Fear-induced suppression of nociceptive behaviour and activation of Akt signalling in the rat periaqueductal grey: role of fatty acid amide hydrolase

Running title: FAAH inhibition and pain-fear interactions

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Abstract

The endocannabinoid system regulates nociception and aversion and mediates fear-conditioned analgesia (FCA). We investigated the effects of the fatty acid amide hydrolase (FAAH) inhibitor URB597, which inhibits the catabolism of the endocannabinoid anandamide and related *N*-acylethanolamines, on expression of FCA and fear- and pain-related behaviour *per se* in rats. We also examined associated alterations in the expression of the signal transduction molecule phospho-Akt in the periaqueductal gray (PAG) by immunoblotting. FCA was modeled by assessing formalin-evoked nociceptive behaviour in an arena previously paired with footshock. URB597 (0.3 mg/kg i.p.) enhanced FCA and increased fear-related behaviour in formalin-treated rats. Conditioned fear *per se* in non-formalin-treated rats was associated with increased expression of phospho-Akt in the PAG. URB597 reduced the behavioural expression of conditioned fear *per se* in the early part of the trial, an effect that was accompanied by attenuation of the fear-induced increase in phospho-Akt expression in the PAG. Intra-plantar injection of formalin also reduced the fear-induced increase in phospho-Akt expression. These data provide evidence for a role of FAAH in FCA, fear responding in the presence or absence of nociceptive tone, and fear-evoked increases in PAG phospho-Akt expression. In addition, the results suggest that fear-evoked activation of Akt signalling in the PAG is abolished in the presence of nociceptive tone.

Introduction

Pain and anxiety disorders are a significant burden for individuals and society and their co-morbidity is common (Asmundson and Katz, 2009). In war veterans, for example, there is evidence both for co-morbidity of persistent pain and anxiety/depressive disorders (Forman-Hoffman et al., 2007) as well as altered nociceptive processing associated with post-traumatic stress disorder (Geuze et al., 2007). Animal models of persistent pain, conditioned fear, and fear-conditioned analgesia (FCA) aid elucidation of the physiological mechanisms underpinning nociception and aversion and the reciprocal relationship which they share.

The endogenous cannabinoid (endocannabinoid) signaling system plays a key role in the regulation of pain (Rea et al., 2007; Guindon and Hohmann, 2009) and conditioned fear (Marsicano et al., 2002; Finn et al., 2004; Chhatwal and Ressler, 2007; Roche et al., 2007; Resstel et al., 2009; Finn, 2010). Furthermore there is a significant body of evidence implicating the endocannabinoid system in mediating analgesia associated with conditioned (fear-conditioned analgesia; FCA) or unconditioned aversive stimuli (Hohmann and Suplita, 2006; Ford and Finn, 2008; Butler and Finn, 2009). For example, we have previously demonstrated that pharmacological blockade of the cannabinoid type 1 (CB_1) receptor attenuates FCA in rats (Finn et al., 2004). Moreover, pharmacological inhibition of fatty acid amide hydrolase (FAAH), the enzyme responsible for the catabolism of the endocannabinoid anandamide and the related *N*-acylethanolamines *N*-oleoyl ethanolamide (OEA) and *N*-palmitoyl ethanolamide (PEA), enhances FCA via CB_1 and CB_2 receptor-dependent mechanisms (Butler et al., 2008). Work from Hohmann and colleagues has

indicated an important role for the endocannabinoid system in the midbrain periaqueductal grey (PAG) in mediating analgesia induced by exposure to the unconditioned aversive stimulus of footshock (Hohmann et al., 2005; Suplita et al., 2005; Connell et al., 2006). There is, however, a paucity of studies investigating potential molecular mediators of FCA and altered fear responding in the presence of nociceptive tone which we sought to address here.

Stimulation of the CB_1 receptor activates protein kinase B/Akt (Gomez del Pulgar et al., 2000) signalling. In addition, Akt activation has been shown to play an important role in nociception (Sun et al., 2006) and conditioned fear (Sui et al., 2008; Dahlhoff et al., 2010). Furthermore, conditioned fear-induced increases in Akt phosphorylation in the basolateral amygdala and dorsal hippocampus are more pronounced in mice lacking the CB_1 receptor compared with wild-type controls, suggesting an inhibitory effect of endocannabinoid-CB_1 receptor signaling on fear-induced activation of Akt (Cannich et al., 2004). To date, however, no studies have assessed the effects of conditioned fear or FCA on Akt activation in the PAG and its modulation by pharmacological inhibition of FAAH. The present study addressed these questions with the aim of enhancing understanding of the supraspinal molecular biology of endocannabinoid-mediated FCA and regulation of fear responding in the presence or absence of nociceptive tone.

