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**Repeated forced swim stress differentially affects formalin-evoked nociceptive
behaviour and the endocannabinoid system in stress normo-responsive and stress
hyper-responsive rat strains**

Elaine M. Jennings^{a, c, +}, Bright N. Okine^{a, c, +}, Weredeselam M. Olango^{a, c}, Michelle Roche^{b, c},

David P. Finn^{a, c, *}

*^aPharmacology and Therapeutics, ^bPhysiology, School of Medicine, ^cGalway Neuroscience
Centre and Centre for Pain Research, NCBES, National University of Ireland, Galway,
Ireland*

⁺ Equal contribution

^{*}Corresponding author

Prof. David P. Finn, Pharmacology and Therapeutics, School of Medicine, University Road,
National University of Ireland, Galway, Ireland. Tel. +353 91 495280; Fax +353 91 495586

Email: david.finn@nuigalway.ie

URL: http://www.nuigalway.ie/pharmacology/professor_david_finn.html

Abstract

Repeated exposure to a homotypic stressor such as forced swimming enhances nociceptive responding in rats. However, the influence of genetic background on this stress-induced hyperalgesia is poorly understood. The aim of the present study was to compare the effects of repeated forced swim stress on nociceptive responding in Sprague-Dawley (SD) rats versus the Wistar-Kyoto (WKY) rat strain, a genetic background that is susceptible to stress, negative affect and hyperalgesia. Given the well-documented role of the endocannabinoid system in stress and pain, we investigated associated alterations in endocannabinoid signalling in the dorsal horn of the spinal cord and amygdala. In SD rats, repeated forced swim stress for 10 days was associated with enhanced late phase formalin-evoked nociceptive behaviour, compared with naive, non-stressed SD controls. In contrast, WKY rats exposed to 10 days of swim stress displayed reduced late phase formalin-evoked nociceptive behaviour. Swim stress increased levels of monoacylglycerol lipase (MAGL) mRNA in the ipsilateral side of the dorsal spinal cord of SD rats, an effect not observed in WKY rats. In the amygdala, swim stress reduced anandamide (AEA) levels in the contralateral amygdala of SD rats, but not WKY rats. Additional within-strain differences in levels of CB₁ receptor and fatty acid amide hydrolase (FAAH) mRNA and levels of 2-arachidonylglycerol (2-AG) were observed between the ipsilateral and contralateral sides of the dorsal horn and/or amygdala. These data indicate that the effects of repeated stress on inflammatory pain-related behaviour are different in two rat strains that differ with respect to stress responsivity and affective state and implicate the endocannabinoid system in the spinal cord and amygdala in these differences.

Keywords: Pain; Endocannabinoid; Formalin; Wistar-Kyoto; Sprague-Dawley; forced swim; stress-induced hyperalgesia

List of Abbreviations:

WKY: Wistar-Kyoto

SD: Sprague-Dawley

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

AEA: Anandamide

2-AG: 2-Arachidonoylglycerol

MAGL: Monoacylglycerol lipase

FAAH: Fatty acid amide hydrolase

CB₁: Cannabinoid receptor 1

SIH: Stress-induced hyperalgesia

PAG: Periaqueductal grey

SIA: Stress-induced analgesia

LC-MS/MS: Liquid chromatography with tandem mass spectrometry

URB597: Cyclohexylcarbamic acid 3'-(Aminocarbonyl)-[1,1'-biphenyl]-3-yl ester

1. Introduction

Clinical and preclinical studies have highlighted a complicated relationship between stress and pain, with the nature, duration and intensity of the stressor all having an important influence on pain responding. Acute, intense stress has been shown in numerous studies to result in a reduction in pain, known as stress-induced analgesia (SIA) (for review see Butler and Finn, 2009). However, repeated or chronic exposure to physical or psychological stress typically results in stress-induced hyperalgesia (SIH), in humans (Gibbons et al., 2012; Kuehl et al., 2010; Jennings et al., 2014; Olango and Finn, 2014) and rodents (Bardin et al., 2009;

Dina et al., 2011; Le Roy et al., 2011; Quintero et al.; 2011, Jennings et al., 2014; Olango and Finn, 2014). For example, studies have shown that exposure to repeated forced swim stress can lead to mechanical hyperalgesia and increased inflammatory and thermal nociceptive behaviour in rats (Quintero et al., 2000; Suarez-Roca et al., 2006, 2008, 2013). These data suggest that repeated swim stress is a useful model to further understand the behavioural and neurochemical basis of SIH.

Genetic background also plays a key role in determining the effect of stress on pain. The Wistar-Kyoto (WKY) rat is an inbred strain that exhibits a stress-hyperresponsive and anxiety/depressive-like phenotype, compared with Sprague-Dawley (SD) rats (Burke et al., 2010). Moreover, WKY rats exhibit a hyperalgesic phenotype, with increased responding to inflammatory and visceral noxious stimuli (Burke et al., 2010; O'Mahony et al., 2013; Rea et al., 2014). Thus, the WKY rat is a useful model to study the influence of genetic background and negative affect on pain processing. However, the effects of exposure to repeated stress on nociceptive responding in the WKY rat remain to be determined and are the focus of the present study.

The endocannabinoid system consists of two receptor subtypes, CB₁ (Matsuda, 1990) and CB₂ (Munro et al., 1993) receptors, their endogenous ligands, with 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) and N-arachidonylethanolamide (anandamide; AEA) (Devane et al., 1992) being the two best characterised, and the enzymes that either synthesise or degrade the endogenous ligands (e.g. the AEA-degrading fatty acid amide hydrolase (FAAH (Cravatt et al., 1996)) or the 2-AG-degrading enzyme monoacylglycerol lipase (MAGL (Dinh et al., 2002))). A wealth of evidence indicates that the

endocannabinoid system plays a key role in modulating pain and anxiety (Finn, 2010, 2012; Hill et al., 2010; Hohmann and Suplita 2006; Lutz, 2009; Viveros et al., 2005). In particular, the endocannabinoid system in the amygdala plays a key role in the regulation of fear- and pain-related behaviour, and their interactions, in rodents (Finn et al., 2003; Martin et al., 1999; Meng et al., 1998; Roche et al., 2007; Olango et al., 2011; Hohmann et al., 2005; Rea et al., 2013; Marsicano et al., 2002). Direct evidence for a role of the endocannabinoid system in SIH comes from work demonstrating a down-regulation of CB₁ receptor expression in the dorsal root ganglion in a rat model of water avoidance SIH (Hong et al., 2009, 2011). Moreover, systemic administration of a CB₁ receptor agonist can attenuate restraint SIH in rats (Shen et al., 2010). Alterations in the endocannabinoid system of WKY rats have been reported (Vinod et al., 2012) and we have recently demonstrated that impaired endocannabinoid signalling likely underlies the hyperalgesic phenotype of WKY rats compared with SD controls (Rea et al., 2014). However, the effect of stress on pain responding in WKY rats, and associated alterations in the endocannabinoid system, have not yet been investigated.

