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# **Endocannabinoid modulation of inflammatory hyperalgesia in the IFN- $\alpha$ mouse model of depression**

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## Abstract

Depression is a well-recognised effect of long-term treatment with interferon-alpha (IFN- $\alpha$ ), a widely used treatment for chronic viral hepatitis and malignancy. In addition to the emotional disturbances, high incidences of painful symptoms such as headache and joint pain have also been reported following IFN- $\alpha$  treatment. The endocannabinoid system plays an important role in emotional and nociceptive processing, however it is unknown whether repeated IFN- $\alpha$  administration induces alterations in this system. The present study investigated nociceptive responding in the IFN- $\alpha$ -induced mouse model of depression and associated changes in the endocannabinoid system. Furthermore, the effects of modulating peripheral endocannabinoid tone on inflammatory pain-related behaviour in the IFN- $\alpha$  model was examined. Repeated IFN- $\alpha$  administration (8,000IU/g/day) to male C57/Bl6 mice increased immobility in the forced swim test and reduced sucrose preference, without altering body weight gain or locomotor activity, confirming development of the depressive-like phenotype. There was no effect of repeated IFN- $\alpha$  administration on latency to respond in the hot plate test on day 4 or 7 of treatment, however, formalin-evoked nociceptive behaviour was significantly increased in IFN- $\alpha$  treated mice following 8 days of IFN- $\alpha$  administration. 2-Arachidonoyl glycerol (2-AG) levels in the periaqueductal grey (PAG) and rostroventromedial medulla (RVM), and anandamide (AEA) levels in the RVM, were significantly increased in IFN- $\alpha$ -, but not saline-, treated mice following formalin administration. There was no change in endocannabinoid levels in the prefrontal cortex, spinal cord or paw tissue between saline- or IFN $\alpha$ -treated mice in the presence or absence of formalin. Furthermore, repeated IFN- $\alpha$  and/or formalin administration did not alter mRNA expression of genes encoding the endocannabinoid catabolic enzymes (fatty acid amide hydrolyase or monoacylglycerol lipase) or endocannabinoid receptor targets (CB<sub>1</sub>, CB<sub>2</sub> or

PPARs) in the brain, spinal cord or paw tissue. Intra plantar administration of PF3845 (1µg/10µl) or MJN110 (1µg/10µl), inhibitors of AEA and 2-AG catabolism respectively, attenuated formalin-evoked hyperalgesia in IFN-α, but not saline-, treated mice. In summary, increasing peripheral endocannabinoid tone attenuates inflammatory hyperalgesia induced following repeated IFN-α administration. These data provide support for the endocannabinoid system in mediating and modulating heightened pain responding associated with IFNα-induced depression.

**Key Words:** Inflammation-induced depression, forced swim test, anhedonia, formalin test, Anandamide, 2-AG, FAAH, MAGL, CB<sub>1</sub> receptor

**Abbreviations:** 2-AG: 2-Arachidonolglycerol; AEA: anandamide; CB<sub>1</sub>: cannabinoid receptor 1; CB<sub>2</sub>: cannabinoid receptor 2; FAAH: Fatty acid amide hydrolase; FST: Forced swim test; IFN-α: interferon alpha; PAG: Periaqueductal Grey; PPAR: peroxisome proliferator-activated receptors; PFC: Prefrontal cortex; RVM: Rostral Ventromedial Medulla; SC: Spinal cord

## Introduction

Interferon-alpha (IFN- $\alpha$ ), a pro-inflammatory cytokine commonly used to treat various viral hepatitis and malignancy, results in depression in up to 50% of patients (Felger et al., 2016, Udina et al., 2012, Raison et al., 2005, Su et al., 2019). Knowledge of this association has led to the development of the IFN- $\alpha$ -induced rodent model of depression (Siddegowda et al., 2011, Ping et al., 2012, Fahey et al., 2007, Hayley et al., 2013), which has been valuable in examining neurobiological mechanisms underlying inflammation-associated depressive-like behaviour (Hoyo-Becerra et al., 2014). Depression and chronic pain are highly co-morbid conditions, and increasing evidence suggests that inflammatory processes may be key biological substrates underlying this association (Walker et al., 2014, Karshikoff et al., 2016, Burke et al., 2015). In addition to the emotional disturbances, repeated IFN- $\alpha$  administration has also been reported to be associated with 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). Furthermore, painful symptoms reported following IFN- $\alpha$  treatment present more frequently in patients also experiencing co-morbid depression (Shakoor et al., 2010). Although acute administration of IFN- $\alpha$  has been shown to exhibit analgesic-like effects in rodents (Blalock and Smith, 1981, Lee et al., 2010, Jiang et al., 2000, Liu et al., 2016), to our knowledge no study has evaluated nociceptive responding preclinically following repeated IFN- $\alpha$  administration.

The endogenous cannabinoid (endocannabinoid) system plays an important role in a wide variety of physiology processes including affective and nociceptive responding and thus, has been proposed as a mediator and modulator of depression-pain interactions [for reviews see (Fitzgibbon et al., 2015, Huang et al., 2016, Corcoran et al., 2015, Boorman et al., 2016)].

Accordingly, preclinical studies have demonstrated that reduced endocannabinoid-cannabinoid receptor (CB)1 signalling in the rostral ventromedial medulla (RVM) underpins inflammatory hyperalgesia in male Wistar Kyoto rats (Rea et al., 2014), a preclinical model of stress hyper-responsivity and depression (for review see (Aleksandrova et al., 2019)). In the chronic unpredictable stress model of depression, thermal (hot plate test) and mechanical (von Frey test) hyperalgesia was attenuated by pre-treatment with a fatty acid amide hydrolyase (FAAH) inhibitor, which enhanced endogenous levels of AEA (Lomazzo et al., 2015). Several lines of evidence have demonstrated that endocannabinoid modulation of neuroimmune processes has potent effects on affective responding (review see (Henry et al., 2016, Crowe et al., 2014)) and recent data from our lab have demonstrated that FAAH inhibition attenuates toll-like receptor 3-induced neuroinflammation and associated alterations in anxiety and nociceptive (mechanical and cold allodynia) responding in female rats (Flannery et al., 2018). Thus, alterations in the endocannabinoid system may underlie, at least in part, changes in nociceptive responding observed in the depressed state.

The aim of this study was to evaluate thermal and inflammatory nociceptive responding in the IFN- $\alpha$  model of depression and associated alterations in expression and levels of various components of the endocannabinoid system in supraspinal, spinal and peripheral nociceptive regions. The endocannabinoid system is highly expressed in all levels of the pain pathway and antinociceptive effects of endocannabinoids, phytocannabinoids, and synthetic cannabinoid receptor agonists have been reported in animal models of acute, inflammatory, and neuropathic pain (for review see (Starowicz and Finn, 2017)). As such, the effects of increasing endocannabinoid tone peripherally on inflammatory hyperalgesia in the IFN- $\alpha$  model was examined.

