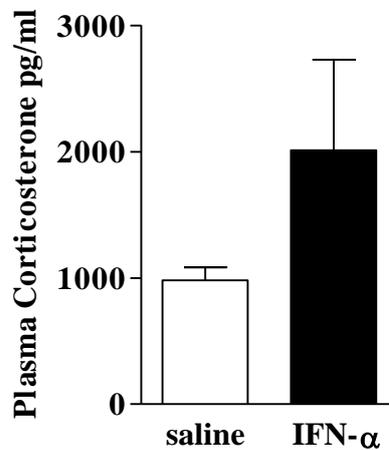


Figure 4.3.4.4. Protein expression of plasma corticosterone following 8 days of IFN- α or saline treatment: Data are expressed as mean \pm SEM; n = 4-5 per group.

4.4 Discussion

Following on from earlier studies in Chapter 3, where a trend towards depressive-like behaviour and thermal hyperalgesia was observed in C57Bl/6 mice repeatedly exposed to IFN- α treatment, and in order to further investigate the neurobiological mechanisms underpinning these effects, the current series of experiments evaluated depressive-like and nociceptive behaviour following repeated administration of a high dose of IFN- α . The data presented demonstrate that a dose of 8,000IU/g IFN- α induces hedonic and depressive-like behaviour as early as 4 days following repeated administration of the drug. Furthermore, depressive-like behaviour is evident up until and including ten days following repeated administration of IFN- α . Investigation of nociceptive responding within this timeframe also indicates hyperalgesic behaviour in response to a noxious inflammatory, but not thermal, stimulus, thus indicating a co-existent depressive-like and hyperalgesic phenotype. Changes in hedonic behaviour and inflammatory nociceptive responding were not evident following a single acute administration of IFN- α , indicating that repeat immune stimulation is required in order to induce the behavioural changes observed. Accordingly, this behavioural phenotype was associated with concurrent increases in leukocyte infiltration (CD11b expression) in the spleen; although changes in inflammatory mediators, BDNF or HPA axis activity were unaltered. Further studies are required in order to evaluate the mechanisms by which repeated IFN- α administration mediates the alterations in emotional and nociceptive behaviour.

The first set of experiments examined if repeated administration of a high dose of IFN- α elicited a depressive-like phenotype. This was achieved by assessing immobility behaviour in the TST and FST and anhedonic behaviour in the SPT. The data revealed that administration of IFN- α induced a significant increase in duration of immobility in the FST following 10 days of treatment, and a reduction in sucrose preference from day 4 to day 7 following administration. In accordance, previous published findings have demonstrated depressive-like behaviour in the FST following 5 to 15 days of IFN- α administration, albeit at lower doses (Siddegowda et al., 2011a, Makino et al., 1998, Makino et al., 2000, Ping et al., 2012). As the FST was only examined at one time point in the current study (day 10), we cannot rule

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out that effects may also be observed at earlier or later time points. Immobility in the TST has been proposed as analogous to enhanced behavioural despair and depressive-like behaviour in the FST. However, although repeated IFN- α administration resulted in enhanced immobility in the FST, a similar effect was not apparent in the TST, following 7 days of IFN- α administration. In comparison, Ping et al. (2012) demonstrated that the duration of immobility in the TST was increased following 6,000IU/g/day IFN- α for 7 days. Discrepancies between our data and that of Ping et al. may relate to methodological issues, time of testing, isotype of IFN- α or prior exposure to the open field. It is also possible that the FST may induce a greater stress response in the mice compared with the TST, which may be required in order to unmask the depressive- or despair-like behaviour. As the aforementioned tests rely on motor responses as readout, it is important to assess the effect of treatments on this parameter. Locomotor activity was assessed throughout each study at various time points using the open field test. Data from this revealed that repeated administration of IFN- α at the current dose (8,000IU/g/day) did not alter locomotor activity at any time points examined. Thus, none of the data generated was impacted by effects on locomotor activity.

The SPT is a recognized measure of anhedonia, a key symptom associated with depressive disorder. As such, in addition to examining depressive-like behaviour in the TST and FST, we also sought to examine the effect of repeated IFN- α on anhedonia in mice. The SPT was carried out prior to and throughout the course of repeated IFN- α (8,000IU/g/day) or saline administration. IFN- α -treated mice exhibited a significant reduction in sucrose preference when compared with saline-treated counterparts by day 4 and was also present on day 7 of administration. However, sucrose preference was re-established in IFN- α treated mice by day 11 of administration. Thus, under the current study conditions, repeated IFN- α induced a transient anhedonic effect. It is possible that the transient nature of the data is due to repeated exposure to the sucrose solution which the animals eventually learn to find more palatable than water. Further studies on separate groups of animals at each time point would be required to determine if this is the case. However, in accordance with our data, previous findings have demonstrated a significant reduction in sucrose

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preference in C57Bl/6 mice, following 7 days of hIFN- α administration at comparable doses (Ping et al., 2012), although this effect was only assessed at one time point. Comparable effects were observed following repeated administration of hIFN- α in rats (Fahey et al., 2007b). In contrast to C57Bl/6 mouse strain, repeated administration of IFN- α in CD1 mice induced no effect on sucrose preference over a three-week period (Kosel et al., 2011). Thus, C57Bl/6 mice may be more susceptible to the anhedonic effect of repeated IFN- α administration when compared to outbred CD1 mice. In rats, acute administration of hIFN- α has also shown to reduce (Sammut et al., 2001) or elicit no effect (De La Garza et al., 2005) on sucrose preference. The design of the current study also enabled us to examine the effect of acute administration of IFN- α on hedonic behaviour in mice and demonstrated that the acute administration elicits no effect on sucrose administration. Thus, taken together, the data in this chapter demonstrate that repeated administration of a high dose of IFN- α to mice results in a depressive-like phenotype which can be revealed in 2 different paradigms (FST and sucrose preference) and indicate a possible window during which effects may be observed (day 4 to 10). Given these data, it was decided to examine the effects of repeated IFN- α on nociceptive responding during this time window.

Although IFN- α therapy is frequently associated with pain complaints in patients, few studies have characterized such behaviour following IFN- α treatment in animals. As described previously, investigations have suggested thermal analgesia results following acute administration of IFN- α in rodents (Jiang et al., 2000, Lee et al., 2010, Blalock and Smith, 1981, Dafny, 1998). The data presented in this chapter did not evaluate thermal nociceptive responding following acute IFN- α administration but demonstrated that formalin-evoked inflammatory pain responding is not altered by acute treatment. Thus, acute IFN- α may modulate pain thresholds to thermal stimuli but not persistent inflammatory pain processes. However, apart from the studies conducted in chapter 3, no published study has evaluated the effect of repeated IFN- α administration on nociceptive responding in rodents. Although, repeated IFN- α (800IU/g/day) elicited thermal hyperalgesia following 21 days of administration (chapter 3), the current study did not observe such a reduction in

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latency to respond or hypersensitivity to a thermal stimulus following 4 or 7 days of repeated IFN- α (8,000 IU/g/day) administration. It is possible that changes in thermal nociceptive responding may be observed with a more prolonged treatment regime (>7days), which remains to be examined. Despite enhanced thermal nociceptive responding following repeated administration of a low dose of IFN- α , data in chapter 3 demonstrated a lack of effect on formalin-evoked nociceptive responding. The formalin test is a widely used test of inflammatory pain behaviour, the early phase (0-5 min) of which is mediated by primary afferent sensory neurons, namely through direct TRPA1 activation in the paw (McNamara et al., 2007) and subsequent C fibre activity, while the later or tonic phase (20-50 min) usually represents a combination of afferent input and central sensitisation in the dorsal horn (McNamara et al., 2007, Puig and Sorkin, 1996, Tjolsen et al., 1992). The present study demonstrates that 8 days of repeated IFN- α administration significantly increases nociceptive behaviour in the formalin test, an effect observed throughout both the early and late phase of response. It remains to be determined the precise mechanism by which repeated IFN- α administration may modulate pain processes resulting in the hyperalgesic phenotype observed, although given that alterations are observed in both the early and late phase of the formalin test, modulation of neuronal processes at any or all levels of the pain pathways may be involved.

IFN- α -induced alterations in peripheral and neuroimmune functioning may underlie the depressive-like behaviour and hyperalgesia as observed in the current study. As such, a further aim of this study was to determine if repeated IFN- α induced persistent inflammatory response which in turn may alter IDO, BDNF and HPA axis activity, at the time when the behavioural changes were observed (following 8 days of administration). Inflammatory processes, BDNF and IDO were examined in the spleen, a key immune organ in the body and the PFC, a key brain region regulating emotional and nociceptive processes. The data revealed that repeated IFN- α administration increases the expression of CD11b in the spleen but did not alter mRNA or protein levels of several pro- or anti-inflammatory cytokines. CD11b is a cell surface marker on several leukocytes including monocytes (Strauss-Ayali et al., 2007, Cifarelli et al., 2007), granulocytes including neutrophils (Weirich et al., 1998,

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Davis et al., 2000) and NK cells (Vosshenrich et al., 2005) as well as spleen cells (Christensen et al., 2001) and bone marrow cells (Postigo et al., 1991, Kasaian et al., 1992). Furthermore, this marker is implicated as having a central role in the migration of leukocytes from peripheral blood to the sites of inflammation (Springer, 1990, Nielsen et al., 1994). Leukocyte invasion of the spleen has been identified in several inflammatory conditions such as following bacterial (Rauch et al., 2012) or viral (Norris et al., 2013) infection. Similarly, in terms of cytokine responses, plasma IL-6 and TNF- α levels were found to be unaltered following chronic administration of hIFN- α administration in rats (De La Garza et al., 2005). Despite the lack of change in cytokine levels in the spleen, the increase in CD11b expression indicates a likely increase in leukocyte invasion and activation and thus confirms an inflammatory response to the repeated IFN- α administration.

IDO is a tryptophan-metabolising enzyme, induced by pro-inflammatory cytokines/mediators and clinical studies have reported increased serum levels and activity of this enzyme in response to initiation of IFN- α therapy (Comai et al., 2011, Chevolet et al., 2015). Similarly, plasma analyses of depressed suicidal patients exhibit reduced tryptophan levels and an elevated kynurenine/tryptophan ratio, both indicative of increased IDO activity (Bradley et al., 2015). Furthermore, IDO levels and activity are enhanced in the plasma of patients with chronic back pain exhibiting symptoms of depression (Kim et al., 2012a). The current study demonstrated a slight trend towards an increase in the mRNA expression of IDO in the spleen 24 hours following the last treatment of IFN- α , although this failed to reach statistical significance. While IDO levels and activity have not previously been characterized following repeated administration of IFN- α in rodents, other inflammatory stimuli such as LPS, a TLR4 agonist, known to induce depressive-like (O'Connor et al., 2009, Salazar et al., 2012) and nociceptive (Kanaan et al., 1996) behaviour in rodent behavioural studies, have induced peripheral and central increases in mRNA expression of this enzyme 24 hours following systemic administration of the stimulus (Fujigaki et al., 2001). It is possible that IDO levels and/or activity were enhanced at a time point earlier than that examined in the current study and in turn this had mediated its downstream effects on central neuronal function. Alternative

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strategies to characterise changes in IDO activity such as to assess enzyme activity itself through quantification of tryptophan and kynurenine levels and analysis of the resultant tryptophan/kynurenine ratio may yield a clearer indication of IDO induction in the model. Brain levels of IDO have also been proposed to play a significant role in co-morbid pain-depression interactions (Kim et al., 2012a). However, in the current study, mRNA levels of IDO were undetected in the PFC indicating that it is likely that this enzyme was not induced in the brain by repeated IFN- α administration, although induction in other brain areas cannot be ruled out.

It has been proposed that the emotional and nociceptive changes following IFN- α treatment are due to neuroinflammation and consequently altered neuronal functioning in key brain regions. Repeated administration of IFN- α did not alter mRNA expression of GFAP (astrocyte marker) or CD11b (microglial/macrophage marker) in the PFC. These data indicate a likely lack of persistent enhancement of glial activation in the brain, however mRNA expression of these genes is a very crude measure of glial activation and more subtle changes may be present that would require more sensitive and/or direct techniques to be employed (e.g. immunohistochemistry). In accordance with this, evidence of enhanced microglial activity has been previously observed in animals repeatedly exposed to IFN- α treatment for 5 weeks as evidenced through immunohistochemical analysis of hippocampal tissue which exhibited increased expression of Iba1, an alternative marker of microglial activity (Zheng et al., 2015). In addition to examining mRNA expression of markers of glial activation, this study also examined the expression of IL-1 β and IL-6 in the PFC and although there was a slight increase in IL-1 β expression, this failed to reach statistical significance. Similarly, De La Garza and colleagues, also reported a lack of central pro-inflammatory response following acute and repeated administration of hIFN- α in rats, albeit whilst employing a much lower dose (De La Garza et al., 2005). However, in contrast, a recent study in mice has reported increases in IL-1 β and IL-6 expression within hippocampal tissue following 5 weeks of treatment with mIFN- α , an effect which was maintained 24 hours following the final dose of IFN- α (Zheng et al., 2015). It is possible that changes in glial and cytokine expression may occur in other regions than the PFC or

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following a longer treatment regime as reported by Zheng and colleagues (Zheng et al., 2015).

As described in the introduction to this chapter, inflammatory processes such as those following IFN- α treatment are known to impact on neuronal function, and one mechanism by which this occurs is through the reduction of BDNF levels, thus reducing neurogenesis and promoting central atrophy (Rajkowska and Miguel-Hidalgo, 2007). IFN- α treatment has been shown to significantly lower serum BDNF levels in patients with chronic hepatitis C following induction of therapy (Lotrich et al., 2013, Kenis et al., 2011), an effect which inversely correlated with depressive symptoms (Kenis et al., 2011). However, in the current study, no such changes in mRNA expression of the neurotrophin were observed peripherally in the spleen or centrally in the PFC, although we cannot exclude the possibility that changes in protein levels may have occurred in the absence of changes in mRNA. However, these data are somewhat in agreement with previous findings in rats whereby BDNF protein levels in the frontal cortex or hippocampus were unaltered following 5 weeks of IFN- α treatment in rats (Fahey et al., 2007b).

Finally, repeated IFN- α administration and the resultant inflammatory state may modulate HPA axis function, which in turn can modulate emotional and nociceptive processes. As such, plasma corticosterone levels were assessed following repeated IFN- α and despite a perceived trend towards an enhanced level of this hormone in IFN- α -treated mice, the data revealed no significant change relative to saline-treated counterparts. This compares with observations in alternative inflammatory conditions, where plasma corticosterone levels have shown to be dramatically enhanced such as that following LPS administration (Kerr et al., 2012). Although we cannot rule out that the ability to mount a HPA response to a stress such as the FST or formalin test is not altered in IFN- α treated mice, the data indicate that basal levels are unaltered at the time when behavioural changes are observed.

Overall, the current data demonstrate that repeated administration of a high dose IFN- α induces a persistent peripheral inflammatory state which results in a depressive-like phenotype and hyperalgesic responding to a noxious inflammatory

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stimulus. Further studies are required in order to elucidate how repeated IFN- α administration mediates these effects on emotional and nociceptive processing and whether these effects are mediated by common or parallel neuroanatomical or neurotransmitter systems. However, the current data demonstrate that it is possible to mimic the clinical situation of emotional and nociceptive changes following repeated IFN- α treatment and provide a means of evaluating the underlying mechanisms and possible therapeutic strategies.

Chapter 5

Characterisation of the endocannabinoid signalling system following repeated administration of IFN- α

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Interferon- α (IFN- α) is a pro-inflammatory cytokine used for the treatment of various infections and cancers. However, this therapy is associated with neuropsychiatric complications including depression and anxiety (Schaefer et al., 2002, Raison et al., 2005) and, more recently, the development of painful symptoms (Shakoor et al., 2010, Nogueira et al., 2012). As discussed in earlier chapters, depressive-like behaviour has been identified in several reports investigating the effects of acute and repeated administration of IFN- α on emotional responding in animals (See Table 3.1). Furthermore, as described in chapter 4, we have also recently observed enhanced formalin-evoked nociceptive responding following repeated IFN administration at a time when depressive-like behaviour in the FST and SPT is also observed. Research to date evaluating the mechanism(s) of inflammation-induced depression suggests that this behavioural phenotype is a pathological consequence of sustained immune activation particularly within the CNS [for review see (Walker et al., 2014, Leonard, 2010, Young et al., 2014) and chapter 1]. As described previously (chapter 1 and 4), inflammatory processes such as that which occur following administration of IFN- α , stimulates overexpression of the tryptophan-degrading enzyme, indoleamine 2, 3, dioxygenase (IDO), thus increasing peripheral and subsequent central levels of its alternative toxic substrate, kynurenine (Comai et al., 2011). This compound can then be metabolized further to produce quinolinic acid, an NMDA receptor agonist prompting glutamate excitotoxicity and associated neuronal impairment (Wichers and Maes, 2004, Mazarei et al., 2013, Walker et al., 2013). Furthermore, IFN- α and associated pro-inflammatory cytokines can also themselves enter the CNS and/or indirectly activate glia in the CNS, promoting a pro-inflammatory environment centrally which in turn modulates neuronal activity that may underlie both emotional (Rajkowska and Miguel-Hidalgo, 2007) and nociceptive (Loggia et al., 2015) processes. Thus taken together, the presence of an inflammatory state can induce alterations in the function of neurons that mediate and modulate emotion and pain.

The hyperalgesia associated with repeated IFN- α administration most likely occurs due to altered nociceptive processing along the ascending and/or descending pain systems.

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These pathways have been described in detail in chapter 1 and therefore will only briefly be referred to again here. Essentially, sensory information from a noxious stimulus is relayed to the dorsal horn of the spinal cord via the primary afferent neurons, which in turn synapse on second order neurons transmitting the nociceptive information via ascending pain pathways which innervate higher brain regions including thalamic nuclei and brainstem nuclei. These in turn send projections to cortical structures such as the somatosensory cortex (pain sensation) as well as other areas including the insular cortex, anterior cingulate and medial prefrontal cortices and limbic areas, for the emotional, motivational and cognitive aspect of pain perception (Casey et al., 1994, Svensson et al., 1997, Hunt and Rossi, 1985, Willis and Westlund, 1997). The descending pain pathway consists primarily of neuronal connections from the PAG to the RVM and onto the dorsal horn of the spinal cord and acts to modulate incoming nociceptive information. The PAG has a prominent inhibitory role in pain sensation transmission, the activity of which can be modulated by input from cortical and limbic area including the amygdala (Hopkins and Holstege, 1978, Oka et al., 2008) and the prefrontal cortex (An et al., 1998, Neugebauer et al., 2009). The RVM receives input from the PAG which acts to modulate the firing of ON- and OFF-cell neurons which in turn facilitate and suppress pain transmission respectively and thus, are involved in the mechanisms modulating descending inhibition (Heinricher et al., 2009). Dysregulation of descending pain facilitation and/or inhibition has been implicated in the development of pathological inflammatory and neuropathic pain [for review see (Porreca et al., 2002)] and thus may underlie the hyperalgesia observed following repeated IFN- α administration.

Nociceptive transmission can be influenced by various mediators such as inflammatory mediators, cortisol, and neurotransmitter systems [for reviews see (Barr and Hunter, 2014, Calvo et al., 2012a, Bomholt et al., 2004, Bannister et al., 2009, Dickenson et al., 1997)]. Over the past decade, there has been a wealth of evidence demonstrating an important role for the endocannabinoid system in modulating pain processing [for reviews see (Ulugol, 2014, Burston and Woodhams, 2014)]. For instance, serum endocannabinoid levels are reportedly reduced in chronic pain patients (Fichna et al.,

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2013). Furthermore, genetic alterations in the CB₁ receptor and FAAH have also been identified in patients exhibiting painful symptoms associated with migraine, Parkinson's disease and irritable bowel syndrome (Juhász et al., 2009, Park et al., 2011, Greenbaum et al., 2012). Data from various animal models have demonstrated that endocannabinoids are synthesized and released throughout the pain pathways and during the expression of painful processes (Alger and Kim, 2011, Cravatt and Lichtman, 2004) and act primarily in an analgesic manner. However, in contrast, recent data from our group demonstrate that formalin-evoked nociception associates with unchanged levels of AEA in the medial (m)PFC (Okine et al., 2014), dorsolateral (dl)PAG (Olango et al., 2012) and RVM (Rea et al., 2014) while 2-AG levels are not altered in the mPFC (Okine et al., 2014) or dlPAG (Olango et al., 2012). We have however reported increases in 2-AG in the rat RVM following formalin exposure (Rea et al., 2014). Additionally, alterations in levels of cannabinoid receptors have been reported in numerous preclinical models of pain including that of a chronic inflammatory nature. For instance, CB₁ receptor expression has been shown to be significantly increased in the dorsal horn of the spinal cord of rats pre-exposed to monosodium iodoacetate (MIA)-induced osteoarthritis (Lim et al., 2003, Malek et al., 2015). Similarly, intraplantar administration of CFA increases CB₂ receptor expression in the DRG and paw (Hsieh et al., 2011) but not within the lumbar spinal cord (Zhang et al., 2003) of rats. While there are limited data available characterising central CB₁ and CB₂ receptor expression levels following the induction of inflammatory pain, a recent study demonstrates significant CB₂ upregulation in the striatum from 4 and up to 14 days following local administration of LPS (Concannon et al., 2015), an immune stimulant which provokes depressive-like behaviour (Pan et al., 2013) and inflammatory hyperalgesia (Zouikr et al., 2014, Zouikr et al., 2015) in rats. In addition, some studies have reported such changes in cannabinoid receptor expression in animal neuropathies. For instance CB₁ receptor expression is increased in thalamic nuclei and hippocampal subregions (CA3 and dentate gyrus) seven days after induction of spinal cord injury while in contrast, CB₁ was found to be downregulated in the amygdaloid nuclei, ACC and PAG (Knerlich-Lukoschus et al., 2011).

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Recent studies examining animal models of co-existent depression and pain have also demonstrated changes in endocannabinoid signaling and cannabinoid receptor expression and/or activity. For instance, mRNA levels of CB₁ have been shown to be downregulated in the DRG in a rat model of repeat stress-induced visceral pain (Hong et al., 2009). The Wistar-Kyoto (WKY) rat is a stress hyperresponsive rat strain with a depressive-like phenotype and exhibits thermal (Burke et al., 2010) and visceral (Gibney et al., 2010, Gosselin et al., 2010, O'Malley et al., 2010) hyperalgesia as well as enhanced formalin-evoked inflammatory pain behaviour (Burke et al., 2010, Rea et al., 2014). Recent studies in our laboratory have found that in WKY rats, formalin administration results in a significant reduction in AEA levels in the RVM of WKY rats, an effect which was not observed in Sprague Dawley (SD) counterparts. In addition, formalin increased levels of 2-AG and mRNA expression of NAPE-PLD and DAGL- α , precursors of AEA and 2-AG respectively, in the RVM of SD rats, an effect not observed in WKY animals (Rea et al., 2014). Furthermore, pharmacological studies demonstrated an important role of AEA-CB₁ receptors interactions in the RVM in the inflammatory hyperalgesic response observed in WKY rats (Rea et al., 2014). Thus, taken together alterations in endocannabinoid tone and function may underlie the hyperalgesia observed in the presence of an anxiety/depressive phenotype and as such may also underlie the behavioural effects following repeated IFN- α administration.

