British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01478.x
www.brjpharmacol.org

Themed Section: Cannabinoids in Biology and Medicine, Part II

RESEARCH PAPER

The endocannabinoid system in the rat dorsolateral periaqueductal grey mediates fear-conditioned analgesia and controls fear expression in the presence of nociceptive tone

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Keywords
pain; fear; cannabinoid type 1 (CB_1) receptor; endocannabinoids; *N*-acylethanolamines; periaqueductal grey; rats

Received
17 March 2011
Accepted
19 April 2011

BACKGROUND AND PURPOSE

Endocannabinoids in the midbrain periaqueductal grey (PAG) modulate 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 CB_1 receptor antagonist/inverse agonist rimonabant, or vehicle, before re-exposure to a context paired 24 h previously with foot shock. Formalin-evoked nociceptive behaviour and fear-related behaviours (freezing and 22 kHz ultrasonic vocalization) were assessed. In a separate cohort, 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 foot shock resulted in FCA. Intra-dlPAG administration of rimonabant significantly attenuated FCA and fear-related behaviours expressed in the presence of nociceptive tone. Conditioned fear without formalin-evoked nociceptive tone was associated with increased levels of 2-AG, AEA, PEA and OEA in the dlPAG. FCA was specifically associated with an increase in AEA levels in the dlPAG.

CONCLUSIONS AND IMPLICATIONS

Conditioned fear to context mobilises endocannabinoids and NAEs in the dlPAG. These data support a role for endocannabinoids in the dlPAG in mediating the potent suppression of pain responding which occurs during exposure to conditioned aversive contexts.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids in Biology and Medicine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.165.issue-8. To view Part I of Cannabinoids in Biology and Medicine visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

Abbreviations

2-AG, 2-arachidonyl glycerol; AEA, anandamide; CPS, composite pain score; dl, dorsolateral; DMSO, dimethylsulphoxide; FAAH, fatty acid amide hydrolyase; FC, fear conditioned; FCA, fear-conditioned analgesia; MAGL, monoacylglycerol lipase; NAEs, *N*-acylethanolamines; NoFC, non-fear conditioned; OEA, *N*-oleoyl ethanolamide; PAG, periaqueductal grey; PEA, *N*-palmitoylethanolamide

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. foot shock) results in conditional analgesia (Ford and Finn, 2008; Butler and Finn, 2009). 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 and Katz, 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 and Keay, 1996). The PAG is a key component both of the circuitry responsible for anxiety-related defence responses (Bandler *et al.*, 1985; Krieger and Graeff, 1985; Schenberg *et al.*, 1990; Carrive *et al.*, 1997; 1999; LeDoux, 1998; Amorapanth *et al.*, 1999) and of the descending inhibitory pain pathway (Helmstetter *et al.*, 1998; Pavlovic and Bodnar, 1998; Oliveira and Prado, 2001; Millan, 2002). 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 and Landeira-Fernandez, 1990). The dorsolateral PAG (dlPAG) is also known to be important in the descending inhibitory control of pain (McMullan and Lumb, 2006; Waters and Lumb, 2008; Haghparast and Ahmad-Molaei, 2009) and modulation of aversive responses (Fontani and Meucci, 1983; Brandão *et al.*, 1999; Canteras and Goto, 1999; Bertoglio and Zangrossi, 2005; Lino-de-Oliveira *et al.*, 2006; Moreira *et al.*, 2007; Lisboa *et al.*, 2008; Resstel *et al.*, 2008; Klein *et al.*, 2010). Furthermore, lesions of the dlPAG (Kinscheck *et al.*, 1984; Helmstetter and Tershner, 1994) 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 (CB_1 and CB_2; receptor nomenclature follows Alexander *et al.*, 2011) (Matsuda *et al.*, 1990; Gerard *et al.*, 1991), endogenous ligands or so-called 'endocannabinoids' that bind to and activate the cannabinoid receptors, the two best characterized 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 (NAEs), *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) (Walker *et al.*, 2002) that, 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.*, 1996; 2001). The endocannabinoid system has recently emerged as an important modulator of many neural functions including the control of fear- and pain-related behaviour (Guindon and Hohmann, 2009; Moreira *et al.*, 2009; Finn, 2010).

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 foot shock, is prevented by systemic administration of the CB_1 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 *et al* 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 foot shock stress (Hohmann *et al.*, 2005). Specifically, this form of analgesia evoked by unconditioned physical stress was blocked by intra-dlPAG administration of rimonabant and enhanced by intra-dlPAG administration of the FAAH inhibitors, arachidonoyl 5-HT (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 that occurs during exposure to conditioned aversive contexts.

Methods

Animals

All animal care and experimental protocols were in accordance with EU Directive 86/609 and approved by 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. Experiments were carried out on adult male Lister–Hooded rats (240–310 g; Charles River, Kent, UK) maintained at a constant temperature (21 ± 2°C) under standard lighting conditions (12:12 h light : dark, lights on from 07:00 to 19:00 h). All experiments were carried out during the light phase between 08:00 h and 17:00 h. Food and water were available *ad libitum*.