The aim of the present study was to test the hypothesis that repeated exposure to forced swim stress would differentially alter nociceptive responding to noxious thermal and inflammatory stimuli and components of the endocannabinoid system in the amygdala and spinal cord of WKY versus SD rats. The amygdala was chosen because of its key roles in (a) the affective/emotional dimension of the pain experience (Zhang et al., 2013) (b) endocannabinoid-mediated modulation of stress and pain (see previous paragraph) and (c) because forced swim stress has been shown to induce alterations in amygdalar neurochemistry (Connor et al., 1997; Kelliher et al., 2000). The spinal cord was chosen

because of the critical role of the dorsal horn in mediating and modulating pain and because it is an important site for swim stress-induced hyperalgesia (Quintero et al., 2003).

2. Methods

2.1 Animals

Experiments were carried out in adult male SD (200-250g, n=16, Charles River, UK) and WKY (200-250g, n=16, Harlan, UK) rats. All rats were housed singly and maintained at a constant temperature ($21 \pm 2^{\circ}\text{C}$) under standard lighting conditions (12:12h light:dark, lights on from 07.00 to 19.00h). All experiments were carried out during the light phase between 08.00h and 18.00h. Food and water were available *ad libitum*. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, and carried out under license from the Department of Health in the Republic of Ireland and in accordance with EU Directive 86/609.

2.2 Experimental design

Rats were randomly assigned to one of 4 different groups: SD-Naive, SD-Stress, WKY-Naive, WKY-Stress (n=8 rats per group). SD and WKY rats were tested in separate experiments. Forced swim stress exposure was carried out for 10 minutes on the first day and 20 minutes on the subsequent 9 days (i.e. 10 days swim stress exposure in total; a shorter 3 day exposure was also investigated but was found to have no significant effect on hot plate test latency or formalin-evoked nociceptive behaviour). As previously described in Quintero et al., 2000, the forced swim stress paradigm involved placing the rat inside a Plexiglas cylinder (25-cm diameter, 50-cm height), filled with water ($24\text{--}26^{\circ}\text{C}$) to a 20-cm level. The

water was changed and the cylinder cleaned before and after each animal. Rats in the naive control groups were not exposed to forced swim stress and remained in their home cages during stress exposure sessions.

2.3 Nociceptive responding

2.3.1 Hot plate test

On day 11, 24 hrs after last swim session, naive and forced swim SD and WKY groups were individually placed on the hot plate at $55\pm 2^{\circ}\text{C}$ to assess thermal responding. Licking or lifting/flinching of either hind paw were used as parameters for determining hot plate latency. A cut-off point of 45 seconds was set to avoid injury. The hot plate was cleaned between animals with 0.5% acetic acid.

2.3.2 Formalin-induced nociceptive behaviour

Nociceptive behaviour in the formalin test was assessed for each animal on day 12, 48 hours after the last swim session for swim-stressed groups. All rats received an intra-plantar injection of 100 μL formalin, 1% in 0.9% saline, into the right hind paw. Rats were then placed in a Perspex observation chamber (30 x 30 x 40cm, L x W x H) and behaviour was recorded for 120 min from a video camera located beneath the observation chamber. The chamber was cleaned between animals with 0.5% acetic acid. Behaviour was analysed with the aid of EthoVision®XT7 software (Noldus, The Netherlands) by a rater blind to experimental conditions. Formalin-evoked nociceptive behaviour was categorized as P1 = duration of time for which little or no weight is placed on the formalin-injected paw; P2 = duration of paw elevation; and P3 = duration of licking/biting/shaking/flinching of paw. A

composite pain score (CPS) was obtained from the following equation: $CPS = (P1 + 2(P2) + 3(P3))/(5 \text{ minute time bin})$ according to the method of Dubuisson and Dennis (Dubuisson and Dennis, 1977). Rats were killed by decapitation 120min post-formalin injection and brains and spinal cords (dorsal quadrant of the lumbar enlargement ipsilateral and contralateral to formalin injected paw) were removed rapidly and snap-frozen on dry ice. Brains were stored at -80°C prior to gross dissection of the amygdala. Both amygdala and spinal cord tissue were ground, in pestle and mortar on dry ice, into a homogenate powder and split into two aliquots for endocannabinoid assay by liquid chromatography with tandem mass spectrometry (LC-MS/MS) or mRNA analysis by qRT-PCR.

2.4 Endocannabinoid levels in the amygdala and spinal cord assessed using LC-MS/MS

The concentrations of the endocannabinoids, AEA and 2-AG, as well as the related *N*-acylethanolamines *N*-palmitoylethanolamide and *N*-oleoylethanolamide, were measured in spinal cord and amygdala tissue by LC-MS/MS as described previously (Butler et al., 2012; Ford et al., 2011; Knaster et al., 2012; Olango et al., 2011; Rea et al., 2014). Briefly, tissue was first homogenized in 400µL of 100% acetonitrile containing known fixed amounts of deuterated internal standards (0.014 nmol AEA-d8, 0.48 nmol 2-AG-d8, 0.016 nmol PEA-d4 and 0.015 nmol OEA-d2). Homogenates were centrifuged at 14,000g for 15 min at 4°C and the supernatant was collected and evaporated to dryness in a centrifugal evaporator. Samples were re-suspended in 40µL of 65% acetonitrile and 2 µL were injected onto a Zorbax C-18 column (150 x 0.5 mm internal diameter) from a cooled autosampler maintained at 4°C (Agilent Technologies Ltd., Ireland). Mobile phases consisted of A (high-performance liquid chromatography [HPLC]-grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 12µL/min. Reverse-phase gradient elution began initially at

65% B and over 10 min was ramped linearly up to 100% B. At 10 min, the gradient was held at 100% B up to 20 min. At 20.1 min, the gradient returned to initial conditions for another 10 min to re-equilibrate the column. Analyte detection was carried out in electrospray-positive ionization mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies, Cork, Ireland). Quantitation of each analyte was performed using MassHunter Quantitative Analysis Software (Agilent Technologies, Cork, Ireland). The limit of quantification was 1.32pmol/g and 12.1pmol/g, 1.5 pmol g⁻¹, 1.4 pmol g⁻¹ for AEA, 2-AG, PEA and OEA, respectively. Quantitation of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was performed by Masshunter Quantitative Analysis Software (Agilent Technologies Ltd). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio with a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard.