Given the proposed role of endocannabinoid signaling in mediating depression-pain interactions, the hypothesis for the current study was that IFN- α -associated alterations in emotional and nociceptive processing are coincident with underlying changes in the endocannabinoid system.

Thus, this study sought to investigate the effect of repeated IFN- α administration on endocannabinoid and *N*-acylethanolamine levels and associated enzyme and receptor expression in the prefrontal cortex, various components of the descending pain pathway (PAG and RVM), the spinal cord and the paw tissue. Furthermore, this study sought to investigate if endocannabinoid levels in these regions differ between saline- and IFN- α -treated animals during expression of nociceptive behaviour in the formalin test.

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5.1.1 Aims

1. Confirm earlier observation of IFN- α -induced enhancement of formalin-evoked nociception in mice
2. Quantify levels of endocannabinoids and *N*-acylethanolamines in the paw, spinal cord and brain regions following repeated administration of IFN- α
3. Characterize brain regional, spinal and paw tissue levels of endocannabinoids and *N*-acylethanolamines during expression of hyperalgesic behaviour in the formalin test following repeated administration of IFN- α
4. Characterize possible changes in the gene expression of endocannabinoid-catabolising enzymes, FAAH and MAGL, following repeated administration of IFN- α
5. Characterize possible changes in expression of cannabinoid receptor targets following repeated administration of IFN- α

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5.2 Materials and Methods

5.2.1 Animals

As before, male C57BL/6 mice (weight 25-30g; Charles River Laboratories, UK) were used in the experiments. Animals were housed singly in plastic-bottomed cages containing wood shavings as bedding. Animals were maintained in a constant temperature (21 +/- 2°C) under standard lighting conditions (12 : 12 hr light-dark, lights on from 07.00 to 19.00 hr). All experiments were carried out during the light phase, between 10.00 and 16.00 hr. Mice were given free access to food and water.

The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

5.2.2 Pharmacological Treatments

Human interferon-alpha (hIFN- α -2a; 3M IU/0.5ml), Roferon-A®, was purchased from Roche Pharmaceuticals. The drug was prepared in saline from stock on a daily basis. This drug/vehicle solution was administered subcutaneously in a volume of 3 μ l/g to give a final concentration of 8,000IU/g/day IFN- α . Dosing occurred daily, between the hours of 10.00 and 12.00. The dose chosen was based on earlier studies whereby repeated administration of 8,000IU/g/day IFN- α induced depressive-like and hyperalgesic behaviour, as described in chapter 4.

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5.2.3 Experimental Design

This study evaluated the effect of repeated administration of a high dose of IFN- α (8,000IU/g/day) on various components of the endocannabinoid system in C57Bl/6 mice in the absence and presence of persistent inflammatory pain behaviour. Animals were administered saline for five days to acclimatize them to repeated injection and handling, followed by repeated administration of IFN- α or saline vehicle every day for eight days. 24 hours following the final injection of saline/IFN- α , a subset of animals were sacrificed, brains removed and the PFC, components of the descending pain pathway (PAG and RVM) as well as the lumbar (L4-L6) region of the spinal cord and left hind paw tissue collected and stored at -80°C for subsequent qRT-PCR analysis of cannabinoid-related genes and quantification of endocannabinoids and *N*-acylethanolamines using mass spectrometry (see chapter 2 for methodology).

A further subset of animals was exposed to the formalin test (see chapter 2 for details) for 35 minutes prior to sacrifice. A period of 35 minutes was chosen based on findings reported in chapter 4 where formalin-evoked hyperalgesia in IFN- α treated animals was most pronounced, and to evaluate endocannabinoid levels at the peak of nociceptive responding. After sacrifice, tissues were removed as above for quantification of endocannabinoid and *N*-acylethanolamine levels.

5.2.4 RNA Extraction, Reverse Transcription and Real-time RT-PCR

RNA was extracted from prefrontal cortical tissue as well as that of the PAG, RVM, spinal cord and plantar region of the left hind paw, as described in chapter 2. In the case of the paw tissue, samples were ground into a fine powder using a mortar and pestle over dry ice, prior to RNA extraction. RNA was reverse transcribed into cDNA and quantitative RT-PCR was then performed using Taqman gene expression assays for FAAH, MAGL, CB₁, CB₂, PPAR- α and PPAR- γ mRNA expression (see chapter 2 for methodology).

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5.2.5 Mass Spectrometry analysis of endocannabinoid and *N*-acylethanolamine levels

Brain regions and spinal cord were assayed for endocannabinoid and *N*-acylethanolamine content, as described in chapter 2. Prior to addition of, and homogenization with internal standards, paw tissue samples were ground into a fine powder using a mortar and pestle over dry ice. This was carried out due to the increased elasticity of the paw tissue which would otherwise make it more difficult to homogenise. The remaining protocol was carried out as described in chapter 2 and analyte quantity was reported as pmol (2-AG) or nmol (AEA, PEA, OEA) per gram of brain or spinal cord. Upon weighing paw tissue samples prior to homogenisation, a large variance in tissue weight was noted, an effect which was evident within all treatment groups (saline, 1.6-6.2mg; IFN- α , 1.9-5.8mg; saline-formalin, 0.4-17.2mg; IFN- α -formalin, 0.5-13mg). This was despite equivalent amounts of tissue taken. As such it was decided that expressing lipid quantity relative to tissue weight would not be a reliable measure of so, therefore lipid quantities were alternatively expressed as that per paw tissue sample.

5.2.6 Statistics

Data were analysed using SPSS statistical package, employing the unpaired t-test, one- or two-way ANOVA followed by Fisher's LSD *post hoc* analysis where appropriate. Where data were not normally distributed or homogenous, data were resolved using the Mann-Whitney U-test and Kruskal-Wallis analysis by ranks. Data were considered significant when $P < 0.05$. Results are expressed as group means \pm standard error of mean (SEM).

5.3 Results

5.3.1 Repeated administration of IFN- α increases nociceptive behaviour in the formalin test of inflammatory pain

In order to confirm IFN- α -related hyperalgesia as observed in chapter 4, a subset of saline- and IFN- α -treated mice were administered an intraplantar injection of formalin into the left hind paw and subsequent nociceptive responding was assessed over the following 35 minutes.

One-way repeated measures ANOVA revealed a significant effect of time [F (6, 96) = 4.656, P=0.000] but not IFN- α treatment [F (1, 16) = 0.351, P=0.562] or time x α treatment interaction [F (6, 96) = 1.123, P=0.355] on elevation of the injected paw (pain '1' behaviour) over the course of the 35-minute test (figure 5.3.1A). Further temporal analysis revealed that IFN- α -treated animals did not exhibit a significant change in pain '1' behaviour during the early (0-5 minutes) or late (20-30 minutes) phase of nociceptive responding in the formalin test when compared with saline-treated (figure 5.3.1B).

One-way repeated measures ANOVA revealed a significant effect of time [F (6, 96) = 19.626, P=0.000] and a trend towards a significant effect of IFN- α treatment [F (1, 16) = 3.944, P=0.064] and time x IFN- α treatment interaction [F (6, 96) = 2.137, P=0.056] on direct shaking, licking, biting or flinching of the affect paw (pain '2' behaviour) (Figure 5.3.1C). Further temporal analysis revealed that IFN- α -treated animals exhibited a significant increase in pain '2' behaviour during the late (20-30 minutes) [P=0.03] but not the early (0-5 minutes) [P=0.78] phase of nociceptive responding in the formalin test when compared with saline-treated counterparts (figure 5.3.1D).

One-way repeated measures ANOVA revealed a significant effect of time [F (6, 96) = 18.557, P=0.000] and a trend towards a significant effect of IFN- α treatment [F (1, 16) = 3.630, P=0.075] and time x treatment interaction [F (6, 96) = 1.935, P=0.083] on composite pain score (CPS) in the formalin test in C57Bl/6 mice (figure 5.3.1E). Further temporal analysis revealed a significant increase in CPS during the late (20-30 minutes)

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[P=0.03] but not the early (0-5 minutes) [P=0.60] phase of nociceptive response in the formalin test in IFN- α -treated animals compared with saline-treated, as seen in figure 5.3.1F.

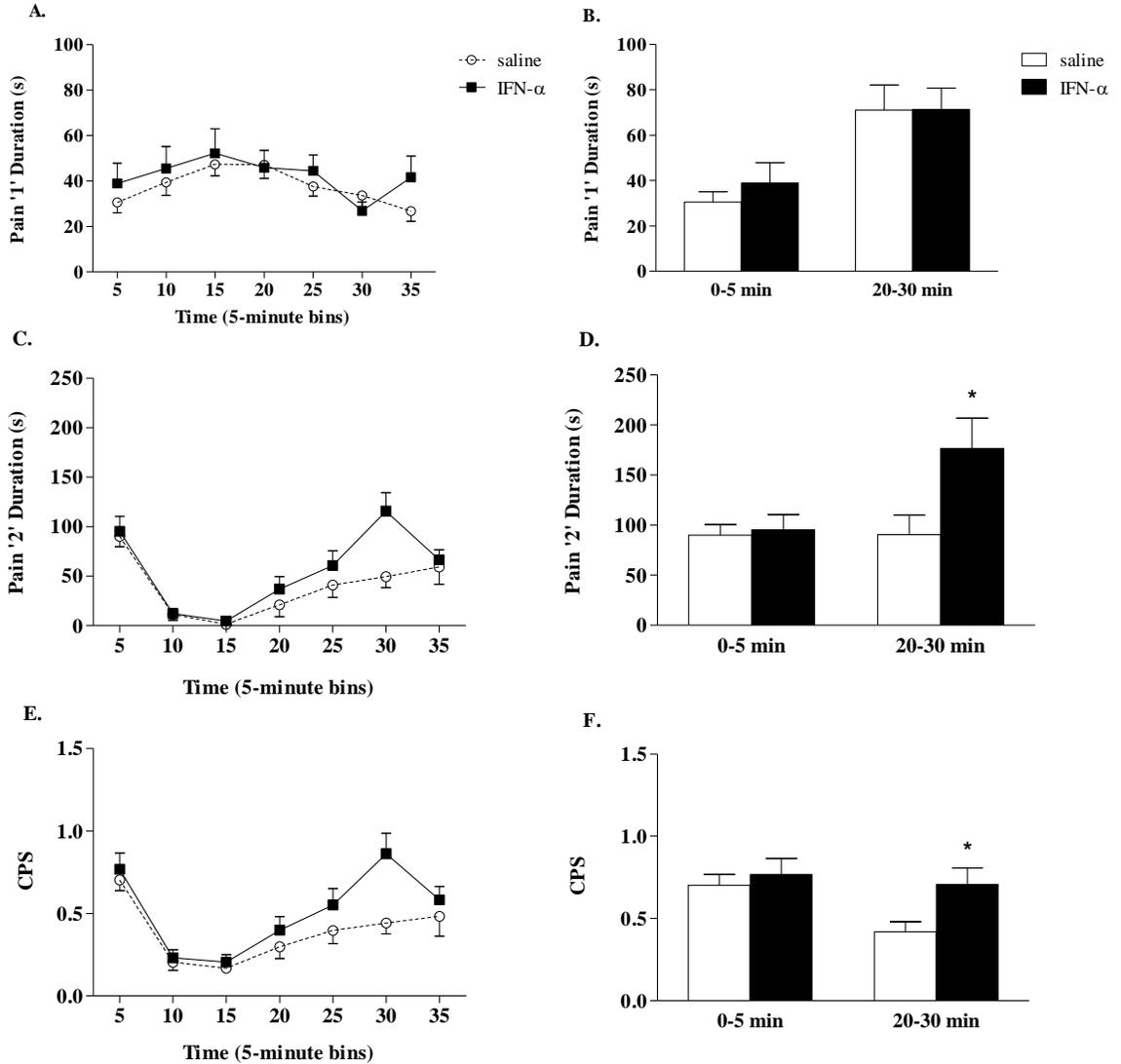


Figure 5.3.1. Nociceptive behaviour in the formalin test: A, Profile of pain '1' behaviour over 35 minutes; B, Duration of pain '1' behaviour in the early (0-5min) and late (20-30min) phase of the formalin test; C, Profile of pain '2' behaviour over 35 minutes; D, Duration of pain '2' behaviour in the early (0-5min) and late (20-30min) phase of the formalin test; E, Profile of composite pain score (CPS) over 35 minutes and F, Duration of composite pain score (CPS) in the early (0-5min) and late (20-30min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group. *P<0.05 vs. saline.

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5.3.2 Intraplantar administration of formalin increases 2-AG and AEA levels in the PAG and RVM in mice repeatedly exposed to IFN- α treatment

Following eight days of saline or IFN- α treatment, mice were either sacrificed or received intraplantar formalin administration 35 minutes prior to sacrifice, the brain removed and discrete brain regions including the prefrontal cortex, PAG, RVM, the spinal cord and paw tissue were dissected out for subsequent quantification of endocannabinoid levels using mass spectrometry.

Two-way ANOVA revealed a significant effect of formalin, but not IFN- α treatment or IFN- α x formalin interaction on 2-AG levels in the PAG and RVM (table 5.3.2). Fisher's LSD *post hoc* analysis revealed that 2-AG levels were significantly increased in both the PAG and RVM in IFN- α -treated animals that received intraplantar formalin administration when compared to non-formalin treated counterparts (figure 5.3.2A). Although a similar trend was evident for saline-treated animal that received formalin, this effect was not statistically significant.

Two-way ANOVA revealed no significant effect of IFN- α treatment, formalin or IFN- α x formalin interaction on 2-AG levels in the prefrontal cortex or spinal cord.

Levels of 2-AG were low in the paw tissue isolated from saline- and IFN- α -treated animals. Kruskal-Wallis analysis by ranks revealed a significant effect of IFN- α treatment but not formalin on 2-AG levels in the paw tissue of mice (table 5.3.2). Although 2-AG levels in the paw tissue of IFN- α -treated animals that received formalin appeared lower than saline-formalin treated counterparts, Fisher's LSD *post hoc* analysis revealed no significant difference in 2-AG levels between groups.

Two-way ANOVA revealed a significant effect of formalin, but not IFN- α treatment or IFN- α x formalin interaction on AEA levels in the PFC and RVM (table 5.3.2). Fisher's LSD *post hoc* analysis revealed that intraplantar formalin administration significantly increased AEA levels in the prefrontal cortex of saline-treated animals only, an effect not observed in IFN- α -treated mice. Analysis also revealed that AEA levels increased in

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the RVM of IFN- α -treated mice that received formalin, an effect not observed in saline-treated counterparts, as seen in figure 5.3.2C.

Two-way ANOVA revealed no significant effect of IFN- α , formalin or IFN- α x formalin interaction on AEA levels in the PAG or spinal cord.

Although AEA levels in the paw tissue of saline-treated animals that received formalin appeared higher than non-formalin treated counterparts, Kruskal-Wallis analysis by ranks revealed no significant effect of IFN- α or formalin treatment on AEA levels in the paw tissue of mice.

	Region	IFN-α	Formalin	Interaction (IFN-α x Formalin)
2-AG	Prefrontal Cortex	0.121	0.404	0.949
	PAG	0.205	0.001^{##}	0.260
	RVM	0.941	0.008^{##}	0.272
	Spinal Cord	0.272	0.266	0.585
	Paw	0.038*	0.085	NA
AEA	Frontal Cortex	0.619	0.012[#]	0.100
	PAG	0.317	0.395	0.111
	RVM	0.577	0.038[#]	0.541
	Spinal Cord	0.230	0.853	0.764
	Paw	0.553	0.491	NA

Table 5.3.2. Effect of repeated IFN- α and/or formalin administration on 2-AG and AEA levels in discrete tissues. Data are expressed as P value; n= 5-9 per group. *P<0.05 vs. saline; ^{##}P<0.01, [#]P<0.05 vs. non-formalin-treated animals.

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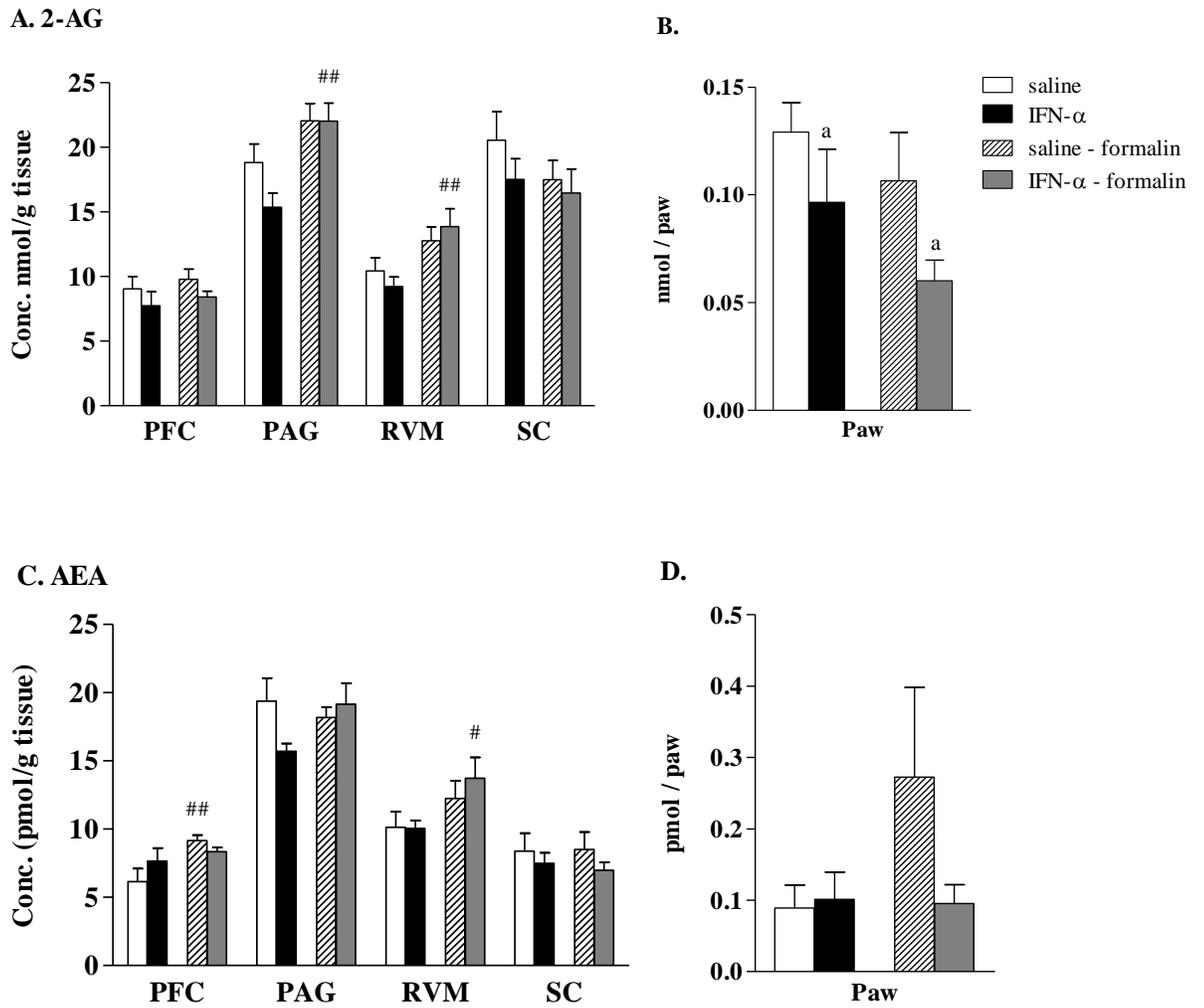


Figure 5.3.2. Quantification of endocannabinoids in discrete tissues following eight days of saline/IFN- α treatment, and following formalin administration: A, 2-AG levels in the prefrontal cortex, PAG, RVM and spinal cord; B, 2-AG levels in the plantar tissue of the left hind paw; C, AEA levels in the prefrontal cortex, PAG, RVM and spinal cord and D, AEA levels in the plantar tissue of the left hind paw. Data are expressed as mean \pm SEM; n= 5-9 per group. $^{##}P < 0.01$, $^{\#}P < 0.05$ vs. non-formalin-treated animals; overall effect of IFN- α in ANOVA.

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5.3.3 Changes in PEA and OEA levels in the brain, spinal cord and paw of saline- and IFN- α , in the presence and absence of a nociceptive stimulus

Levels of the *N*-acylethanolamines OEA and PEA were also assessed in saline- and IFN- α - treated mice, with or without exposure to the formalin test.

Two-way ANOVA revealed a significant effect of formalin, but not IFN- α treatment or IFN- α x formalin interaction on PEA levels in the PFC (table 5.3.3). Fisher LSD *post hoc* analysis revealed that intraplantar formalin administration significantly increased PEA levels in the PFC of IFN- α -treated mice, an effect not observed in saline-treated counterparts (figure 5.3.3A).

Two-way ANOVA revealed a significant effect of IFN- α treatment, but not formalin administration or IFN- α x formalin interaction on PEA levels in the spinal cord. Fisher's LSD *post hoc* analysis revealed that mice which were repeatedly administered IFN- α exhibited a significant decreased in PEA levels in the spinal cord in comparison with saline-treated counterparts (figure 5.3.3A). Further analysis revealed that IFN- α treated mice which also received formalin tended to have lower PEA levels in the spinal cord in comparison with saline- treated counterparts, although this just failed to reach statistical significance [P = 0.060].

Although there was a trend toward a significant effect of IFN- α on PEA levels in the PAG, two-way ANOVA revealed no significant effect of IFN- α , formalin or IFN- α x formalin interaction on PEA levels in the PAG or RVM (table 5.3.3).

Kruskal-Wallis analysis by ranks revealed a significant effect of formalin, but not IFN- α treatment on PEA levels in the left hind paw tissue. Although PEA levels in the paw tissue of saline-treated animals tended to increase following formalin administration, an effect not seen in IFN- α treated mice, this effect did not reach statistical significance [P = 0.083] (figure 5.5.3.B).

Two-way ANOVA revealed a significant effect of formalin, but not IFN- α or IFN- α x formalin interaction, on OEA levels in the PFC (see table 5.3.3). Fisher's LSD *post hoc* analysis revealed that intraplantar formalin administration significantly increased OEA levels in the PFC in both saline- and IFN- α -treated animals (figure 5.3.3.C).

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Two-way ANOVA revealed a significant effect of IFN- α , but not formalin administration or IFN- α x formalin interaction, on OEA levels in the spinal cord. Although OEA levels appeared decreased in IFN- α -treated animals in comparison with saline-treated counterparts, this effect did not reach statistical significance.

Two-way ANOVA revealed no significant effect of IFN- α -treatment, formalin or IFN- α x formalin interaction on OEA levels in the PAG and RVM.

Kruskal-Wallis analysis by ranks revealed no significant effect of IFN- α -treatment or formalin or IFN- α x formalin interaction on OEA levels in the paw tissue.