## **2. Materials and Methods**

### **2.1 Animals**

Male C57Bl/6 mice (weight 25-30g; Charles River Laboratories, UK) were housed in plastic-bottomed cages containing wood shavings as bedding. 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). Mice were given free access to food and water. All experimental procedures were carried out during the light phase, between 07.00 and 16.00 hrs and animals were randomly assigned to the various treatment groups. 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 ARRIVE guidelines and the European Communities Council directive 2010/63/EU.

### **2.2 Pharmacological Treatments**

Human interferon-alpha (hIFN- $\alpha$ -2a; 3M IU/0.5ml), Roferon-A®, Roche Pharmaceuticals) was diluted in sterile saline and administered subcutaneously in a volume of 3 $\mu$ l/g to give a final concentration of 8000IU/g/day IFN- $\alpha$ . Saline control mice received an equivalent volume of saline vehicle. The dose and duration of IFN- $\alpha$  administration was chosen based on pilot data and published research demonstrating effect on depressive-like behaviour (Ping et al., 2012). All animal were administered saline injections for 5 days prior to treatment to avoid any effects of injection procedure or handling on behavioural outcomes. Body weight was assessed daily at the time of injection to confirm that repeated IFN- $\alpha$  did not elicit any adverse

effect on body weight gain. All behavioural testing was carried out at least 24 hours after the last IFN- $\alpha$  administration to eliminate any acute effects of treatment.

The FAAH and monoacylglycerol lipase (MAGL) inhibitors PF3845 and MJN110 (gifted from Benjamin Cravatt, US), were dissolved in ethanol: saline: cremophor at a 1:1:18 dilution to give a final concentration of 1 $\mu$ g/10 $\mu$ l. The dose of PF3845 was chosen based on previous studies demonstrating anti-allodynic efficacy 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 (Wilkerson et al., 2016, Guindon et al., 2011).

### **2.3 Formalin test of inflammatory nociceptive behaviour**

The formalin test is a widely used test of inflammatory pain consisting of three phases: animals show a short period of nociceptive behaviours in phase I (0-5min), which is followed by a quiescent or attenuation of nociceptive responses in interphase (5 -15 min), and then a longer lasting period of nociceptive behaviour from 20 min up to 60 minutes (Hunskar et al., 1986, Hunskar and Hole, 1987). As previously described (Burke et al., 2010), the test animals were placed in a perspex transparent, square arena (30 x 30 x 30 cm) for 10 minutes, to reduce the effects of novelty stress on nociceptive responding. Mice then received a 1% solution of formalin (Sigma Aldrich; Ireland) into the left hind-paw (20 $\mu$ l) after which the animal was then placed back into the arena. Behaviour was recorded and later rated with the aid of EthoVision XT v11.5 software (Noldus, Netherlands). Formalin-evoked nociceptive behaviour was calculated as duration of licking, biting, shaking or flinching of the injected paw over 5-minute intervals over the course of the testing period. Formalin-induced oedema was assessed by



measuring the change in diameter (mm) of the left hind paw before and after formalin administration, using a Vernier callipers as previously described (Burke et al., 2010).

## **2.4 Quantitative RT-PCR for endocannabinoid receptors and catabolic enzymes**

RT-qPCR was carried out as previously described (Flannery et al., 2018). In brief, RNA was extracted from tissues using Nucleospin® RNA II total isolation kit (Macherey-Nagel, Germany) and reverse transcribed into cDNA using a high capacity complementary DNA (cDNA) kit (Applied Biosystems, Warrington, UK). Taqman gene expression assays were used to assess expression of FAAH (Mm00515684\_m1), monoacylglycerol lipase (MAGL) (Mm00449274\_m1), CB<sub>1</sub> (Mm01212171\_s1), CB<sub>2</sub> (Mm02620087\_s1), peroxisome proliferator-activated receptors (PPAR) $\alpha$  (Mm00440939\_m1) and PPAR $\gamma$  (Mm00440939\_m1) using an ABI Prism 7500 qPCR machine (Applied Biosystems, Warrington, UK).  $\beta$ -actin gene expression was used as an endogenous control. Expression was analysed using the  $\Delta\Delta$ CT method and expressed as %Saline-treated controls.

## **2.5 Mass spectrometry analysis of levels of endocannabinoid levels**

Mass spectrometry was carried out as previously described (Kerr et al., 2012, Kerr et al., 2013, Henry et al., 2014). In brief, samples were homogenised in 400 $\mu$ L 100% acetonitrile containing deuterated internal standards (0.014 nmol anandamide-d8 and 0.48nmol 2-AG-d8) and lyophilised samples re-suspended in 40 $\mu$ L 65% acetonitrile and separated by reversed-phase gradient elution. Analyte detection was carried out in electrospray-positive ionisation and multiple reaction monitoring (MRM) mode on an Agilent 1100 HPLC system coupled to a triple

quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Quantification of 2-AG and AEA was performed by ratiometric analysis and expressed as nmol/g or pmol/g of tissue respectively.

## **2.6 Experimental Design**

### **2.6.1 Effect of repeated IFN- $\alpha$ on depressive-like behaviours and nociceptive responding**

The effect of IFN- $\alpha$  (8,000 IU/g/day) on depressive-like behaviour was assessed in the forced swim test (FST) (cohort 1), sucrose preference test (SPT) (cohort 2), hot plate and formalin test (cohort 3) as previously described (Flannery et al., 2018, Burke et al., 2010). Separate cohorts of animals were used to avoid potential impacts of repeated testing and stress on the behavioural outcomes. In brief, cohort 1 mice were placed in a novel arena (30 x 30 x 30cm) to assess locomotor activity prior to and on day 1, 3, 5, 7 and 10 days of IFN- $\alpha$  administration. On day 10 of treatment, mice were placed in a FST arena for 6 minutes and duration of immobility (s) scored for the last 4 min of the trial. Cohort 2 mice were singly housed and offered the choice of two bottles, one filled with tap water and the other with 1% sucrose solution, in their homecage for 3 days prior to saline/IFN $\alpha$  administration to establish a baseline sucrose preference. IFN- $\alpha$  or saline was administered once daily thereafter and sucrose preference assessed on day 1, 4 and 7. % sucrose preference was calculated as (sucrose intake/total fluid intake)\*100. Based on the establishment of the anhedonic response from day 4 of IFN- $\alpha$  administration onwards, nociceptive responding was assessed from day 4 to day 8 of treatment. Cohort 3 mice were administered saline or IFN- $\alpha$  daily and exposed to the hot plate test on day 4 and 7 and latency to lick the hind paw was recorded as an index of thermal nociceptive responding. Inflammatory nociceptive responding was

assessed in the formalin test over a 60 min period on day 8 (see section 2.3 for details). All behavioural analysis was carried out by an experimenter blinded to group identity.

#### **2.6.2 Effect of a single administration of IFN- $\alpha$ on nociceptive responding in the formalin test**

Mice were administered saline or IFN- $\alpha$  and 24 hours later were exposed to the formalin test.

