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**The endocannabinoid system in the rat dorsolateral periaqueductal grey
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Running title: Endocannabinoids, PAG and conditional analgesia

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Summary

Background and purpose: Endocannabinoids in the midbrain periaqueductal grey (PAG) are involved in modulating nociception and unconditioned stress-induced analgesia, however, their role in fear-conditioned analgesia (FCA) has not been examined. The present study examined the role of the endocannabinoid system in the dorsolateral (dl) PAG in formalin-evoked nociceptive behaviour, conditioned fear and FCA in rats.

Experimental approach: Rats received intra-dlPAG administration of the CB₁ receptor antagonist/inverse agonist rimonabant, or vehicle, prior to re-exposure to a context paired 24hrs previously with footshock. Formalin-evoked nociceptive behaviour and fear-related behaviours (freezing and 22kHz ultrasonic vocalisation) were assessed. In a separate cohort, alterations in levels of endocannabinoids (2-arachidonoyl glycerol [2-AG] and *N*-arachidonoyl ethanolamide [anandamide; AEA]) and the related *N*-acylethanolamines (NAEs) (*N*-palmitoyl ethanolamide [PEA] and *N*-oleoyl ethanolamide [OEA]) were measured in dlPAG tissue following re-exposure to conditioned context in the presence or absence of formalin-evoked nociceptive tone.

Key results: Re-exposure of rats to the context previously associated with footshock resulted in FCA. Intra-dlPAG administration of rimonabant significantly attenuated FCA and fear-related behaviours expressed in the presence of nociceptive tone. Conditioned fear in the absence of formalin-evoked nociceptive tone was associated with increased levels of the endocannabinoids (2-AG and AEA) and NAEs (PEA and OEA) in the dlPAG. FCA was specifically associated with an increase in AEA levels in the dlPAG.

Conclusions and implications: These data suggest that conditioned fear to context mobilises endocannabinoids and NAEs in the dlPAG and support a role for the endocannabinoid system in the dlPAG in mediating the potent suppression of pain responding which occurs during exposure to conditioned aversive contexts.

Keywords: pain; fear; cannabinoid type 1 (CB₁) receptor; endocannabinoids; *N*-acylethanolamines; periaqueductal grey; rats

Abbreviations

2-AG, 2-arachidonyl glycerol; AEA, anandamide; CB₁, cannabinoid type 1 receptor; CB₂, cannabinoid type 2 receptor; CPS, composite pain score; DMSO, dimethylsulfoxide; FAAH, fatty acid amide hydrolyase; FCA, fear-conditioned analgesia; FC, fear conditioned; MAGL, monoacylglycerol lipase; NAEs, *N*-acylethanolamines; NoFC, non-fear conditioned; OEA, *N*-oleoyl ethanolamide; PAG, periaqueductal grey; PEA, *N*-palmitoylethanolamide; Rim, rimonabant; Veh, vehicle

Introduction

Though pain is part of a global defence response initiated upon exposure to noxious stimuli, maladaptive persistent/chronic pain is a major unmet clinical need. Conditioned fear is known to suppress nociceptive behaviour potently, resulting in fear-conditioned analgesia (FCA), the phenomenon by which re-exposure of an animal to a context previously paired with an aversive stimulus (e.g. footshock) results in conditional analgesia (Butler *et al.*, 2009; Ford *et al.*, 2008). A large body of evidence suggests overlap in the neural substrates mediating pain and conditioned fear. Recent studies have also described significant co-morbidity of anxiety disorders with persistent pain conditions (Asmundson *et al.*, 2009) and altered pain processing in patients with anxiety disorders, including post-traumatic stress disorder (Geuze *et al.*, 2007; Kraus *et al.*, 2009). In light of this evidence, detailed understanding of the neurobiology underpinning the relationship between fear and pain is of fundamental physiological and potential therapeutic significance.

The periaqueductal grey (PAG) is a mesencephalic structure that can be divided into four columns along its rostro-caudal axis: the dorsomedial, dorsolateral, lateral and ventrolateral columns (Bandler *et al.*, 1996). The PAG is a key component both of the circuitry responsible for anxiety-related defence responses (Amorapanth *et al.*, 1999; Bandler *et al.*, 1985; Carrive *et al.*, 1999; Carrive *et al.*, 1997; Krieger *et al.*, 1985; LeDoux, 1998; Schenberg *et al.*, 1990) and of the descending inhibitory pain pathway (Helmstetter *et al.*, 1998; Millan, 2002; Oliveira *et al.*, 2001; Pavlovic *et al.*, 1998). The PAG is also known to play a key role in mediating analgesia induced by stress or fear, with direct administration of the μ -opioid receptor antagonist naltrexone into the ventrolateral PAG attenuating FCA in rats (Helmstetter *et al.*, 1990). The

dorsolateral PAG (dlPAG) is also known to be important in the descending inhibitory control of pain (Haghparast *et al.*, 2009; McMullan *et al.*, 2006; Waters *et al.*, 2008) and modulation of aversive responses (Bertoglio *et al.*, 2005; Brandão *et al.*, 1999; Canteras *et al.*, 1999; Fontani *et al.*, 1983; Klein *et al.*, 2010; Lino-de-Oliveira *et al.*, 2006; Lisboa *et al.*, 2008; Moreira *et al.*, 2007; Resstel *et al.*, 2008). Furthermore, lesions of the dlPAG (Helmstetter *et al.*, 1994; Kinscheck *et al.*, 1984) have been shown to reduce or abolish the expression of FCA in rats, and stimulation of the dorsal PAG can be used to induce FCA which is attenuated by injection of the benzodiazepine midazolam into this region (Castilho *et al.*, 2002).