	Region	IFN-α	Formalin	Interaction (IFN-α x Formalin)
PEA	Frontal Cortex	0.707	0.005^{##}	0.649
	PAG	0.067	0.098	0.733
	RVM	0.678	0.809	0.869
	Spinal Cord	0.005^{**}	0.082	0.581
	Paw	0.453	0.047[#]	NA
OEA	Frontal Cortex	0.669	0.001^{##}	0.974
	PAG	0.062	0.065	0.907
	RVM	0.868	0.150	0.684
	Spinal Cord	0.023[*]	0.549	0.871
	Paw	0.343	0.123	NA

Table 5.3.3. Effect of repeated IFN- α and/or formalin administration on PEA and OEA levels in discrete tissues. Data are expressed as P value; n= 5-9 per group. ^{**}P<0.01, ^{*}P<0.05 vs. saline-treated; ^{##}P<0.01, [#]P<0.05 vs. non-formalin-treated animals.

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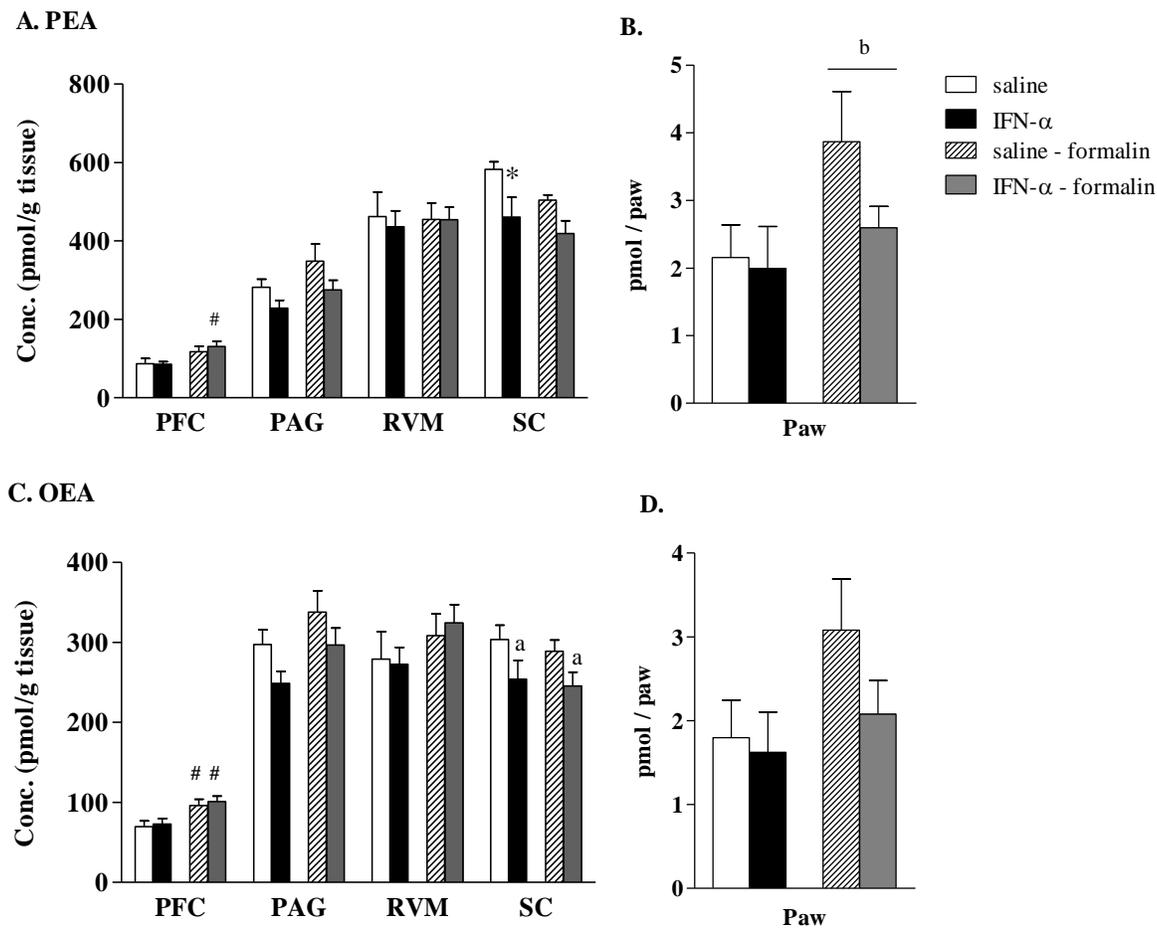


Figure 5.3.3. Quantification of *N*-acyl ethanolamines OEA and PEA in discrete tissues following eight days of IFN- α treatment: A, PEA levels in the prefrontal cortex, PAG, RVM and spinal cord; B, PEA levels in the plantar tissue of the left hind paw; C, OEA levels in the prefrontal cortex, PAG, RVM and spinal cord and D, OEA levels in the plantar tissue of the left hind paw. Data are expressed as mean \pm SEM; n= 5-9 per group. *P<0.05 vs. saline, [#]P<0.05 vs. non-formalin-treated animals; a, overall effect of IFN- α in ANOVA; b, overall effect of formalin in Kruskal-Wallis.

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5.3.4 Repeated administration of IFN- α does not alter expression of FAAH or MAGL mRNA in the brain, spinal cord or paw tissue

Following eight days of saline or IFN- α treatment, animals were sacrificed without any prior exposure to intraplantar formalin administration and discrete brain regions, spinal cord and paw tissue dissected out for subsequent quantitative RT-PCR analysis of mRNA for FAAH, the enzyme primarily responsible for the enzymatic degradation of AEA, PEA and OEA; and MAGL, the enzyme primarily responsible for metabolising 2-AG.

Unpaired t-tests revealed no significant effect of repeated administration of IFN- α on FAAH expression in the prefrontal cortex [P=0.17], PAG [P=1.00], RVM [P=0.84], spinal cord [P=0.97] or paw tissue [P=0.11]. Similarly, unpaired t-tests revealed no significant effect of repeated administration of IFN- α on MAGL expression in the prefrontal cortex [P=0.57], PAG [P=0.80], RVM [P=0.50], spinal cord [P=0.74] or paw tissue [P=0.91].

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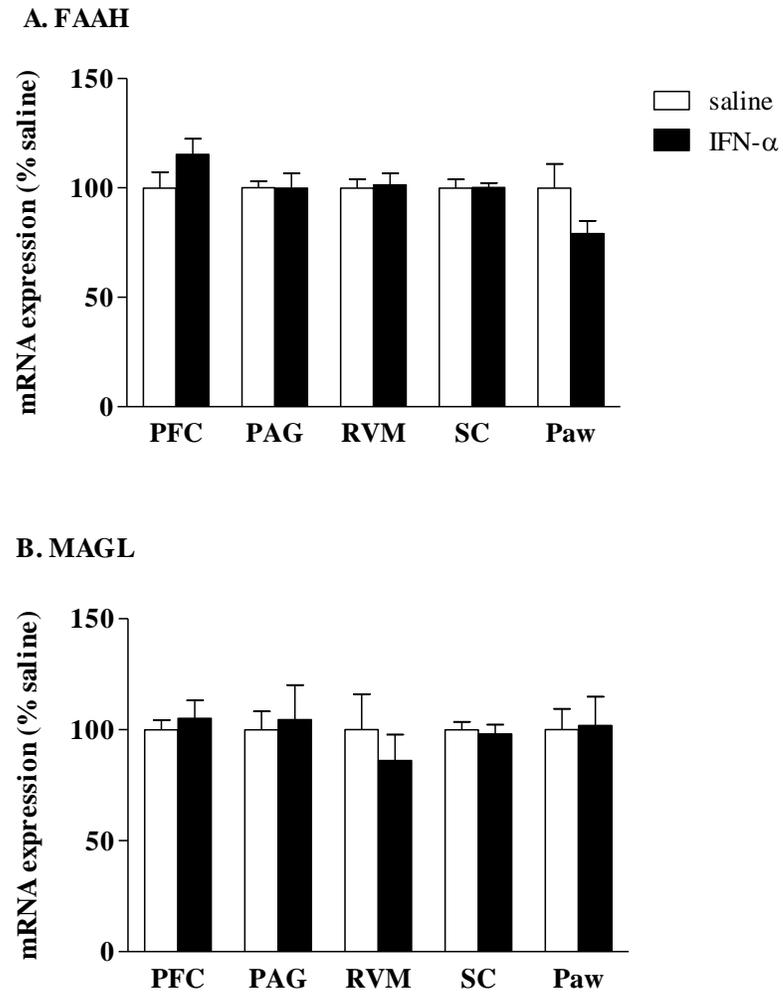


Figure 5.3.4. mRNA expression of endocannabinoid metabolising enzymes following 8 days of IFN- α or saline treatment: A, FAAH and B, MAGL. Data are expressed as mean \pm SEM; n = 6-8 per group.

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5.3.5 Repeated administration of IFN- α does not alter CB₁, CB₂, PPAR- α or PPAR- γ receptor expression in discrete brain regions, the spinal cord or paw tissue

Quantitative RT-PCR analysis was used to examine the expression of the cannabinoid receptors, CB₁ and CB₂ as well as other known targets of the endocannabinoids, PPAR- α and PPAR- γ .

Unpaired t-tests revealed no significant effect of repeated administration of IFN- α on CB₁ receptor expression in the PFC [P=0.71], PAG [P=0.28], RVM [P=0.49], spinal cord [P=0.29] or paw tissue [P=0.97].

Despite an observed trend towards increased expressional levels of CB₂ in the PFC and PAG, unpaired t-tests also revealed no significant effect of IFN- α treatment on CB₂ expression in the PFC [P=0.35] or PAG [P=0.15]. Mann-Whitney U-tests revealed no significant effect of repeated IFN- α administration on CB₂ receptor gene expression in the RVM [U=0.755] or spinal cord [U=0.959]. CB₂ receptor expression was below the limit of detection in the paw tissue isolated from both saline- and IFN- α -treated mice.

Unpaired t-tests revealed no significant effect of repeated administration of IFN- α on PPAR- α expression in the PFC [P=0.38], PAG [P=0.97], RVM [P=0.74], spinal cord [P=0.82] or paw tissue [P=0.33] following eight days of treatment.

Similarly, unpaired t-tests revealed no significant effect of repeated administration of IFN- α on PPAR- γ expression in the PFC [P=0.98], PAG [P=0.87], RVM [P=0.55], spinal cord [P=0.45] or paw tissue [P=0.86].

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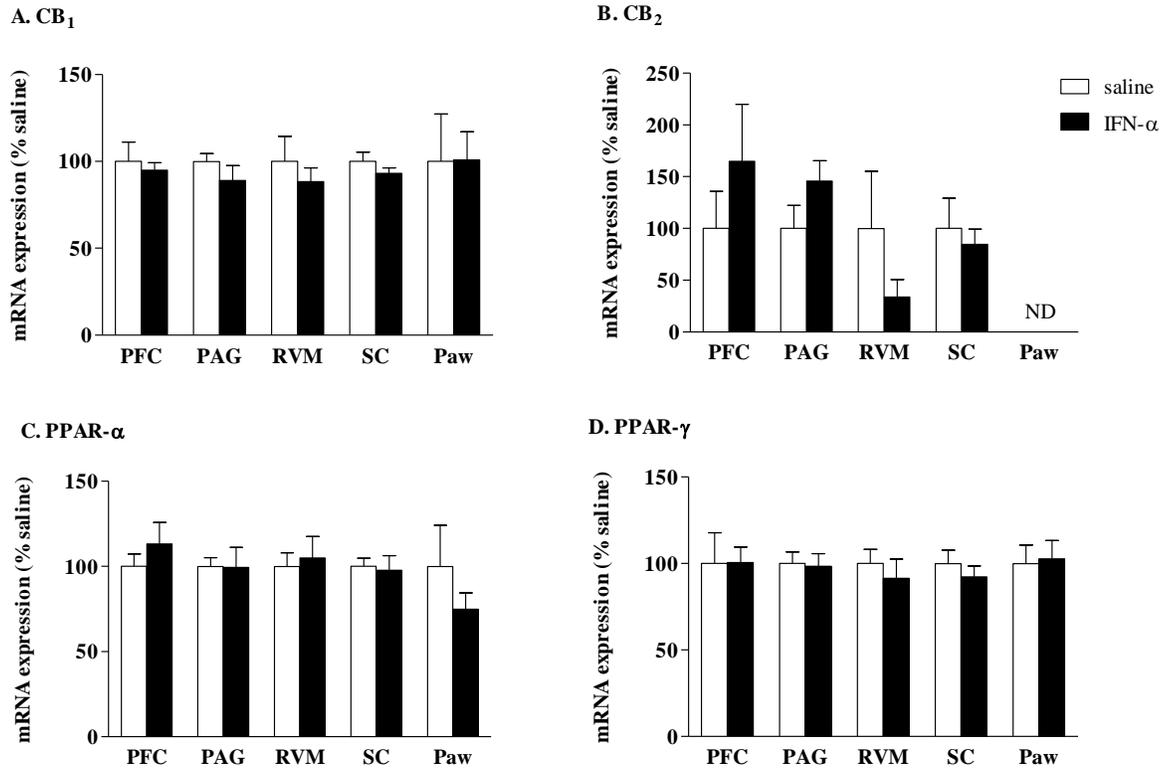


Figure 5.3.5. mRNA expression of endocannabinoid and PPAR receptors following 8 days of IFN- α or saline treatment: A, CB₁ receptor; B, CB₂ receptor; C, PPAR- α receptor and D, PPAR- γ receptor. Data are expressed as mean \pm SEM; n = 6-8 per group. Abbreviations: ND, not detected.

5.4 Discussion

The endocannabinoid system has been demonstrated to play an important modulatory role in emotional and nociceptive processing, alterations in which have been reported in several preclinical models of pain and/or depression. This study aimed to examine if repeated IFN- α treatment, which we have shown to induce both a depressive phenotype and inflammatory hyperalgesia, results in alterations in various components of the endocannabinoid system at a peripheral, spinal and supraspinal level. The current data demonstrated that repeated IFN- α administration results in increases in 2-AG levels in the PAG and RVM, as well as AEA levels in the RVM, following formalin administration, compared with saline-treated counterparts. Furthermore, repeated IFN- α treatment was associated with significant reductions in PEA and OEA in the spinal cord, an effect which was not altered following formalin treatment. Furthermore, intraplantar formalin administration tended to enhance FAAH substrate levels in the paw tissue of saline-treated animals, an effect not observed in IFN- α -treated counterparts. These effects were not associated with alterations in the expression of mRNA coding for the endocannabinoid metabolic enzymes or receptors. Taken together these data indicate alterations in the endocannabinoid system in peripheral, spinal and supraspinal regions of mice repeatedly treated with IFN- α , effects which may underlie, at least in part, the hyperalgesic effects observed in these mice.

As demonstrated previously, intraplantar injection of formalin induces a state of tonic persistent inflammatory pain in rodents, evident through a biphasic and directed response to paw injury (Hunskar and Hole, 1987, Burke et al., 2010, Rea et al., 2014), As observed in the previous chapter, the formalin-evoked nociceptive response was significantly increased in animals pre-exposed to IFN- α treatment in comparison with saline-treated counterparts. However, it should be noted that in the present study, inflammatory hyperalgesia was evident only in the second phase of the formalin test and not in the early phase, as was previously observed (chapter 4). Methodological differences between the two studies may account, in part, for the lack of effect in the

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first phase. Notably, mice in the current study were not exposed to the hot plate test prior to the formalin test, and the shortened duration of formalin exposure meant that there was increased movement and activity in the holding rooms which may have induced stress/reactivity in the animals prior to testing. Despite this, the current data confirm that repeated IFN- α results in enhanced formalin-evoked nociceptive behaviour in mice.

Alterations in the endocannabinoid system in key sites of the ascending and descending pain pathway have been reported in several preclinical pain models and may underlie the development of hyperalgesia and/or allodynia. As such, the effect of repeated IFN- α administration on the levels of endocannabinoids and the expression of the catabolic enzymes and receptor targets were examined peripherally, spinally and supraspinally. Quantification of endocannabinoid and N-acylethanolamine levels in the frontal cortex, components of the descending pain pathway and the paw tissue of saline-treated animals revealed that levels were comparable with those shown previously (Kinsey et al., 2009, Caprioli et al., 2012, Niphakis et al., 2012, Niphakis et al., 2013, Khasabova et al., 2008, Booker et al., 2012). Repeated administration of IFN- α did not significantly alter levels of AEA or 2-AG in any of the regions examined, although a slight decrease in the levels of both endocannabinoids was observed in the PAG when compared to saline-treated counterparts. Given the important role for the PAG in the descending modulation of pain, changes in endocannabinoid levels in this region may have an impact on the ability of IFN- α treated mice to modulate nociceptive processes. Accordingly, the data in the current study demonstrate that the formalin-evoked hyperalgesia observed in IFN- α -treated mice is associated with increased 2-AG levels in the PAG, with a similar but non-significant effect observed for AEA. In addition, intraplantar formalin resulted in increases in the levels of AEA and 2-AG in the RVM of IFN- α -treated mice. Although there was a trend for similar increases in AEA and 2-AG in the PAG and RVM of saline-treated mice following formalin administration, these effects failed to reach statistical significance. These data are in accordance with previous published data from our group and other demonstrating that AEA reportedly remains unchanged in the

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dorsolateral (dl) PAG (Olango et al., 2012), RVM (Rea et al., 2014) and paw tissue (Beaulieu et al., 2000) and 2-AG levels are not altered in the dlPAG (Olango et al., 2012) or paw tissue (Beaulieu et al., 2000) upon intraplantar administration of formalin to rats. However, it should be noted that there have been studies demonstrating enhanced AEA levels in the PAG (Walker et al., 1999) and 2-AG in the RVM (Rea et al., 2014) following intraplantar exposure to formalin in rats. Furthermore, the latter study reported alterations in endocannabinoid tone in the RVM of the WKY rat, a further model of anxiety/depression associated with formalin-evoked hyperalgesia. More specifically, this study demonstrated that formalin administration results in a significant reduction in AEA levels in the RVM of the WKY rat, an effect which was not observed in Sprague Dawley (SD) counterparts. Additionally, the aforementioned formalin-induced increases in 2-AG in the RVM of SD rats was not observed in WKY animals (Rea et al., 2014). Thus, taken together, the current study and that of Rea et al., suggest that alterations in endocannabinoid tone in the RVM may underlie formalin-evoked hyperalgesia in the presence of an anxiety/depressive phenotype.

It should also be noted that the current study demonstrated that intraplantar formalin induced an increase in AEA levels in the PFC of saline-, but not IFN- α -, treated animals, although the levels of AEA in the PFC of IFN- α -formalin and saline-formalin groups were comparable. This effect of formalin administration is somewhat in contrast to that previously observed in our lab, whereby no change in AEA levels was observed in the medial (m)PFC in rats following a 30-minute exposure to the test (Okine et al., 2014). The increases as observed in naïve animals in the present study were seen during the expression of pain behaviour following formalin administration at a slightly different time point (35min vs. 30min) and reflect changes in the entire PFC and not that within a specific subregion as was previously evaluated (Okine et al., 2014). The PFC is an important region in modulating emotional responses and in the affective dimension of pain. It is possible that formalin may not be able to further enhance the levels of AEA in this region in IFN- α as they are already at a maximum level. The inability to further

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enhance AEA levels in the PFC in response to a noxious stimulus may underlie some of the behavioural changes observed in IFN- α treated mice.

In comparison to the heightened 2-AG and AEA levels in the PAG and RVM of IFN- α treated animals following formalin, the current data also suggest that under these conditions, animals may concurrently be unable to mobilize endocannabinoids at the level of the paw. In particular and although not reaching statistical significance, AEA and 2-AG levels are lower in the paw of IFN- α , when compared to saline-treated animals, following formalin administration. Thus an inability to mobilize endocannabinoids at the site of inflammatory pain may contribute to the hyperalgesia observed. Taken together the data presented herein indicate altered mobilization of endocannabinoids in key components of the pain pathway in IFN- α treated mice exposed to a noxious inflammatory stimulus. These alterations may underlie the development of the hyperalgesic response observed or alternatively act as a compensatory mechanism in an attempt to normalize the response, with further studies required to evaluate this.

It is possible that the changes in endocannabinoid levels observed may be due to altered expression or activity of the enzymes responsible for the metabolism of these lipids. As such, we examined the mRNA expression of these metabolic enzymes namely FAAH and MAGL and found that these were unaltered in all of the regions examined. This is somewhat in contrast with the clinical scenario whereby it has recently been revealed that expressional levels of the AEA-metabolising enzyme, FAAH2, are increased in the peripheral serum of patients upon initiation of IFN- α therapy (Zajkowska et al., 2015). Importantly, this effect was only evident in patients exhibiting treatment-induced depression. Such increases in expressional levels of the endocannabinoid-degrading enzyme, could potentially predispose thus treated patients to associated reductions in substrate levels, namely that of AEA, a trait shown to be evident in depressed patients. It is important to consider that while a similar increase is not observed in the discrete peripheral and brain regions investigated in the current study, analysis of murine serum

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levels or indeed, subjection to a longer treatment time as was the case in the clinical study (4 weeks vs. 8 days), may reveal comparable changes in expressional levels of similar degrading enzyme(s). Moreover, it should also be noted that changes in the activity of these enzymes cannot be ruled out and this also account for the changes in endocannabinoid levels observed. Alternatively, alterations in the expression and activity of the endocannabinoid biosynthetic enzymes may have occurred and this warrants examination in future studies.

In order to mediate their effects, endocannabinoids must bind to and activate receptors, with activity primarily attributed to activation of the CB₁ and/or CB₂ receptor. However, endocannabinoids have also been shown to have activity at several other receptor targets, including peroxisome proliferators-activated receptors PPARs. As such a further aim of the study was to evaluate the effect of repeated IFN- α administration on cannabinoid and PPAR expression in the PFC, descending pain pathway and the paw tissue. The resulting data show that repeated exposure to IFN- α does not alter expression of the cannabinoid receptor, CB₁, or the PPARs in any of the regions/tissues examined. While there was a trend towards an increase in CB₂ receptor expression in the PFC and PAG of animals which had been exposed to continuous IFN- α treatment, this effect did not reach statistical significance and no changes in CB₂ receptor expression were noted in the RVM, spinal cord or paw tissue of IFN- α -treated animals when compared with saline-treated animals. However, lack of change in expression does not rule out possible changes in receptor activity/tone.