#### **2.6.3. Alterations in the endocannabinoid system in saline- and IFN-treated mice and effects of intra-plantar formalin administration**

Mice were administered IFN- $\alpha$  or saline for eight days after which they were divided into 4 groups (Saline, IFN- $\alpha$ , Saline-formalin and IFN- $\alpha$ -formalin). Mice were taken from their homecage and immediately sacrificed (Saline, IFN- $\alpha$ ) or subjected to the formalin test for 30min and sacrificed there after (Saline-formalin and IFN- $\alpha$ -formalin). Brain was removed and discrete brain regions (prefrontal cortex (PFC), periaqueductal grey (PAG), rostral ventromedial medulla (RVM)) were dissected, the spinal cord was removed and L5-6 was dissected and paw skin tissue (between the foot pads) of the left hindpaw was carefully removed. All tissues were snap frozen on dry ice and stored at -80°C until analysis.

#### **2.6.4 Effect of increasing endocannabinoid tone on formalin-induced inflammatory pain in saline- and IFN- $\alpha$ treated mice.**

Mice were administered IFN- $\alpha$  or saline for eight days after which they were divided into 6 groups (Saline-Vehicle, Saline-PF3845, Saline-MJN110, IFN- $\alpha$ -Vehicle, IFN- $\alpha$ -PF3845 or IFN- $\alpha$ -MJN110). Vehicle, PF3845 or MJN110 was administered into the plantar region of the left hind paw in a volume of 10 $\mu$ l and 20 minutes later mice were placed into the perspex square

arena for 10 minutes and locomotor activity assessed. Mice then received an intraplantar injection of formalin into the same hindpaw and were placed back in the arena and behaviour recorded for 60 minutes.

## **2.7 Statistical Analysis**

SPSS statistical package (IBM SPSS v20.0 for Microsoft Windows; SPSS Inc., Chicago, IL, USA) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene's test, respectively. Parametric data were analysed using repeated measures ANOVA, two-way ANOVA or t-test. *Post-hoc* analysis was performed using Fisher's *LSD* test where appropriate. Kruskal-Wallis ANOVA by ranks was employed for analysis of AEA and 2-AG levels in the paw tissue. The level of significance was set at  $p < 0.05$ . Graphs were constructed using Graphpad Prism 5 and results are expressed as group means + SEM or median  $\pm$  interquartile range.

### **3. Results**

#### **3.1 Repeated administration of IFN- $\alpha$ increases immobility in the FST and reduces sucrose preference.**

Repeated IFN- $\alpha$  administration did not alter body weight gain over the course of 10 days of administration (Fig 1a). Although locomotor activity was significantly reduced from baseline on each of the test days (repeated measures ANOVA: effect of day [ $F(5, 70) = 24.20, P < 0.001$ ], this did not differ between saline and IFN- $\alpha$  treated animals (Fig 1b). IFN- $\alpha$  treated animals exhibited a significant increase in immobility in the forced swim test [t-test  $P < 0.01$ ] (Fig1c). Sucrose preference did not differ at baseline or 24 hours following the first IFN- $\alpha$  administration. However, preference was significantly reduced on days 4 and 7 of administration in IFN- $\alpha$  treated mice (repeated measures ANOVA: IFN $\alpha$  treatment  $F(1, 12) = 42.76, P < 0.001$ , day [ $F(3, 36) = 12.705, P < 0.001$ ], IFN $\alpha$  x day interaction [ $F(3, 36) = 10.866, P < 0.001$ ]) (Fig1d). There was no significant effect of IFN- $\alpha$  administration on total fluid intake (data not shown).

#### **3.2 Repeated administration of IFN- $\alpha$ enhances inflammatory, but not thermal, nociceptive responding**

Saline and IFN- $\alpha$  treated animals were exposed to a noxious thermal stimulus (hot plate test) on day 4 and 7 of administration. Repeated measures ANOVA revealed that latency to respond to the heat stimulus did not differ between saline and IFN- $\alpha$  treated rats on either of the test days (Fig 2a). Animals were subsequently exposed to the formalin test of inflammatory pain on day 8. Analysis revealed that IFN- $\alpha$ -treated mice exhibited a significant increase in nociceptive behaviour during the first 5 minutes and during the 40-50 minute period of the test, when compared with saline-treated counterparts (repeated measures

ANOVA: effect of IFN- $\alpha$  [ $F(1, 16) = 14.702, P=0.001$ ] and time [ $F(11, 176) = 19.732, P<0.001$ ] (Fig 2b). Locomotor activity did not differ between saline and IFN- $\alpha$  treated mice during the formalin test ( $1094 \pm 81\text{cm}$  vs  $1263 \pm 117\text{cm}$  respectively; t-test  $P>0.05$ ).

### **3.3 Single acute administration of IFN- $\alpha$ does not alter formalin-evoked inflammatory nociceptive behaviour**

The data so far indicate that repeated IFN- $\alpha$  administration elicits depressive-like behaviour and greater formalin-evoked nociceptive responding. Anhedonic-like behaviour was not observed following a single acute administration of IFN- $\alpha$ , but rather develops following repeated administration (Fig1d). However, it is unknown if inflammatory nociceptive responding would be altered following a single administration of IFN- $\alpha$ . Analysis revealed that formalin-induced nociceptive behaviour was not altered 24hrs following a single administration of IFN- $\alpha$  (Fig3).

### **3.4 Increased formalin-evoked nociceptive behaviour in IFN- $\alpha$ treated mice is associated with changes in AEA and 2-AG levels**

In order to determine if endocannabinoid levels are altered when IFN- $\alpha$ -related hyperalgesia is maximally expressed, formalin evoked nociceptive behaviour was assessed in saline and IFN- $\alpha$  treated mice for 30 minutes and endocannabinoid levels measured in discrete tissues thereafter. Assessment of behavioural responding following formalin administration confirmed an increase in nociceptive responding by IFN- $\alpha$  treated mice of the 25-30 minute time period of the formalin trial (ANOVA: time [ $F(6, 96) = 19.626, P=0.000$ ], IFN- $\alpha$  [ $F(1, 16) = 3.944, P=0.064$ ] and time x IFN- $\alpha$  treatment interaction [ $F(6, 96) = 2.137, P=0.056$ ]) (Fig4a).

Analysis of AEA levels in the PFC, PAG, RVM, spinal cord and paw skin tissue revealed that intraplantar formalin administration significantly increased AEA levels in the PFC of saline, but not IFN- $\alpha$ , treated animals (Fig 4b and Table 1). AEA levels were increased in the RVM of IFN- $\alpha$ -treated mice that received formalin, an effect not observed in saline-treated counterparts (Fig4b). There was no significant effect of IFN- $\alpha$  and/or formalin on AEA levels in the PAG or spinal cord. Although AEA levels in the paw skin tissue of saline-treated animals that received formalin tended to be higher than non-formalin counterparts or formalin treated IFN- $\alpha$  mice, Kruskal-Wallis analysis by ranks revealed no significant effect of IFN- $\alpha$  or formalin treatment on AEA levels in the paw skin tissue (Fig 4b and Table 1).