2.5 Quantitative real-time PCR

qRT-PCR was carried out as described previously (Rea et al., 2014; Burke et al., 2014 and Kerr et al., 2012). RNA was extracted from the tissue using the Machery-Nagel NucleoSpin[®] RNA II extraction kit (Nucleospin RNA II, Technopath, Ireland), according to the manufacturer's instructions. RNA quality (260/280 ratio: 1.8-2) and quantity was assessed using a Nanodrop spectrophotometer (ND-1000, Nanodrop, Labtech International, UK) and samples were normalised for equal RNA quantity. RNA samples were subjected to a reverse transcription step using SuperScript[®] III Reverse Transcriptase (Bio-Sciences, DunLaoghaire, Ireland). Single stranded cDNA products were then analysed by real-time

quantitative PCR using the Applied Biosystems StepOne Plus Real Time PCR System (Applied Biosystems, UK). Taqman gene expression assays (Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probe were used (Bio-Sciences, Ireland). Assay IDs for the genes examined were as follows for rat *CBI* (Rn00562880_m1), *FAAH* (Rn00577086_m1), *MAGL* (Rn00593297_m1), and VIC-labelled *GAPDH* (4308313) as the house keeping gene and endogenous control. A ‘no template’ control (NTC) reaction was included in all assays. The relative expression of target genes to endogenous control was calculated using the formula $2^{-\Delta Ct}$, where ΔCt represents the magnitude of the difference between target and endogenous cycle threshold (Ct) values. mRNA data were expressed as a percentage of the contralateral naive control values for each strain.

2.6 Data analysis

SPSS statistical package (IBM SPSS v19.0 for Microsoft Windows; SPSS, Inc., IL, USA) was used to analyse all data. All data passed normality testing (Shapiro-Wilk test). For each strain, hot plate test data were analysed by Student’s unpaired, two-tailed t-test and formalin-evoked nociceptive behaviour data were analysed using repeated measures analysis of variance (ANOVA) with stress as between-subject factors and time as the within-subject factor. Neurochemical and gene expression data for each strain were analysed using two-way ANOVA with stress and side (ipsilateral versus contralateral) as factors. Pairwise group comparisons were made with Tukey’s post-hoc test where appropriate. Data were considered significant when $P < 0.05$. Results are expressed as group means \pm standard error of the mean (\pm SEM).

3. Results

3.1 Effect of swim-stress on thermal nociceptive responding in SD and WKY rats

Forced swim stress once daily for 10 days had no significant effect on response latency in the hot plate test in either SD or WKY rats, compared with non-stressed naive control rats (Figure 1).

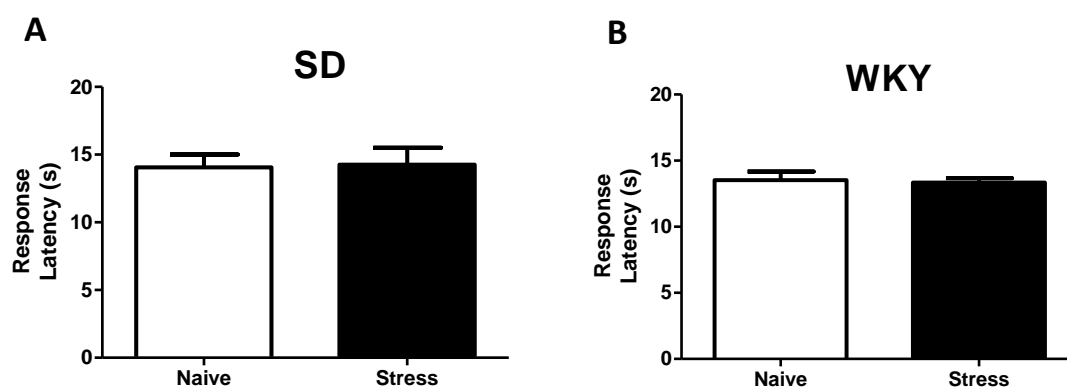


Figure 1. The effects of once daily exposure to forced swim stress for 10 days on response latency in the hot plate test in (A) SD and (B) WKY rats. Data expressed as mean \pm SEM; n=8 per group.

3.2 Effect of swim-stress on formalin-evoked nociceptive behaviour in SD and WKY rats

Following daily exposure to forced swim stress for 10 days, SD and WKY rats received an intra-plantar injection of formalin 48 hours after the last swim stress session and nociceptive

behaviour was assessed from which the CPS was calculated. Data are presented in 5 minute time bins over the 2 hour period of the test (Figure 2). Repeated measures ANOVA revealed a significant effect of time (SD: $F_{23,322}=38.07$ $p<0.01$; WKY: $F_{23,322}=42.63$ $p<0.01$) on formalin-evoked nociceptive behaviour in SD and WKY rats. Post-hoc analysis revealed an increase in formalin-evoked nociceptive behaviour towards the latter part of the 2 hour formalin trial in SD rats exposed to 10 days of swim stress, compared with non-stressed, naive SD rats ($p<0.05$, Figure 2A). In contrast, 10 day swim-stress exposure in WKY rats resulted in a significantly reduced formalin-evoked nociceptive behaviour in the latter part of the formalin trial, compared with non-stressed, naive WKY rats ($p<0.05$, Figure 2B). Thus, swim-stress had a differential effect on formalin-evoked nociceptive behaviour in SD (increase) versus WKY (decrease) rats.