Alternative endogenous ligands which are known to provoke potent activity at PPAR receptors and act as entourage compounds to AEA include the FAAH substrates and *N*-acylethanolamines, PEA and OEA. PEA in particular has been shown to be altered in several pain models and play an important role in modulating pain responding when administered into discrete brain regions or spinally. For example, recent studies in our lab found a significant reduction in PEA levels in the mPFC following the 30-minute formalin test in rats (Okine et al., 2014). Direct administration of PEA into the PAG has

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been shown to reduce latency to respond in the tail-flick test, an effect mediated by reducing ON/OFF neuronal cell firing in the RVM (de Novellis et al., 2012). With regard to the spinal cord, topical or spinal application of PEA permanently abolishes formalin-induced spontaneous and evoked neuronal activity of spinal nociceptive neurons in mice (Luongo et al., 2013). Upon local administration, PEA also attenuates pain behaviour throughout both stages of formalin-evoked pain behaviour as observed following intraplantar administration of the lipid in mice (Calignano et al., 1998). While less is known about the role of OEA in the context of pain, similar to PEA, levels of OEA are also reduced in the mPFC following formalin administration.(Okine et al., 2014). The current study demonstrated that OEA was significantly increased in the PFC of both saline- and IFN- α -treated animals upon formalin exposure, while PEA levels were significantly increased in IFN- α -formalin treated animals only. However although not significant, a similar trend for an increase in PEA in saline-treated animals following formalin administration was evident. These data are somewhat in contrast to the reduction observed in these *N*-acylethanolamines in the PFC of rats following formalin administration (Okine et al., 2014). Differences in the species used (mice vs rats) and the time post formalin at which these lipids were assessed (35min vs. 30min post administration) may account for the discrepancy between the studies. However, the current data suggest that formalin evokes an increase in the mobilization of OEA (and PEA in IFN- α treated mice) in the PFC, and although further studies are required, these changes which may be involved in mediating and/or modulate nociceptive responding. Examining levels in other components of the descending pain pathway, revealed that while trends towards formalin-induced increases in PEA and/or OEA were observed in the PAG and RVM of both treatment groups, neither effect reached statistical significance. However, a significant reduction in PEA and OEA levels in the spinal cord of IFN- α treated animals was observed, an effect which was maintained though not significant, upon exposure to the formalin test. Given the homeostatic importance of PEA in physiological response to pain, particularly in the spinal cord, where PEA has shown to normalize electrophysiological parameters associated with spinal nociceptive activity and formalin-induced glial activation (Luongo et al., 2013), it is conceivable

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that such deficiencies may sensitize or allow IFN- α -treated animals to be more sensitive to the nociceptive effects of formalin administration and thus precipitating the hyperalgesia response as observed in these animals.

Finally and similarly to AEA, intraplantar formalin exposure produced an overall significant increase in PEA levels (with a similar trend for OEA) in the paw tissues of saline, but not IFN- α -treated animals. While these findings are in contrast to previous published findings whereby intraplantar administration of formalin (1-5%) failed to enhance PEA levels in the affected paw skin of naïve rats (Beaulieu et al., 2000), a contrasting observation possible due to the differing species employed (mice vs. rats) or time of assay assessment post formalin (35min vs. 60min), the data herein suggest an inability of IFN- α -treated animals to mobilize these FAAH substrates in the paw tissue in response to formalin administration, which may underlie the hyperalgesic response observed in IFN- α -treated animals.

Overall, the findings from this study demonstrate that IFN- α treated animals exhibit alterations in endocannabinoid and *N*-acylethanolamine tone in peripheral, spinal and supraspinal sites, in the presence and absence of formalin-evoked nociception. Although further studies are required in order to determine their functional significance, these changes may account, at least in part, for the hyperalgesic and/or altered emotional response following repeated IFN- α administration.

Chapter 6

The effect of intraplantar administration of FAAH or MAGL inhibitors on formalin-evoked nociceptive behaviour in saline- and IFN- α -treated mice

Chapter 6: Effect of local FAAH or MAGL inhibition on IFN- α -induced hyperalgesia

Cannabis has been used as a medicine throughout the world for several thousand years. Evidence of its use in treating various ailments including pain appears as early as 2600 B.C. The principal psychoactive ingredient of cannabis, Δ^9 -THC, was first identified in 1964 (Gaoni and Mechoulam, 1971) and subsequent attempts to understand its mechanism of action have led to characterization of the endogenous cannabinoid (endocannabinoid) system. The system consists of the cannabinoid receptors (CB₁ and CB₂) (Matsuda et al., 1990, Munro et al., 1993, Devane et al., 1988), their naturally occurring endogenous ligands, the best characterized of which are anandamide (AEA) (Devane et al., 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam et al., 1995), and the enzymes involved in their biosynthesis and degradation. Fatty acid amide hydrolase (FAAH) is the main enzyme responsible for the catabolism of AEA (Cravatt et al., 1996), while in comparison, 2-AG is primarily catabolised by monoacylglycerol lipase (MAGL) (Blankman et al., 2007). While CB₁ and CB₂ represent the classical receptors for cannabinoid-mediated activity, recent evidence also suggests that cannabinoids have affinity for and activity at other receptor targets including peroxisome proliferator-activated receptors (PPARs), TRPV1 and GPR55 (Alexander and Kendall, 2007).

As highlighted in earlier chapters (chapter 1 and 5), given the distribution of the endocannabinoid system throughout spinal, supraspinal and peripherpheral regions, it is in a prime position to regulate neurophysiological activities such as nociceptive processing. This has been a very active area of research over the past decade with a number of excellent reviews synthesising the data supporting a role for the endocannabinoid system in modulating nociception [for reviews see (Boychuk et al., 2015, Rani Sagar et al., 2012, Ulugol, 2014)]. Despite the medicinally beneficial properties of Δ^9 -THC and other direct CB₁ receptor agonists, the associated cognitive impairment and other neuropsychiatric side effects (Moreira et al., 2009) have encouraged the pursuit of alternative strategies to enhance endocannabinoid function. As such, more subtle methods of targeting the system, such as through enhancement of endocannabinoid tone, is now a valid consideration. Increasing endogenous levels of AEA and 2-AG, through genetic or pharmacological inhibition of FAAH or MAGL

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respectively, has been shown to elicit analgesic activity in several animal models, effects comparable with that of direct CB₁ receptor agonism (Kathuria et al., 2003, Ahn et al., 2009, Kinsey et al., 2009). Importantly, such effects occur without disruption of motility, cognition or thermoregulation (Kathuria et al., 2003, Cravatt et al., 2001), suggesting that FAAH and/or MAGL inhibition regulates a discrete subset of behavioural processes.

Several studies have reported on the peripheral modulation of nociceptive processing by cannabinoids, an effect that has the advantage of avoidance of the aforementioned psychiatric and cognitive side effects associated with central CB₁ receptor activation. For example, with particular regard to inflammatory models of pain behaviour, carrageenan-evoked mechanical and thermal hyperalgesia has shown to be suppressed following local intraplantar administration of CB₁ or CB₂ receptor agonists (Gutierrez et al., 2007). Additionally, intraplantar administration of the endocannabinoid, AEA, dose-dependently reduces mechanical hyperalgesia in the PGE₂-induced model of inflammatory pain in rats (Romero et al., 2013), an effect reversed by blockade of the CB₁ receptor, while local administration of 2-AG alleviates inflammatory pain behaviour throughout the late phase of formalin-evoked nociceptive responding in rats (Guindon et al., 2007), an effect mediated by specific activity at the CB₂ receptor. Furthermore, pharmacological inhibition of FAAH and/or MAGL in the paw, thus elevating local endocannabinoid levels, similarly alleviates inflammatory pain behaviour. For example, intraplantar administration of the FAAH inhibitor, URB597, alleviates mechanical hyperalgesia in the CFA-treated paw an effect accompanied by significant increases in both AEA and 2-AG in carrageenan-treated rats (Jhaveri et al., 2008), while in the same model, intraplantar administration of URB597 also blocks carrageenan-evoked peripheral receptor field expansion of wide dynamic range (WDR) neurons (Sagar et al., 2008). The recently-developed inhibitor, PF3845, produces a similar anti-allodynic effect in the LPS-treated paw, an observation shown to be both CB₁- and CB₂-receptor mediated (Booker et al., 2012). Local administration of the MAGL inhibitor, JZL184, also dose-dependently reduces pain behaviour throughout the

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early and late phases of formalin-evoked nociceptive response in rodents, an event occurring in the absence of any local change in endocannabinoid levels (Guindon et al., 2011). Furthermore in animal models of neuropathic pain, intraplantar administration of AEA or 2-AG, or local enhancement of these endocannabinoids following FAAH or MAGL inhibition, results in anti-nociceptive efficacy against mechanical (Vera et al., 2012, Khasabova et al., 2008, Khasabova et al., 2011) and thermal hyperalgesia (Johanek and Simone, 2004), effects primarily mediated via CB₁ and CB₂ receptor activity.

The above evidence demonstrates an important role for peripheral FAAH and MAGL substrates in modulating physiological responding to various stimuli in the presence of underlying inflammatory and neuropathic pain. The previous studies in this thesis have demonstrated that repeated administration of IFN- α induces inflammatory hyperalgesia in mice (chapters 4 and 5), an effect associated with inability to mobilize AEA, 2-AG and *N*-acylethanolamines within the plantar tissue of the paw in response to formalin administration, as observed in saline-treated counterparts (chapter 5). This inability to mobilize endocannabinoids may, in part, underlie the hyperalgesic response to formalin in IFN- α -treated animals. As such, we hypothesized that enhancing endocannabinoid tone via inhibition of FAAH or MAGL would attenuate the hyperalgesia observed.

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6.1.1 Aims

1. Investigate if inhibiting the activity of FAAH or MAGL directly within the paw modulates formalin-induced nociceptive behaviour.
2. Evaluate if formalin-evoked hyperalgesia observed following repeated IFN- α administration is altered by peripheral enhancement of endocannabinoid tone.

6.2 Materials and Methods

6.2.1 Animals

Male C57BL/6 mice (weight 25-30g; Charles River Laboratories, UK) were housed in groups of four in plastic-bottomed cages containing wood shavings as bedding. One day prior to initiation of experimental procedures, animals were individually housed and were kept as so or the remainder of the study. Animals were maintained in a constant temperature (21 +/- 2°C) under standard lighting conditions (12 : 12 hr light-dark, lights on from 07.00 to 19.00 hr). All experiments were carried out during the light phase, between 08.00 and 18.00 hr. Mice were given free access to food and water.

The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

6.2.2 Pharmacological Treatment

Human interferon-alpha (hIFN- α -2a; 3M IU/0.5ml), Roferon-A®, was purchased from Roche Pharmaceuticals. The drug was prepared in saline from stock on a daily basis. This drug/vehicle solution was administered subcutaneously in a volume of 3 μ l/g to give a final concentration of 8,000IU/g IFN- α or equivalent volume of saline vehicle. Dosing occurred daily, between the hours of 10.00 and 12.00. The dose chosen was based on previous studies demonstrating depressive-like and hyperalgesic behaviour following repeated administration of IFN- α in mice (chapters 4 and 5).

The FAAH inhibitor, PF3845 (from the NIMH drug synthesis scheme), and MAGL inhibitor, MJN110 (gifted from Benjamin Cravatt, U.S.), were each dissolved in ethanol: saline: cremophor at a 1:1:18 dilution to give a final concentration of 1 μ g/10 μ l. This drug/vehicle solution was administered into the plantar region of the left hind paw in a volume of 10 μ l and dosing occurred 30 minutes prior to the formalin test. The dose of PF3845 chosen was based on previous studies demonstrating anti-allodynic efficacy

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following LPS-induced mechanical allodynia in mice (Booker et al., 2012). The dose of MJN110 was chosen based on its relative potency and in line with concentrations and activity of other MAGL inhibitors following intraplantar administration (Khasabova et al., 2011, Guindon et al., 2011).

6.2.3 Experimental Design

Following acclimatization to the animal unit (5 days), mice were housed singly and 24 hrs later, administered saline vehicle (s.c.) for 5 days to acclimatize to handling and injection procedure. Saline or IFN- α administration began the following day for 8 days. 24 hours following the final saline/IFN- α administration, animals received an intraplantar injection of the FAAH inhibitor PF3845, the MAGL inhibitor MJN110 or vehicle (10 μ l/paw) and 20 minutes later were placed in the Perspex testing arena and locomotor activity assessed for 10 minutes. Following this, animals received intraplantar formalin and nociceptive behaviour was recorded onto DVD for 1 hr and rated using Ethovision software as previously described (chapter 2). At the end of behavioural testing, animals were sacrificed following deep isoflurane anesthesia, the brain was removed and the PFC dissected out. The plantar region of the paw tissue was also removed and both were snap-frozen on dry ice and stored at -80°C until determination of endocannabinoid and *N*-acylethanolamine levels using LC-MS-MS.

As before (chapter 5), upon weighing paw tissue samples prior to analysis using LC-MS-MS, a large variance in tissue weight was noted, an effect which was evident within all treatment groups (saline-vehicle, 1.9-7.5mg; saline-PF3845, 1.2-60mg; saline-MJN110, 0.9-6mg, IFN- α -vehicle, 0.6-6.8mg, IFN- α , 0.5-14mg, IFN- α -MJN110, 0.6-8.6mg). This was despite equivalent amounts of tissue taken. As such it was decided that expressing lipid quantity relative to tissue weight would not be a reliable measure of so, therefore lipid quantities were alternatively expressed as that per paw tissue sample.

6.2.7 Statistical Analysis

Data were analysed using SPSS statistical package, two-way ANOVA followed by Fisher's LSD *post hoc* analysis where appropriate. Where data were not normally

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distributed or homogenous, data were resolved using Kruskal-Wallis analysis by ranks. Data were considered significant when $P < 0.05$. Results are expressed as group means \pm standard error of mean (SEM).

6.3 Results

6.3.1 Intraplantar administration of PF3845 or MJN110 does not alter locomotor activity of saline- or IFN- α -treated mice

Locomotor activity was assessed in the formalin arena for a period of ten minutes. This was carried out twenty minutes following intraplantar administration of PF3845, MJN110 or vehicle and ten minutes prior to administration of the noxious inflammatory stimulus.

Two-way ANOVA revealed no significant effect of IFN- α [$F(1, 50) = 0.024, P=0.877$], drug [$F(2, 50) = 0.836, P=0.439$] or IFN- α x drug treatment interaction [$F(2, 49) = 0.213, P=0.809$] on locomotor activity over a 10-minute period on exposure to a novel arena

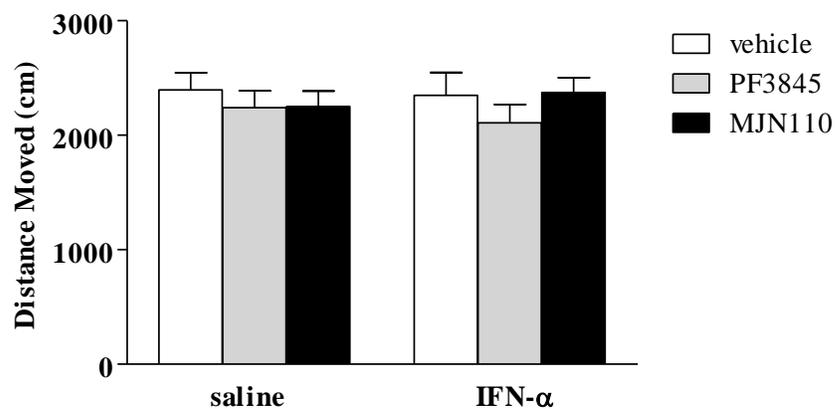


Figure 6.3.1. The effect of intraplantar administration of PF-3845, MJN-110 or vehicle on locomotor activity of saline- or IFN- α -treated mice. Data are expressed as mean \pm SEM; n= 9-10 per group.

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6.3.2 Intraplantar administration of PF3845 or MJN110 alleviates nociceptive responding in the formalin test in IFN- α - but not saline-treated mice

Immediately following assessment of locomotor activity, mice received intraplantar formalin and were returned to the same arena where nociceptive responding was assessed over the following 60 minutes.

Two-way repeated measures ANOVA revealed a significant effect of time [$F(11, 539) = 37.074, P=0.000$] but not IFN- α [$F(1, 49) = 3.142, P=0.083$], drug treatment [$F(2, 49) = 0.134, P=0.875$] or any interaction effects on elevation of the affected paw (pain '1' behaviour) in the formalin test. Upon further temporal analysis, two-way ANOVA revealed no significant effect of IFN- α [$F(1, 49) = 0.340, P=0.562$] or drug treatment [$F(2, 49) = 2.853, P=0.067$] on paw elevation within the early phase (0-5 min) of the test, although a trend towards an increase in this behaviour was observed in MJN110-treated animals in comparison with vehicle-treated as evident in figure 6.3.2.1.C. Two-way ANOVA also revealed no significant effect of IFN- α [$F(1, 49) = 0.345, P=0.560$] or drug treatment [$F(2, 49) = 1.737, P=0.187$] on paw elevation during the second phase (20-25 min) of formalin-evoked response (see figure 6.3.2.1.D).

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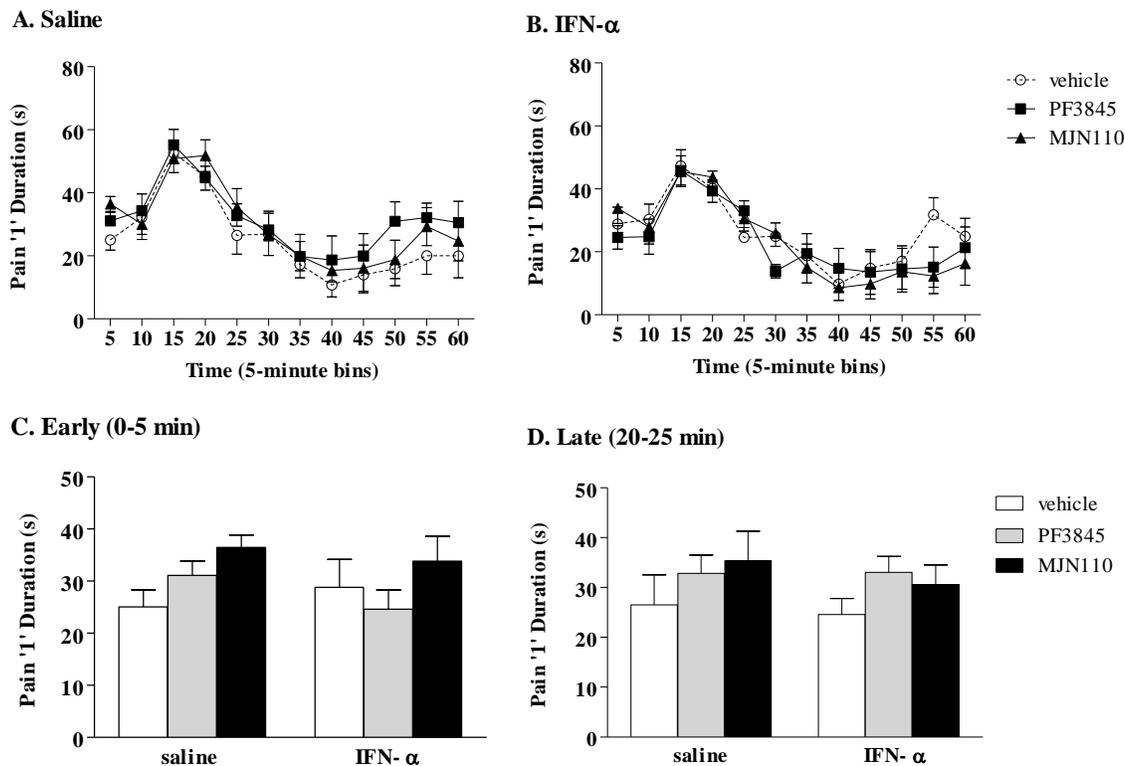


Figure 6.3.2.1. Effect of intraplantar PF3845 or MJN110 on pain '1' nociceptive behaviour in the formalin test: A, Profile of pain '1' behaviour over 60 minutes in saline-treated animals; B, Profile of pain '1' behaviour over 60 minutes in IFN- α -treated animals; C, Duration of pain '1' behaviour in the early (0-5min) phase of the formalin test; D, Duration of pain '1' behaviour in the late (20-25min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group.

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Two-way repeated measures ANOVA revealed a significant effect of time [F (11, 539) = 70.968, P=0.000], drug treatment [F (2, 49) = 4.179, P=0.021] and time x drug interaction [F (11, 539) = 2.052, P=0.022] but not IFN- α [F (1, 49) = 0.270] on pain '2' behaviour i.e. direct shaking, licking, biting or flinching of the affected paw in the formalin test. Fisher's LSD *post hoc* analysis revealed that in IFN- α -treated animals, MJN110, reduced pain '2' behaviour during the initial 5 minutes of the test, while PF3845 and MJN110 reduced pain '2' behaviour at discrete time points throughout the test, namely 20-25min and 45-50min, as can be seen in figure 6.3.2.2B.

Upon further temporal analysis, two-way ANOVA revealed no effect of drug treatment [F (2, 49) = 2.954, P=0.061], IFN- α [F (1, 49) = 2.164, P=0.148] or interaction on pain '2' behaviour throughout the early phase (0-5min) of the test. In comparison, two-way ANOVA also revealed a significant effect of drug treatment [F (2, 49) = 3.521, P=0.037] but not IFN- α treatment [F (1, 49) = 2.712, P=0.106] on pain '2' behaviour throughout the later phase of formalin-evoked response. Fisher's LSD *post hoc* analysis revealed that PF3845 and MJN110 reduced pain '2' behaviour throughout the second phase (20 -25 min) of the test in IFN- α - but not saline-treated animals (see figure 6.3.2.2 C, D).

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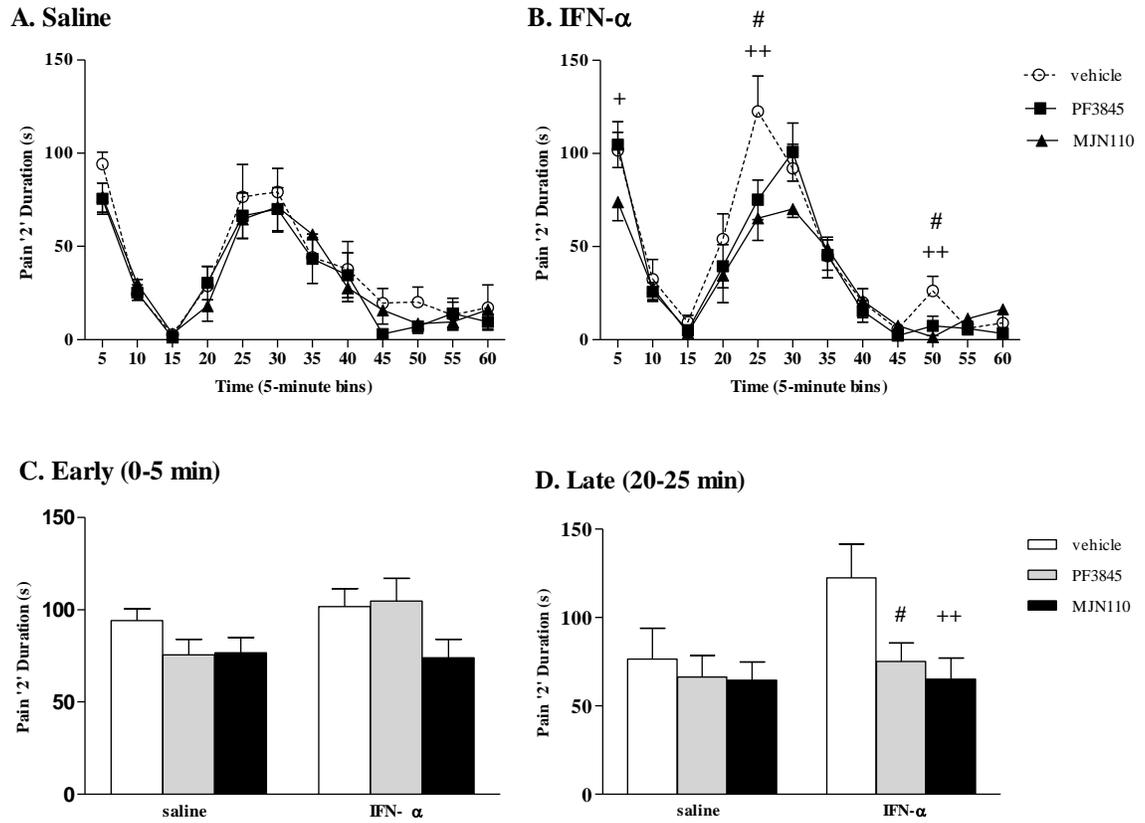


Figure 6.3.2.2. Effect of intraplantar PF3845 or MJN110 on pain '2' nociceptive behaviour in the formalin test: A, Profile of pain '2' behaviour over 60 minutes in saline-treated animals; B, Profile of pain '2' behaviour over 60 minutes in IFN- α -treated animals; C, Duration of pain '2' behaviour in the early (0-5min) phase of the formalin test; D, Duration of pain '2' behaviour in the late (20-25min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group. #P<0.05 vs. vehicle in PF3845-treated; ++P<0.01, +P<0.05 vs. vehicle in MJN110-treated.