Analysis of 2-AG levels in the PFC, PAG, RVM, spinal cord and paw tissue revealed that 2-AG levels were significantly increased in the PAG and RVM of IFN- $\alpha$ , but not saline,-treated animals that received intraplantar formalin administration when compared to non-formalin treated counterparts (Fig4c and Table 1). There was no significant effect of IFN- $\alpha$  and/or formalin on 2-AG levels in the PFC or spinal cord. Kruskal-Wallis analysis by ranks revealed a significant effect of IFN- $\alpha$  treatment on 2-AG levels in the paw skin tissue of mice (Fig4c and Table 1).

### **3.5 Repeated administration of IFN- $\alpha$ does not alter expression of endocannabinoid catabolic enzymes or receptor targets in the PFC, descending pain pathway or in paw tissue**

In order to determine if the changes in AEA and 2-AG in response to formalin administration are due to IFN- $\alpha$  induced changes in FAAH and MAGL, the primary metabolic enzymes for the endocannabinoids, the expression of these enzymes was assessed in the brain, spinal cord and paw tissue 8 days following saline or IFN- $\alpha$  administration. Data revealed no significant

difference in the expression of either FAAH (Fig 5a) or MAGL (Fig 5b) in any of the regions examined. In addition expression of endocannabinoid receptor targets was also examined revealing no significant effect of repeated IFN- $\alpha$  administration on expression of CB<sub>1</sub>, CB<sub>2</sub>, PPAR $\alpha$  or PPAR $\gamma$  in any of the regions examined (Fig 5c-f).

### **3.6 Enhancing endocannabinoid tone at site of peripheral nociceptor activation attenuates IFN- $\alpha$ related inflammatory hyperalgesia**

Given the decrease in 2-AG, and trend for a decrease in AEA, in paw tissue of IFN- $\alpha$  treated mice following formalin administration, we proposed that IFN- $\alpha$  related hyperalgesia may, in part, be due to reduced endocannabinoid tone at primary afferent nerve endings during inflammatory pain. If this was the case then enhancing endocannabinoid tone by inhibiting the degradation of the endocannabinoids may attenuate IFN- $\alpha$  induced hyperalgesia. Intraplantar administration of the FAAH inhibitor PF3845 or the MAGL inhibitor MJN110 did not alter locomotor activity (Fig 6a) or paw oedema induced by intraplantar formalin (Fig6b) in either saline or IFN- $\alpha$  treated animals. Repeated measures ANOVA revealed an effect of drug treatment [ $F(2, 49) = 4.18, P=0.021$ ], time x IFN- $\alpha$  [ $F(11, 539) = 2.05, P=0.022$ ] and time [ $F(11, 539) = 70.97, P<0.001$ ] on nociceptive responding in the formalin trail. *Post hoc* analysis revealed that MJN110 did not alter formalin-evoked nociceptive behaviour in saline-treated animals (Fig 6c). Intraplantar administration of PF3845 reduced formalin-evoked nociceptive behaviour of saline-treated animals at one discrete time point (40-45min). Vehicle-IFN- $\alpha$  treated animals exhibited significantly greater nociceptive responding in the 10-15 and 20-25 minute period when compared to saline-treated counterparts (Fig6d). Intraplantar PF3845 attenuated nociceptive responding in IFN- $\alpha$ -treated mice in the 20-25



and 45-50 minute period, while MJN110 attenuated nociceptive behaviour of IFN- $\alpha$  treated mice in 0-5, 20-25 and 45-50 minute periods of the test (Fig 6d).

#### 4.4 Discussion

An increasing body of data indicates a high co-morbidity between depression and pain, with inflammatory mechanisms likely to underlie this association. Accordingly, IFN- $\alpha$  treatment has been shown to be associated with a high incidence of depression and painful somatic symptoms (Felger et al., 2016, Shakoor et al., 2010, Capuron et al., 2002). The current findings add to this body of work demonstrating that in addition to a depressive-like phenotype, repeated IFN $\alpha$  administration in mice results in inflammatory, but not thermal, hyperalgesia. This heightened inflammatory nociceptive responding is associated with alterations in AEA and 2-AG levels both supraspinally and peripherally. Furthermore, enhancing local/peripheral AEA or 2-AG tone attenuated inflammatory hyperalgesia following repeated IFN- $\alpha$  administration. Taken together, the data indicate a role for the endocannabinoid system in mediating and modulating inflammatory hyperalgesia associated with repeated IFN- $\alpha$  treatment.

Nociceptive responding has been demonstrated to be altered in genetic, pharmacological, lesion and stress-induced models of depression [for reviews see (Burke et al., 2017, Burke et al., 2015, Jennings et al., 2014)]. Furthermore, an acute immune challenge with TLR agonists induces both depressive-like behaviours (O'Connor et al., 2009, Salazar et al., 2012, Gibney et al., 2013) and alterations in nociceptive thresholds (Pitychoutis et al., 2009, Flannery et al., 2018). Although repeated IFN $\alpha$  administration has been demonstrated to induce a depressive-like phenotype in both rats and mice (Siddegowda et al., 2011, Ping et al., 2012, Fahey et al., 2007, Hayley et al., 2013), to our knowledge this is the first study to evaluate effects on nociceptive responding. The data presented herein confirm the depressive-like phenotype following repeated, but not acute, IFN- $\alpha$  administration indicated as an increase

in immobility in the forced swim test (behavioural despair) and reduced sucrose preference (anhedonia). Acute administration of IFN- $\alpha$  has previously been shown to exhibit antinociceptive effects in rodents mediated by mu-opioid receptor activation (Lee et al., 2010, Jiang et al., 2000, Wang et al., 2006). However, the results presented herein demonstrated that a single acute administration of IFN- $\alpha$  did not alter inflammatory nociceptive responding 24hrs post administration. Intrathecal administration of IFN- $\alpha$  has been shown to attenuate carrageenan-induced nociceptive responding for up to 2 hours, which was not observed 4 hours post administration (Liu et al., 2016). The lack of effect of a single acute administration in the current study is most likely due to the route of IFN- $\alpha$  administration (spinal vs systemic), the time at which nociceptive responding was examined (minutes vs 24hrs post administration) and type of nociceptive responding examined (thermal vs inflammatory). Thus, while IFN- $\alpha$  signalling in supraspinal and spinal sites is important in modulating acute (min-hrs) nociceptive responding, effects are not observed 24hrs post a single administration. In comparison, the data presented herein demonstrate that repeated IFN- $\alpha$  administration results in inflammatory, but not thermal, hyperalgesia, at a time when the depressive-like phenotype is also apparent. Thus, repeated IFN- $\alpha$  administration results in changes in the neurobiological circuitry underlying emotional and nociceptive processing giving rise to a depressive-like and hyperalgesic state. To our knowledge this is the first study to demonstrate altered nociceptive responding in the IFN- $\alpha$ -model of depression.