Materials and Methods

Animals

Male Lister-hooded rats (220-260 g weight on arrival; Charles River, Margate, Kent, UK) were housed in groups of 3 in plastic bottomed cages (45 x 20 x 20 cm) containing wood shavings as bedding. Rats were habituated to the new environment and to handling for a minimum of four days after arrival. Animal housing rooms were maintained at a constant temperature (22 ± 2° C) under standard lighting conditions (12 : 12 h light : dark, lights on from 08.00 to 20.00 h). Access to food and water was provided *ad libitum*. Rats weighed 250-310 g on the experimental days. All *in vivo* experiments were 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 FAAH inhibitor URB597 [(3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) Cayman Europe, Tallinn, Estonia] was dissolved in ethanol:cremaphor:saline (1:1:18) vehicle on the day of use. The drug was administered at a volume of 1 ml/kg via the intra-peritoneal (i.p.) route at a dose of 0.3 mg/kg.

Experimental procedures

The procedure for inducing fear-conditioned analgesia (FCA) was identical to that which we have described previously (Finn et al., 2004; Finn et al., 2006; Roche et al., 2007; Butler et al., 2008; Roche et al., 2009). It consisted of two phases, conditioning and testing, occurring 24 h apart. Subjects were randomly assigned to groups and the sequence of testing was randomised in order to minimise any confounding effects of the testing procedure.

On the conditioning day, rats were placed in a Perspex fear-conditioning / observation chamber (30 x 30 x 30 cm) and after 15 s they received the first of 10 footshocks (0.4 mA, 1 s duration; LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK) spaced 60 s apart. Fifteen seconds after the last footshock, rats were returned to their home cage. The Perspex arena and stainless steel bars were cleaned with 0.5% v/v acetic acid after each conditioning session. Of the 8 experimental groups (full details below) in this study, 4 groups were exposed to the arena and received footshocks while another 4 groups were exposed to the arena for an equivalent 9.5 min period without receiving footshocks.

The second phase of the behavioural experiments began 23 h later when rats received an i.p. injection of the FAAH inhibitor, URB597 (0.3 mg/kg), or vehicle. The dose and time of administration of URB597 were chosen based on previous studies with this compound from our laboratory and others (Kathuria et al., 2003; Jayamanne et al., 2006; Butler et al., 2008). Thirty min after the i.p. injection, the diameter of the right hindpaw was measured with Vernier calipers immediately prior to intra-plantar

injection of 50 µl formalin (2.5% v/v formaldehyde in 0.9% saline) or 0.9% w/v saline into the right hindpaw while under brief isofluorane anesthesia. Exactly 30 min after the intra-plantar injection (i.e. 60 min post-URB597/Vehicle injection and 24 h following conditioning), rats were returned to the Perspex arena. Before re-exposure of animals, the stainless steel bars and the arena were cleaned with 0.5% acetic acid. There were 8 experimental groups in total: No fear-conditioning + vehicle + saline (No FC-Veh-Sal), No fear-conditioning + URB597 + saline (No FC-URB597-Sal), No fear-conditioning + vehicle + formalin (No FC-Veh-Form), No fear-conditioning + URB597 + formalin (No FC-URB597-Form), Fear-conditioning + vehicle + saline (FC-Veh-Sal), Fear-conditioning + URB597 + saline (FC-URB597-Sal), Fear-conditioning + vehicle + formalin (FC-Veh-Form), and Fear-conditioning + URB597 + formalin (FC-URB597-Form). A bat detector (Batbox Duet, Batbox Ltd, West Sussex, UK) was used to detect ultrasonic vocalisation in the 22 kHz range and behaviours were recorded for 15 min with a video camera located beneath the observation chamber. This 30-45 min post-formalin interval was chosen on the basis of previous studies demonstrating that nociceptive behaviours during the formalin test are relatively stable during this time period and that FCA and conditioned fear is CB_1-dependent in this time-frame (Finn et al., 2003; Finn et al., 2004; Roche et al., 2007). Following the 15 min re-exposure, rats were removed from the arena and rapidly decapitated. The brains were quickly removed within 1 min, snap-frozen on dry ice and stored at -80°C prior to tissue isolation for Western immunoblotting and mass spectrometry using Palkovits' punch methodology as described below. A post-mortem measurement of the diameter of the right hindpaw was made using Verniers calipers. Recorded behaviour was scored for nociceptive, aversive, and general behaviours.