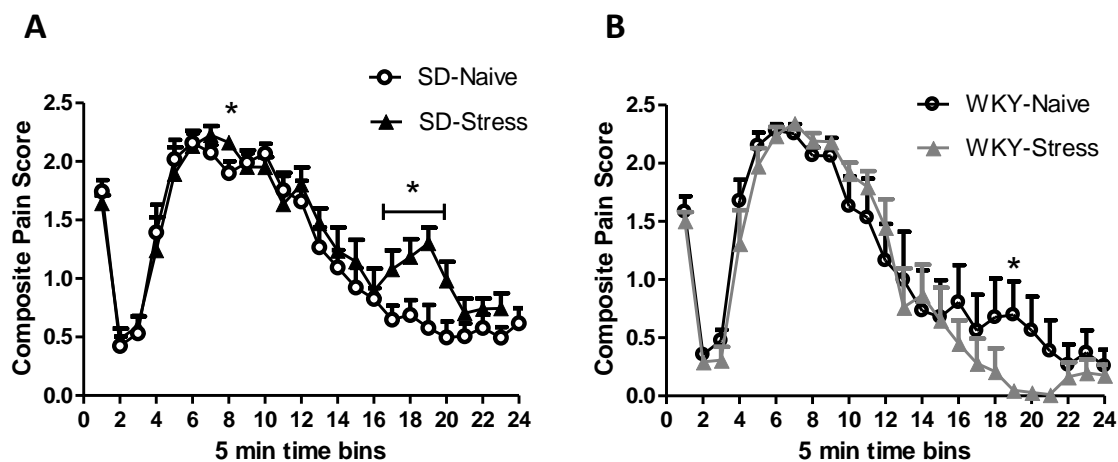


Figure 2. The effects of once daily exposure to forced swim stress for 10 days on formalin-evoked nociceptive behaviour in (A) SD and (B) WKY rats. Data are expressed as mean \pm SEM; $n=8$ per group. $*p<0.05$ for Stress vs. Naive (SD: Sprague-Dawley, WKY:Wistar-Kyoto)

3.3 Post-mortem characterisation of the endocannabinoid system in the dorsal horn of the spinal cord

Levels of endocannabinoids (AEA and 2-AG) were measured in the dorsal quadrant of the lumbar enlargement of the spinal cord, on both the ipsilateral and contralateral sides relative to formalin injection. Repeated swim stress did not significantly alter the levels of AEA or 2-AG in either SD or WKY rats compared with non-stressed, naive controls. There were no significant differences between levels of AEA or 2-AG on the ipsilateral versus contralateral sides of the spinal cord (Figure 3). In addition we also measured the levels of the related *N*-acylethanolamines, PEA and OEA in the dorsal horn of both rat strains. Repeated swim stress had no effect on PEA or OEA levels in SD or WKY rats (data not shown).

There were no significant effects of swim-stress or differences between ipsilateral versus contralateral sides, on CB₁ receptor or FAAH mRNA expression in the spinal cord of SD rats, compared with non-stressed, naive controls (Figure 4A and 4B). However, 2-way ANOVA revealed a significant effect of side ($F_{1,28}=5.18$; $p<0.05$) on levels of MAGL mRNA in SD rats. Post-hoc analysis revealed that MAGL mRNA levels were significantly increased in the ipsilateral side of the spinal cord in swim-stressed SD rats, compared with naive SD controls ($p<0.05$, Figure 4C).

In WKY rats, swim stress did not affect CB₁ receptor, FAAH or MAGL mRNA levels, but there was a significant effect of amygdala side on CB₁ receptor ($F_{1,24}=29.98$; $p<0.01$) and FAAH ($F_{1,26}=31.62$; $p<0.01$) mRNA levels. Post-hoc testing revealed significant increases in

CB₁ receptor and FAAH mRNA levels on the ipsilateral versus the contralateral side of the spinal cord of WKY rats ($p \leq 0.01$, Figure 4D and 4E). These differences were seen in both naive and swim-stressed WKY rats. To further address the potential contribution of changes in astrocytes or microglia to these changes in CB₁ and FAAH expression presented in Figure 4D and 4E of the manuscript, we determined the expression of the astrocytic marker GFAP, and the microglial marker CD11b in the ipsilateral versus contralateral sides of the dorsal spinal cord of WKY rats. There were no significant ipsilateral versus contralateral differences in the GFAP expression between the groups or in CD11b in naive WKY rats (data not shown). CD11b expression was decreased in the ipsilateral side of WKY-stress rats compared with the contralateral side (data not shown), a finding that was in the opposite direction to change in CB₁ receptor and FAAH mRNA expression. Additionally, we did not find any correlation between CB₁ or FAAH with either GFAP or CD11b mRNA expression (data not shown). Collectively these data suggest that there is no relationship between the changes in CB₁ and FAAH mRNA expression presented in Figure 4 D and 4E and changes in markers of astrocytes or microglia.

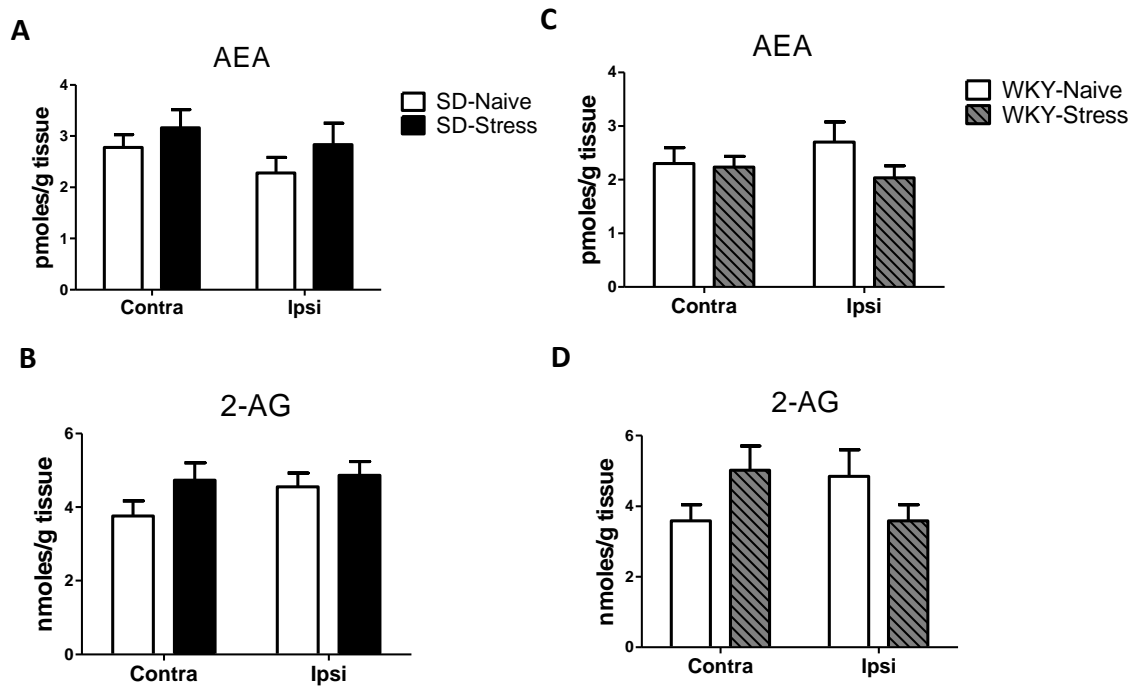


Figure 3. Levels of the endocannabinoids AEA and 2-AG in the ipsilateral and contralateral sides (relative to intra-plantar formalin injection) of the dorsal spinal cord (lumbar enlargement) in formalin-treated SD (A, B) and WKY (C, D) rats that were exposed daily to swim-stress for 10 days, versus naive controls. Data are expressed as mean \pm SEM; n=8 per group. (AEA: anandamide, 2-AG: 2-Arachidonoylglycerol, Contra: contralateral, Ipsi: ipsilateral, SD: Sprague-Dawley, WKY: Wistar-Kyoto).