The endocannabinoid system plays an important regulatory role in affective and nociceptive responding and therefore alterations in this system may underlie the co-morbidity between depression and chronic pain (Fitzgibbon et al., 2015, Huang et al., 2016, Corcoran et al., 2015, Boorman et al., 2016). Inflammation-induced models of depression have been demonstrated to exhibit alterations in the endocannabinoid system. For example, CB<sub>1</sub> receptor expression

in the hippocampus and brain stem is reduced following TLR4 activation at a time when depressive-like behaviour is also evident (Hu et al., 2012). Unpublished data from our own lab has demonstrated a decrease in CB<sub>2</sub> receptor expression in the hypothalamus following TLR3 activation (Flannery and Roche; unpublished observations). FAAH inhibition has been shown to attenuate TLR4- and TLR3-induced neuroinflammation and modulate associated affective and nociceptive (mechanical and thermal) responding (Henry et al., 2017, Flannery et al., 2018). IFN- $\alpha$  induced anorexia, nausea and vomiting in hepatitis C patients has been shown to be reduced by oral cannabinoid-containing medications (Costiniuk et al., 2008) and IFN- $\alpha$  therapy was associated with a significant increase in serum levels of FAAH and N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (the biosynthetic enzyme of AEA) in patients that went on to develop depression (Zajkowska et al., 2015). Thus, IFN- $\alpha$  treatment may alter endocannabinoid tone, which then may in turn underlie the associated depression and/or hyperalgesia. The data herein demonstrate that 2-AG levels were reduced in the paw skin tissue of IFN- $\alpha$  treated mice, both in the absence and presence of inflammatory pain. The expression of the 2-AG metabolic enzyme MAGL in the paw skin tissue was not altered in IFN- $\alpha$  treated mice, however we cannot rule out that activity of this enzyme may be increased resulting in the reduced levels of 2-AG. In order to further investigate this possibility and evaluate the effect of enhancing 2-AG tone at the primary afferent nerve endings on IFN- $\alpha$  related hyperalgesia, the MAGL inhibitor MJN110 was administered directly into the paw. The data demonstrated that MJN110 attenuated IFN- $\alpha$  related hyperalgesia during the first (0-5min) and second (20-25min) phase of the formalin test, without altering nociceptive responding in saline-treated counterparts. It is possible that the MJN110-induced increase in 2-AG attenuated the initial inflammatory response to formalin at the peripheral primary afferent nerve endings, which in turn attenuates the development of secondary

centrally mediated hyperalgesic response. The lack of effect in saline-treated counterparts confirms that repeated IFN- $\alpha$  induces changes in the 2-AG system peripherally, which are, at least partially, responsible for the expression of inflammatory hyperalgesia.

AEA levels in the paw of saline-, but not IFN- $\alpha$ -, treated mice tended to be increased following intraplantar formalin administration. Thus, it is possible that IFN- $\alpha$ -treated mice may be unable to mobilise AEA at the level of the paw in response to a noxious inflammatory stimulus, resulting in the hyperalgesia. In line with this hypothesis, studies have shown that FAAH substrate levels are enhanced in response to an inflammatory noxious stimulus (Maccarrone et al., 2001) and enhancing AEA levels in the paw has been shown to attenuate second phase formalin-evoked nociceptive responding (Schreiber et al., 2012). The data herein demonstrate that intraplantar administration of the FAAH inhibitor PF3845 attenuated IFN- $\alpha$  related hyperalgesia during the second phase (20-25 min) of the formalin test, without altering nociceptive responding in saline-treated counterparts during this time. PF3845 reduced formalin-evoked nociceptive responding in saline-treated mice at the 40-45 minute time period. It is possible that the lack of effect on formalin-evoked nociceptive responding over a longer period may be due to PF3845 inducing an increase in a number of other FAAH substrates, and not just AEA, which may elicit competing effects or mask AEA mediated analgesic effects. In comparison, repeated IFN- $\alpha$  treatment impairs AEA/FAAH substrate activity peripherally in response to a noxious inflammatory stimulus, which is partially responsible for IFN- $\alpha$  related hyperalgesia.

AEA and 2-AG levels, the expression of the metabolic enzymes FAAH and MAGL, and the cannabinoid receptors, were also assessed in key supraspinal nociceptive regions and in the spinal cord following repeated IFN- $\alpha$  administration. Antinociceptive effects of

endocannabinoid, phytocannabinoids and synthetic cannabinoids have been demonstrated via modulation of nociceptive processing at these spinal and supraspinal sites (for review see (Starowicz and Finn, 2017)). Although basal levels/expression did not differ between saline and IFN- treated mice, under conditions of inflammatory pain, 2-AG levels in the PAG and RVM, and AEA levels in the RVM; were significantly increased in IFN- $\alpha$ -, but not saline-, treated mice. It is possible that increasing endocannabinoid levels in these key regions of the descending pain pathway may underlie the IFN- $\alpha$  related hyperalgesic response or could be an attempt to engage endogenous analgesic mechanism in an attempt to control heightened nociceptive responding in these animals. Further studies are required to decipher the role of the endocannabinoid system in the descending pain pathway in IFN- $\alpha$ -related hyperalgesia.

In conclusion, the data demonstrate that in addition to the depressive-like phenotype, repeated IFN- $\alpha$  administration also results in inflammatory hyperalgesia. This novel model now provides a means of evaluating the neurobiological substrates and treatments for inflammation-associated depression and/or pain. Moreover, the data identified alterations in the endocannabinoid system associated with IFN- $\alpha$  induced hyperalgesia. While the role of spinal and supraspinal pathways remain to be deciphered, these data indicate that local enhancement of endocannabinoid tone is capable of attenuating the IFN- $\alpha$  related hyperalgesia. This may have important implications for the treatment of inflammation-related hyperalgesia with local or topical cannabinoid-based medicines that would be devoid of central and adverse psychotropic effects.