Drug preparation

The CB_1 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 dissolved on the day of use at a concentration of 2 mM in dimethylsulphoxide (DMSO, 100%). This concentration of rimonabant was chosen based on pilot studies in our laboratory and previous work demonstrating that it attenuated 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 O_2, 0.5 L·min^{-1}) anaesthesia, a stainless steel guide cannula (9 mm length, Plastics One Inc., Roanoke, VA) was stereotaxically implanted 1 mm above the right dlPAG of each rat (coordinates: AP = −6.3 mm from bregma, ML = +1.9 mm at an angle of 16°, DV = 4 mm 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.) was inserted into the guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (1.25 mg/25 µL, s.c., Rimadyl, Pfizer, Kent, UK), was administered before the surgery to provide postoperative analgesia. Animals received a single daily dose of the antimicrobial agent enrofloxacin (10 mg·kg^{-1}, s.c., Batyril, 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 after surgery before experimentation. During this recovery period, the rats were handled, and their body weight and general health were monitored daily.

Experimental procedure

The experimental procedure was essentially as described previously (Finn *et al.*, 2004; 2006; Roche *et al.*, 2007; 2010; Butler *et al.*, 2008). In brief, it consisted of two phases, conditioning and testing, occurring 24 h apart. Animals 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 × 30 × 40 cm), and after 15 s received the first of 10 foot shocks spaced 60 s apart (0.4 mA, 1 s duration; LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK). Fifteen seconds after the last foot shock, rats were returned to their home cage. Controls not receiving foot shock were exposed to the chamber for an equivalent 9.5 min period.

Experiment 1. The test phase commenced 23.5 h later when the animals received an intra-plantar injection of 50 µL formalin (2.5% in 0.9% saline) into the right hind paw under brief isoflurane anaesthesia (3% in O_2; 0.5 L·min^{-1}). Rats were returned to their home cage for a further 15 min, after which time they received a single intra-dlPAG microinjection (0.2 µL) of rimonabant (0.4 nmol) 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-dlPAG injection, rats were returned to their home cage until 30 min after the formalin injection when 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 22 kHz range and behaviours were recorded for 15 min with the aid of a video camera located beneath the observation chamber. The 30–45 min 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; 2006; Roche *et al.*, 2007; 2010; Rea *et al.*, 2009), and our previous work demonstrating that such FCA expressed during this period is CB_1 receptor-mediated (Finn *et al.*, 2004; Butler *et al.*, 2008).

Rats were decapitated at the end of the test trial and 0.2 µL 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 23.5 h later by intra-plantar injection of formalin or saline as described above. Rats were returned to their home cage until 30 min after intra-plantar injection, when 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 3 min and then the animals were removed, decapitated and the brains 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 min 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 before histological analysis. The block of tissue containing the PAG was cryosectioned (30 µm), and the brain sections marked with green dye were mounted on glass slides and counterstained with cresyl 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, unaware of 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 22 kHz 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)) / (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 related NAEs in dlPAG tissue using liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Frozen coronal brain sections (300 µm) containing the dlPAG from rats in Experiment 2 were cut on a cryostat. Tissue from the right dlPAG was punched from the frozen sections (between bregma, –5.6 mm and bregma, –7.64 mm) (Paxinos and Watson, 1997) using cylindrical brain punchers (Harvard Apparatus, Edenbridge, Kent, UK internal diameter 2 mm). Each punched tissue sample was kept frozen throughout the collection procedure, weighed (average weight of punched tissue = 7.5 mg) and stored at –80°C before extraction for and determination of, the concentrations of the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) and the related NAEs *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) by LC-MS/MS. Tissue extraction was carried out using a lipid extraction method as follows: each brain tissue sample was first homogenized in 400 µL 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, 0.015 nmol OEA-d2). Homogenates were centrifuged at 14 000× *g* for 15 min at 4°C, and the supernatant was collected and evaporated to dryness in a centrifugal evaporator. Lyophilized samples were resuspended in 40 µL 65% acetonitrile, and 2 µL was injected onto a Zorbax® C18 column (150 × 0.5 mm 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 12 µL·min^{-1}. Reversed-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 a further 10 min to re-equilibrate the column. The total run time was 30 min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 11.36 min, 12.8 min, 14.48 min and 15.21 min respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd). Instrument conditions and source parameters including fragmentor voltage and collision energy were optimised for each analyte of interest before 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). 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 18.75 ng to 71.5 fg except for 2-AG, which was 187.5 ng–715 fg. The limit of quantification was 1.32 pmol·g^{-1}, 12.1 pmol·g^{-1}, 1.5 pmol·g^{-1}, 1.41 pmol·g^{-1} for AEA, 2-AG, PEA and OEA respectively.

Statistical analysis

The SPSS 17.0 statistical package (SPSS Ireland, Dublin, Ireland) 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 ± standard error of the mean (±SEM).

Materials

The standard and deuterated forms of AEA, 2-AG, PEA and OEA were obtained from Cayman Chemical Company, Tallinn, Estonia.