The endogenous cannabinoid system is a novel lipid signalling system comprised of at least two G-protein coupled cannabinoid receptors (CB₁ and CB₂) (Gerard *et al.*, 1991; Matsuda *et al.*, 1990), endogenous ligands or so-called 'endocannabinoids' that bind to and activate the cannabinoid receptors, the two best characterised being 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoyl ethanolamide (anandamide; AEA) (Devane *et al.*, 1992; Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995) and the enzymes regulating the biosynthesis and degradation of these endocannabinoids. Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are two key enzymes catalysing the degradation of AEA and 2-AG, respectively (Ahn *et al.*, 2008). Additional fatty acid amides related to the endocannabinoids include the *N*-acylethanolamines, *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) (Walker *et al.*, 2002) which, despite having little or no affinity for, or activity at, the CB receptors, are believed to enhance endocannabinoid signalling by competing with AEA for the catalytic site of FAAH (Cravatt *et al.*, 2001; Cravatt *et al.*, 1996). The endocannabinoid system has recently emerged as an important

modulator of many neural functions including the control of fear- and pain-related behaviour (Finn, 2010; Guindon *et al.*, 2009; Moreira *et al.*, 2009).

A role for the endocannabinoid system in the suppression of pain responding during or following exposure to either unconditioned or conditioned stress has been demonstrated. For example, our previous work has shown that FCA modelled in rats by assessing formalin-evoked nociceptive behaviour in a context previously paired with footshock, is prevented by systemic administration of the CB₁ receptor antagonist/inverse agonist rimonabant (Finn *et al.*, 2004) and enhanced by systemic administration of the FAAH inhibitor URB597 (Butler *et al.*, 2008). Work by Hohmann and colleagues has demonstrated an important role for the endocannabinoid system in the dlPAG in mediating unconditioned stress-induced analgesia expressed as a suppression of tail-flick responding following exposure of rats to unconditioned footshock stress (Hohmann *et al.*, 2005). Specifically, this form of analgesia evoked by unconditioned physical stress was blocked by intra-dlPAG administration of the CB₁ receptor antagonist/inverse agonist rimonabant and enhanced by intra-dlPAG administration of the FAAH inhibitors, arachidonoyl serotonin (AA-5-HT) and URB597, or the MAGL inhibitor, URB602 (Hohmann *et al.*, 2005; Suplita *et al.*, 2005). However, the role of the endocannabinoid system in the PAG in analgesia induced by conditioned psychological stress/fear (FCA) has not been examined. In addition, no studies to date have investigated the role of the endocannabinoid system in the PAG in fear expressed in the presence of nociceptive tone. Thus, we sought here to determine the role of the endocannabinoid system in the dlPAG in the expression of FCA, formalin-evoked nociceptive behaviour *per se* and fear expression in the presence of formalin-evoked nociceptive tone. The results suggest that conditioned fear to context mobilises endocannabinoids and NAEs in the dlPAG and

support a role for the endocannabinoid system in the dlPAG in mediating the potent suppression of pain responding which occurs during exposure to conditioned aversive contexts.

Methodology

Animals

Experiments were carried out on adult male Lister-Hooded rats (240–310g; Charles River, Kent, UK) 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 17.00h. Food and water were available *ad libitum*. The experimental protocol was carried out following approval from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Department of Health and Children in the Republic of Ireland and in accordance with EU Directive 86/609.

Drug preparation

The CB₁ receptor antagonist/inverse agonist rimonabant (SR141716A; (N- [piperidin-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1-H-pyrazole-3-carboxamide], NIMH Chemical Synthesis Programme Batch 10937-163-1) was prepared on day of use to a concentration of 0.4nmol / 0.2 μ l (2mM) in DMSO (dimethylsulfoxide, 100%). This concentration of rimonabant was chosen based on pilot studies in our laboratory and previous work demonstrating that it attenuates unconditioned stress-induced analgesia in rats when injected into the dorsal PAG (Hohmann et al., 2005; Suplita et al., 2005).

Cannula implantation

Under isoflurane (2-3% in O₂, 0.5L/min) anaesthesia, a stainless steel guide cannula (9mm length, Plastics One Inc., Roanoke, Virginia, USA) was stereotaxically implanted 1mm above the right dlPAG of each rat (coordinates: AP = - 6.3mm from bregma, ML = + 1.9mm at an angle of 16°, DV = 4mm from the meningeal dura matter according to the rat brain atlas published by Paxinos and Watson (1997). The cannulae were permanently fixed to the skull using stainless steel screws and carboxylate cement. A stylet made from stainless steel tubing (Plastics One Inc., Roanoke, Virginia, USA) was inserted into the guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (1.25mg/25µL, s.c., Rimadyl, Pfizer, Kent, UK), was administered before the surgery to manage postoperative analgesia. Animals received a single daily dose of the antimicrobial agent enrofloxacin (10mg/kg, s.c., Batyryl, Bayer plc, Berkshire, UK) for 5 days to prevent postoperative infection. Following cannula implantation, the rats were housed singly and at least 6 days were allowed for recovery post surgery prior to experimentation. During this recovery period, the rats were handled, and their body weight and general health monitored once daily.