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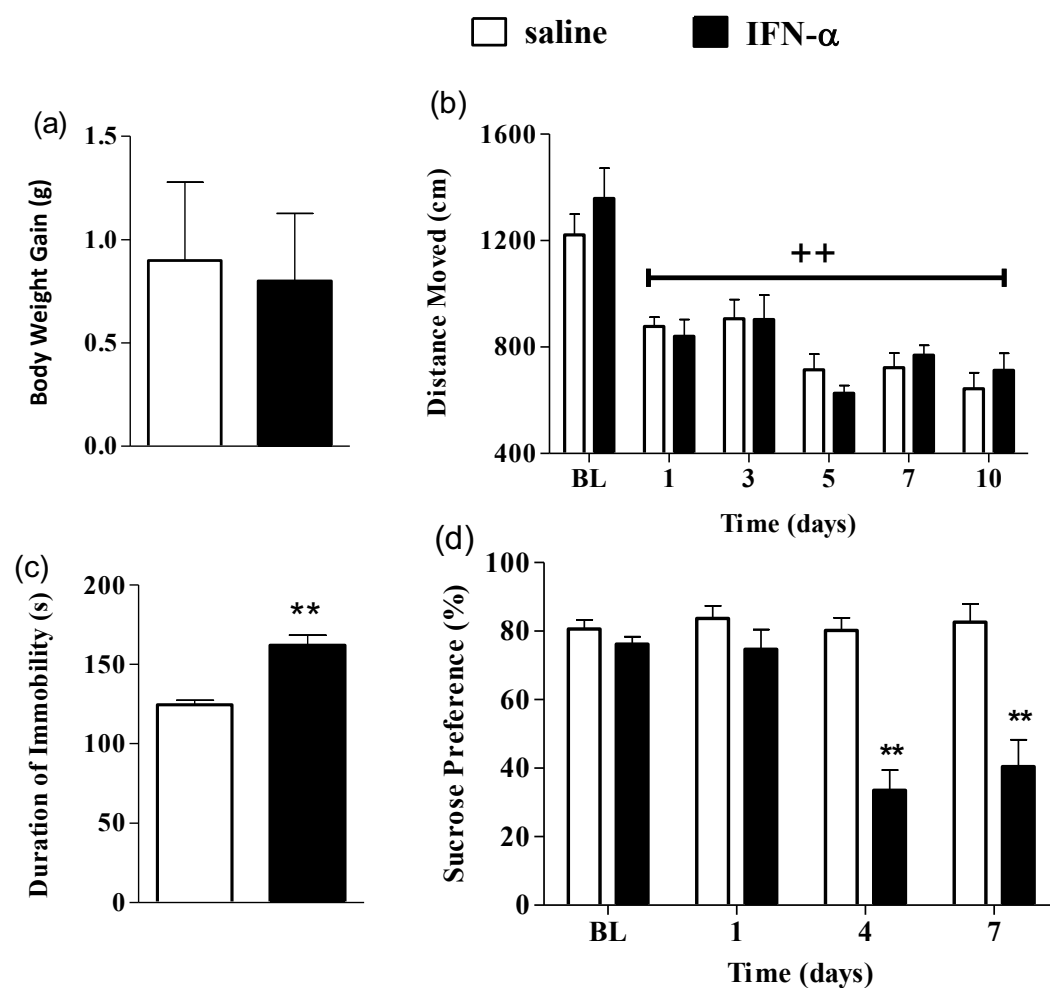
**Table 1: Results of statistical analysis on effect of repeated IFN- $\alpha$  and/or formalin administration on 2-AG and AEA levels in discrete tissues.**

	Region	IFN- $\alpha$	Formalin	Interaction (IFN- $\alpha$ x Formalin)
<b>AEA</b>	<b>PFC</b>	0.619	<b>0.012</b>	0.100
	<b>PAG</b>	0.317	0.395	0.111
	<b>RVM</b>	0.577	<b>0.038</b>	0.541
	<b>Spinal cord</b>	0.230	0.853	0.764
	<b>Paw Skin</b>	0.553	0.491	NA
<b>2-AG</b>	<b>PFC</b>	0.121	0.404	0.949
	<b>PAG</b>	0.205	<b>0.001</b>	0.260
	<b>RVM</b>	0.941	<b>0.008</b>	0.272
	<b>Spinal Cord</b>	0.272	0.266	0.585
	<b>Paw Skin</b>	<b>0.038</b>	0.085	NA

Data are expressed as P value from two-way ANOVA or Kruskal-Wallis analysis by ranks (paw skin tissue). n= 5-9 per group. Numbers in **bold** indicate significant effects. Anandamide (AEA); 2-Arachidonolglycerol (2-AG); Prefrontal cortex (PFC); Periaqueductal Grey (PAG), Rostral Ventromedial Medulla (RVM)

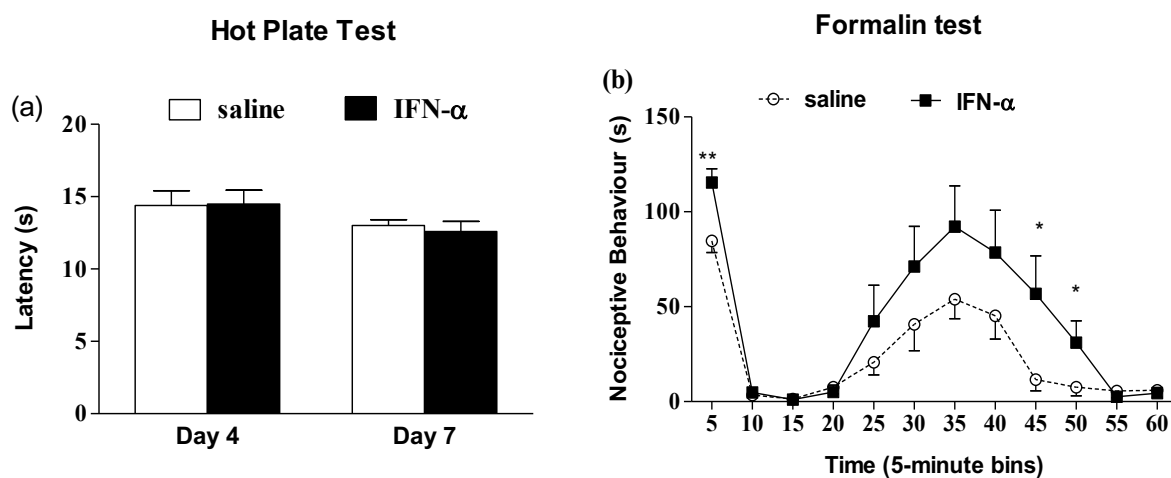
## Figure Legends

**Figure 1**



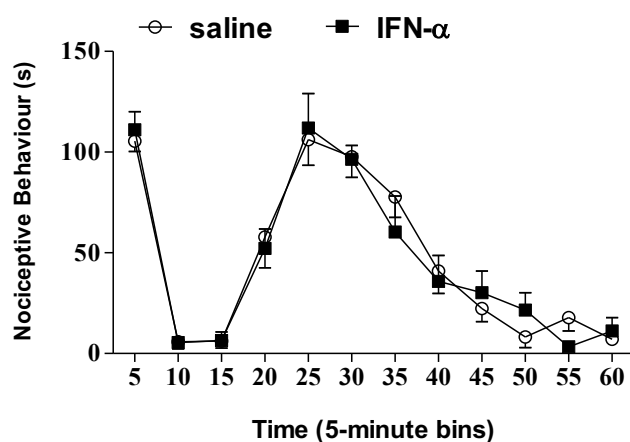
**Figure 1.** The effect of repeated IFN- $\alpha$  administration on (A) Body weight gain over 10 days, (b) locomotor activity (c) immobility in the FST and (d) sucrose preference. ++P<0.01 vs baseline (BL) \*\*P<0.01 vs saline. Data are expressed as mean  $\pm$  SEM; n= 9-10 per group.

**Figure 2**



**Figure 2.** (a) Latency to respond in the hot plate test following 4 or 7 days of saline or IFN- $\alpha$  treatment. (b) Nociceptive behaviour over a 60 minute period in the formalin test following 8 days of saline or IFN- $\alpha$  treatment. Data are expressed as mean  $\pm$  SEM; n= 10 per group; \*\*p<0.01, \*p<0.05 vs. saline-treated mice.