Results

Experiment 1

Histological verification of injector placement. Eighty percent of the injections were placed within the borders of the right dlPAG (Figure 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 mm ± 0.28) and produced robust licking, biting, shaking, flinching and elevation of the injected paw as indicated by the CPS during the entire 15 min trial period (Figure 2). Re-exposure of rats to the arena previously paired with foot shock resulted in a significant reduction of formalin-evoked nociceptive behaviour, compared with non-fear-conditioned, vehicle-treated counterparts (CPS: FC-Veh vs. NoFC-Veh, $P < 0.05$, Figure 2), confirming expression of FCA. Intra-dlPAG administration of rimonabant significantly attenuated FCA (FC-Veh vs. FC-Rim, $P < 0.05$; Figure 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 (Figure 3A) or 22 kHz ultrasonic vocalization (Figure 3B) during the test trial. In contrast, fear conditioning was associated with significant increases in the duration of freezing and 22 kHz ultrasonic vocalization upon re-exposure to the context (NoFC-Veh vs. FC-Veh, $P < 0.01$, Figure 3A and B). Intra-dlPAG administration of rimonabant significantly reduced the duration of contextually induced freezing and 22 kHz ultrasonic vocalization (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-dlPAG 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).

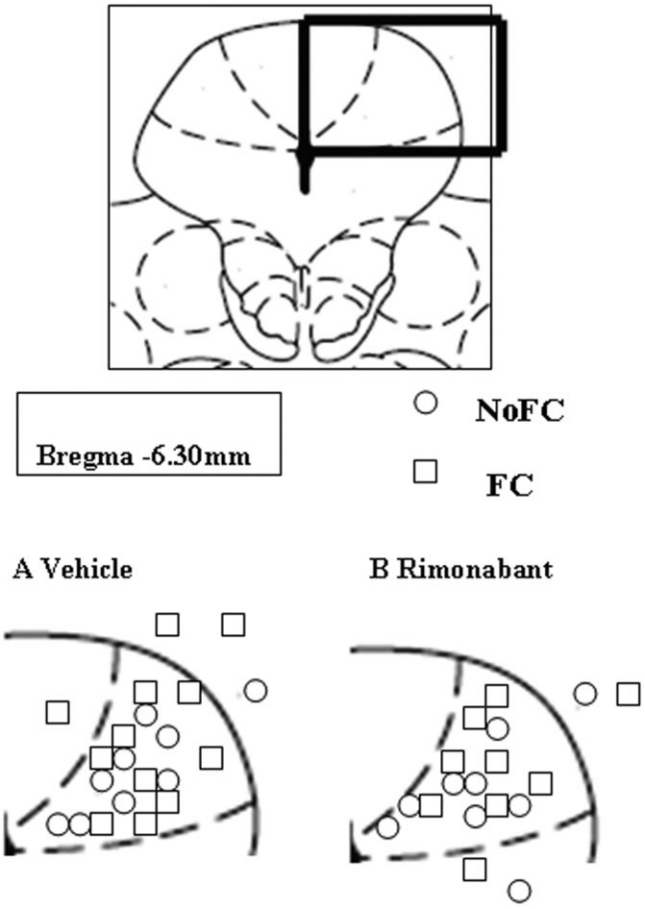

Figure 1

Diagram of the sites of injection of (A) vehicle (100% DMSO) or (B) the CB_1 receptor antagonist/inverse agonist rimonabant in the right dlPAG. Adapted from Paxinos and Watson (1997).

Experiment 2

Effects of fear conditioning and intra-plantar formalin injection on tissue levels of endocannabinoids and related NAEs in the dlPAG. Non-fear-conditioned rats receiving intra-plantar saline displayed little or no nociceptive behaviour or contextually induced fear behaviour during the 3 min test trial (Figure 4A–C). In comparison, intra-plantar injection of formalin resulted in right hind paw 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, $P < 0.01$; Figure 4A). Levels of endocannabinoids (AEA and 2-AG) and the related NAEs

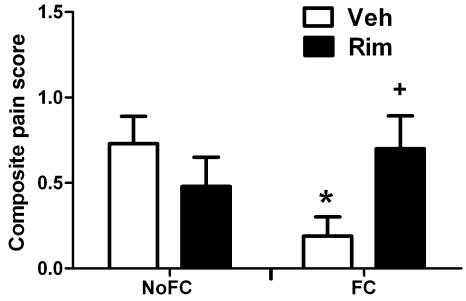

Figure 2

Effect of fear conditioning and intra-dlPAG administration of rimonabant (Rim, 0.4 nmol/0.2 μL) on formalin-evoked nociceptive behaviour in rats during a 15 min re-exposure to an observation chamber paired 24 h previously with foot shock. *P < 0.05 significantly different from NoFC-Veh; ^{+}P < 0.05 significantly different from FC-Veh (Fisher's LSD *post hoc* test following ANOVA: drug × fear conditioning; $F_{(1,29)}$ = 5.16, P = 0.03). Data expressed as mean ± SEM (n = 6–9).

(PEA and OEA) in the right dlPAG 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 (Figure 4D–G).

FCA was expressed over the 3 min trial (FC-Form vs. NoFC-Form, P < 0.05; Figure 4A). Levels of AEA in the right dlPAG 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; Figure 4D).