Behavioural analysis

Behaviour was analysed using the Observer® 5.0 software package (Noldus Information Technology, Wageningen, The Netherlands), which allowed for continuous event recording over each 15 min trial. A trained observer blind to the experimental conditions assessed behaviour. Formalin-evoked nociceptive behaviour was scored according to the weighted composite pain scoring technique (CPS-$WST_{0,1,2}$) described by Watson et al. (1997). According to this method, pain behaviours are categorised as time spent raising the paw above the floor without contact with any other surface (pain 1) and holding, licking, biting, shaking or flinching the paw (pain 2) to obtain a composite pain score [CPS = (pain 1 + 2(pain 2))/(total duration of analysis period)]. Oedema of the formalin-treated hindpaw was assessed by measuring the difference between the post-mortem diameter and that measured before formalin administration. Duration of freezing (cessation of all visible movement except that necessary for respiration), emission of 22 kHz ultrasonic vocalisations, or their co-occurrence, were measured as indices of conditioned aversive behaviour. General exploratory and locomotor behaviours assessed included duration of sniffing, walking, grooming, rearing duration and rearing frequency.

Tissue isolation by Palkovits' punch

Frozen coronal brain sections (300 μm) were cut on a cryostat and collected through the PAG and dorsal hippocampus. The dorsal and ventrolateral PAG and dorsal

hippocampus were punched from the frozen sections using cold cylindrical brain punchers (Harvard Apparatus, internal diameter 0.75mm for dorsal PAG, 1.0mm for ventrolateral PAG, 2mm for dorsal hippocampus). The length of tissue punched was approximately 1.8mm for PAG (start: bregma -5.8 mm end: bregma -7.64 mm) and 0.9mm for the dorsal hippocampus (start: bregma -2.80 mm end: bregma -3.70 mm (Palkovits, 1983; Paxinos and Watson, 1998). Punched tissue was stored at -80^{o}C prior to Western immunoblotting (PAG) or liquid chromatography coupled to tandem mass spectrometry (dorsal hippocampus).

Western immunoblotting

Frozen tissue punches obtained from the dorsal and ventrolateral PAG were lysed in 12.5 µl lysis buffer (80 mM sodium β-glycerophosphate, 1 mM dithiothreitol, 1 mM sodium fluoride, pH at 7.6 at 4°C) containing protease inhibitor cocktail (Sigma Aldrich Ireland Ltd., Dublin, Ireland) and phosphatase inhibitor cocktails 1 and 2 (Sigma Aldrich Ireland Ltd., Dublin, Ireland) to release cytoplasmic proteins. Tissue was homogenized in a 1.5 ml microcentrifuge tube using a pellet pestle cordless motor with polypropylene attachment (Sigma Aldrich Ireland Ltd., Dublin, Ireland) and centrifuged at 14,000 g for 15 minutes at 4°C and the supernatant collected. The supernatants from the dorsal and ventrolateral PAG were pooled due to low tissue quantities. Sample buffer (50 mM Tris-HCl, 1.84% SDS, 8% glycerol, 2% bromophenol blue, and 5% 2-mercaptoethanol) was added to the lysate and the solution was boiled for 5 minutes. The denatured protein solution was then loaded onto a Phastgel Homogenous 20 (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) using 8 x 1 µl sample applicators (GE Healthcare UK Ltd.,