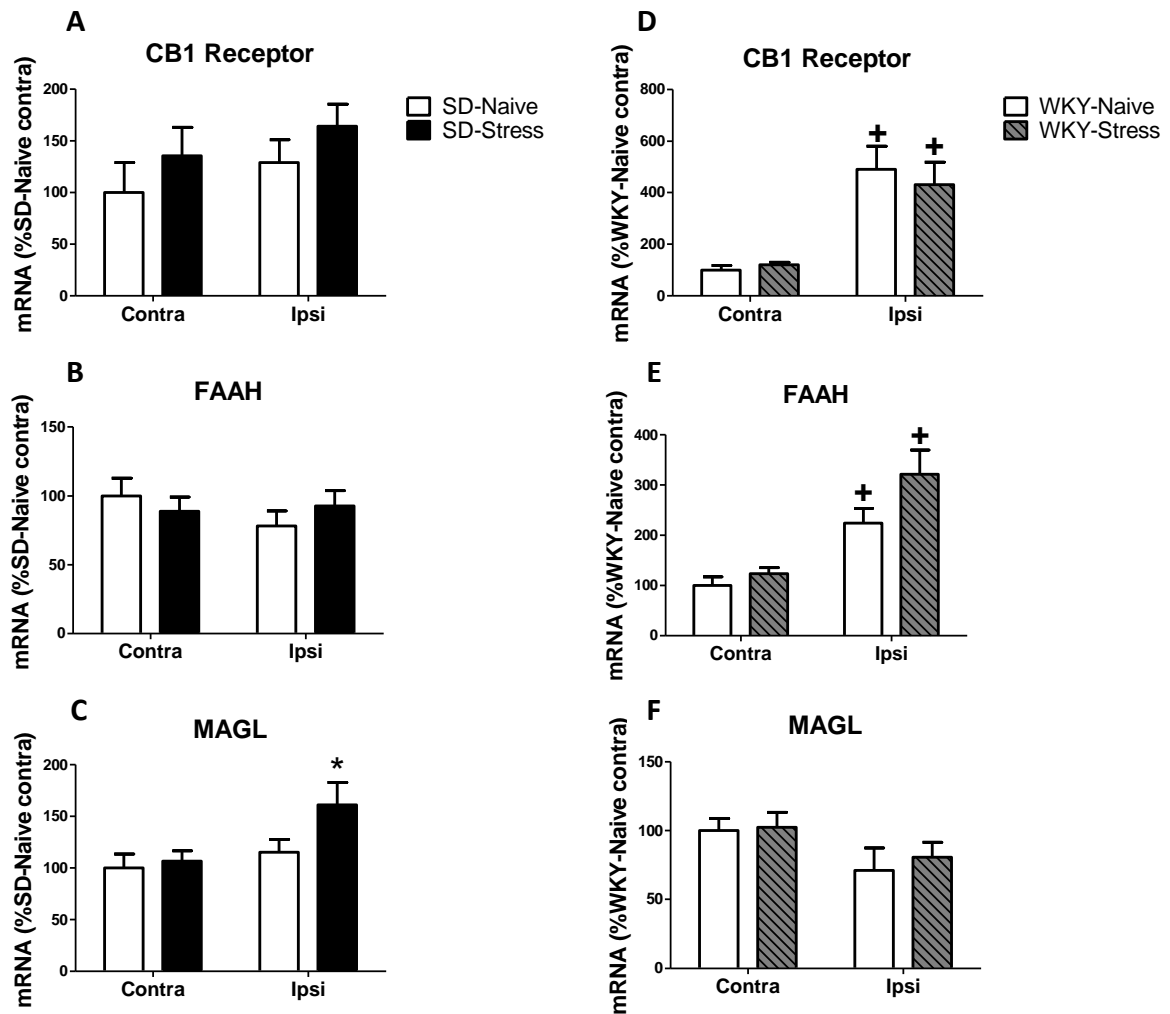


Figure 4. The expression of CB₁, FAAH and MAGL mRNA in the ipsilateral and contralateral sides (relative to intra-plantar formalin injection) of the dorsal spinal cord (lumbar enlargement) in SD (A, B, C) and WKY (D, E, F) rats that were exposed daily to swim-stress for 10 days, versus naive controls. * $p < 0.05$ vs. SD-Naive Ipsi; + $p < 0.05$ vs corresponding group on contra side. Data are expressed as mean \pm SEM; $n = 8$ per group. (CB: cannabinoid receptor, Contra: contralateral, FAAH: fatty acid amide hydrolase, Ipsi: ipsilateral, MAGL: monoacylglycerol lipase, SD: Sprague-Dawley, WKY: Wistar-Kyoto)

3.4 Post-mortem characterisation of the endocannabinoid system in the amygdala

Levels of endocannabinoids and related *N*-acylethanolamines, PEA and OEA were measured in the ipsilateral and contralateral amygdala (relative to intra-plantar formalin injection) in both strains. Two-way ANOVA revealed a significant effect of side ($F_{1,28}=9.80$; $p<0.01$) and stress ($F_{1,28}=8.9$; $p<0.01$) on levels of AEA in SD rats. Swim-stressed SD rats exhibited a significant reduction in AEA levels in the contralateral amygdala, compared with non-stressed naive SD controls ($p<0.05$, Figure 5A). Levels of AEA were significantly lower on the side of the amygdala ipsilateral to formalin injection in naive SD rats, compared with the contralateral side in this group ($p<0.05$, Figure 5A). Two-way ANOVA revealed a significant effect of side ($F_{1,27}=14.82$; $p<0.01$) on levels of 2-AG in SD rats. Levels of 2-AG were significantly higher on the side of the amygdala ipsilateral to formalin injection in both the SD-Naive and SD-Stress groups, compared with the contralateral side in these groups ($p<0.05$, Figure 5B). There were no effects of side or stress on levels of AEA or 2-AG in the amygdala of WKY rats (Figure 5C and 5D). Levels of PEA and OEA were not altered by swim stress and did not differ between amygdala side in either rat strain (data not shown). To determine whether the ipsilateral/contralateral differences in AEA and 2-AG found in SD rats were due to intra-plantar formalin injection, we measured levels of these endocannabinoids in the left and right amygdala of non-formalin treated male SD rats from another experiment in our laboratory where the only treatment that the rats had received was a single intraperitoneal injection for three days with brain tissue collected 4 hours after the final injection. We found no significant differences for AEA (right amygdala: 13.42 ± 0.64 , left amygdala: 14.62 ± 1.2 pmoles/gram tissue; $n=4$ per side) or 2-AG levels (right amygdala: 30.25 ± 2.6 , left amygdala: 33.64 ± 3.6 nmoles/gram tissue; $n=4$ per side).