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

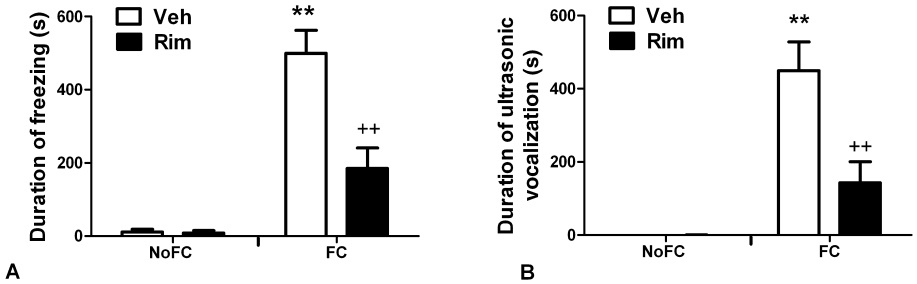

Figure 3

Effects of fear conditioning and intra-dlPAG administration of rimonabant (Rim; 0.4 nmol/0.2 μL) 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 × fear conditioning interaction $F_{(1,29)}$ = 9.75, P = 0.005) and (B) the duration of 22 kHz ultrasonic vocalization (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 15 min re-exposure to an observation chamber paired 24 h previously with foot shock. ** P < 0.01 significantly different from NoFC-Veh; ^{++}P < 0.01 significantly different from FC-Veh (Fisher's LSD) Data expressed as mean ± SEM (n = 6–9).

Table 1

Effects of fear conditioning and intra-dlPAG administration of rimonabant on locomotor activity

Group	Locomotor activity(s)	Defecation (number of pellets)
NoFC- Veh	205.3 ± 27.9	0 ± 0
NoFC- Rim	273.3 ± 53	0 ± 0
FC-Veh	80.4 ± 16.9**	3.25 ± 0.81**
FC-Rim	89.3 ± 19.8	2 ± 0.5

Effects of fear conditioning (FC) and intra-dlPAG administration of rimonabant (Rim) 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 that was paired with foot shock 24 h previously. **P < 0.01 significantly different from 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 ± SEM (n = 6–9).

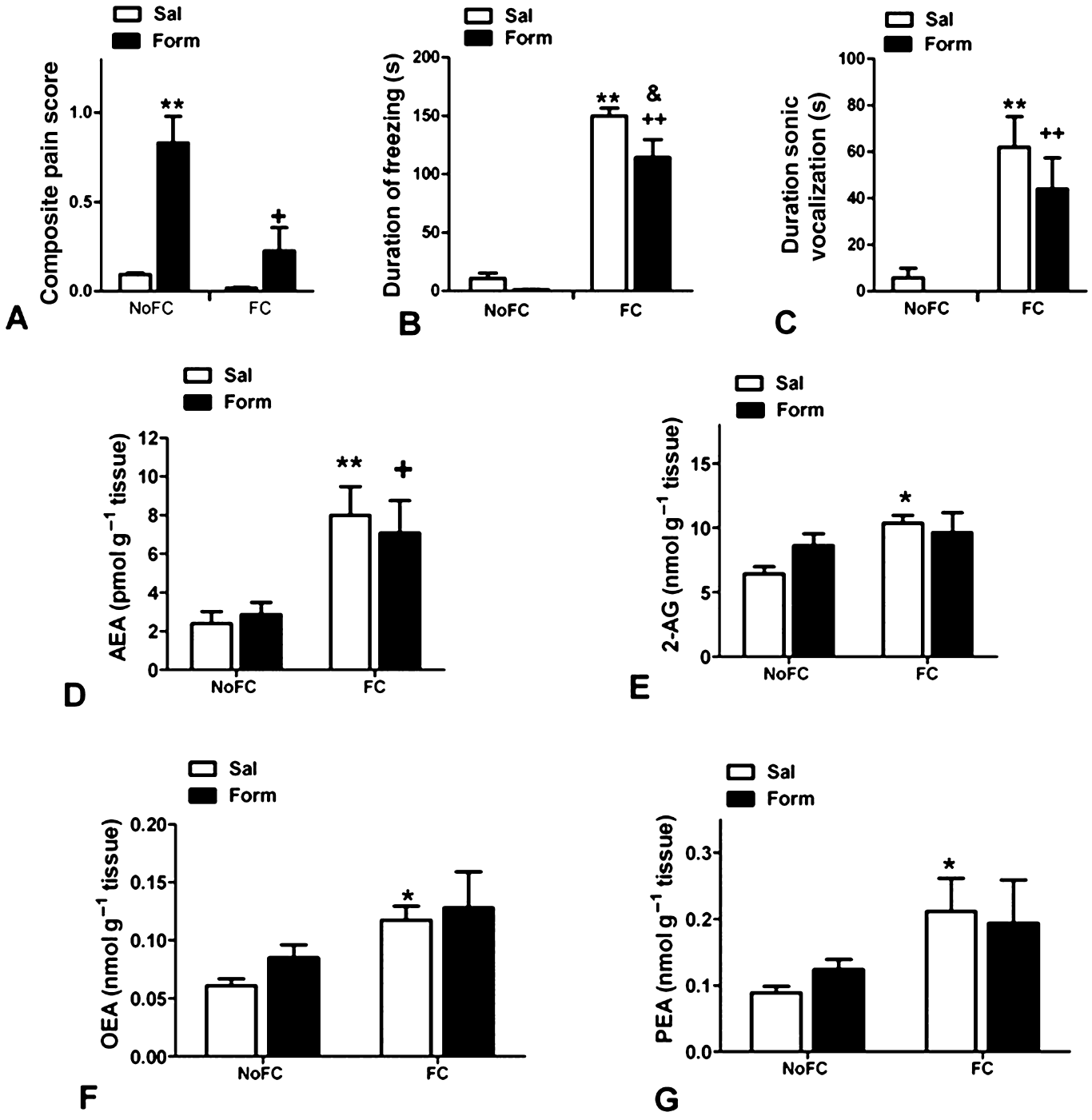

Figure 4

Effects of fear conditioning and intra-plantar formalin (Form), 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 × 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 22 kHz ultrasonic vocalization (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 × 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 × 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 × form: $F_{(1,20)} = 0.138$, $P = 0.714$) in the right dlPAG 3 min following re-exposure to an observation chamber paired 24 h previously with foot shock. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from NoFC-Sal, $^{+}P < 0.05$, $^{++}P < 0.01$ significantly different from NoFC-Form, $^{\&}P < 0.05$ significantly different from FC-Sal (Fisher's LSD). Data expressed as mean ± SEM ($n = 12$, A-C; $n = 5$–6, D–G).