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Two-way repeated measures ANOVA revealed a significant effect of time [F (11, 539) = 81.870, P=0.000] and time x IFN- α interaction [F (11, 539) = 2.32, P=0.009], but not IFN- α treatment [F (1, 49) = 0.039, P=0.845] or drug [F (2, 49) = 2.903, P=0.064] or time x drug interaction [F (22, 539) = 0.988, P=0.477] effects on CPS in the formalin test. Fisher's LSD *post hoc* analysis revealed that CPS was significantly increased in IFN- α -vehicle-treated animals at one discrete time bin in the test (20-25 min) when compared to saline-treated counterparts (figure 6.3.2.3.A, B).

Following further temporal analysis, two-way ANOVA revealed no significant effect of IFN- α [F (1, 49) = 2.282, P=0.137] or drug treatment [F (2, 49) = 2.242, P=0.117] on CPS during the early phase of formalin-evoked response, but revealed a significant effect of drug treatment [F (2, 49) = 3.341, P=0.040] but not IFN- α [F (1, 49) = 2.986, P=0.090] on CPS during the second phase of response (20-25 min) of the formalin response (figure 6.3.2.3.C, D). Fisher's LSD *post hoc* analysis revealed that PF3845 and MJN110 reduced CPS during the second phase (20-25 min) of nociceptive response in IFN- α -treated animals, an effect not observed in saline-treated counterparts [figure 6.3.2.3.D].

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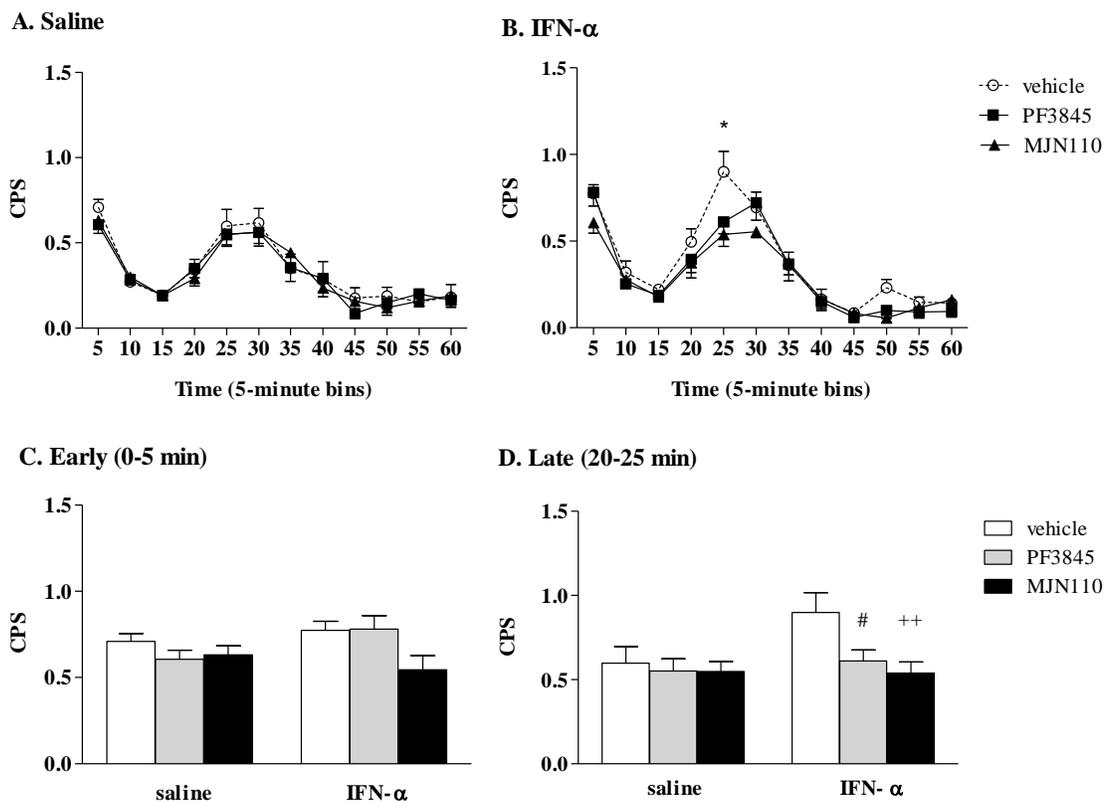


Figure 6.3.2.3. Effect of intraplantar PF3845 or MJN110 on CPS in the formalin test: A, Profile of composite pain score (CPS) over 60 minutes in saline-treated animals; B, Profile of composite pain score (CPS) over 60 minutes in IFN- α -treated animals; C, composite pain score (CPS) in the early (0-5min) phase of the formalin test; D, composite pain score (CPS) in the late (20-25min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group. *P<0.05 vs. saline (in figure 6.3.2.3.A.); #P<0.05; ++P<0.01 vs. vehicle-treated counterparts.

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6.3.3 Intraplantar administration of PF3845 or MJN110 does not attenuate formalin-evoked paw oedema in saline- or IFN- α -treated mice

Two-way ANOVA revealed no significant effect of IFN- α [$F(1, 49) = 0.641, P=0.427$], drug treatment [$F(2, 49) = 1.381, P=0.261$] or IFN- α x drug interaction [$F(2, 49) = 0.591, P=0.558$] on paw diameter following intraplantar injection of PF3845, MJN110 or vehicle just prior to formalin administration (data not shown). Intraplantar formalin administration induced paw oedema in all animals exemplified as increase in paw diameter. Two-way ANOVA also revealed no significant effect of IFN- α [$F(1, 49) = 0.199, P=0.657$], drug treatment [$F(2, 49) = 1.406, P=0.255$] or IFN- α x drug interaction [$F(2, 49) = 0.177, P=0.838$] on formalin-evoked paw oedema.

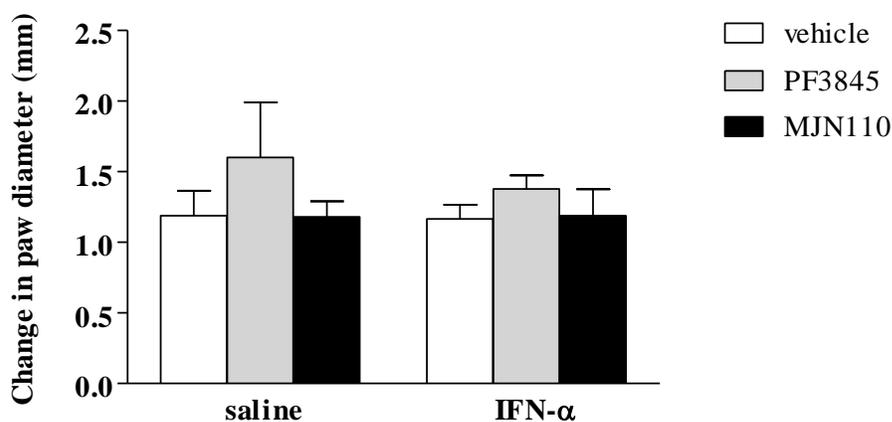


Figure 6.3.3. Effect of intraplantar PF3845 or MJN110 on formalin-induced increase in paw diameter in saline- or IFN- α -treated mice. Data are expressed as mean \pm SEM; n= 8-10 per group.

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6.3.4 Intraplantar administration of PF3845 or MJN110 exhibits differential effects on endocannabinoid levels in the paw tissue and PFC of saline- and IFN- α -treated animals following formalin administration

Following the one-hour formalin test, animals were sacrificed and the PFC (contralateral) and hindpaw (ipsilateral) tissue removed for assay of endocannabinoid levels using mass spectrometry.

Two-way ANOVA revealed a significant effect of intraplantar drug treatment, but not IFN- α or IFN- α x drug interaction, on 2-AG levels in paw tissue of mice following the formalin test. Fisher's LSD *post hoc* analysis revealed that MJN110 significantly increased tissue levels of 2-AG in the paw of saline-, but not IFN- α -treated animals when compared with vehicle-treated counterparts (figure 6.3.4.A). Kruskal-Wallis ANOVA by ranks revealed no significant effect of IFN- α or drug treatment on AEA levels in the paw of mice following the formalin test (see table 6.3.4 and figure 6.3.4).

Two-way ANOVA revealed a significant effect of intraplantar drug treatment, but not IFN- α or IFN- α x drug interaction on 2-AG levels in the PFC of mice following the formalin test. Fisher's LSD *post hoc* analysis revealed MJN110 significantly increased tissue levels of 2-AG in the PFC of saline-, but not IFN- α -treated animals (figure 6.3.4.B). Kruskal-Wallis ANOVA by ranks revealed no significant effect of IFN- α or drug treatment on AEA levels in the PFC of mice following the formalin test as recorded in table 6.3.4 and illustrated in figure 6.3.4.

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	Region	IFN-α	Drug treatment	Interaction (IFN-α x Drug)
2-AG	Paw	0.082	0.021[#]	0.423
	PFC	0.930	0.041[#]	0.125
AEA	Paw	0.260	0.095	NA
	PFC	0.337	0.133	NA

Table 6.3.4. Results of statistical analysis examining effect of repeated IFN- α and intraplantar PF3845 or MJN110 administration on 2-AG and AEA levels in the frontal cortex and paw tissue following formalin administration. Data are expressed as P value; n= 8-10 per group. ^{##}P<0.01, [#]P<0.05, effect of drug (vehicle/ PF3845/ MJN110) treatment.

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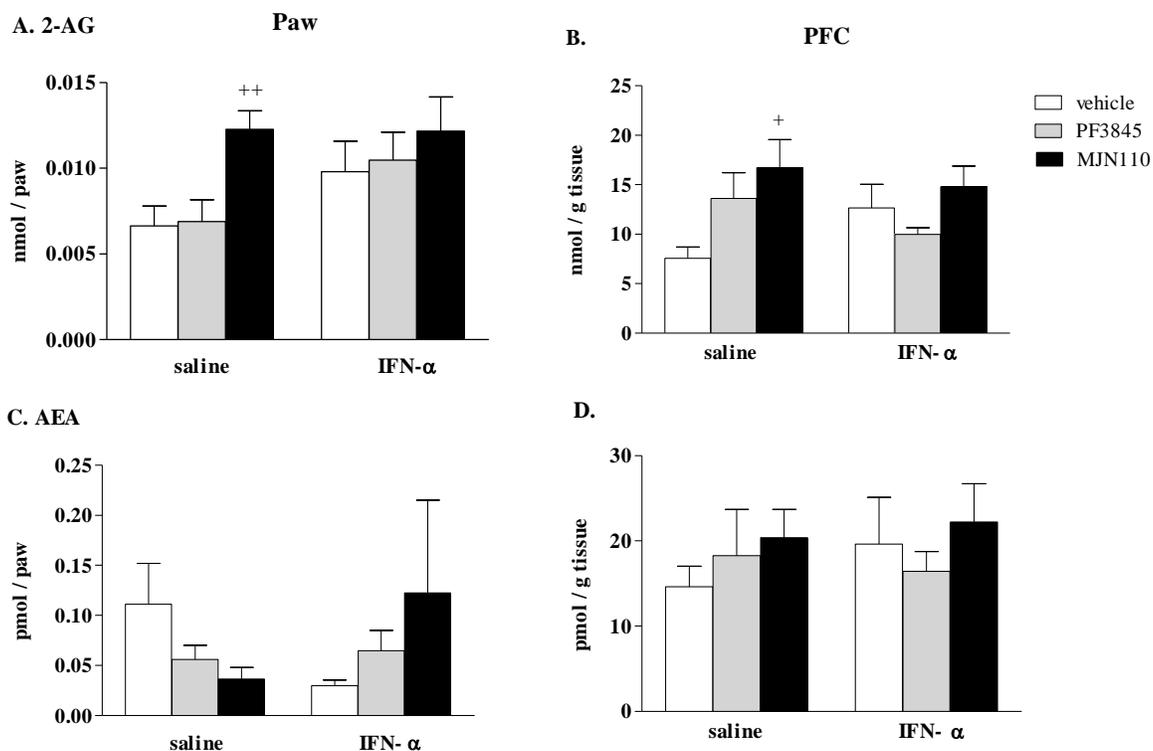


Figure 6.3.4. The effect of PF3845 and MJN110 on levels of 2-AG and AEA levels in the paw tissue and PFC of formalin-treated mice previously exposed to repeated saline or IFN- α treatment. A, 2-AG levels in the paw tissue (plantar tissue of the left hind paw); B, 2-AG levels in the PFC (prefrontal cortex); C, AEA levels in the plantar tissue of the left hind paw; D, AEA levels in the PFC. Data are expressed as mean \pm SEM; n= 8-10 per group. ⁺⁺P<0.01, ⁺P<0.05 vs. vehicle-treated counterparts.

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6.3.5 Intraplantar administration of PF3845 or MJN110 exhibits differential effects on *N*-acylethanolamine levels in the PFC and paw tissue of saline- and IFN- α -treated animals following formalin administration

Following the one-hour formalin test, animals were sacrificed and the PFC (contralateral) and left hind paw (ipsilateral) tissue removed for assay of *N*-acylethanolamine levels using mass spectrometry.

Two-way ANOVA revealed a significant effect of IFN- α x drug treatment but not IFN- α or intraplantar drug treatment on PEA levels in the paw (see table 6.3.5). Fisher's LSD *post hoc* analysis revealed intraplantar PF3845 increased PEA levels in the paw tissue of IFN- α -, but not saline-treated animals (see figure 6.5.3.A). Two-way ANOVA also revealed a significant effect of intraplantar drug treatment, but not IFN- α , on OEA levels in the paw. Fisher's LSD *post hoc* analysis revealed that intraplantar PF3845 increased OEA levels in the paw tissue of IFN- α -, but not saline-treated animals (see figure 6.3.5.C).

Two-way ANOVA revealed a significant effect of intraplantar drug treatment, but not IFN- α or drug x IFN- α interaction on PEA levels in the PFC. Fisher's LSD *post hoc* analysis revealed that intraplantar PF3845 increased PEA levels in the PFC of saline-, but not IFN- α -treated animals (see figure 6.3.5.B). Although there appeared to be a slight increase in PEA levels in the PFC of saline-treated mice following intraplantar administration of MJN110, analysis revealed no significant effect of the MAGL inhibitor on PEA levels in the PFC. Two-way ANOVA revealed no significant effect of IFN- α or intraplantar drug treatment on OEA levels in the PFC following the formalin test.

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	Region	IFN- α	Drug treatment	Interaction (IFN- α x Drug)
PEA	Paw	0.318	0.075	0.043⁺
	PFC	0.840	0.024[#]	0.339
OEA	Paw	0.369	0.008^{##}	0.061
	PFC	0.805	0.119	0.401

Table 6.3.5. Effect of repeated IFN- α and intraplantar PF3845 or MJN110 administration on 2-AG, AEA, PEA and OEA levels in the PFC and paw tissue. Data are expressed as P value; n= 7-10 per group. ##P<0.01, #P<0.05, effect of drug (vehicle/ PF3845/ MJN110) treatment; +P<0.05, effect of IFN- α and drug treatment.

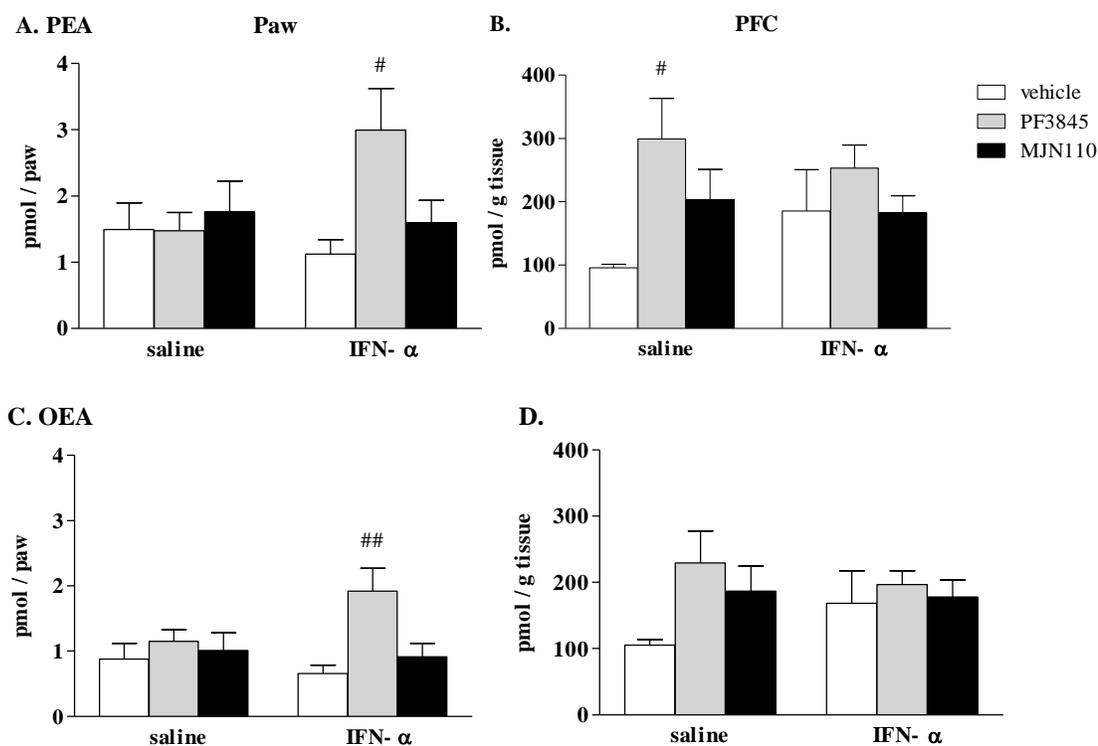


Figure 6.3.5. Quantification of *N*-acylethanolamines in the PFC and paw tissue following eight days of IFN- α treatment, and following formalin administration: A, PEA levels in the PFC (prefrontal cortex); B,

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PEA levels in the plantar tissue of the left hind paw; C, OEA levels in the PFC (prefrontal cortex); D, OEA levels in the plantar tissue of the left hind paw. Data are expressed as mean \pm SEM; n= 7-10 per group. ^{##}P<0.01, [#]P<0.05 vs. vehicle-treated counterparts.

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6.4. Discussion

The present chapter sought to examine the effect of enhancing endocannabinoid tone at the site of the inflammatory noxious stimulus (paw) and evaluate the effect on nociceptive responding and IFN- α associated hyperalgesia. As shown in previous chapters, repeated administration of IFN- α enhanced late phase formalin-evoked nociceptive responding. Intraplantar administration of the FAAH inhibitor PF3845 significantly attenuated IFN- α associated hyperalgesia, while failing to alter nociceptive responding in saline-treated mice. This anti-nociceptive effect of PF3845 was associated with significantly increased levels of PEA and OEA in the paw tissue of IFN- α -treated animals. Similarly, intraplantar administration of the MAGL inhibitor MJN110, attenuated both early phase formalin evoked nociceptive behaviour and the late phase hyperalgesic behaviour in IFN- α -treated animals, without altering formalin-evoked nociceptive responding in saline-treated mice. The MJN110-associated behavioural changes observed in IFN- α -treated animals occurred in the absence of any significant increase in 2-AG levels in the paw. Furthermore, the effect of FAAH and MAGL inhibition on formalin-evoked nociceptive responding was not associated with any change in paw oedema or locomotor activity. Taken together these data provide further evidence of altered endocannabinoid tone at a peripheral level following repeated IFN- α treatment, effects which appear to, at least in part, underlie the hyperalgesic phenotype of these mice.

In the current study, IFN- α -induced hyperalgesia was identified in the late (20-25 min) phase of nociceptive responding in the formalin test of inflammatory pain behaviour. While this observation is similar to that observed in the chapter 4 and 5, albeit at an earlier time (chapter 4, 40-50 min; chapter 5, 25-30 min), the hyperalgesic effect observed in the early phase of the trial identified in chapter 4 was again not noted in the current study. As highlighted in chapter 5, lack of change in first phase nociceptive responding may be due to differences in experimental conditions such as lack of pre-exposure to the hotplate test or prior intraplantar injection. Despite this, the current data

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again demonstrate that repeated IFN- α administration induces an increase in formalin-evoked nociceptive behaviour, thus highlighting the reproducibility of the model.

A key aim of the current study was to examine the effect of peripheral FAAH and MAGL inhibition on formalin-evoked nociceptive behaviour. The current data demonstrated that intraplantar administration of the FAAH inhibitor PF3845, did not alter formalin-evoked nociceptive responding in saline-treated animals. While the effect of intraplantar PF3845 had not been previously evaluated with regard to formalin-evoked nociception, intraplantar administration of PF3845 has been shown to partially reverse LPS-induced mechanical allodynia (Booker et al., 2012), an alternative model of inflammatory pain behaviour. In addition, intraplantar administration of an alternative FAAH inhibitor, URB597, has been shown to inhibit carrageenan-induced receptive field expansions, but not mechanical-evoked responses of spinal neurons, in rats with hind paw inflammation (Sagar et al., 2008). Furthermore, the generation of a FAAH knockout mouse with specific deletion of the gene coding for FAAH in peripheral tissues has indicated a function of peripheral FAAH in the modulation of nociceptive processing in the carrageenan model of inflammatory pain (Cravatt et al., 2004). Recent studies have demonstrated that the peripherally-restricted FAAH inhibitor URB937 exhibits anti-nociceptive efficacy throughout the later phase of the formalin test (Clapper et al., 2010). Thus, taken together, these data indicate a role for peripheral FAAH substrates in modulating nociceptive responding associated with inflammatory pain. It is possible that the inability of PF3845 to modulate nociceptive responding in saline-treated animals in the current study may be due to the lack of increase in FAAH substrates at the level of the paw following administration. Such opposing effects in the paw of saline vs. IFN- α -treated animals are difficult to interpret. However, in light of a recent finding from the clinic whereby initiation of IFN- α therapy induces a profound increase in peripheral FAAH expression (Zajkowska et al., 2015), it could be the case that such upregulation of FAAH expression and/or activity as a result of chronic IFN- α treatment thus makes the enzyme more susceptible to inhibition. In comparison, PF3845 resulted in a significant increase in *N*-acylethanolamine (PEA) levels in the

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PFC, but not AEA levels. Similar to our data, Booker et al. also demonstrated that intraplantar administration of PF3845 did not alter AEA levels in the brain tissue; with levels in the paw tissue below the limit of detection of their mass spectrometry system. Additionally, this study did not report if any such effects were observed in PEA and OEA levels (Booker et al., 2012). We cannot rule out that AEA may have been increased at a time point earlier than that examined in our study (90 min post administration). However, the increase in PEA (and to a lesser degree OEA) in the brain may indicate that intraplantar administration of the dose of PF3845 used in the current study (1 μ g) entered the systemic circulation and consequently the CNS resulting in global FAAH inhibition. Thus, the data indicate that an overall increase in FAAH substrate does not alter formalin-evoked nociceptive behaviour.