Swim stress had no significant effect on levels of CB₁ receptor, FAAH or MAGL mRNA in the ipsilateral or contralateral amygdala of SD or WKY rats (Figure 6). Two-way ANOVA revealed a

significant effect of side on levels of CB₁ receptor mRNA in WKY rats ($F_{1,24}=29.98$; $p<0.01$). In naive WKY rats, levels of CB₁ receptor mRNA were significantly lower on the side of the amygdala ipsilateral to formalin injection, compared with the contralateral side ($p<0.05$; Figure 6D). A similar trend was observed in the WKY-Stress group, but just failed to reach statistical significance (Figure 6D).

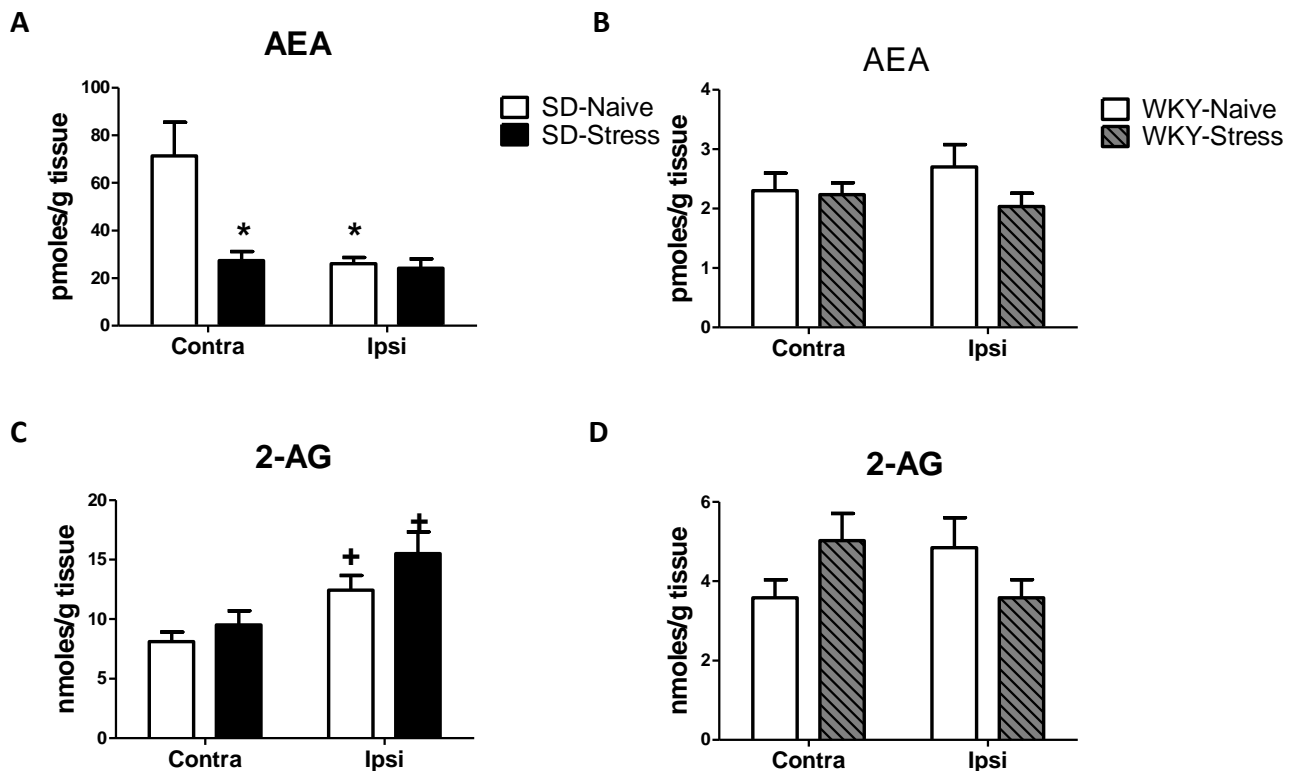


Figure 5. Levels of endocannabinoids AEA and 2-AG in the ipsilateral and contralateral sides of the amygdala (relative to intra-plantar formalin injection) of SD (A,B) and WKY (C,D) rats that were exposed daily to swim-stress for 10 days, versus naive controls. * $p<0.05$ vs. SD-Naive Contra; + $p<0.05$ vs corresponding group on contra side. Data are expressed as mean \pm SEM; $n=8$ per group. (AEA: anandamide, 2-AG: 2-Arachidonoylglycerol, Contra: contralateral, Ipsi: ipsilateral, SD: Sprague-Dawley, WKY: Wistar-Kyoto).