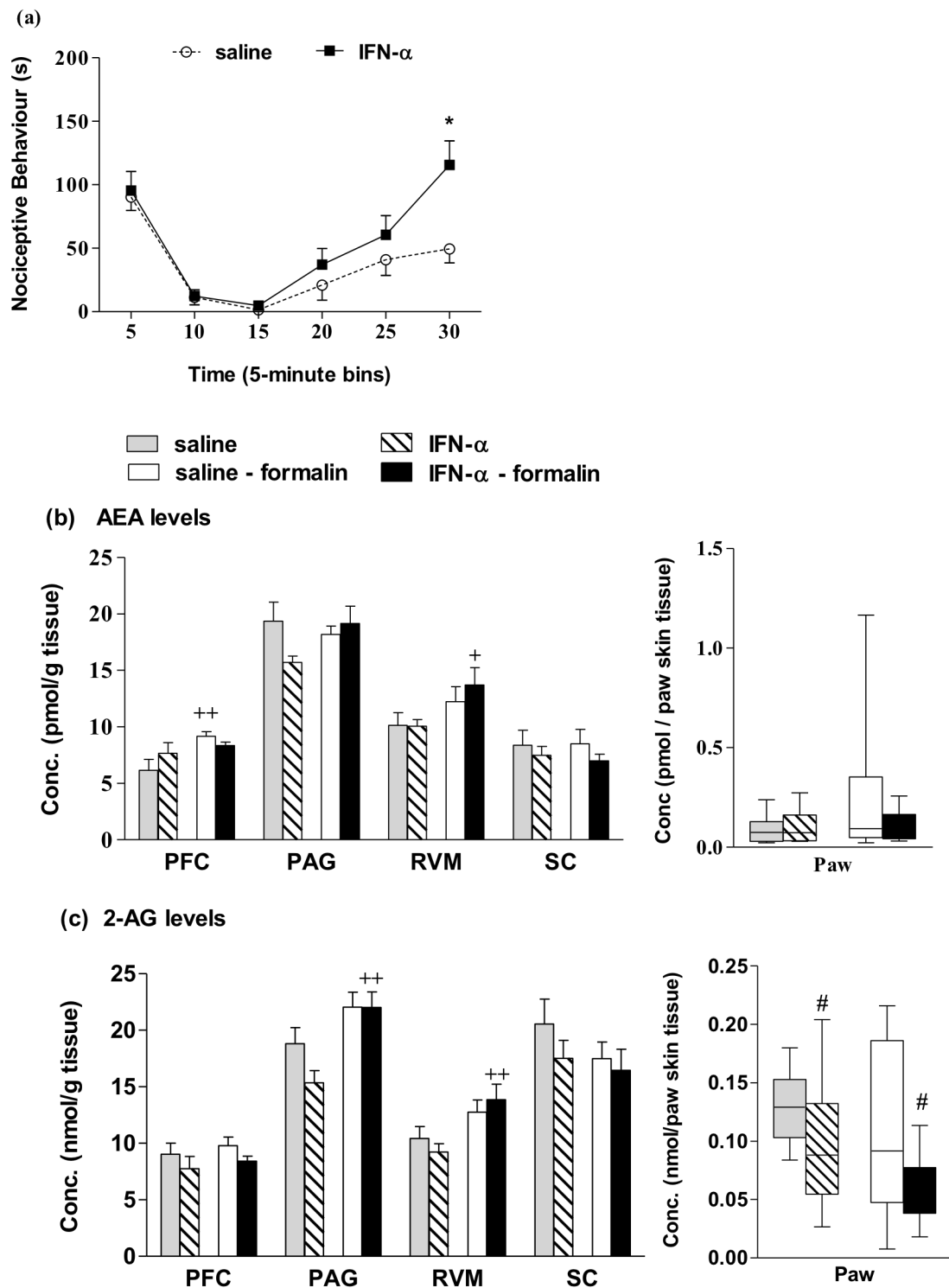
**Figure 3**



**Figure 3.** (a) Nociceptive behaviour over a 60 minute period in the formalin test 24hrs following a single administration of saline or IFN- $\alpha$ . Data are