In contrast to saline-treated mice, PF3845 significantly reduced late phase formalin-evoked nociceptive behaviour in repeated IFN- α -treated mice. Upon assay of endocannabinoid and *N*-acylethanolamine levels, it was discovered that while AEA levels remained unchanged in all treated groups, the other FAAH substrates, PEA and OEA, were significantly increased in the paw of IFN- α -treated animals, an effect which occurred in the absence of any central changes of the aforementioned substrates. Thus, the anti-hyperalgesic effect of PF3845 in IFN- α -treated mice appears to be mediated by enhancing FAAH substrate levels in the paw. Several studies have reported an analgesic effect of PEA on formalin-evoked nociception following local (LoVerme et al., 2006) and spinal administration (Naderi et al., 2012, LoVerme et al., 2006, Luongo et al., 2013). Previous findings in chapter 5 indicated that mice that received repeated IFN- α treatment exhibited an inability to mobilize FAAH substrates in the paw in response to formalin administration. In comparison, in the present study, there was no difference in FAAH substrate levels in the paw between formalin-treated, saline- and IFN- α -treated mice. It is possible that the lack of difference is due to time at which the endocannabinoid levels were assessed in this study, (1 hour post-formalin when nociceptive responding was minimal), in comparison to chapter 5 where endocannabinoid levels were assessed during the second phase of the test and just after

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the hyperalgesic profile was observed. In any case, the data provided in this chapter support this theory that IFN- α treated mice may be unable to mobilize FAAH substrates in response to formalin and build on this, demonstrating that enhancing FAAH substrate levels in the paw can inhibit inflammation-related hyperalgesia in IFN- α -treated mice. Examining the effects of enhancing 2-AG tone on formalin-evoked nociceptive responding revealed that intraplantar administration of the MAGL inhibitor, MJN110, failed to elicit anti-nociceptive effects in saline-treated animals, despite significant increases in 2-AG levels in the paw and PFC tissue. This, to our knowledge, is the first study investigating the effects of intraplantar MJN110 on formalin-evoked nociceptive behaviour, although systemic administration of MJN110 has been shown to reduce mechanical allodynia both in a rat model of diabetic neuropathy (Niphakis et al., 2013) and following chronic constrictive injury (CCI) of the sciatic nerve in mice (Ignatowska-Jankowska et al., 2015). In comparison, intraplantar administration of an alternative MAGL inhibitor, JZL184, has been shown to alleviate nociceptive behaviour throughout the early and later stages of formalin-evoked responding in rats, although 2-AG levels in the paw were reported to be unaltered in this study (Guindon et al., 2011). The discrepancies between the current study and the latter may be due to experimental design including the species of rodent (mice vs. rat), concentration of formalin, level of MAGL inhibition and 2-AG enhancement. Furthermore, it should be noted that, intraplantar JZL184, at doses comparable to that employed in the current study, failed to modulate mechanical thresholds in naïve animals, but attenuates mechanical allodynia in a model of cisplatin-induced neuropathy (Khasabova et al., 2014). Thus, it is possible that MAGL inhibitors may be more effective in modulating nociceptive responding under conditions of hyperalgesia where alterations in endocannabinoid tone may already exist.

In support of this latter suggestion and in contrast to the lack of effect of MJN110 in saline-treated mice, local administration of MJN110 attenuated formalin-evoked nociceptive behaviour throughout the early and late phase in IFN- α -treated animals. This behavioural effect occurred in the absence of any significant changes in local or

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central levels of 2-AG in IFN- α -treated animals, although it is possible that levels of 2-AG may have been increased at a time point prior to our tissue collection. The lack of significant increase in 2-AG levels as observed in IFN- α -treated animals may also be due to the absolute levels of 2-AG in the paw of IFN- α -treated animals already at a maximum (levels of 2-AG in the paw of saline-MJN110 vs. IFN-MJN110 animals not different). The present study remains an early evaluation of the analgesic activity of the novel MAGL inhibitor, MJN110, and while the current somewhat paradoxical behavioural and post-mortem findings are difficult to interpret, this report would suggest that IFN- α -treated animals may exhibit altered peripheral nociceptive circuits and effects associated with an inability to mobilize endocannabinoids in response to an inflammatory noxious stimulus. Enhancing local endocannabinoid tone either via FAAH or MAGL inhibition is associated with analgesic effects in IFN- α -treated mice, again suggesting an important role for this system in mediating the hyperalgesic profile of these mice.

Overall, the data presented in this chapter provide compelling evidence which suggests that deficits in peripheral PEA and/or OEA, may mediate IFN- α -induced hyperalgesia. The data identify a local site of action for the anti-nociceptive effects observed following intraplantar administration of the FAAH inhibitor PF3845, specifically through increased levels of endogenous PEA and OEA in the paw tissue of IFN- α -treated animals. In addition, local administration of MJN110 reduces formalin-evoked nociceptive behaviour in IFN- α -treated animals. Thus, taken together the data presented in this chapter further support a role for altered endocannabinoid tone in mediating the hyperalgesia associated with repeated IFN- α administration.

Chapter 7

The effect of systemic administration of FAAH or MAGL inhibitors on formalin-evoked nociceptive behaviour in saline- and IFN- α -treated mice

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Several studies over the past decade have demonstrated that genetic or pharmacological deletion/inhibition of FAAH or MAGL and subsequent enhancement of their endogenous substrates, is associated with anti-allodynic and anti-nociceptive properties in several animal models of both inflammatory and neuropathic pain [for review see (Jhaveri et al., 2007, Ulugol, 2014)]. For example, preclinical studies investigating the behavioural efficacy of enhancing AEA tone following systemic administration of the FAAH inhibitor URB597 have identified anti-allodynic and analgesic activity in rodents subjected to a noxious immune challenge. For instance, systemic administration of URB597 has been shown to reverse thermal hyperalgesia (Naidu et al., 2010) and mechanical hyperalgesia (Naidu et al., 2010, Booker et al., 2012) associated with intraplantar injection of LPS, while repeated administration attenuates paw oedema in this model (Naidu et al., 2010). These anti-nociceptive effects of URB597 were evident upon administration of a drug dose previously shown to significantly enhance central and spinal levels of AEA (Kinsey et al., 2009) and were mediated via activity of the CB₁ and CB₂ receptors (Naidu et al., 2010, Booker et al., 2012). Furthermore, these behavioural effects are accompanied by an attenuation of pro-inflammatory cytokines IL-1 β and TNF- α in the LPS-treated paw (Naidu et al., 2010). With regard to formalin-evoked nociception, intraperitoneal administration of URB597 has also been shown to reverse chemical hyperalgesia in diabetic rats throughout the formalin test, in the absence of such an effect in non-hyperglycemic counterparts (Hasanein et al., 2009). Additionally, studies from our group have previously shown that systemic administration of URB597 does not alter formalin-evoked nociceptive responding, but rather enhances fear-associated suppression of nociceptive responding at a discrete time point in rats (Butler et al., 2012). Furthermore, systemic URB597 also alleviates formalin-evoked nociceptive behaviour in WKY animals, a stress-hyperresponsive genetic model of depression, without such an effect in the SD strain (Rea et al., 2014). This study also demonstrated that inhibition of FAAH directly within the RVM attenuates formalin-evoked responding in WKY rats, an effect accompanied by increased AEA levels within the RVM and prevented by local CB₁ receptor antagonism (Rea et al., 2014). This evidence highlights an important role of FAAH substrates,

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including AEA, in discrete brain regions such as the RVM in mediating and modulating the hyperalgesic phenotype observed in this stress sensitive/depressive-like rat strain.

PF3845 is a more recently developed inhibitor of FAAH with less off-target effects compared with URB597. Initial findings have demonstrated that oral administration of this FAAH inhibitor instigates a dose dependant inhibition of complete Freund's adjuvant (CFA)-induced mechanical allodynia, an effect concurrent with significant central and peripheral increases in FAAH substrate levels (Ahn et al., 2009). In addition, this behavioural effect was also shown to occur through a cannabinoid receptor dependant mechanism (Ahn et al., 2009). PF3845 has also displayed similar effects in alternative models of inflammatory pain behaviour such as the acetic acid writhing test where mice exhibit a dose-dependent anti-nociceptive response to the FAAH inhibitor (Sakin et al., 2015) while systemic administration of PF3845 also attenuates LPS-induced tactile allodynia (Booker et al., 2012) as well as carrageenan-induced mechanical allodynia (Ghosh et al., 2015). Thus, taken together, global systemic inhibition of FAAH, and consequently enhancement of AEA tone, both peripherally and centrally, is associated with anti-nociceptive effects in several models; effects which may be dependent on the underlying tone of the system.

Although considerably more research has been conducted into the effects of FAAH inhibition on nociceptive responding, with the advent of new more selective MAGL inhibitors, studies on the effects of increasing levels of 2-AG on behavioural and neurochemical changes associated with neuropathic and inflammatory pain have been growing steadily. Systemic administration of irreversible inhibitors of MAGL including JZL184, exhibit analgesic efficacy in acute models of inflammatory pain in rodents. For example, systemic JZL184 has been shown to abolish carrageenan-induced receptor field expansion of wide dynamic range (WDR) neurons (Woodhams et al., 2012) and elicit anti-edematous and anti-allodynic effects in the mouse carrageenan model of inflammatory pain (Ghosh et al., 2013). Reductions in paw oedema were shown to be

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CB₂ receptor dependent while anti-allodynic behaviour was shown to be both CB₁ and CB₂ receptor dependent (Ghosh et al., 2013). Similarly, a newly developed selective MAGL inhibitor KML29, an analogue of JZL184 (Chang et al., 2012), has been shown to attenuate carrageenan-induced paw oedema and mechanical allodynia following systemic administration, effects associated with increased whole brain levels of 2-AG (Ignatowska-Jankowska et al., 2014). Thus, enhancing central (and likely peripheral) 2-AG tone elicits anti-nociceptive effects following a noxious inflammatory stimulus. Novel MAGL inhibitors continue to be developed, with recent studies demonstrating that MJN110, a potent and selective inhibitor of MAGL, attenuates mechanical allodynia in animal models of neuropathic pain including that following CCI of the sciatic nerve (Ignatowska-Jankowska et al., 2015) and diabetic neuropathy (Niphakis et al., 2013), effects synonymous with central increases in 2-AG in levels. An early study by Bisogno and colleagues has reported inhibition of pain behaviour throughout the second phase of response in the formalin test following systemic administration of OMDM169, a potent inhibitor of 2-AG hydrolysis, and this effect was accompanied through concomitant increases in 2-AG levels in the ipsilateral formalin-treated paw (Bisogno et al., 2009). While the data presented in chapter 6 demonstrate that intraplantar administration of MJN110 attenuates formalin-evoked nociceptive behaviour in IFN- α -, but not saline-treated mice, there have been no studies to our knowledge examining the effects of systemic administration of MJN110 on formalin-evoked nociceptive responding. Furthermore, it is unknown if the effects of MAGL inhibition on inflammatory pain responding may be altered in the presence of an affective state such as observed following repeated IFN- α administration.

Data from the previous chapters indicate that formalin-evoked nociceptive behaviour in saline-treated animals is unaffected by local FAAH or MAGL inhibition, thus prompting the hypothesis that systemic administration of the drugs and subsequent increases in central substrate levels may be required for the induction of anti-nociceptive behaviour. In addition, repeated IFN- α has been shown to result in alteration in endocannabinoid tone peripherally, spinally and supraspinally in the absence and presence of nociceptive

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tone, effects which may underlie formalin-evoked hyperalgesia in these animals (chapter 5). Local enhancement of these *N*-acylethanolamines and 2-AG in the paw was found to attenuate inflammatory pain behaviour in these animals (chapter 6), highlighting a role for locally produced endocannabinoids in mediating the IFN- α induced hyperalgesia. However, a role may also exist for FAAH and/or MAGL substrates at spinal or supraspinal sites in mediating or modulating the hyperalgesia observed in these animals.

Thus, it was hypothesised that systemic inhibition of FAAH or MAGL and the consequential enhancement of central AEA and 2-AG tone, attenuates formalin-evoked nociceptive behaviour in mice repeatedly administered IFN- α or saline.

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7.1.1 Aims

1. Investigate the effect of systemic administration of the FAAH or MAGL inhibitors PF3845 and MJN110, respectively, on formalin-evoked nociceptive behaviour in saline-treated animals.
2. Evaluate if formalin-evoked nociceptive behaviour in mice repeatedly administered IFN- α is altered by systemic administration of the FAAH and MAGL inhibitors PF3845 and MJN110 respectively.

7.2 Materials and Methods

7.2.1 Animals

Male C57BL/6 mice (weight 25-30g; Charles River Laboratories, UK) were used in this experiment. Animals were all housed in groups of four in plastic-bottomed cages containing wood shavings as bedding. One day prior to initiation of experimental procedures, animals were individually housed and were kept as so or the remainder of the study. Animals were maintained in a constant temperature (21 +/- 2°C) under standard lighting conditions (12 : 12 hr light-dark, lights on from 07.00 to 19.00 hr). All experiments were carried out during the light phase, between 08.00 and 18.00 hr. Mice were given free access to food and water.

The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 2010/63/EU.

7.2.2 Pharmacological Treatment

Human interferon-alpha (rhIFN- α -2a; 3M IU/0.5ml), Roferon-A®, was purchased from Roche Pharmaceuticals. The drug was prepared in sterile saline from stock on a daily basis. This drug/vehicle solution was administered subcutaneously in a volume of 3 μ l/g to give a final concentration of 8,000IU/g IFN- α . Dosing occurred daily, between the hours of 10.00 and 12.00. The dose chosen was based on previous studies demonstrating depressive-like and hyperalgesic behaviour following repeated administration of IFN- α in mice (chapter 4).

The FAAH inhibitor, PF3845 (from the NIMH drug synthesis scheme), and MAGL inhibitor, MJN110 (gifted from Benjamin Cravatt, U.S.), were prepared in ethanol: saline: cremophor; 1:1:18 on a daily basis. This drug/vehicle solution was administered through the intraperitoneal route in a volume of 10 μ l g⁻¹ to give a final concentration of 10mg kg⁻¹ of PF3845 and 5mg kg⁻¹ of MJN110. Dosing occurred two hours prior to the

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formalin test. The PF3845 dose and time of administration was chosen based on previous studies demonstrating increased brain levels of AEA and anti-allodynic efficacy on the LPS-induced mechanical allodynia in mice (Booker et al., 2012). Similarly, the chosen dose and time of administration of MJN110 previously alleviated mechanical allodynia in a rat model of diabetic neuropathy and was shown to increase 2-AG levels in the brain (Niphakis et al., 2013).

7.2.3 Experimental Design

Following acclimatization to the animal unit (5 days), mice were singly housed and 24hr later were administered saline vehicle for 5 days to acclimatize to handling and injection procedure. IFN- α or saline administration began the following day for 8 days. 22 hours following the final dose, animals received an i.p. injection of PF3845, MJN110 or vehicle and were returned to their homecage. One hour and fifty minutes later, animals were placed in the Perspex arena for a 10-minute habituation period, after which subjects received an intraplantar injection of formalin and nociceptive behaviour was evaluated for one hour thereafter. At the end of behavioural testing, animals were sacrificed by decapitation following deep isoflurane anesthesia, the brain was removed and PFC dissected out, snap frozen on dry ice and stored at -80°C for subsequent analysis of endocannabinoid and *N*-acylethanolamine levels using LC-MS-MS (see chapter 2 for methodology).

7.2.7 Statistical Analysis

Data were analysed using SPSS statistical package, employing two-way ANOVA or two-way repeated measures ANOVA followed by Fisher's LSD *post hoc* analysis where appropriate. Data were considered significant when $P < 0.05$. Results are expressed as group means \pm standard error of mean (SEM).

7.3 Results

7.3.1 Systemic administration of MJN110, but not PF3845, increases locomotor activity of saline- and IFN- α -treated mice

Locomotor activity was assessed in the formalin arena for a period of ten minutes. This was carried out 1hr 50 min following systemic administration of PF3845 or MJN110 and directly prior to intraplantar administration of formalin.

Two-way ANOVA revealed a significant effect of systemic drug treatment [F (2, 53) = 52.318, P=0.000], but not IFN- α [F (1, 53) = 0.596, P=0.444] or treatment x IFN- α interaction [F (2, 53) = 0.105, P=0.901], on locomotor activity in the 10-minute exposure to the arena. Fisher's LSD *post hoc* analysis revealed that MJN110 significantly increased distance moved of both saline- and IFN- α -treated animals when compared to vehicle treated counterparts (figure 7.3.1).

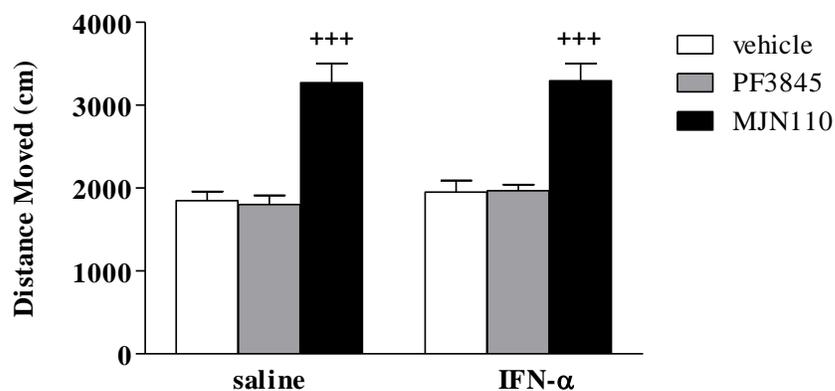


Figure 7.3.1. The effect of systemic administration of PF-3845, MJN-110 or vehicle on locomotor activity of saline- or IFN- α -treated mice. Data are expressed as mean \pm SEM; n= 9-10 per group; ⁺⁺⁺P<0.001, MJN110 vs. vehicle-treated counterparts.

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7.3.2. Systemic administration of MJN110, but not PF3845, attenuates formalin-evoked nociceptive behaviour of saline- and IFN- α -treated mice

Animals were administered PF3845, MJN110 or vehicle two hours prior to formalin administration and subsequent nociceptive responding was assessed over the following 60 minutes.

Two-way repeated measures ANOVA revealed a significant effect of time [F (11, 550) = 95.38, P=0.000] and drug treatment [F (2, 50) = 19.58, P=0.000], but not IFN- α [F (1, 50) = 0.000, P=0.985] on elevation of the affected paw (pain '1' behaviour) in the formalin test. Fisher's LSD *post hoc* analysis revealed that MJN110 increased paw elevation in saline- and IFN- α -treated animals at discrete time points throughout the test, namely the initial 30 to 35 minutes. Fisher's LSD *post hoc* analysis also revealed that PF3845 reduced paw elevation in both groups during the later stage of formalin-evoked response (45-50 min), as evident in figure 7.3.2.1.A, B.

Upon further temporal analysis, two-way ANOVA revealed a significant effect of drug treatment [F (2, 50) = 33.307, P=0.000], but not IFN- α [F (1, 50) = 0.002, P=0.966] or drug treatment x IFN- α interaction [F (2, 50) = 1.130, P=0.331] on paw elevation within the early phase (0-5 min) of the formalin test. Fisher's LSD *post hoc* analysis revealed that MJN110 significantly increased this behaviour in saline- and IFN- α -treated animals in comparison with vehicle-treated counterparts (figure 7.3.2.1.C). Two-way ANOVA revealed a significant effect of drug treatment [F (2, 50) = 28.677, P=0.000], but not IFN- α [F (1, 50) = 0.130, P=0.720] or drug x IFN- α interaction [F (2, 50) = 2.287, P=0.112] on paw elevation during the second phase (20-30 min) of formalin-evoked response. Fisher's LSD *post hoc* analysis revealed that MJN110 significantly increased this behaviour in saline- and IFN- α -treated animals in comparison with vehicle-treated counterparts (figure 7.3.2.1.D).

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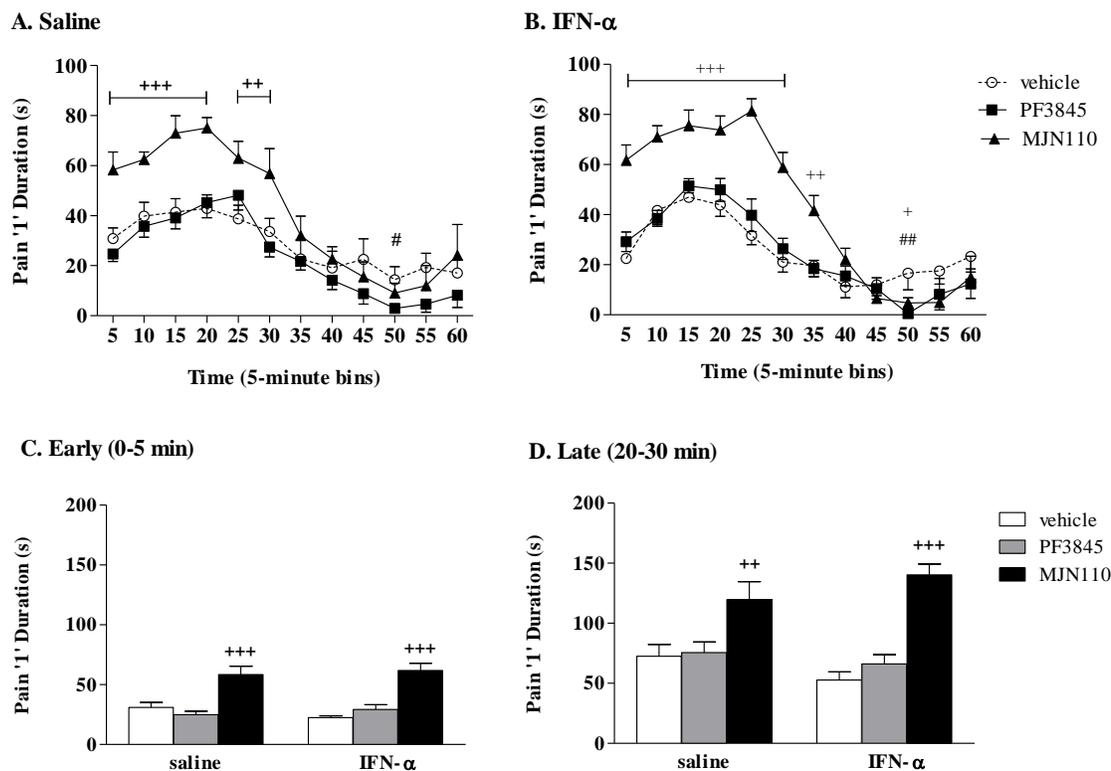


Figure 7.3.2.1. Effect of systemic PF3845 or MJN110 on pain '1' nociceptive behaviour in the formalin test: A, Profile of pain '1' behaviour over 60 minutes in saline-treated animals; B, Profile of pain '1' behaviour over 60 minutes in IFN- α -treated animals; C, Duration of pain '1' behaviour in the early (0-5min) phase of the formalin test; D, Duration of pain '1' behaviour in the late (20-30min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group. +++P<0.001, ++P<0.01, +P<0.05, MJN110 vs. vehicle-treated counterparts; ###P<0.01, #P<0.05, PF3845 vs. vehicle-treated counterparts.