Experimental procedure

The experimental procedure was essentially as described previously (Butler *et al.*, 2008; Finn *et al.*, 2004; Finn *et al.*, 2006; Roche *et al.*, 2009; Roche *et al.*, 2007). In brief, it consisted of two phases, conditioning and testing, occurring 24h apart. Subjects were randomly assigned to groups, and the sequence of testing was randomized in order to minimize any confounding effects of testing procedure. On the conditioning day, rats were placed in a Perspex fear conditioning / observation

chamber (30 x 30 x 40cm), and after 15s received the first of 10 footshocks spaced 60s apart (0.4mA, 1s duration; LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK). Fifteen seconds after the last footshock, rats were returned to their home cage. Controls not receiving footshock were exposed to the chamber for an equivalent 9.5 min period.

Experiment 1: The test phase commenced 23.5h later when the subjects received an intraplantar injection of 50 μ L formalin (2.5% in 0.9% saline) into the right hind-paw under brief isoflurane anaesthesia (3% in O₂; 0.5L/min). Rats were returned to their home cage for a further 15min, after which time they received a single intra-dIPAG microinjection (0.2 μ L) of rimonabant (0.4nmol) or vehicle (100% DMSO) using an injector and Hamilton syringe as described previously (Finn et al., 2003, Roche et al., 2007).

This design resulted in four experimental groups: fear-conditioning + vehicle (FC-Veh); fear-conditioning + rimonabant (FC-Rim); no-fear-conditioning + vehicle (NoFC-Veh); and no-fear-conditioning + Rimonabant (NoFC-Rim). Following intra-dIPAG injection, rats were returned to their home cage until 30min post-formalin injection after which time they were placed back in the perspex observation chamber to which they had been exposed during the conditioning phase. A bat detector (Batbox Duet, Batbox, Steyning, West Sussex, UK) was used to detect ultrasonic vocalization in the 22kHz range, and behaviours were recorded for 15min with the aid of a video camera located beneath the observation chamber. The 30–45min post-formalin interval was chosen on the basis of previous studies demonstrating robust suppression of formalin-evoked nociceptive behaviour upon re-exposure to an aversively conditioned context during this part of the second phase formalin response

(Finn *et al.*, 2004; Finn *et al.*, 2006; Rea *et al.*, 2009; Roche *et al.*, 2009; Roche *et al.*, 2007), and our previous work demonstrating that such fear-conditioned analgesia expressed during this period is CB₁ receptor-mediated (Butler *et al.*, 2008; Finn *et al.*, 2004).

Rats were decapitated at the end of the test trial and 0.2µL 2% fast-green dye (dissolved in DMSO) was microinjected via the guide cannula to mark the site of injection. Following removal of the brain, a block of tissue either side of the injection site (PAG) was removed, snap-frozen on dry ice and stored at -80°C for subsequent histological verification of cannula positioning in the right dlPAG.

Experiment 2: A separate cohort of rats underwent a similar experimental protocol to that described above but without intra-PAG cannulation or administration of rimonabant. This design comprised a conditioning phase on day 1 followed 23.5hrs later by intra-plantar injection of formalin or saline as described above. Rats were returned to their home cage until 30min post-intraplantar injection, after which they were placed back in the Perspex observation chamber to which they had been exposed during the conditioning phase. This design resulted in four experimental groups: fear-conditioning + Saline (FC-Sal); fear-conditioning + formalin (FC-Form); no-fear-conditioning + Saline (NoFC-Sal) and no-fear-conditioning + Formalin (NoFC-Form). Behaviours were recorded for 3min, following which animals were removed, decapitated and the brain removed rapidly, snap-frozen on dry ice and stored at -80°C for subsequent cryo-sectioning and collection of dlPAG tissue for quantitation of endocannabinoids and the entourage NAEs. The 3 minute post-fear induction time-point was chosen based on the data from Experiment 1 demonstrating robust

expression of FCA at this time-point and published work demonstrating fear-induced increases in brain endocannabinoid concentrations at this time-point (Marsicano *et al.*, 2002).

Histology

The site of injection was determined prior to data analysis. The block of tissue containing the PAG was cryo-sectioned (30µm), and the brain sections with green dye mark were mounted on glass slides and counter-stained with creysl violet in order to determine the precise location of the site of microinjection using a light microscope.

Behavioural analysis

Behaviour was analysed using the Observer XT 7.0 software package (Noldus Technology, Wageningen, the Netherlands), which allowed for continuous event recording over the duration of the trial. A trained observer blind to the experimental conditions assessed behaviour including the duration of freezing (defined as the cessation of all visible movement except that necessary for respiration), duration of 22kHz ultrasound emission, and general behaviours (walking, rearing and grooming). Formalin-evoked nociceptive behaviour was scored according to the weighted composite pain scoring (CPS) technique described by Watson *et al.* (1997). According to this method, pain behaviours are categorized 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 [$CPS = (C1 + 2(C2)) / (\text{total duration of analysis period})$]. Post-formalin oedema was assessed by measuring the hind-paw diameter before and after formalin injection using Vernier callipers.