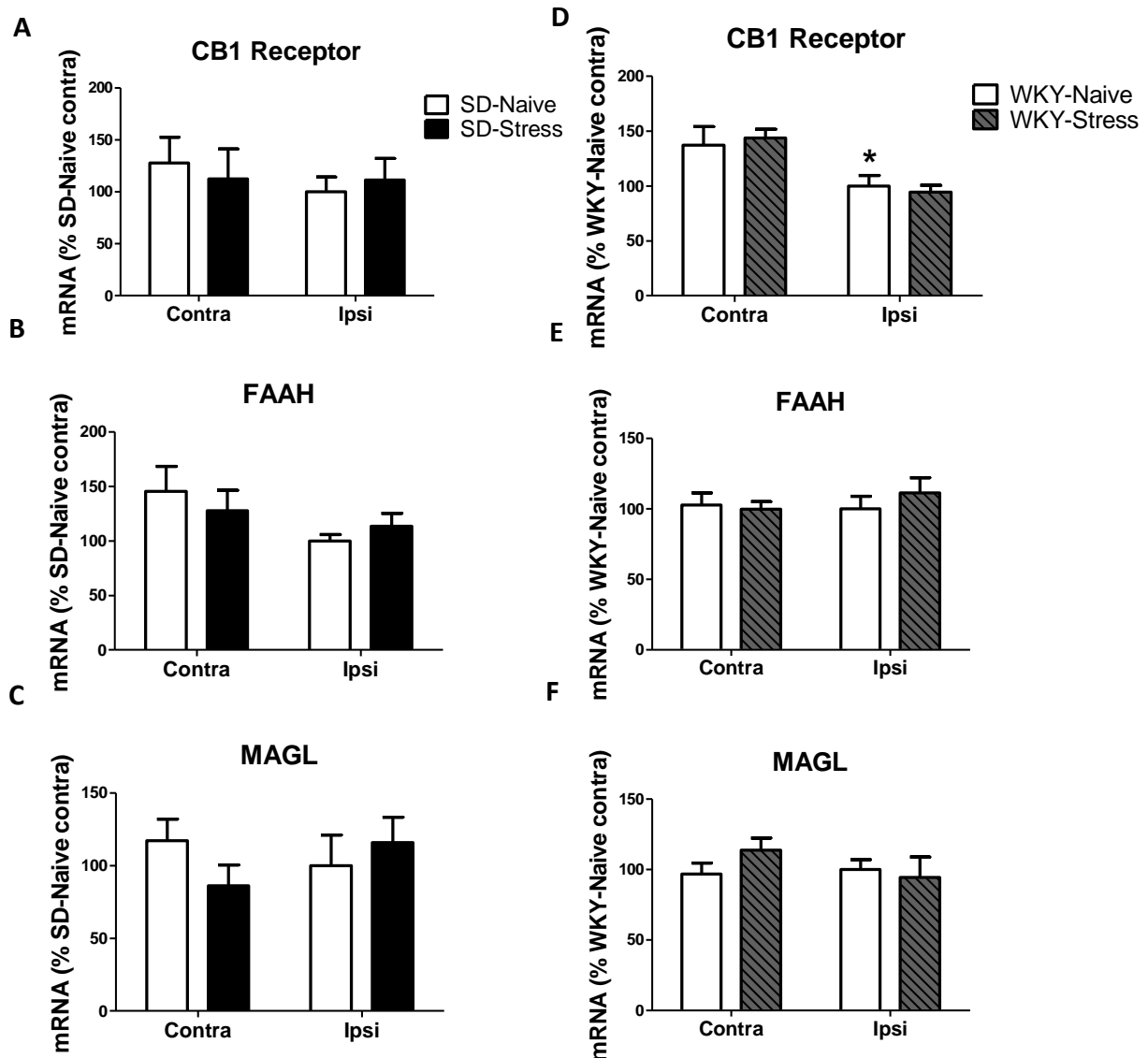


Figure 6. The expression of CB₁, FAAH and MAGL mRNA in ipsilateral and contralateral sides of the amygdala (relative to intra-plantar formalin injection) of SD (A,B,C) and WKY (D,E,F) rats that were exposed to daily swim-stress for 10 days, versus naive control. * $p < 0.05$ vs. SD-Naive. Data expressed as mean \pm SEM; $n = 8$ per group. (CB: cannabinoid receptor, Contra: contralateral, FAAH: fatty acid amide hydrolase, Ipsi: ipsilateral, MAGL: monoacylglycerol lipase, SD: Sprague-Dawley, WKY: Wistar-Kyoto).

4. Discussion

The data presented here suggest that repeated exposure to homotypic stress of forced swimming has differential effects on inflammatory pain-related behaviour in SD versus WKY rats. Specifically, daily exposure to forced swim stress for 10 days increased formalin-evoked nociceptive behaviour towards the end of a 2 hour formalin trial in SD rats, but had the opposite effect in WKY rats. Moreover, neurochemical and molecular analysis of post mortem spinal cord and amygdala tissue also revealed differential effects of formalin and/or swim-stress on components of the endocannabinoid system in the two rat strains. These data indicate that the effects of repeated stress on inflammatory pain-related behaviour are different in two rat strains that differ with respect to stress responsivity and affective state and implicate the endocannabinoid system in the spinal cord and amygdala in these differences.

Daily exposure of SD rats to forced swim stress for 10 days produced a discrete increase in formalin-evoked nociceptive behaviour for 20 minutes during the second phase of the formalin trial. Previously it has been shown that 3 days of swim stress can be sufficient to induce enhanced nociceptive behaviour to formalin injection in SD rats (Quintero et al., 2011; 2003; 2000) however initial experiments in our laboratory failed to demonstrate SIH to formalin after 3 days of swim stress (data not shown) and therefore we used a 10 day exposure in the present study. In contrast to the effects of swim stress in SD rats, swim-stressed WKY rats exhibited a decrease in formalin-evoked nociceptive behaviour, over the same 20 minute period of the second phase as that during which hyperalgesia was seen in SD rats. It is interesting that although WKY rats normally exhibit a hyperalgesic phenotype relative to SD rats (Burke et al., 2010; O'Mahony et al., 2013), exposure to repeated forced swim stress resulted in a hypoalgesic effect in WKY rats. This finding suggests that exposure to repeated homotypic stress has differential effects in WKY versus SD rats on the

neurobiological circuitry and systems that regulate formalin-evoked nociceptive behaviour and supports other studies that have reported differential effects of repeated stress on nociceptive responding in SD versus WKY rats (Robbins et al., 2007). The effects of forced swim stress exposure on pain responding were dependent on the pain modality and/or pain test under investigation because response latency in the hot plate test was unaffected by swim stress in both SD and WKY rats. These results contrast with those of Quintero and colleagues who have previously demonstrated hyperalgesic responses on the hot plate test in swim-stressed SD rats (Quintero et al., 2000). Discrepancies between the work of Quintero et al and our study may be due to differences in the source/supplier of the rats and/or differences in environmental or husbandry conditions in the animal facilities. The influence of external factors such as breeding techniques and environmental and husbandry conditions on behavioural outcomes of animal studies has been well recognised and reported in previous studies. For example variations in anxiety-like behavior and epileptogenesis in outbred female Wistar rats obtained from different breeders have been reported previously (Honndorf et al., 2011). In addition environmental factors such as variations in environmental enrichment or lack thereof can have an impact not only on rodent behaviour but also on brain neurochemistry (for review see review Simpson and Kelly, (2011)). It is well established that early-life stress can alter nocieptive responding in adulthood. Neonatal limited bedding results in reduced muscle nociceptive threshold in adult rats that can be further exacerbated by stress (Alvarez et al., 2013, Green et al., 2011). In addition, supplier and diet can result in variations in the development of hyperalgesia and allodynia in a rat model of neuropthic pain (Perez et al., 2004). Thus, subtle differences in breeding and environmental practices between suppliers and laboratories can have an important impact on behavioural outcomes in animal studies and such differences can account for discrepancies in research findings. Differences between animal suppliers (Harlan UK versus Charles River UK) and/or the fact that the two rat strains were studied in separate experiments herein rather than concurrently probably also

explain why the hyperalgesia that we have reported previously (Burke et al., 2010, Rea et al., 2014) for WKY rats compared with SD rats was not observed in the present study.