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Two-way repeated measures ANOVA revealed a significant effect of time [F (11, 550) = 53.27, P=0.000], drug treatment [F (2, 50) = 11.92, P=0.000] and time x drug treatment interaction [F (22, 550) = 4.44, P=0.000], but not IFN- α [F (1, 50) = 1.50, P=0.226], on pain '2' behaviour i.e. direct shaking, licking, biting or flinching of the affected paw in the formalin test. Fisher's LSD *post hoc* analysis revealed that in both saline- and IFN- α -treated animals, MJN110 significantly reduced pain '2' behaviour at discrete time points throughout the test, namely 0-5 min, 15-20 min, 20-25 min and 25-30 min time bins, as depicted in figure 7.3.2.2.A, B.

Upon further temporal analysis, two-way ANOVA revealed a significant effect of drug treatment [F (2, 50) = 22.376, P=0.000], but not IFN- α [F (1, 50) = 0.860, P=0.358] or interaction thereof [F (2, 50) = 2.469, P=0.095] on pain '2' behaviour throughout the early phase (0-5min) of the formalin test. Fisher's LSD *post hoc* analysis revealed that MJN110 significantly attenuated pain '2' behaviour in saline- and IFN- α -treated animals when compared with vehicle-treated counterparts (figure 7.3.2.2.C). Two-way ANOVA also revealed a significant effect of drug treatment [F (2, 50) = 16.707, P=0.000], but not IFN- α treatment [F (1, 50) = 0.269, P=0.606] or interaction [F (2, 50) = 0.111, P=0.895] on pain '2' behaviour throughout the later phase of formalin-evoked response. Fisher's LSD *post hoc* analysis revealed that MJN110 reduced pain '2' behaviour during the second phase (20 -30 min) of formalin-evoked response in saline- and IFN- α -treated animals (see figure 7.3.2.2.D)

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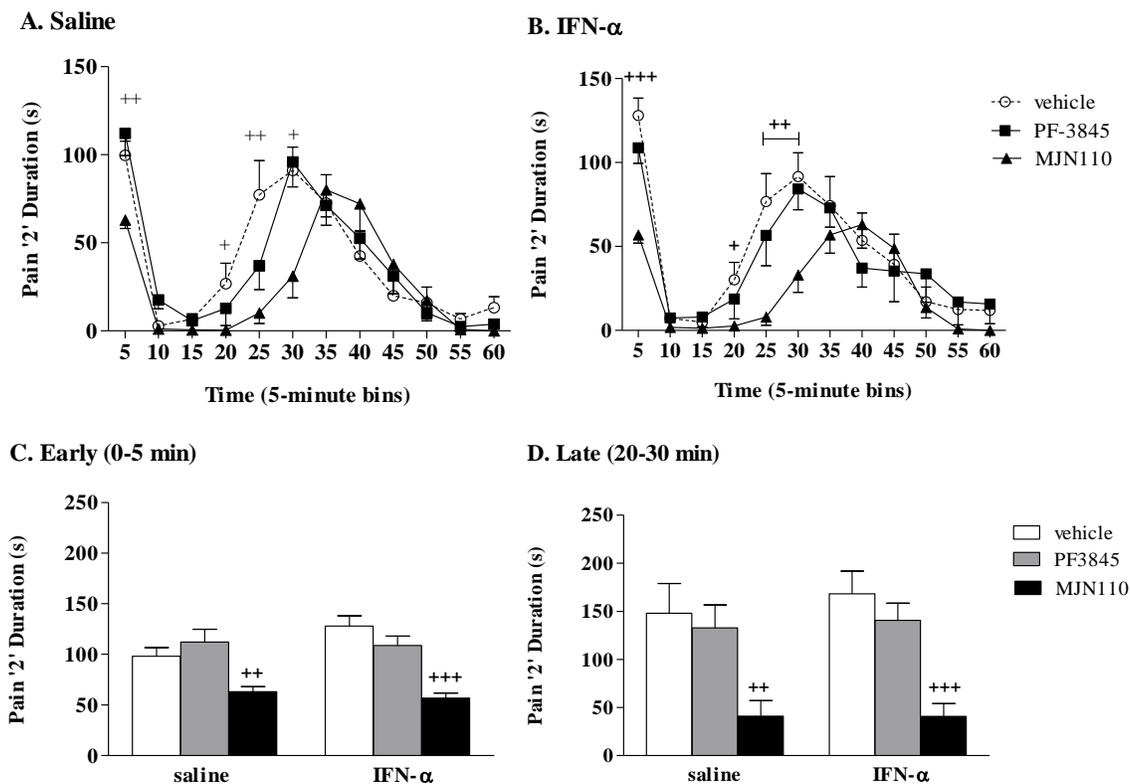


Figure 7.3.2.2. Effect of systemic PF3845 or MJN110 on pain '2' nociceptive behaviour in the formalin test: A, Profile of pain '2' behaviour over 60 minutes in saline-treated animals; B, Profile of pain '2' behaviour over 60 minutes in IFN- α -treated animals; C, Duration of pain '2' behaviour in the early (0-5min) phase of the formalin test; D, Duration of pain '2' behaviour in the late (20-30min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group. *P<0.05 vs. saline; +++P<0.001, ++P<0.01, +P<0.05, MJN110 vs. vehicle-treated counterparts; ###P<0.001, PF3845 vs. vehicle-treated counterparts.

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Two-way repeated measures ANOVA revealed a significant effect of time [F (11, 550) = 60.091, P=0.000] and time x drug interaction [F (22, 550) = 3.11, P=0.000], but not drug treatment [F (2, 50) = 2.35, P=0.106] or IFN- α [F (1, 50) = 1.35, P=0.250], on CPS in the formalin test. Fisher's LSD *post hoc* analysis revealed that MJN110 reduced CPS in saline- and IFN- α -treated animals at discrete time points throughout the test. More specifically, MJN110 reduced formalin-evoked nociceptive behaviour during the early (0-5 min) and late (20-30 min) phase of response in IFN- α -treated animals and reduced the late phase (20-25 min) response in saline-treated animals. In addition, Fisher's LSD *post hoc* analysis also revealed that PF3845 slightly increased CPS during the interphase (5-10 min) in saline-treated animals while MJN110 induced the same (5-15 min) in both saline- and IFN- α -treated [figure 7.3.2.3.A, B].

Following further temporal analysis, two-way ANOVA revealed a significant effect of drug treatment [F (2, 50) = 10.588, P=0.000], but not IFN- α [F (1, 50) = 0.860, P=0.358] or interaction [F (2, 50) = 1.669, P=0.199] on CPS during the early phase of formalin-evoked response. Fisher's LSD *post hoc* analysis revealed that MJN110 significantly reduced formalin-evoked nociceptive behaviour in IFN- α -, but not saline-treated animals when compared to vehicle-treated counterparts. Two-way ANOVA also revealed a significant effect of drug treatment [F (2, 50) = 9.952, P=0.000], but not IFN- α [F (1, 50) = 0.226, P=0.637] or interaction thereof [F (2, 50) = 0.018, P=0.982] on CPS during the late phase (20-30 min) of formalin-evoked response. Fisher's LSD *post hoc* analysis revealed that MJN110 significantly reduced formalin-evoked nociceptive behaviour during the second phase in saline- and IFN- α -treated animals when compared with vehicle-treated counterparts (figure 7.3.2.3.D).

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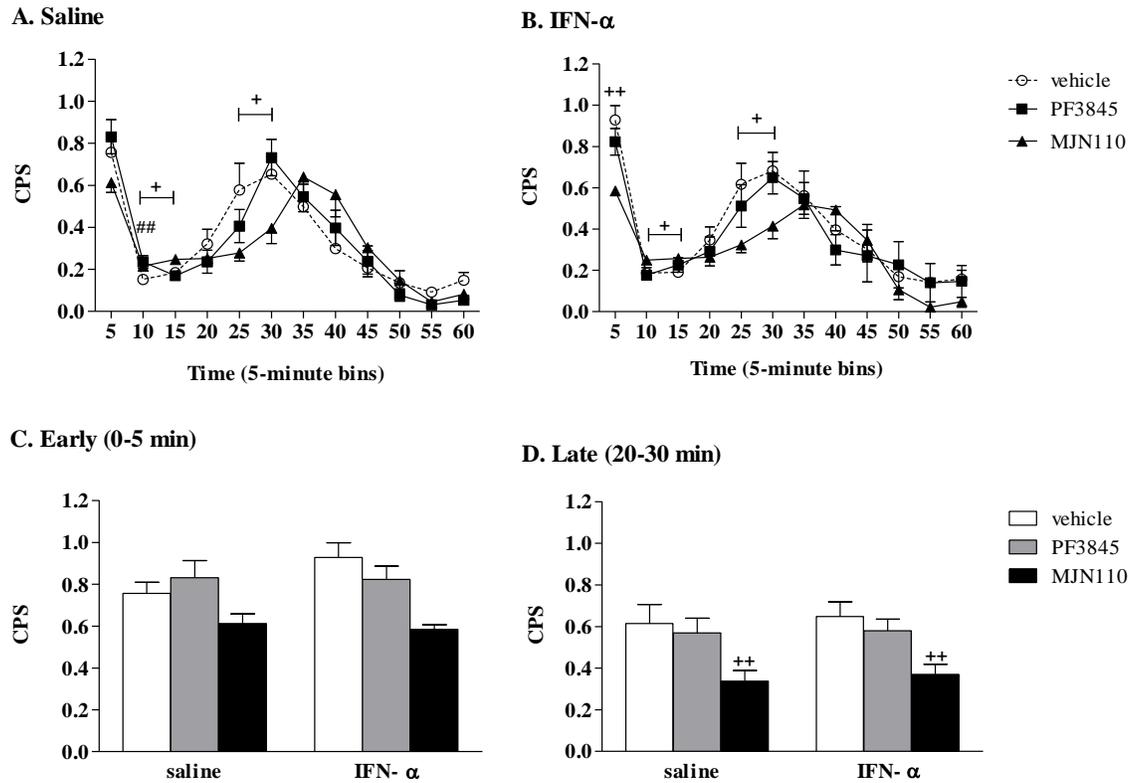


Figure 7.3.2.3. Effect of systemic PF3845 or MJN110 on CPS in the formalin test: A, Profile of composite pain score (CPS) over 60 minutes in saline-treated animals; B, Profile of composite pain score (CPS) over 60 minutes in IFN- α -treated animals; C, composite pain score (CPS) in the early (0-5min) phase of the formalin test; D, composite pain score (CPS) in the late (20-30 min) phase of the formalin test. Data are expressed as mean \pm SEM; n= 9-10 per group. $##P<0.01$, PF3845 vs. vehicle-treated counterparts, $+++P<0.001$, $++P<0.01$, $+P<0.05$, MJN110 vs. vehicle-treated counterparts.

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7.3.3 Systemic administration of the PF3845 or MJN110 does not attenuate formalin-evoked paw oedema

Changes in diameter of the left hind paw were evaluated following completion of the formalin test and compared with pre-formalin paw diameter.

Two-way ANOVA revealed no significant effect of IFN- α [$F(1, 50) = 3.843, P=0.056$], drug treatment [$F(2, 50) = 0.735, P=0.485$] or IFN- α x drug treatment interaction [$F(2, 50) = 0.953, P=0.392$] on paw oedema following the formalin test.

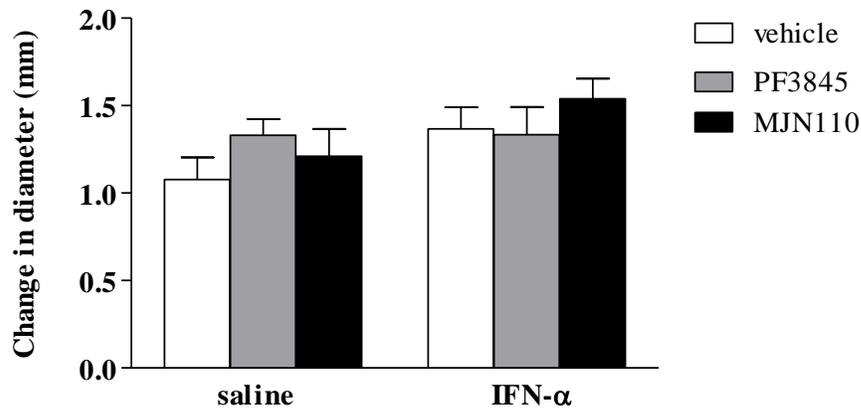


Figure 7.3.3. Effect of systemic PF3845 or MJN110 on formalin-induced increase in paw diameter in saline- or IFN- α -treated mice. Data are expressed as mean \pm SEM; n= 9-10 per group.

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7.3.4. Systemic administration of PF3845 or MJN110 increase AEA and 2-AG levels in the prefrontal cortex of saline- and IFN- α -treated mice following formalin administration

Immediately following the formalin test, animals were sacrificed and the contralateral PFC was dissected from the brain and removed for assay of endocannabinoid and *N*-acylethanolamine levels using mass spectrometry.

Two-way ANOVA revealed an effect of drug treatment [$F(2, 49) = 117.328, P=0.000$] but not IFN- α [$F(1, 49) = 0.061, P=0.805$] or drug x IFN- α interaction [$F(2, 49) = 0.026, P=0.974$] on 2-AG levels in the PFC of mice following the formalin test. *Post hoc* analysis revealed that systemic administration of MJN110 significantly increased PFC tissue levels of 2-AG in both saline- and IFN- α -treated animals, when compared with vehicle-treated counterparts as evident in figure 7.3.4.A.

Two-way ANOVA revealed an effect of drug treatment [$F(2, 49) = 52.071, P=0.000$] but not IFN- α [$F(1, 49) = 1.272, P=0.265$] or drug x IFN- α interaction [$F(2, 49) = 0.549, P=0.581$] on AEA levels in the PFC of mice following the formalin test. *Post hoc* analysis revealed that systemic administration of PF3845 significantly increased PFC tissue levels of AEA in both saline- and IFN- α -treated animals, when compared with vehicle-treated counterparts, as illustrated in figure 7.3.4.A.

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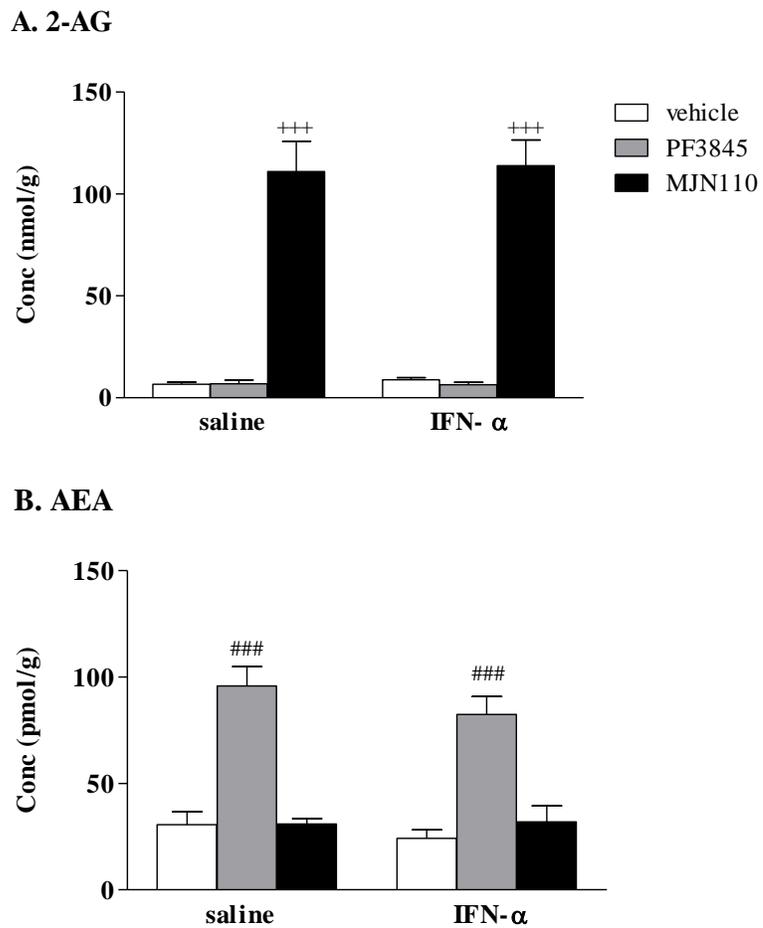


Figure 7.3.4. The effect of PF3845 and MJN110 on levels of (A) 2-AG and (B) AEA levels in the PFC of formalin-treated mice previously exposed to repeated saline or IFN- α treatment. Data are expressed as mean \pm SEM; n= 9-10 per group. ⁺⁺⁺P<0.001, MJN110 vs. vehicle-treated counterparts, ^{###}P<0.001, PF3845 vs. vehicle-treated counterparts.

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7.3.5 Systemic administration of PF3845, but not MJN110, increases *N*-acylethanolamine levels in the PFC of saline- and IFN- α -treated animals following formalin administration

Two-way ANOVA revealed an effect of drug treatment [$F(2, 49) = 467.657, P=0.000$] but not IFN- α [$F(1, 49) = 0.099, P=0.755$] or drug x IFN- α interaction [$F(2, 49) = 0.166, P=0.847$] on PEA levels in the PFC of mice following the formalin test. Fisher's LSD *post hoc* analysis revealed that PF3845 increased brain tissue levels of PEA in both saline- and IFN- α -treated animals in comparison with vehicle-treated counterparts.

Two-way ANOVA revealed an effect of drug treatment [$F(2, 49) = 413.421, P=0.000$] but not IFN- α [$F(1, 49) = 0.226, P=0.637$] or drug x IFN- α interaction [$F(2, 49) = 0.303, P=0.740$] on OEA levels in the PFC of mice following the formalin test. Fisher's LSD *post hoc* analysis revealed that PF3845 increased brain tissue levels of OEA in both saline- and IFN- α -treated animals in comparison with vehicle-treated counterparts (see figure 7.3.5).

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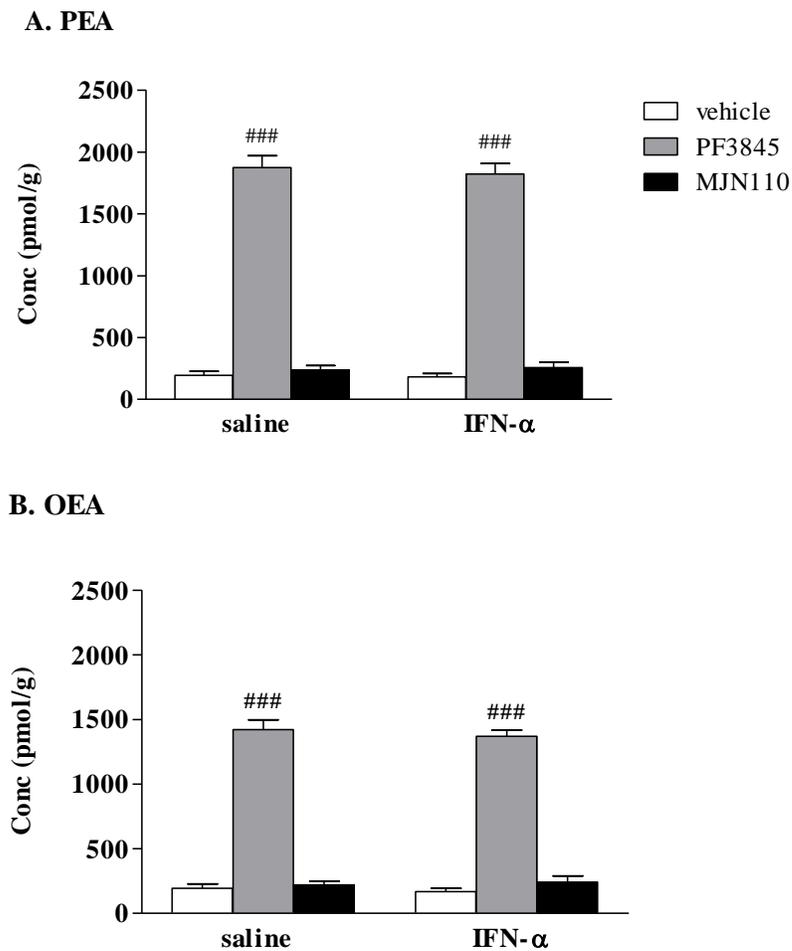


Figure 7.3.5. The effect of PF3845 and MJN110 on levels of A, PEA and B, OEA levels in the PFC of formalin-treated mice previously exposed to repeated saline or IFN- α treatment. Data are expressed as mean \pm SEM; n= 9-10 per group. ###P<0.001, PF3845 vs. vehicle-treated counterparts.

7.4 Discussion

The present study aimed to examine the effects of enhancing AEA or 2-AG tone both peripherally and centrally, on formalin-evoked nociceptive responding in saline- and IFN- α -treated mice. The data revealed that formalin-evoked nociceptive behaviour but not paw oedema, of both saline- and IFN- α -treated animals, was reduced following systemic administration of the MAGL inhibitor, MJN110, an effect associated with a significant increase in 2-AG in the PFC. However, this treatment regime was also associated with an increase in locomotor activity in all animals. Furthermore, although FAAH inhibition following PF3845 enhanced cortical levels of AEA, PEA and OEA, this failed to alter formalin-evoked nociceptive behaviour in saline- or IFN- α -treated mice. Taken together, the current data demonstrate that increasing levels of endogenous 2-AG, but not FAAH substrates, attenuates inflammatory pain behaviour, an effect not altered in IFN- α -treated animals.