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

Frozen coronal brain sections (300µm) containing the dIPAG from rats in Experiment 2 were cut on a cryostat. Tissue from the right dIPAG was punched from the frozen sections (between bregma -5.6mm and bregma -7.64mm) (Paxinos & Watson, 1998) using cylindrical brain punchers (Harvard Apparatus, internal diameter 2mm). Each punched tissue sample was kept frozen throughout the collection procedure, weighed: (average weight of punched tissue = 7.5mg) and stored at -80°C prior to extraction for and determination of the concentrations of the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) and the related *N*-acylethanolamines (NAEs) or so-called “entourage compounds” *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Tissue extraction was carried out using a lipid extraction method as follows: Each brain tissue sample was first homogenised in 400µL 100% acetonitrile containing known fixed amounts of deuterated internal standards (0.014 nmol AEA-d8, 0.48nmol 2-AG-d8, 0.016nmol PEA-d4, 0.015nmol OEA-d2). Homogenates were centrifuged at 14,000 g for 15 minutes at 4°C and the supernatant was collected and evaporated to dryness in a centrifugal evaporator. Lyophilised samples were re-suspended in 40µL 65% acetonitrile and 2µL were injected onto a Zorbax® C18 column (150 × 0.5mm internal diameter) from a cooled autosampler maintained at 4°C (Agilent Technologies Ltd, Cork, Ireland). Mobile phases consisted of A (HPLC grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 12µL/min. Reversed-phase gradient elution began initially at 65% B and over 10min was ramped linearly up to 100% B. At 10min, the gradient was held at

100% B up to 20min. At 20.1min, the gradient returned to initial conditions for a further 10mins to re-equilibrate the column. The total run time was 30min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 11.36min, 12.8min, 14.48min and 15.21min respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Instrument conditions and source parameters including fragmentor voltage and collision energy were optimised for each analyte of interest prior to assay of samples. Quantitation of target endocannabinoids was achieved by positive ion electrospray ionization and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [M + H⁺] of the analytes of interest and the deuterated forms of the internal standards. Quantitation of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was performed using Masshunter Quantitative Analysis Software (Agilent Technologies Ltd, Cork, Ireland). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard. Linearity (regression analysis determined R^2 values of 0.99 or greater for each analyte) was determined over a range of 18.75ng to 71.5fg except for 2-AG which was 187.5ng-715fg. The limit of quantification was 1.32pmol/g, 12.1pmol/g, 1.5pmol/g, 1.41pmol/g for AEA, 2-AG, PEA and OEA respectively.

Statistical analysis

The SPSS 17.0 statistical package was used to analyse all data. Behavioural data from the test day and neurochemical data were analysed using two-factor analysis of variance (ANOVA), with the factors being fear-conditioning and drug (Experiment 1) or fear-conditioning and formalin (Experiment 2). Post-hoc pairwise comparisons were made with Fisher's LSD when appropriate. Data were considered significant when $P < 0.05$. Results are expressed as group means \pm standard error of the mean (\pm SEM).

Results

Experiment 1

Histological verification of injector placement

Eighty percent of the injections were placed within the borders of the right dlPAG (Fig. 1) with the remaining 20% positioned in the superior colliculus, ventral PAG or dorsomedial PAG. Only the results of experiments in which injections were correctly positioned in the dlPAG were included in the analysis.

Effect of intra-dlPAG administration of rimonabant on formalin-induced nociceptive behaviour and FCA

Intra-plantar injection of formalin induced right hind-paw oedema (change in paw diameter in NoFC-Veh group = $1.35\text{mm} \pm 0.28$), and produced robust licking, biting, shaking, flinching and elevation of the injected paw as indicated by the CPS during the entire 15min trial period (Fig. 2). Re-exposure of rats to the arena previously

paired with footshock resulted in a significant reduction of formalin-evoked nociceptive behaviour, compared with non-fear-conditioned, vehicle-treated counterparts (CPS: FC-Veh vs. NoFC-Veh, $p < 0.05$, Fig. 2), confirming expression of FCA. Intra-dlPAG administration of rimonabant (Rim) significantly attenuated FCA (FC-Veh vs. FC-Rim, $p < 0.05$; Fig. 2) without altering formalin-evoked nociceptive behaviour in non-fear-conditioned rats, thus indicating a specific effect on FCA rather than on nociceptive behaviour *per se*.

Effect of intra-dlPAG administration of rimonabant on conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

Non-fear-conditioned rats displayed little or no contextually induced freezing (Fig. 3A) or 22kHz ultrasonic vocalisation (Fig. 3B) during the test trial. In contrast, fear-conditioning was associated with significant increases in the duration of freezing and 22kHz ultrasonic vocalisation upon re-exposure to the context (NoFC-Veh vs. FC-Veh, $p < 0.01$, Fig. 3A and 3B). Intra-dlPAG administration of rimonabant significantly reduced the duration of contextually-induced freezing and 22kHz ultrasonic vocalisation (FC-Veh vs. FC-Rim, $p < 0.01$).