the NAEs (PEA and OEA) in the right dlPAG (NoFC-Sal vs. FC-Sal, $P < 0.05$, Figure 4D–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; Figure 4D–G).

Discussion

The present study demonstrated that the CB_1 receptor antagonist/inverse agonist rimonabant, injected directly into the right dlPAG, prevented conditioned fear-induced suppression of formalin-evoked nociceptive behaviour, which followed re-exposure of rats to a context previously paired with aversive foot shock (i.e. prevented 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 or 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 foot shock is similar in its nature and magnitude to previous reports demonstrating FCA using related or identical paradigms (Helmstetter and Fanselow, 1987; Finn *et al.*, 2004; 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 dlPAG 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 that combines the tail-flick test of acute nociceptive responding with unconditioned foot shock stress (Hohmann *et al.*, 2005). These authors also demonstrated a key role for the endocannabinoid system in the dlPAG in mediating this form of unconditioned stress-induced analgesia (Hohmann *et al.*, 2005). Their work demonstrated that intra-dlPAG administration of rimonabant attenuated unconditioned stress-induced analgesia, while intra-dlPAG 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-dlPAG administration of rimonabant, support these earlier findings and extend our understanding by suggesting that the endocannabinoid system in the dlPAG 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, 2010). 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 (Bandler *et al.*, 1985; Krieger and Graeff, 1985; LeDoux *et al.*, 1988; Schenberg *et al.*, 1990; Carrive *et al.*, 1997, 1999; Amorapanth *et al.*, 1999; Vianna and Brandão, 2003), and there is good evidence that endocannabinoid signalling in the PAG plays an important role in the modulation of behavioural responses to unconditioned (Kathuria *et al.*, 2003; Bortolato *et al.*, 2006; Patel and Hillard, 2006; Lafenêtre *et al.*, 2007; Moreira *et al.*, 2007; Lisboa *et al.*, 2008) and conditioned (Fendt *et al.*, 1996; Marsicano *et al.*, 2002; Finn *et al.*, 2004; Chhatwal and Ressler, 2007; Lafenêtre *et al.*, 2007; Broiz *et al.*, 2008; Lisboa *et al.*, 2008; 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 22 kHz ultrasonic vocalization. This result corroborates previous reports of an inverse relationship between fear and pain responding (Fanselow and Helmstetter, 1988; Helmstetter, 1993; Finn *et al.*, 2004; Butler *et al.*, 2008; Roche *et al.*, 2010) and provides novel evidence that CB_1 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 CB_1 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 NAEs, PEA and OEA, in the right dlPAG of rats killed 3 min 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.

Increased levels of 2-AG and AEA in the rat dlPAG 2–7 min and 7–25 min, respectively, following foot shock have already been reported (Hohmann *et al*., 2005). Moreover, re-exposure to a 3 min tone paired previously with foot shock 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 compounds in the brain. Though themselves devoid of significant activity at the CB_1 receptor, by competing as substrates for FAAH, OEA and PEA may in turn enhance the actions of AEA at CB_1 receptors 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 CB_1 receptors in this region. Interestingly, our results revealed that although there was a fear-related elevation in all four 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 CB_1 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 (Vaughan *et al*., 2000; de Novellis *et al*., 2005). It is also possible that alternative targets such as transient receptor potential vanilloid 1 (TRPV1) channels 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 (Maione *et al*., 2006; Palazzo *et al*., 2008) 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 CB_1 receptor signalling in the dlPAG is necessary and sufficient for the expression of FCA in rats. Future studies in our laboratory will investigate the role of TRPV1 and indeed other non-CB_1 receptor targets of endocannabinoids, such as 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 that the effects of conditioned fear on levels of 2-AG, OEA and PEA are affected by the presence of nociceptive tone. The mechanism(s) responsible for such a state-dependent alteration in the responses of these analytes to conditioned fear are not known. However, it does not appear to be due to formalin-evoked alterations in absolute levels of the analytes, as there were no significant effects of intra-plantar formalin injection on tissue concentrations of any of the four analytes in the dlPAG under the present experimental conditions. These results differ from previous studies that 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 *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 vs. formalin test), dose of the formalin administered (4%, 150 μL into both hind paws vs. 2.5%, 50 μL 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 (FCA). Pharmacological blockade of CB_1 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.

Acknowledgements

This work was supported by a research grant from Science Foundation Ireland. WM Olango is a recipient of an EMBARK Postgraduate Fellowship from The Irish Research Council for Science, Engineering and Technology.

Conflicts of interest

None.