expressed as mean  $\pm$  SEM; n= 9-10 per group

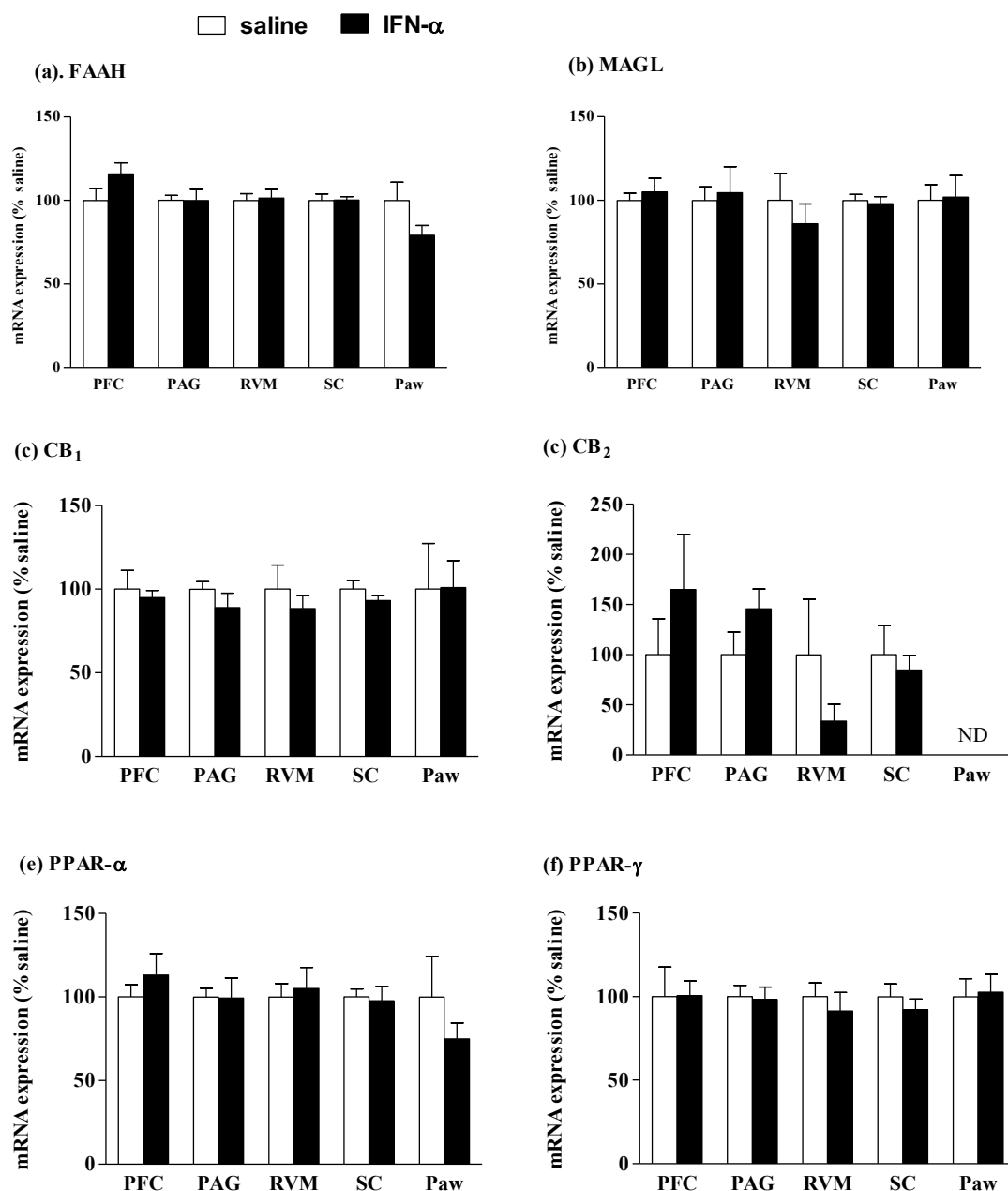
**Figure 4**



**Figure 4.** Effect of repeated administration of IFN- $\alpha$  for 8 days on formalin evoked nociceptive behaviour and endocannabinoid levels in brain, spinal cord and paw skin tissue. (a) IFN $\alpha$ -treated mice exhibit an increase nociceptive behaviour 25-30 minutes post formalin administration. (b) AEA and (c) 2-AG levels in the PFC, PAG, RVM, spinal cord (SC) and plantar tissue of the left hind paw of saline- and IFN- $\alpha$ -treated mice with or without intraplantar formalin. Data are expressed as mean  $\pm$  SEM except for AEA and 2-AG levels in paw tissue which are expressed as median and interquartile range; n= 5-9 per group. \*P<0.05 vs Saline-treated counterpart. ++P<0.01, +P<0.05 vs. non-formalin-treated animals; # overall effect of IFN- $\alpha$ . Anandamide (AEA); 2-Arachidonolglycerol (2-AG); Prefrontal cortex (PFC); Periaqueductal Grey (PAG), Rostral Ventromedial Medulla (RVM); Spinal cord (SC)

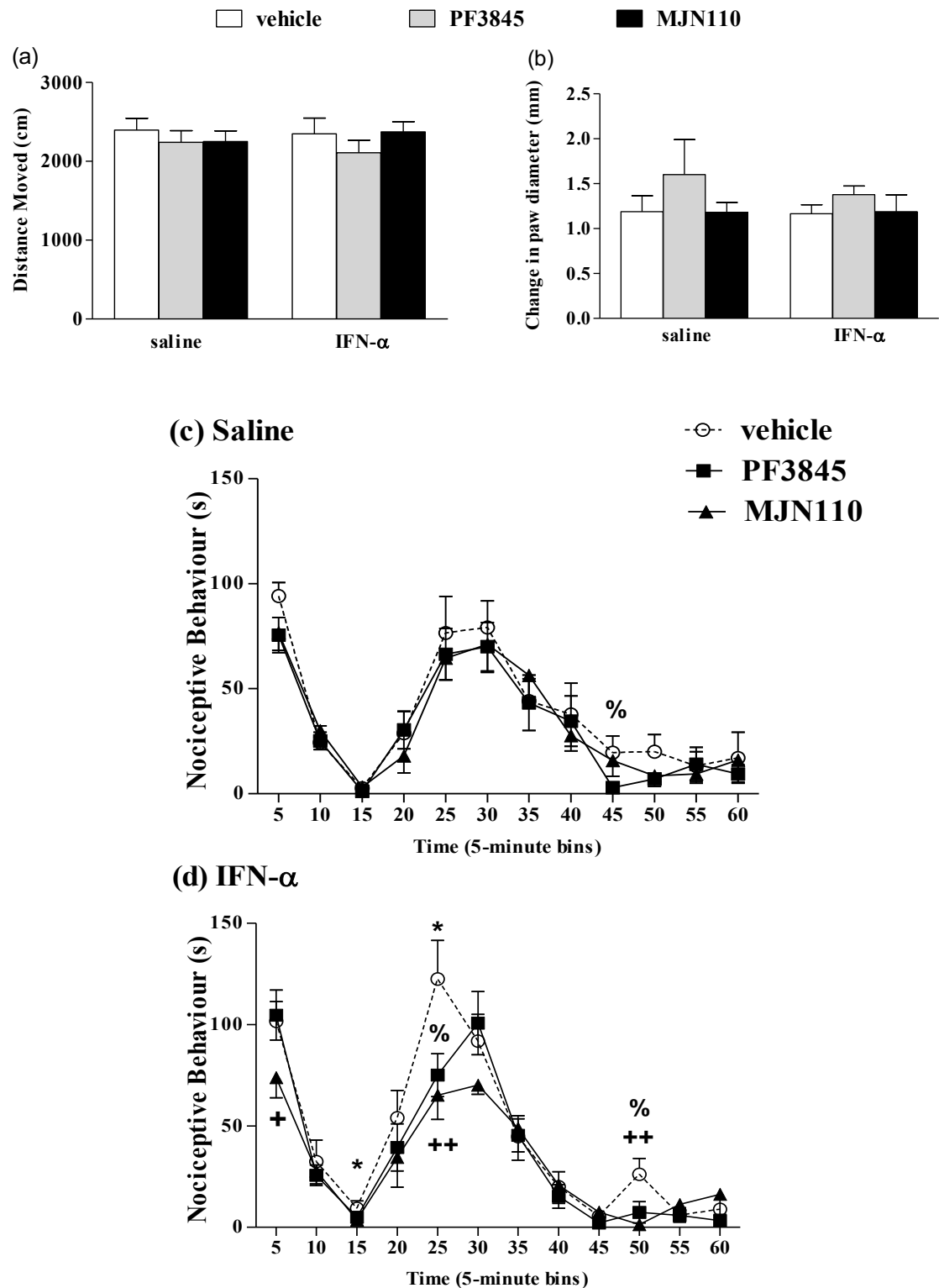


**Figure 5**



**Figure 5:** The effect of repeated IFN- $\alpha$  administration for 8 days on the mRNA expression of (a) FAAH, (b) MAGL, (c) CB<sub>1</sub>, (d) CB<sub>2</sub>, (e) PPAR $\alpha$  and (f) PPAR $\gamma$ . Data are expressed as mean  $\pm$  SEM; n = 6-8 per group. ND, not detected. Fatty Acid Amide Hydrolyase (FAAH); Monoacylglycerol Lipase (MAGL); Prefrontal cortex (PFC); Periaqueductal Grey (PAG), Rostral Ventromedial Medulla (RVM); Spinal cord (SC)

Figure 6



**Figure 6.** Effect of intraplantar PF3845 or MJN110 on (a) locomotor activity and (b) paw oedema post formalin injection in saline and IFN- $\alpha$ -treated mice. Nociceptive behaviour of (c) saline- and (d) IFN- $\alpha$ -treated animals over the 60 min trial. Data are expressed as mean  $\pm$  SEM; n= 8-10 per group. \*P<0.05 IFN- $\alpha$ -vehicle vs Saline-vehicle. %P<0.05 PF3845 vs vehicle-treated counterpart. +P<0.05 \*\*P<0.01 MJN110 vs vehicle-treated counterpart