Effects of fear conditioning or rimonabant on locomotor activity and defecation in formalin-treated rats

Fear-conditioning was associated with a significant reduction in the duration of locomotor activity measured as the sum of time spent rearing, grooming and walking, (NoFC-Veh vs. FC-Veh, $p < 0.01$; Table 1) and a concurrent increase in defecation (NoFC-Veh vs. FC-Veh, $p < 0.01$; Table 1) during re-exposure to the context. Intra-

dIPAG administration of rimonabant had no significant effect on these fear-induced alterations and had no significant effect *per se* in non-fear-conditioned rats (Table 1).

Experiment 2

Effects of fear-conditioning and intra-plantar formalin injection on tissue levels of endocannabinoids and related N-acylethanolamines in the dIPAG

Non-fear-conditioned rats receiving intra-plantar saline displayed little or no nociceptive behaviour or contextually-induced fear behaviour during the 3min test trial (Fig. 4(A-C)). In comparison, intra-plantar injection of formalin resulted in right hindpaw oedema (Change in paw diameter: NoFC-Sal 0.9 ± 0.09 mm vs. NoFC-Form 1.66 ± 0.07 mm; $P < 0.001$) and significant nociceptive responding (CPS) (NoFC-Sal vs. NoFC-Form, $P < 0.01$; Fig. 4A). Levels of endocannabinoids (AEA and 2-AG) and the entourage N-acylethanolamines (PEA and OEA) in the right dIPAG of either non-fear-conditioned (NoFC-Sal vs. NoFC-Form) or fear-conditioned (FC-Sal vs. FC-Form) rats were not significantly altered following formalin administration, compared with rats receiving intra-plantar saline (Fig.4(D-G)).

FCA was expressed over the 3min trial (FC-Form vs. NoFC-Form, $P < 0.05$; Fig. 4A). Levels of AEA in the right dIPAG were significantly increased in those rats expressing FCA when compared with formalin-injected rats that were not fear-conditioned (FC-Form vs. NoFC-Form, $P < 0.05$; Fig. 4D).

Contextual fear-conditioning was associated with significant increases in the duration of freezing and 22kHz ultrasonic vocalisation in both saline-injected (NoFC-Sal vs. FC-Sal, $P < 0.01$; Fig. 4B and 4C) and formalin-injected (NoFC-Form vs. FC-Form, P

< 0.01; Fig. 4B and 4C) rats re-exposed to the context. The increased duration of freezing in fear-conditioned rats was partially attenuated by intra-plantar formalin administration (FC-Sal *vs.* FC-Form, $P < 0.05$, Fig. 4B). In the absence of nociceptive tone (i.e. in saline-treated rats), conditioned fear was associated with increased levels of endocannabinoids (AEA and 2-AG) and the entourage compounds (PEA and OEA) in the right dlPAG (NoFC-Sal *vs.* FC-Sal, $p < 0.05$, Fig. 4(D-G)). In comparison, in the presence of formalin-evoked nociceptive tone, conditioned fear was associated with a significant ($P < 0.05$) increase in levels of AEA only (NoFC-Form *vs.* FC-Form; Fig. 4[D-G]).

Discussion

The present study demonstrated that the CB₁ receptor antagonist/inverse agonist rimonabant, injected directly into the right dlPAG, prevents conditioned fear-induced suppression of formalin-evoked nociceptive behaviour which results following re-exposure of rats to a context previously paired with aversive footshock (i.e. prevents fear-conditioned analgesia; FCA). This blockade of FCA by intra-PAG rimonabant was accompanied by a rimonabant-induced attenuation of conditioned fear responding in the presence of formalin-evoked nociceptive tone. Expression of conditioned fear *per se* was associated with increased tissue levels of AEA, 2-AG, PEA and OEA in the right dlPAG and expression of FCA was specifically associated with increased levels of AEA in this region. Together, these results represent the first demonstration of an important role for the endocannabinoid system in the dlPAG in mediating analgesia induced by conditioned psychological stress/fear and in regulating fear expression during pain responding.

The suppression of formalin-evoked nociceptive behaviour observed here upon re-exposure to a context previously paired with footshock is similar in its nature and magnitude to previous reports demonstrating FCA using related or identical paradigms (Finn *et al.*, 2004; Helmstetter *et al.*, 1987; Roche *et al.*, 2007). Systemic (i.p.) administration of rimonabant has previously been shown to prevent FCA in rats (Finn *et al.*, 2004). Furthermore, our recent work has demonstrated enhancement of FCA following systemic administration of the FAAH inhibitor URB597, and blockade of this URB597-induced enhancement by rimonabant (Butler *et al.*, 2008). We now demonstrate for the first time that direct administration of rimonabant into the right dIPAG prevents the fear-induced suppression of formalin-evoked nociceptive responding in rats without affecting the formalin-evoked response in non-fear-conditioned rats, confirming a specific effect on FCA. The endocannabinoid system has also been shown to mediate a form of unconditioned stress-induced analgesia in a rat model which combines the tail-flick test of acute nociceptive responding with unconditioned footshock stress (Hohmann *et al.*, 2005). Hohmann and colleagues also demonstrated a key role for the endocannabinoid system in the dIPAG in mediating this form of unconditioned stress-induced analgesia (Hohmann *et al.*, 2005). Their work demonstrated that intra-dIPAG administration of rimonabant attenuated unconditioned stress-induced analgesia while intra-dIPAG administration of FAAH or MAGL inhibitors enhanced unconditioned stress-induced analgesia (Hohmann *et al.*, 2005). Our results here, demonstrating a similar attenuation of FCA following intra-dIPAG administration of rimonabant, support the findings of Hohmann and colleagues and extend our understanding by suggesting that the endocannabinoid system in the dIPAG also plays a role in analgesia resulting from