References

Ahn K, McKinney MK, Cravatt BF (2008). Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. Chem Rev 108: 1687–1707.

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Amorapanth P, Nader K, LeDoux JE (1999). Lesions of periaqueductal gray dissociate-conditioned freezing from conditioned suppression behavior in rats. Learn Mem 6: 491–499.

Asmundson GJ, Katz J (2009). Understanding the co-occurrence of anxiety disorders and chronic pain: state-of-the-art. Depress Anxiety 26: 888–901.

Bandler R, Keay K (1996). Columnar organization in the midbrain periaqueductal gray and the integration of emotional expression. Prog Brain Res 107: 285–300.

Bandler R, Depaulis A, Vergnes M (1985). Indentification of midbrain neurones mediating defensive behaviour in the rat by microinjections of excitatory amino acids. Behav Brain Res 15: 107–119.

Bertoglio LJ, Zangrossi JH (2005). Involvement of dorsolateral periaqueductal gray cholecystokinin-2 receptors in the regulation of a panic-related behavior in rats. Brain Res 1059: 46–51.

Bortolato M, Campolongo P, Mangieri RA, Scattoni ML, Frau R, Trezza V *et al*. (2006). Anxiolytic-Like Properties of the Anandamide Transport Inhibitor AM404. Neuropsychopharmacology 31: 2652–2659.

Brandão ML, Anseloni VZ, Pandóssio JE, De Araújo JE, Castilho VM (1999). Neurochemical mechanisms of the defensive behavior in the dorsal midbrain. Neurosci Biobehav Rev 23: 863–875.

Broiz AC, Oliveira LC, Brandão ML (2008). Regulation of conditioned and unconditioned fear in rats by 5-HT1A receptors in the dorsal periaqueductal gray. Pharmacol Biochem Behav 89: 76–84.

Butler RK, Finn DP (2009). Stress-induced analgesia. Prog Neurobiol 88: 184–202.

Butler R, Rea K, Lang Y, Gavin A, Finn D (2008). Endocannabinoid-mediated enhancement of fear-conditioned analgesia in rats: opioid receptor dependency and molecular correlates. Pain 140: 491–500.

Canteras NS, Goto M (1999). Fos-like immunoreactivity in the periaqueductal gray of rats exposed to a natural predator. Neuroreport 10: 413–418.

Carrive P, Leung P, Harris J, Paxinos G (1997). Conditioned fear to context is associated with increased Fos expression in the caudal ventrolateral region of the midbrain periaqueductal gray. Neuroscience 78: 165–177.

Carrive P, Lee J, Su A (1999). Lidocaine blockade of amygdala output in fear-conditioned rats reduces Fos expression in the ventrolateral periaqueductal gray. Neuroscience 95: 1071–1080.

Castilho V, Macedo C, Brandão M (2002). Role of benzodiazepine and serotonergic mechanisms in conditioned freezing and antinociception using electrical stimulation of the dorsal periaqueductal gray as unconditioned stimulus in rats. Psychopharmacology (Berl) 165: 77–85.

Chhatwal J, Ressler K (2007). Modulation of fear and anxiety by the endogenous cannabinoid system. CNS Spectr 12: 211–220.

Connell K, Bolton N, Olsen D, Piomelli D, Hohmann AG (2006). Role of the basolateral nucleus of the amygdala in endocannabinoid-mediated stress-induced analgesia. Neurosci Lett 397: 180–184.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384: 83–87.

Cravatt B, Demarest K, Patricelli M, Bracey M, Giang D, Martin B *et al*. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signalling in mice lacking fatty acid amide hydrolase. Proc Natl Acad Sci USA 98: 9371–9376.

Devane W, Hanus L, Breuer A, Pertwee R, Stevenson L, Griffin G *et al*. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258: 1946–1949.

Fanselow M, Helmstetter F (1988). Conditional analgesia, defensive freezing, and benzodiazepines. Behav Neurosci 102: 233–243.

Fendt M, Koch M, Schnitzler H (1996). Lesions of the central gray block conditioned fear as measured with the potentiated startle paradigm. Behav Brain Res 74: 127–134.

Finn DP (2010). Endocannabinoid-mediated modulation of stress responses: physiological and pathophysiological significance. Immunobiology 215: 629–646.

Finn DP, Beckett S, Richardson D, Kendall D, Marsden C, Chapman V (2004). Evidence for differential modulation of conditioned aversion and fear-conditioned analgesia by CB1 receptors. Eur J Neurosci 20: 848–852.

Finn DP, Jhaveri M, Beckett S, Madjd A, Kendall D, Marsden C *et al*. (2006). Behavioral, central monoaminergic and hypothalamo-pituitary-adrenal axis correlates of fear-conditioned analgesia in rats. Neuroscience 138: 1309–1317.

Finn D, Jhaveri M, Beckett S, Roe C, Kendall D, Marsden C *et al*. (2003). Effects of direct periaqueductal grey administration of a cannabinoid receptor agonist on nociceptive and aversive responses in rats. Neuropharmacology 45: 594–604.

Fontani G, Meucci M (1983). Dorsolateral PAG neurons: tonic immobility and morphine effect in freely moving rabbits. Physiol Behav 31: 213–218.

Ford GK, Finn DP (2008). Clinical correlates of stress-induced analgesia: evidence from pharmacological studies. Pain 140: 3–7.