As mentioned in the Introduction, the endocannabinoid system plays an important role in pain and stress processes. Exposure to noxious stimuli and stress triggers endocannabinoid release to suppress pain (Walker et al., 1999; Hohmann et al., 2005). Stress-induced dysfunction in the endocannabinoid system can lead to altered responses to stress and functional changes in neural circuitry (Hu et al., 2011; Wamstecker et al., 2010) that could affect endocannabinoid-induced antinociception. Swim stress increased levels of mRNA coding for the 2-AG catabolising enzyme MAGL on the ipsilateral side of the spinal cord of formalin-injected SD rats, but not WKY counterparts. This increase in MAGL mRNA was not seen in the contralateral side of the spinal cord (relative to formalin injection), suggesting that it may reflect an interaction between prior stress exposure and formalin injection. However, although there was increased expression of the MAGL mRNA, no stress-induced changes in levels of 2-AG were detected. This discrepancy likely reflects the different temporal profiles of mRNA transcription (and subsequently translation to protein) versus the rapid on-demand, synthesis, release and degradation of 2-AG. It is also worth remembering that the present study measured tissue levels of 2-AG, rather than the extracellular, signalling pool of 2-AG. Increased levels of MAGL may have resulted in lower levels of extracellular/signalling 2-AG in the spinal cord, which in turn would mean less CB₁ receptor-mediated antinociception, with consequent SIH, as was observed behaviourally.

It has previously been shown that swim stress-induced hyperalgesia in SD is associated with reduced spinal GABA release (Suarez-Roca et al., 2008). Intrathecal administration of a GABA_B receptor agonist, baclofen, produces analgesia but this is attenuated by the CB₁

receptor antagonist, rimonabant suggesting that the spinal antinociceptive effects of GABA_B receptor activation are influenced by CB₁ receptor modulation (Naderi et al., 2005). Thus, if repeated swim stress results in altered endocannabinoid system functioning at the spinal level, this could attenuate GABA-induced antinociception resulting in increased nociceptive responding. In addition, swim stressed and naive WKY rats, but not SD rats, exhibited increases in CB₁ receptor and FAAH mRNA expression on the side of the spinal cord ipsilateral to formalin injection. Further studies are required to determine the extent to which these alterations may contribute to the behavioural differences observed between the two strains.

Several studies highlight the role of the endocannabinoid system in the amygdala in pain (Hasanein et al., 2007; Ji and Neugebauer, 2014) and its modulation by stress (Connell et al., 2006; Hill et al., 2013). Thus, we assessed the endocannabinoid system in the amygdala in the present study. We found that swim stress resulted in reduced AEA levels in the contralateral amygdala of SD rats, but not WKY rats, a result that could also potentially account for the differential effects of swim stress on formalin-evoked nociceptive behaviour between the two strains. Direct injection of a CB₁ agonist into the amygdala produces antinociception in rats (Hasanein et al., 2007). Thus, this reduction in AEA levels may play an important role in the enhanced pain behaviour of stressed rats. Restraint stress in rats has been shown to reduce levels of AEA in the amygdala, an effect that was inversely correlated with increased serum corticosterone levels (Hill et al., 2009). In addition, direct injection of the FAAH inhibitor, URB597, into the basolateral amygdala attenuated stress-induced increases in circulating corticosterone (Hill et al., 2009). Maladaptive swim stress-induced changes in amygdala endocannabinoid signalling may underlie the hyperalgesic effects of repeated swim stress in SD rats. That this alteration in AEA levels was not observed in WKY

rats supports our previous findings of differential endocannabinoid system responsivity in SD versus WKY rats (Rea et al., 2014).

Differences in levels/expression of components of the endocannabinoid system on the contralateral versus ipsilateral sides of the amygdala (relative to formalin injection) were also observed in WKY versus SD rats. We observed lower levels of AEA and higher levels of 2-AG in the ipsilateral amygdala compared with the contralateral amygdala of SD, but not WKY, rats. Moreover, in WKY rats, lower levels of CB₁ receptor mRNA were observed in the ipsilateral (right) amygdala than in the contralateral (left) side; this difference was not seen in the SD rats. These differences between left and right amygdala may be due to either formalin-induced changes or hemispheric lateralisation of the endocannabinoid system in the amygdala of WKY rats versus SD rats. Hemispheric lateralisation of neuronal activity in the amygdala is seen after the introduction of a noxious stimulus and may explain the side differences. Studies have found that there is functional lateralisation in the amygdala, with the right more important in pain processing (Ji and Neugebauer, 2009; Kolber et al., 2010) and the left in emotional modulation (Phelps et al., 2001). To examine this, we measured AEA and 2-AG levels in non-formalin treated SD rats. We found no differences between the right and left amygdala suggesting that formalin injection most probably accounts for the side differences reported herein.

The clinical relevance of alterations in central endocannabinoid signalling to the development of SIH remains largely unknown due to the paucity of clinical studies within the field. However, evidence from clinical studies does suggest a link between alterations in levels of circulating endocannabinoids and the development of chronic pain conditions that are often associated with stress-related psychiatric disorders. For example, patients with irritable bowel syndrome had higher levels of plasma 2-AG (Fichna et al., 2013) and fibromyalgia patients

had higher levels of circulating AEA compared to healthy controls (Kaufmann et al., 2008). Furthermore, patients with post traumatic stress disorder have higher AEA and 2-AG levels in circulating plasma compared to controls (Hauer et al., 2013). Collectively, these data further substantiate endocannabinoid signalling as a target for the future development of therapeutic agents for more effective treatment of stress-related pain and psychiatric disorders, and their co-morbidity.

In conclusion, exposure of SD rats to 10 days of swim stress causes enhanced formalin-evoked nociceptive behaviour that is associated with increased ipsilateral spinal expression of the endocannabinoid-catabolising enzyme MAGL and a reduction in levels of AEA in the left amygdala. Conversely, exposure of WKY rats to the same swim stress paradigm reduced formalin-evoked nociceptive behaviour, with no associated effect on the spinal or amygdalar endocannabinoid system. Stress-induced and side-related (ipsilateral versus contralateral) alterations in the endocannabinoid system at the level of the spinal cord and amygdala may contribute to the differential effects of swim stress on nociceptive behaviour in WKY versus SD rats.

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