exposure to Pavlovian conditioned psychological stress. Previous studies suggest differences with respect to rimonabant's effects in the basolateral amygdala in unconditioned versus conditioned stress-induced analgesia (Connell *et al.*, 2006, Roche *et al.*, 2007, 2009). It appears, however, that the dlPAG is a common neural substrate for endocannabinoid-mediated analgesia induced by exposure to either unconditioned or conditioned stress. Moreover, our data provide the first evidence of a role for the endocannabinoid system in the dlPAG in the modulation of tonic, persistent inflammatory pain by stress.

The PAG is critically involved in coordinating the defence response to aversive stimuli (Amorapanth *et al.*, 1999; Bandler *et al.*, 1985; Carrive *et al.*, 1999; Carrive *et al.*, 1997; Krieger *et al.*, 1985; LeDoux *et al.*, 1988; Schenberg *et al.*, 1990; Vianna *et al.*, 2003) and there is good evidence that endocannabinoid signalling in the PAG plays an important role in the modulation of behavioural responses to unconditioned (Bortolato *et al.*, 2006; Kathuria *et al.*, 2003; Lafenêtre *et al.*, 2007; Lisboa *et al.*, 2008; Moreira *et al.*, 2007; Patel *et al.*, 2006) and conditioned (Broiz *et al.*, 2008; Chhatwal *et al.*, 2007; Fendt *et al.*, 1996; Finn *et al.*, 2004; Lafenêtre *et al.*, 2007; Lisboa *et al.*, 2008; Marsicano *et al.*, 2002; Resstel *et al.*, 2008) stress. The present experimental design enabled assessment of conditioned fear responding in the presence of formalin-evoked nociceptive tone. Our results revealed that attenuation of FCA by intra-dlPAG rimonabant was associated with a rimonabant-induced attenuation of conditioned fear responding, measured as the duration of contextually induced freezing and 22kHz ultrasonic vocalization. This result corroborates previous reports of an inverse relationship between fear and pain responding (Butler *et al.*, 2008; Fanselow *et al.*, 1988; Finn *et al.*, 2004; Helmstetter, 1993; Roche *et al.*, 2009)

and provides novel evidence that CB₁ receptors in the dlPAG may represent a key neural substrate regulating the reciprocal relationship shared by fear and pain. Previous work has shown that intra-PAG administration of the CB₁ receptor antagonist/inverse agonist, AM251, blocked the anxiolytic effects of exogenous AEA (Moreira *et al.*, 2007) and prevented the attenuation of conditioned fear responses elicited by exogenous AEA (Resstel *et al.*, 2008) but failed to produce an effect on anxiety or fear responses by itself (Moreira *et al.*, 2007; Resstel *et al.*, 2008). It is possible that endocannabinoids in the dlPAG have a differential effect on fear responses depending on the presence or absence of nociception. Importantly, our data also demonstrate that while fear-induced suppression of nociceptive behaviour was prevented by intra-dlPAG rimonabant, fear-induced suppression of general locomotor/exploratory behaviour was not. These data suggest that the fear-induced suppression of formalin-evoked behaviour, and its blockade by intra-dlPAG rimonabant, represent specific effects on nociception rather than non-specific effects on general locomotor activity.

To further investigate the neurochemical mechanisms underpinning the behavioural effects observed we measured tissue concentrations of the endocannabinoids, AEA and 2-AG, and the related 'entourage' NAEs, PEA and OEA, in the right dlPAG of rats sacrificed 3min following re-exposure to context, a time-point where maximal expression of fear-related behaviour and FCA was noted. We report a fear-related increase in levels of AEA and 2-AG in the right dlPAG at this time point. Previously, Hohmann and colleagues have shown increased levels of 2-AG and AEA in the rat dlPAG 2-7min and 7-25min, respectively, following footshock (Hohmann *et al.*, 2005). Moreover, re-exposure to a 3min tone paired previously with footshock