Gerard C, Mollereau C, Vassart G, Parmentier M (1991). Molecular cloning of a human cannabinoid receptor which is also expressed in testis. Biochem J 279: 129–134.

Geuze E, Westenberg HGM, Jochims A, de Kloet CS, Bohus M, Vermetten E *et al*. (2007). Altered Pain Processing in Veterans With Posttraumatic Stress Disorder. Arch Gen Psychiatry 64: 76–85.

Guindon J, Hohmann AG (2009). The endocannabinoid system and pain. CNS Neurol Disord Drug Targets 8: 403–421.

Haghparast A, Ahmad-Molaei L (2009). Effects of electrolytic lesion of dorsolateral periaqueductal gray on analgesic response of morphine microinjected into the nucleus cuneiformis in rat. Neurosci Lett 451: 165–169.

Helmstetter FJ (1993). Stress-induced hypoalgesia and defensive freezing are attenuated by application of diazepam to the amygdala. Pharmacol Biochem Behav 44: 433–438.

Helmstetter F, Fanselow M (1987). Effects of naltrexone on learning and performance of conditional fear-induced freezing and opioid analgesia. Physiol Behav 39: 501–505.

Helmstetter FJ, Landeira-Fernandez J (1990). Conditional hypoalgesia is attenuated by Naltrexone applied to the periaqueductal gray. Brain Res 537: 88–92.

Helmstetter F, Tershner S (1994). Lesions of the periaqueductal gray and rostral ventromedial medulla disrupt antinociceptive but not cardiovascular aversive conditional responses. J Neurosci 14: 7099–7108.

Helmstetter FJ, Tershner SA, Poore LH, Bellgowan PSF (1998). Antinociception following opioid stimulation of the basolateral amygdala is expressed through the periaqueductal gray and rostral ventromedial medulla. Brain Res 779: 104–118.

Herkenham M, Lynn A, Johnson M, Melvin L, de Costa B, Rice K (1991). Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J Neurosci 11: 563–583.

Hill MN, Miller GE, Carrier EJ, Gorzalka BB, Hillard CJ (2009). Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress. Psychoneuroendocrinology 34: 1257–1262.

Hohmann A, Suplita R, Bolton N, Neely M, Fegley D, Mangieri R *et al.* (2005). An endocannabinoid mechanism for stress-induced analgesia. Nature 435: 1108–1112.

Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A *et al.* (2003). Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9: 76–81.

Kinscheck IB, Watkins LR, Mayer DJ (1984). Fear is not critical to classically conditioned analgesia: the effects of periaqueductal gray lesions and administration of chlordiazepoxide. Brain Res 298: 33–44.

Klein S, Nicolas L, Lopez-Lopez C, Jacobson L, McArthur S, Grundschober C *et al.* (2010). Examining face and construct validity of a noninvasive model of panic disorder in Lister-hooded rats. Psychopharmacology 211: 197–208.

Kraus A, Geuze E, Schmahl C, Greffrath W, Treede R-D, Bohus M *et al.* (2009). Differentiation of pain ratings in combat-related posttraumatic stress disorder. Pain 143: 179–185.

Krieger J, Graeff F (1985). Defensive behavior and hypertension induced by glutamate in the midbrain central gray of the rat. Braz J Med Biol Res 18: 61–67.

Lafenêtre P, Chaouloff F, Marsicano G (2007). The endocannabinoid system in the processing of anxiety and fear and how CB1 receptors may modulate fear extinction. Pharmacol Res 56: 367–381.

LeDoux J (1998). Fear and the brain: where have we been, and where are we going? Biol Psychiatry 44: 1229–1238.

LeDoux J, Iwata J, Cicchetti P, Reis D (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. J Neurosci 8: 2517–2529.

Lino-de-Oliveira C, de Oliveira RMW, Pádua Carobrez A, de Lima TCM, Bel EAD, Guimarães FS (2006). Antidepressant treatment reduces Fos-like immunoreactivity induced by swim stress in different columns of the periaqueductal gray matter. Brain Res Bull 70: 414–421.

Lisboa SF, Resstel LBM, Aguiar DC, Guimarães FS (2008). Activation of cannabinoid CB1 receptors in the dorsolateral periaqueductal gray induces anxiolytic effects in rats submitted to the Vogel conflict test. Eur J Pharmacol 593: 73–78.

McMullan S, Lumb BM (2006). Midbrain control of spinal nociception discriminates between responses evoked by myelinated and unmyelinated heat nociceptors in the rat. Pain 124: 59–68.

Maione S, Bisogno T, de Novellis V, Palazzo E, Cristino L, Valenti M *et al.* (2006). Elevation of endocannabinoid levels in the ventrolateral periaqueductal grey through inhibition of fatty acid amide hydrolase affects descending nociceptive pathways via both cannabinoid receptor type 1 and transient receptor potential vanilloid type-1 receptors. J Pharmacol Exp Ther 316: 969–982.

Marsicano G, Wotjak C, Azad S, Bisogno T, Rammes G, Cascio M *et al.* (2002). The endogenous cannabinoid system controls extinction of aversive memories. Nature 418: 530–534.

Matsuda L, Lolait S, Brownstein M, Young A, Bonner T (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346: 561–564.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR *et al.* (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol 50: 83–90.

Millan M (2002). Descending control of pain. Prog Neurobiol 66: 355–474.