Previous studies in this thesis have demonstrated that repeated IFN- α administration was associated with a hyperalgesic response to formalin, particularly in the second phase of the test (chapter 4-6). However, it should be noted that in the current study, although a trend towards IFN- α -induced hyperalgesia was identified throughout the early (0-5 min) phase of the formalin test, no hyperalgesia was observed in the second phase of the trial when compared to saline-treated counterparts (saline-vehicle vs. IFN- α -vehicle). Similarly, IFN- α -induced hyperalgesia was observed in the early phase of the formalin trial in chapter 4, although not in subsequent studies (chapter 5 and 6). The lack of effect throughout the later stage of formalin-evoked responding may be due to the higher CPS recorded for saline-treated animals in the current study (20-30 min, average 0.61) compared with earlier studies (average 0.23), rather than a reduction in the hyperalgesic response observed in IFN- α treated mice. This increase in nociceptive behaviour as observed among saline-treated subjects may be a consequence of changes in experimental procedures such as the prior systemic injection in the current study. Due to the lack of hyperalgesic effect in IFN- α treated mice in the current study, it is difficult to

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determine if the effects of enhancing AEA or 2-AG tone would modulate IFN- α -induced hyperalgesia per se, despite the observed anti-nociceptive effect in both saline- and IFN- α -treated animals.

Systemic administration of the FAAH inhibitor, PF3845, exhibited no significant effect on formalin-evoked nociceptive responding in saline- or IFN- α -treated animals in the formalin test, although pain behaviour was slightly increased between initial and later phases of formalin-evoked response, an effect possibly indicative of a transient tolerant state following initial enhancement of endocannabinoid availability and/or momentary depletion of the protective lipids. This lack of effect on nociceptive responding was evident despite significant increases in FAAH substrate levels, AEA, PEA and OEA, in the PFC. Although the effect of PF3845 has not been previously evaluated in the formalin test of inflammatory pain, intraperitoneal administration of an alternative inhibitor URB597, also does not alter formalin-evoked nociceptive behaviour (Butler et al., 2012, Hasanein et al., 2009) but has been shown to reverse nociceptive behaviour of WKY rats (Rea et al., 2014) as well as diabetic hyperalgesia as observed in the formalin test (Hasanein et al., 2009). This highlights that under conditions of inflammation and/or negative affect, FAAH inhibition may modulate nociceptive responding to an inflammatory stimulus. However, the current study demonstrated that systemic administration of the FAAH inhibitor in IFN- α -treated mice does not alter nociceptive responding in the formalin test, indicating that, under the conditions of the present study central enhancement of FAAH substrate levels is unable to alter formalin-evoked responding in saline or animals previously exposed to repeated IFN- α treatment. Taking this together with the data previously presented (chapter 6), whereby local inhibition of FAAH in the paw reduced formalin-evoked nociceptive responding in IFN- α -treated animals, it would appear that PF3845-mediated effects on nociceptive responding in IFN- α -treated animals may not be due to enhancement of central FAAH substrate levels but rather confined to the peripheral modulation.

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The current study also evaluated the effect of global enhancement of 2-AG tone on nociceptive responding and demonstrated that systemic administration of MJN110 attenuated late phase (20-30 min) formalin-evoked nociceptive responding, but not paw oedema, in saline-treated animals. Although a trend towards a reduction in CPS in MJN110-treated mice was observed in the first five minutes, this failed to reach significance. The potent anti-nociceptive effects of MJN110 on formalin-evoked responding in saline- (and IFN- α -) treated animals, concurrent with increased 2-AG levels in the PFC of all thus treated, suggests a pivotal role for central 2-AG enhancement in promoting MJN110-associated anti-nociception. This is further exemplified by previous findings (chapter 6) whereby local administration and confinement of drug activity failed to elicit any such effect on overall formalin-evoked nociception. In the current study, similar effects to that in saline-treated were also observed in IFN- α -treated mice, although the attenuation in the first phase did reach significance in this group. The anti-nociceptive effect of MJN110 throughout the early phase of formalin-evoked response in IFN- α -treated animals may indicate alterations in activity of nociceptors or local endocannabinoid modulation of nociceptive responding as a result of repeated exposure to IFN- α , such that enhancing 2-AG tone following MAGL inhibition with MJN110 exerts an early phase analgesic-like activity in these animals. Interestingly, MJN110-treated animals (saline and IFN- α) exhibited a significant increase in locomotor activity when compared with that of vehicle-treated counterparts. This locomotor stimulatory property of MJN110 would appear at odds with the reported locomotor depressant effects of cananbinoids however this effect has been previously shown in a mouse model of CCI-induced neuropathy (Ignatowska-Jankowska et al., 2015). Thus, while MJN110-induced increases in 2-AG elicit potent effects on formalin-evoked (current study) and neuropathic pain (Ignatowska-Jankowska et al., 2015), alterations in locomotor activity may represent a possible confounding factor for the anti-nociceptive behaviour observed. To our knowledge, the current study is the first to evaluate systemic MAGL inhibition on pain behaviour associated with intraplantar formalin administration, although an alternative MAGL inhibitor, JZL184, has been shown to elicit anti-nocicpetive effects in other paradigms of inflammatory

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pain including intraplantar carrageenan administration (Woodhams et al., 2012, Ghosh et al., 2013) .

Collectively, the data indicate that global inhibition of MAGL, but not FAAH, attenuates formalin-evoked nociception in both saline- and IFN- α -treated animals. While a role for systemic FAAH or MAGL inhibition in attenuating IFN- α -induced hyperalgesia could not be concluded from the current study, the data demonstrate that inflammatory pain in both the presence and absence of a chronic inflammatory and depressive-like state can be attenuated by overall enhancement of 2-AG tone. Although further studies are required, these data would suggest that MAGL inhibition and enhancing 2-AG tone may be an effective analgesic strategy both in the presence and absence of an altered affective state.

Chapter 8
General Discussion

Interferon- α (IFN- α) is a pro-inflammatory cytokine used for the treatment of hepatitis B and C through direct induction of anti-viral responding (Wang et al., 2002). IFN- α is also efficacious against various cancers through its associated induction of apoptosis (Asmana Ningrum, 2014) and alterations in cell cycle and differentiation in susceptible malignant cells (Sangfelt et al., 2000, Caraglia et al., 2013). However, IFN- α therapy is also associated with neuropsychiatric side effects, including depression and anxiety (Schaefer et al., 2002, Raison et al., 2005) as well as the development of pain (Shakoor et al., 2010, Nogueira et al., 2012, Capuron et al., 2002). Furthermore, recent studies have indicated that painful symptoms reported following IFN- α treatment present more frequently in patients also experiencing co-morbid depression (Shakoor et al., 2010). In order to improve our understanding of repeated cytokine administration and subsequent immune system activation and associated modulation of emotional processes, several animal models of inflammation-induced depression have been established. However, preclinical studies employing IFN- α as the primary inducer of the inflammatory response has yielded varying results (as illustrated in table 3.1). Furthermore, although acute administration of IFN- α has been shown to exhibit analgesic-like effects in rodents (Blalock and Smith, 1981, Lee et al., 2010, Jiang et al., 2000), to our knowledge no study has evaluated the effect of repeated IFN- α administration on nociceptive responding. Therefore, a key aim of this thesis was to devise a protocol that would mimic the clinical situation and provide a robust and reproducible model of IFN- α -induced depression and hyperalgesia. Initial studies employing a published protocol for IFN- α -induced depression (Siddegowda et al., 2011b) revealed that under our experimental conditions repeated administration of IFN- α exhibited no effect on depressive-like behaviour in two different mouse strains, and only slight thermal hyperalgesia in one mouse strain (C57Bl/6J) (chapter 3). However, repeated administration of a higher concentration of IFN- α resulted in depressive-like behaviour as evident in two behavioural paradigms, effects accompanied by concurrent inflammatory hyperalgesia. Establishment of this model of IFN- α -induced depression and pain mimics the clinical scenario and allows for the evaluation of possible neurobiological substrates that may underpin the changes in emotional and nociceptive responding. While inflammation-induced depression is frequently accompanied by

peripheral and central alterations in immune signalling, the data presented in this thesis demonstrate that at the time of behavioural testing, IFN- α -treated animals have minimal alterations in inflammatory mediators either in the periphery (spleen and plasma) or brain. It is likely that the inflammatory response occurred earlier than that examined in the current study (24 hours after IFN- α administration) and inflammation-induced changes in neurotransmitter and/or neuroendocrine systems may underlie the behavioural changes observed.

This thesis then proposed to examine if repeated IFN- α administration resulted in alterations in the endocannabinoid system, which is known to play a key role in emotional and nociceptive processing. The data demonstrated that IFN- α induced changes in this system, and in particular the levels of the endocannabinoids, 2-AG and AEA, and related N-acyl ethanolamines, OEA and PEA, in peripheral, spinal and supraspinal areas following a formalin administration (chapter 5). In particular the data indicated that while formalin evoked an increase in AEA and 2-AG levels in the paw tissue of saline-treated animals, this failed to be observed in IFN- α -treated counterparts. Inhibition of FAAH or MAGL at the level of the paw attenuated IFN- α -induced hyperalgesia, indicating a role for peripheral FAAH and MAGL substrates such as AEA and 2-AG in mediating the hyperalgesia in these animals. Furthermore, systemic MAGL, but not FAAH, inhibition resulted in an attenuation of formalin-evoked nociceptive behaviour in both saline- and IFN- α -treated animals, indicating that inflammatory pain in both the presence and absence of a chronic inflammatory and depressive-like state can be attenuated by enhancing 2-AG tone. Taken together, these findings demonstrate for the first time that it is possible to establish a preclinical mouse model of IFN- α -induced depression-pain and highlight a role for the endocannabinoid system in mediating and modulating IFN- α -related hyperalgesia.

8.1 Repeated IFN- α administration induces depressive-like behaviour concurrent with inflammatory hyperalgesia in mice

The data presented in chapter 3 and 4 collectively demonstrate an emergence of depressive-like and hyperalgesic behaviour in mice following repeated treatment with IFN- α . While initial reports (chapter 3) displayed a tendency towards depressive-like behaviour and significant thermal hyperalgesia following repeated administration of IFN- α in C57Bl6J mice, the employment of a higher dose of IFN- α produced a more robust phenotype of depressive behaviour as observed in the sucrose preference and forced swim tests, and concurrent hyperalgesia in the formalin test of inflammatory pain. While previous studies have similarly identified comparable depressive-like effects following chronic administration of IFN- α in mice (Siddegowda et al., 2011b, Ping et al., 2012, Fahey et al., 2007a), this is the first reporting of IFN- α -induced hyperalgesia in a preclinical study. Furthermore, this study is the first to demonstrate that both depressive-like behaviour and hyperalgesia occur after the same duration of treatment with IFN- α . The current findings add to the body of work previously indicating increases in pain sensitivity in the presence of altered affect in genetic (Burke et al., 2010, Gibney et al., 2010, Gosselin et al., 2010, O'Malley et al., 2010, Rea et al., 2014), pharmacological (Liu et al., 2014b, Nagakura et al., 2009), lesion (Burke et al., 2010, Burke et al., 2013a) and stress-induced (Bardin et al., 2009, Shi et al., 2010, Bravo et al., 2012, Imbe et al., 2012, Bravo et al., 2014) models of depression. LPS is an immunomodulatory stimulus known to induce depressive behaviours (O'Connor et al., 2009, Salazar et al., 2012), and while one report has identified alterations in thermal nociceptive thresholds in the hot plate test (Pitychoutis et al., 2009), further studies are required in order to determine if inflammation-induced depression also results in alterations in nociceptive responding to other stimuli such as mechanical and cold hypersensitivity as well as inflammatory, neuropathic and visceral pain responding. Importantly, the work presented in this thesis, to our knowledge, is one of the first to portray co-occurring hyperalgesic behaviour in a model of inflammation-induced depression (chapter 3 and 4). As highlighted throughout this thesis, clinical IFN- α therapy itself induces depression which can appear in up to 60% of patients (Raison et al., 2005, Capuron et al., 2002) as well as recent reports of somatic symptoms such as

body pain, myalgias, headache, joint pain and abdominal pain (Shakoor et al., 2010, Nogueira et al., 2012, Capuron et al., 2002). Thus, our behavioural findings clearly mimic the depression and pain behaviour seen in the clinic and thus provide a valid means for investigation of the neurobiology underpinning the emergence of these co-morbidities and, more generally, the mechanism underlying the occurrence of inflammation-induced depression and associated development of painful symptomatology.

8.2 IFN- α -induces a mild peripheral immune response at a time when co-occurring depressive-like behaviour and hyperalgesia is observed

Numerous reports from the clinic have identified increased levels of pro-inflammatory cytokines, namely IL-1 β , IL-6 and TNF- α in the plasma and CSF of depressed patients (Lichtblau et al., 2013, Young et al., 2014), while such alterations have similarly been shown in the plasma of chronic pain patients (Koch et al., 2007, Ludwig et al., 2008, Kadetoff et al., 2012). Similarly, many preclinical models of co-occurring depression and pain sensitivity frequently report central increases in levels of pro-inflammatory cytokines. For example, allodynic behaviour following SNL in the OB rat model of depression positively correlates with pro-inflammatory IL-6 mRNA expression in the amygdala (Burke et al., 2013b), while expression of pro-inflammatory chemokines are also increased in the PFC, when compared to either OB or SNL alone (Burke et al., 2014c, Burke et al., 2014a). Additionally, the WKY rat, exhibits exacerbated allodynia following the induction of peripheral nerve injury, an effect correlated with increased mRNA levels of IL-1 β in the brainstem and PFC (Apkarian et al., 2006), while the early life stress model of depression has been shown to exhibit heightened mechanical and cold allodynic sensitivity following peripheral nerve injury an effect concurrent with increased expressional levels of IL-6 and TNF- α in the hippocampus (Burke et al., 2013c). However, in inflammatory models of depression, inflammatory mediators drive the changes in emotional processing via modulation of neurotransmitter levels and neuroendocrine functioning to name but a few (see chapter 1 for full overview). Accordingly, immune activation with the bacterial or viral antigens LPS or poly I:C induces an increase in the production of inflammatory mediators and acute sickness

behaviour lasting up to 6-8 hrs and the emergence of depressive-like behaviour 24 hours post administration with no change in pro-inflammatory mediator levels observed at this time (Gibney et al., 2013, O'Connor et al., 2009). Similarly, the current study is the first to demonstrate that 24 hours following the last IFN- α administration on day 8, a depressive-like and hyperalgesic phenotype is observed with only a small but significant increase in CD11b expression, likely indicating leukocyte invasion and activation in the spleen. Similarly, pro-inflammatory cytokines (IL-6 and TNF- α) were unaltered in peripheral and central compartments following 14 days of IFN- α treatment, as assessed directly after exposure to the FST which was carried out 15 minutes post final IFN- α treatment in rats (De La Garza et al., 2005). However, it is also possible that changes may occur in brain regions other than those examined in the current study, such as the hippocampus, where a increases IL-1 β and IL-6 expression have recently been reported following 5 weeks of treatment with mIFN- α (Zheng et al., 2015), or be revealed by using other techniques for evaluating protein expression and localisation such as immunohistochemistry. Taken together, it would appear that the immune response to IFN- α administration in the mouse is transient and subsequent behavioural effects observed are likely a consequence of such immune response on neurotransmission, neurogenesis and neuroendocrine activity. A full evaluation of all of the possible changes in these systems was beyond the scope of this thesis and as such it was decided to focus on possible changes in the endocannabinoid system in IFN- α -treated mice and if such changes may underlie at least some of the behavioural changes observed in this model.

8.3 The role of the endocannabinoid system in IFN- α -induced hyperalgesia

The endocannabinoid system is a neurotransmitter system known to play an important modulatory role in emotional and nociceptive processing. Recent evidence has identified changes within this system in response to noxious painful stimuli in some of the aforementioned animal models of depression and co-morbid pain hypersensitivity, effects which have been proposed to underlie observed alterations in pain behaviour (Rea et al., 2014, Lomazzo et al., 2015). To our knowledge only one study to date has evaluated the effect of IFN- α on endocannabinoid levels or function of this system. This

study firstly highlighted baseline deficits in serum levels of NAPE-PLD and DAGL- α , biosynthetic enzymes of AEA and 2-AG respectively, which were observed in hepatitis C patients who, upon induction of IFN- α treatment, developed depressive symptoms. Additionally, initiation of IFN- α therapy itself induced a significant increase in serum levels of the AEA-metabolising enzyme, FAAH, and that of NAPE-PLD (Zajkowska et al., 2015), effects observed in depressed patients only. Concurrently, initiation of therapy also induced a significant increase in serum levels of 2-AG in both depressed and non-depressed patients (Zajkowska et al., 2015). However, the system has not yet been evaluated in a preclinical model and as such, the establishment of the model in the current project allowed us to examine this relationship further.

The data presented in this thesis identify few changes within this system in terms of endocannabinoid or *N*-acylethanolamine levels or gene expression of the receptors or metabolic enzymes for these endocannabinoids, following eight days of repeated IFN- α treatment. However, in the presence of formalin-evoked pain, the levels of endocannabinoids and *N*-acylethanolamines in peripheral, spinal and supraspinal sites were altered between saline- and IFN- α -treated mice (chapter 5). Alterations in endocannabinoid tone such as that observed in this study may act as a compensatory mechanism against formalin administration in IFN- α -treated animals, attempting to dampen down or normalise the evoked response, or alternatively that such alterations in endocannabinoid tone within these brain regions underlie the formalin-evoked hyperalgesia observed in the presence of this depressive phenotype. In relation to AEA and the other FAAH substrates, the data demonstrated that AEA levels are enhanced in the RVM of IFN- α -treated mice post-formalin with a concurrent reduction in other FAAH substrate levels in the spinal cord and paw tissue (chapter 5). As any or all of these changes may underpin the altered nociceptive responding observed in IFN- α -treated mice, the next 2 chapters evaluated the effect of enhancing FAAH substrate levels at a peripheral/local (chapter 6) vs. global (chapter 7) level on nociceptive responding. The data demonstrated that enhancing FAAH substrate levels directly at the level of the paw, but not globally, attenuated IFN- α -induced hyperalgesia without altering nociceptive responding in saline-treated mice. Taken together, the data

presented herein provides support for an inability of IFN- α -treated animals to mobilise FAAH substrates at the level of the paw in response to a noxious inflammatory stimulus, resulting in the hyperalgesic response observed in these animals. The precise mechanism by which this occurs remains to be determined, however studies have shown that FAAH substrate levels are enhanced in response to an inflammatory noxious stimulus (Maccarrone et al., 2001) and that enhancing AEA or PEA levels individually at the level of the paw has been shown to attenuate second phase formalin-evoked nociceptive responding (LoVerme et al., 2006, Schreiber et al., 2012). Although hypothetical at present, it is possible that repeated IFN- α administration induces an inflammatory state that sensitizes peripheral nociceptive terminals and/or initially increases AEA and FAAH substrate production and release, but over time is desensitized to further release by an inflammatory (noxious) stimulus. The ability of the system (cannabinoid receptor targets) to respond to elevation of FAAH substrates at the peripheral nociceptor is not altered and thus inhibition of FAAH and subsequent increases in FAAH substrate levels attenuates the hyperalgesia in IFN- α -treated animals. It cannot be ruled out that discrete changes in AEA (and or FAAH substrate) tone in specific parts of the ascending or descending pain pathway may also mediate or modulate IFN- α -mediated hyperalgesia, the role of which remains to be determined. However taken together, the data presented here provides evidence for a role of FAAH substrates at a peripheral level in modulating IFN- α -induced hyperalgesia. Understanding the physiological mechanisms underpinning the hyperalgesia associated with repeated IFN- α is important for informing on possible treatment strategies and thus this data indicate that local or peripheral enhancement of FAAH substrate tone may be a possible therapeutic strategy for patients with this co-morbidity. Importantly such a treatment strategy would be devoid of the cannabinoid-related adverse effects associated with central CB₁ receptor agonism including weight gain, hypolocomotion and cognitive impairment. However, it should also be noted that while effective in treating the co-morbid pain in patients receiving IFN- α treatment (or other inflammatory cytokine therapies), enhancing local/peripheral FAAH substrates would not likely modulate the emotional disturbances such as depression, which are centrally mediated. Thus, the effects of IFN- α treatment on depression and pain may be mediated and modulated by separate neurobiological processes.

In a similar fashion to FAAH substrates, 2-AG tone was also found to be altered in IFN- α -treated mice post formalin administration. Essentially, 2-AG levels were increased in the PAG and RVM of IFN- α -formalin treated rats while concurrently, levels failed to increase in the paw. Enhancing peripheral/local 2-AG tone attenuated the IFN- α -induced hyperalgesia while global enhancement of 2-AG levels resulted in analgesic effects in both saline- and IFN- α -treated mice. As described above, the data indicate that IFN- α -treated mice exhibit an inability to mobilise 2-AG at the level of the nociceptor in response to an inflammatory noxious stimulus, resulting in hyperalgesia. It is likely that a similar mechanism as described for FAAH substrate enhancement mediates this effect and again targeting MAGL at a peripheral/local level may provide a novel therapeutic target for co-morbid pain in IFN- α -treated subjects. However, in contrast to effects observed following systemic FAAH inhibition, MAGL inhibition results in analgesic activity in both saline- and IFN- α -treated mice. Although an alternative MAGL inhibitor JZL184, has been shown to exhibit anti-nociceptive effects in other paradigms of inflammatory pain (Woodhams et al., 2012, Ghosh et al., 2013) and recent studies have demonstrated analgesic effects of the MAGL inhibitor, MJN110, which was used in this study in animal model of neuropathic pain (Ignatowska-Jankowska et al., 2015, Niphakis et al., 2013), to our knowledge this is the first to report effects of MJN110 in a model of inflammatory pain. It should be noted that these effects were also associated with an increase in locomotor activity, and thus it is difficult to determine if the effects on pain are directly mediated via effects on the pain pathways or indirect via enhanced motor responses. While a clinical role for MAGL inhibition in analgesic therapy has yet to be evaluated, these data would suggest that MAGL inhibition and enhancing 2-AG tone may be an effective strategy in treating pain conditions both in the presence and absence of chronic inflammation and/or an altered affective state, as would occur following IFN- α therapy. However, as in the case of FAAH inhibition, it remains to be determined if such a treatment strategy would also modulate the emotional/depressive-like co-morbidity associated with repeated IFN- α treatment.

While many questions remain, collectively the data presented in this thesis identify, for the first time, the emergence of depressive-like and hyperalgesic responding as a result of repeated IFN- α treatment in mice. This novel model now provides a mean of evaluating the neurobiological substrates that underpin and mediate pain and depression in IFN- α -treated subjects, the results of which would likely also be applicable to other cytokine or immune therapies. Moreover, the current data identify a potential role for altered endocannabinoid signalling in the manifestation of the hyperalgesic response associated with IFN- α treatment. We show that pharmacological inhibition of FAAH and MAGL activity and consequently enhanced substrate levels at the site of formalin injection is sufficient to abolish inflammatory hypersensitivity, a treatment strategy that would be devoid of the unwanted side effects of central CB₁ agonism. In this way, the data presents the peripheral endocannabinoid system as a viable target in treating pain hypersensitivities, while avoiding side effects which have traditionally hindered the clinical development of cannabinoid-based therapies. Additionally, we provide initial evidence of a role for a new MAGL inhibitor, MJN110, in the treatment of inflammatory pain in the presence and absence of chronic inflammation and an altered affective state. The work presented therefore creates a foundation upon which to design much needed future mechanistic and translation studies investigating the role of the endocannabinoid system in depression-pain interactions. In conclusion, the work presented herein adds to the body of knowledge on inflammation-induced depression-pain co-morbidities; placing the endocannabinoid system as a possible substrate in mediating the interaction therein.

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