resulted in increased AEA and 2-AG levels in the basolateral amygdala of mice (Marsicano *et al.*, 2002). Our data support and extend these findings by demonstrating that Pavlovian conditioned fear to context mobilises endocannabinoids in the dlPAG. We also observed fear-related increases in the non-endocannabinoid NAEs, OEA and PEA, in the dlPAG. Though Hill *et al.* (2009) demonstrated that peripheral NAEs are responsive to stress, to our knowledge, the present results represent the first report on the effects of conditioned fear on levels of these ‘entourage’ compounds in the brain. Though themselves devoid of significant activity at the CB₁ receptor, by competing as substrates for FAAH, OEA and PEA may in turn enhance the actions of AEA at CB₁ by limiting its degradation. It seems reasonable to speculate that the fear-related increases in one or more of these lipids in the dlPAG may play a key role in mediating FCA, and that the rimonabant-induced blockade of FCA may be mediated by a blockade of the actions of AEA and/or 2-AG on CB₁ receptors in this region. Interestingly, our results revealed that although there was a fear-related elevation in all 4 analytes in rats not receiving intra-plantar formalin injection, only AEA displayed a fear-related elevation in rats that received intra-plantar injection of formalin. These results suggest that elevations in AEA accompany the expression of FCA and that it may be the key endocannabinoid in the dlPAG mediating the rimonabant-sensitive expression of FCA, possibly through activation of CB₁ receptors known to be expressed in the PAG (Herkenham *et al.*, 1991; Tsou *et al.*, 1997) with subsequent disinhibition of output neurons and activation of the descending inhibitory pain pathway (de Novellis *et al.*, 2005; Vaughan *et al.*, 2000). It is also possible that alternative targets such as transient receptor potential vanilloid 1 channels (TRPV1) could mediate the effects of AEA in the dlPAG on FCA. There is good evidence for an important role of AEA activity at

TRPV1 in the PAG in the regulation of both pain (Palazzo et al, 2008; Maione et al 2006) and aversion (Moreira et al, 2009; Terzian et al, 2009). However, Suplita et al. (2005) showed that TRPV1 was not involved in mediating unconditioned stress-induced analgesia in rats. In addition, our results here demonstrate that intra-dlPAG administration of rimonabant prevented FCA completely rather than partially, suggesting that CB₁ receptor signalling in the dlPAG is necessary and sufficient for the expression of fear-conditioned analgesia in rats. Future studies in our laboratory will investigate the role of TRPV1, and indeed other non-CB₁ targets of endocannabinoids (e.g. peroxisome proliferator activated receptors [PPARs] and GPR55) in FCA.

Intra-dlPAG levels of 2-AG, OEA and PEA were significantly increased in saline injected rats following fear conditioning, whereas, in formalin treated rats, levels of these same analytes were not significantly altered by fear conditioning (though some trends towards an increase were seen). These data suggest differential effects of conditioned fear on levels of 2-AG, OEA and PEA in the presence versus absence of nociceptive tone. The mechanism(s) responsible for such a state-dependent alteration in the responsivity of these analytes to conditioned fear are unknown, however it does not appear to be due to formalin-evoked alterations in absolute levels of the analytes since there were no significant effects of intra-plantar formalin injection on tissue concentrations of any of the 4 analytes in the dlPAG under the present experimental conditions. These results differ from previous studies which have reported pain-related increases in tissue levels of endocannabinoids in discrete brain regions including the PAG. For example, in rodents, mechanical allodynia and thermal hyperalgesia following spinal nerve ligation were accompanied by increased levels of

AEA and 2-AG in the PAG, rostral ventromedial medulla and dorsal raphe magnus (Mitrirattanakul *et al.*, 2006; Petrosino *et al.*, 2007). Using an approach employing *in vivo* microdialysis, Walker *et al.* (1999) demonstrated increased levels of extracellular AEA in the rat dorsal and lateral PAG following formalin injection. However, direct comparisons between these earlier studies and the present study are difficult to make due to differences in the models used (spinal nerve ligation versus formalin test), dose of the formalin administered (4%, 150µl into both hind paws *vs.* 2.5%, 50µl into right hind paw), time-points and sub-regions assayed and method of analysis (microdialysis *vs.* tissue levels).

In conclusion, the results reported here provide evidence to support the contention that the endocannabinoid system in the dlPAG is a key neural substrate mediating analgesia expressed during or following exposure to stress, including that evoked by Pavlovian conditioned fear to context. Pharmacological blockade of CB₁ receptors in the dlPAG prevented FCA and reduced fear responding in the presence of nociceptive tone. Furthermore, the results suggest state-dependent alterations in the effects of fear on 2-AG, OEA and PEA in the dlPAG dependent on nociceptive tone. Together these data suggest a key role for the endocannabinoid system in the dlPAG in endogenous analgesia and modulation of fear responding during pain.

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Tables

Table 1. Effects of fear conditioning and intra-dIPAG administration of rimonabant on locomotor activity

Group	Locomotor activity(S)	Defecation (Number of pellets)
NoFC- Veh	205.25±27.91	0±0
NoFC- Rim	273.25±53	0±0
FC-Veh	80.44±16.93**	3.25±0.81**
FC-Rim	89.25±19.76	2±0.5

Effects of fear conditioning and intra-dIPAG administration of rimonabant on locomotor activity (measured as the sum of time spent rearing, grooming and walking) and defecation in rats during a 15 min re-exposure to an observation chamber which was paired with footshock 24h previously. ** $p < 0.01$ vs. corresponding NoFC control; Two-way ANOVA locomotor activity (effect of fear conditioning $F_{(1,29)} = 23.3$, $p < 0.001$); defecation: (effect of fear conditioning $F_{(1,29)} = 26.8$, $p < 0.001$). All data are expressed as Mean \pm SEM (n=6-9). FC, fear-conditioned; NoFC, non-fear conditioned; Veh, vehicle; Rim, rimonabant

Figure Legends

Fig. 1 Schematic depicting the sites of injection of (A) vehicle (100% DMSO) or (B) the CB₁ receptor antagonist/inverse agonist rimonabant in the right dorsolateral periaqueductal grey (dlPAG). FC, fear-conditioned; NoFC, non-fear conditioned. Adapted from Paxinos & Watson (1997)