Mitrirattanakul S, Ramakul N, Guerrero AV, Matsuka Y, Ono T, Iwase H *et al.* (2006). Site-specific increases in peripheral cannabinoid receptors and their endogenous ligands in a model of neuropathic pain. Pain 126: 102–114.

Moreira FA, Aguiar DC, Guimarães FS (2007). Anxiolytic-like effect of cannabinoids injected into the rat dorsolateral periaqueductal gray. Neuropharmacology 52: 958–965.

Moreira FA, Aguiar DC, Campos AC, Lisboa SF, Terzian AL, Resstel LB *et al.* (2009). Antiaversive effects of cannabinoids: is the periaqueductal gray involved? Neural Plast 2009: 625469.

de Novellis V, Mariani L, Palazzo E, Vita D, Marabese I, Scafuro M *et al.* (2005). Periaqueductal grey CB1 cannabinoid and metabotropic glutamate subtype 5 receptors modulate changes in rostral ventromedial medulla neuronal activities induced by subcutaneous formalin in the rat. Neuroscience 134: 269–281.

Oliveira MA, Prado WA (2001). Role of PAG in the antinociception evoked from the medial or central amygdala in rats. Brain Res Bull 54: 55–63.

Palazzo E, Rossi F, Maione S (2008). Role of TRPV1 receptors in descending modulation of pain. Mol Cell Endocrinol 286 (1–2, Suppl. 1): S79–S83.

Patel S, Hillard C (2006). Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. J Pharmacol Exp Ther 318: 304–311.

Pavlovic ZW, Bodnar RJ (1998). Opioid supraspinal analgesic synergy between the amygdala and periaqueductal gray in rats. Brain Res 779: 158–169.

Paxinos G, Watson C (1997). *The Rat Brain in Stereotaxic Coordinates*. Academic Press: San Diego, CA.

Petrosino S, Palazzo E, de Novellis V, Bisogno T, Rossi F, Maione S *et al.* (2007). Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats. Neuropharmacology 52: 415–422.

Rea K, Lang Y, Finn DP (2009). Alterations in Extracellular Levels of Gamma-Aminobutyric Acid in the Rat Basolateral Amygdala and Periaqueductal Gray During Conditioned Fear, Persistent Pain and Fear-Conditioned Analgesia. J Pain 10: 1088–1098.

Resstel L, Lisboa S, Aguiar D, Corrêa F, Guimarães F (2008). Activation of CB1 cannabinoid receptors in the dorsolateral periaqueductal gray reduces the expression of contextual fear conditioning in rats. Psychopharmacology (Berl) 198: 405–411.

Roche M, O'Connor E, Diskin C, Finn D (2007). The effect of CB(1) receptor antagonism in the right basolateral amygdala on conditioned fear and associated analgesia in rats. Eur J Neurosci 26: 2643–2653.

Roche M, Johnston P, Mhuircheartaigh ON, Olango WM, Mackie K, Finn DP (2010). Effects of intra-basolateral amygdala administration of rimonabant on nociceptive behaviour and neuronal activity in the presence or absence of contextual fear. Eur J Pain 14: 487–495.

Schenberg LC, Costa MB, Borges PCL, Castro MFS (1990). Logistic analysis of the defense reaction induced by electrical stimulation of the rat mesencephalic tectum. Neurosci Biobehav Rev 14: 473–479.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Sindo A, Itoh K *et al*. (1995). 2-arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215: 89–97.

Suplita R, Farthing J, Gutierrez T, Hohmann A (2005). Inhibition of fatty-acid amide hydrolase enhances cannabinoid stress-induced analgesia: sites of action in the dorsolateral periaqueductal gray and rostral ventromedial medulla. Neuropharmacology 49: 1201–1209.

Terzian ALB, Aguiar DC, Guimarães FS, Moreira FA (2009). Modulation of anxiety-like behaviour by Transient Receptor Potential Vanilloid Type 1 (TRPV1) channels located in the dorsolateral periaqueductal gray. Eur Neuropsychopharmacol 19: 188–195.

Tsou K, Brown S, Sañudo-Peña MC, Mackie K, Walker JM (1997). Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83: 393–411.

Vaughan CW, Connor M, Bagley EE, Christie MJ (2000). Actions of Cannabinoids on Membrane Properties and Synaptic Transmission in Rat Periaqueductal Gray Neurons In Vitro. Mol Pharmacol 57: 288–295.

Vianna D, Brandão M (2003). Anatomical connections of the periaqueductal gray: specific neural substrates for different kinds of fear. Braz J Med Biol Res 36: 557–566.

Walker JM, Krey JF, Chu CJ, Huang SM (2002). Endocannabinoids and related fatty acid derivatives in pain modulation. Chem Phys Lipids 121: 159–172.

Walker JM, Huang SM, Strangman NM, Tsou K, Sanudo-Pena MC (1999). Pain modulation by release of the endogenous cannabinoid anandamide. Proc Natl Acad Sci U S A 96: 12198–12203.

Waters AJ, Lumb BM (2008). Descending control of spinal nociception from the periaqueductal grey distinguishes between neurons with and without C-fibre inputs. Pain 134: 32–40.

Watson G, Sufka K, Coderre T (1997). Optimal scoring strategies and weights for formalin test in rats. Pain 70: 53–58.