Fig. 2 Effect of fear-conditioning and intra-dlPAG administration of rimonabant (Rim, 0.4nmol/0.2μL) on formalin-evoked nociceptive behaviour in rats during a 15min re-exposure to an observation chamber paired 24h previously with footshock. * $p < 0.05$ vs. NoFC-Veh; + $p < 0.05$ vs. FC-Veh (Fisher's LSD post hoc test following ANOVA: drug x fear conditioning $F_{(1,29)} = 5.16$, $p = 0.03$). Data expressed as Mean \pm SEM (n=6-9). CPS, composite pain score; FC, fear-conditioned; NoFC, non-fear conditioned; Veh, vehicle; Rim, rimonabant

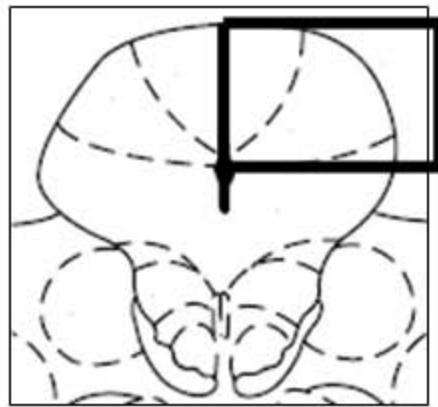
Fig. 3 Effects of fear-conditioning and intra-dlPAG administration of rimonabant (0.4nmol/0.2μL) on (A) the duration of freezing [ANOVA: drug $F_{(1,29)} = 13.85$, $p = 0.001$; fear-conditioning $F_{(1,29)} = 48.55$, $p < 0.001$; drug x fear conditioning interaction $F_{(1,29)} = 9.75$, $p = 0.005$] and (B) the duration of 22kHz ultrasonic vocalisation [ANOVA: drug $F_{(1,29)} = 8.92$, $p = 0.01$; fear-conditioning $F_{(1,29)} = 33.45$, $p < 0.001$, drug x fear-conditioning $F_{(1,29)} = 8.97$, $p = 0.01$] in formalin-injected rats

during the 15min re-exposure to an observation chamber paired 24h previously with footshock. ** $p < 0.01$ vs. NoFC-Veh ; ++ $p < 0.01$ vs. FC-Veh (Fisher's LSD); b); Data expressed as Mean \pm SEM (n=6-9). FC, fear-conditioned; NoFC, non-fear conditioned; Veh, vehicle; Rim, rimonabant

Fig. 4 Effects of fear-conditioning and intra-plantar formalin, alone or in combination, on behaviour and endocannabinoid concentrations in the right dlPAG over the 3-min trial period A) formalin-evoked nociceptive behaviour and FCA (ANOVA: formalin: $F_{(1,44)}=22.77$, $p < 0.001$; fear-conditioning: $F_{(1,44)}=11.85$, $p=0.001$ and formalin x fear-conditioning interaction: $F_{(1,44)}=7.13$, $p=0.011$), B) duration of freezing (ANOVA: fear-conditioning: $F_{(1,44)}=209.62$, $p < 0.001$; formalin: $F_{(1,44)}=6.83$, $p=0.012$) and C) duration of 22kHz ultrasonic vocalisation (ANOVA: fear-conditioning $F_{(1,44)}=27.50$, $p < 0.001$) and on levels of D) AEA (ANOVA: Fear-conditioning: $F_{(1,20)}=16.506$, $p=0.001$; formalin: $F_{(1,20)}=0.035$, $p=0.853$; fear-conditioning x formalin: $F_{(1,20)}=0.332$, $p=0.571$ E) 2-AG (ANOVA: Fear-conditioning: $F_{(1,20)}=6.168$, $p=0.022$; form: $F_{(1,20)}=0.557$, $p=0.464$; form x fear-conditioning $F_{(1,20)}=2.201$, $p=0.154$ and G) OEA (ANOVA: fear-conditioning: $F_{(1,20)}=7.82$, $p=0.011$; form: $F_{(1,20)}=0.94$, $p=0.344$; fear-conditioning x form: $F_{(1,20)}=0.138$, $p=0.714$) in the right dlPAG 3min following re-exposure to an observation chamber paired 24h previously with footshock. * $p < 0.05$, ** $p < 0.01$ vs. NoFC-Sal, + $p < 0.05$, ++ $p < 0.01$ vs NoFC-Form, & $p < 0.05$ vs. FC-Sal (Fisher's LSD); Data expressed as Mean \pm SEM (n=12, A-C; n=5-6, D-G). CPS, composite pain score; FC, fear-conditioned; NoFC, non-fear conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, *N*-palmitoyl ethanolamide; OEA, *N*-oleoyl ethanolamide.

Statement of conflicts of interest

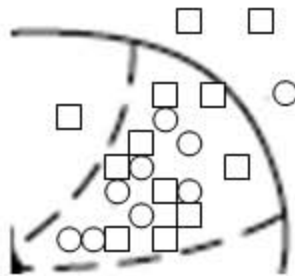
None.



Bregma -6.30mm

○ NoFC
□ FC

A) Vehicle



B) Rimonabant