Little Chalfont, Buckinghamshire, UK). The Phastsystem (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) was used for high-throughput Western blotting of the low quantities of protein present in the punched tissue. Samples from each of the experimental groups were loaded in each gel run . A current was applied to the gel for 25 minutes at 250V, 10 mA, 3W, 65 Vh at 15°C. The gel was then transferred for 1 hour at 20V, 25 mA, 1W, at 15°C onto Hybond-P PVDF membrane. Following the transfer, the membrane was rocked in blocking solution [5% BSA, 0.1% Tween 20 in Tris-Buffered Saline [TBS (20 mM Trizma-base, 137 mM NaCl, pH to 7.6)]] for 1 hour. The membrane was washed twice for 5 minutes in washing solution (0.25% Tween20 in TBS) and then rocked in phospho-Akt rabbit anti-rat antibodies [1/1000 dilution in primary antibody diluent (5% BSA, 0.05% Tween20 in TBS), Cell Signaling Technologies, Boston, Massachusettes, USA] overnight at 4°C. The membrane was washed twice in washing solution and then rocked in peroxidase-conjugated AffiniPure mouse anti-rabbit IgG heavy and light antibody [1/10000 dilution in secondary antibody solution (5% condensed milk, 0.05% Tween20 in TBS), Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK] for 1.5 hours at room temperature. The membrane was washed twice in washing solution and then in TBS. Chemiluminescence and film development were performed under safe-light conditions. Chemiluminescent detection was achieved using ECL Plus Western blotting detection reagents (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK). Membranes were then exposed to Hyperfilm ECL (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) for 30 minutes. The film was developed for 30 seconds, briefly dipped in water, and fixed for 2 minutes (Kodak Rapid Access Chemicals, Sigma Aldrich Ireland Ltd., Dublin, Ireland). Immediately following the development of the image, the membrane was placed in

Restore Western Blot stripping buffer (Pierce Biotechnology, Rockford, Illinois, USA) and spun in a rotary oven at 37°C for 30 minutes. The membrane was washed 4 times in TBS followed by blocking solution for 1 hour and then washed twice in washing solution. The stripped membranes which had previously been probed for phospho-Akt were then rocked in Akt antibody solution (1/1000 dilution, Cell Signaling Technologies, Boston, Massachusetts, USA) to detect total levels of the respective proteins. The subsequent procedures leading to the development of films were similar to those already described above with an exposure time of 2-5 min. Finally, membranes were re-stripped and re-probed with pan-Actin antibody (1/1000 dilution, Cell Signaling Technologies, Boston, Massachusettes, USA) and films developed as described above with an exposure time of 2-5 min. The bands for all photographs were quantified in the linear range of the signal using densitometric analysis on ImageJ (http://rsb.info.nih.gov/ij/). Background integrated density values were computed and subsequently subtracted from band integrated density values to obtain corrected integrated density values. Normalized values for the Western blot data were obtained by dividing the corrected integrated density values for the phospho-Akt bands by the corrected integrated density value for the total Akt bands. The normalized values were divided by the control group (No FC-Veh-Sal) to obtain a percentage control value. Minor modifications to brightness and contrast were made for presentation purposes only.

Measurement of endocannabinoids and related N-acylethanolamines by liquid chromatography- tandem mass spectrometry (LC-MS-MS)

To confirm the ability of URB597 (at the dose and time-point administered here) to elevate FAAH substrates in the brain, concentrations of the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) and the related *N*-acylethanolamines *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) were measured in dorsal hippocampal tissue from the following two treatment groups: No fear-conditioning + vehicle + saline (No FC-Veh-Sal) and No fear-conditioning + URB597 + saline (No FC-URB597-Sal). Dorsal hippocampal tissue was obtained via Palkovits' punch methodology as described above. Punched tissue was first homogenised for 3 seconds using a ultrasonicator probe in ice-cold in 100% acetonitrile containing deuterated internal standards added in fixed amounts to all samples (0.014 nmol AEA-d8, 0.48 nmol 2-AG-d8, 0.016 nmol PEA-d8, 0.015 nmol OEA-d8). 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 resuspended in 40µl 65% acetonitrile and 2µl 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 (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 10 min was ramped linearly up to 100% B. At 10min, the gradient was held at 100% B up to 20 min. At 20.1min, the gradient returned to initial conditions for a further 15 mins to re-equilibrate the column. The total run time was 35 min. Under these conditions, AEA, 2-AG, PEA and OEA, eluted at the following retention times: 11.5 min, 12.9 min, 14.2 min and 15.2 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, Cork, Ireland). Instrument conditions, in particular source parameters such as fragmentor voltage and collision energy, were optimised for each analyte by infusing standards separately. 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 form of the internal standard. Precursor and product ion mass-to-charge (m/z) ratios for all analytes and their corresponding deuterated forms were as follows: PEA (m/z = 300.3-62.1); PEA-d4 (m/z = 304.3-62.1); OEA (m/z = 326.3-62.1); OEA-d2 (m/z = 328.3-62.1); AEA (m/z = 348.3-62.1); AEA-d8 (m/z = 356.3-63.1); 2-AG (m/z = 379.3-287.2); 2-AG-d8 (m/z = 387.3-294.2). 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 calculated 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. The values obtained from the Masshunter Quantitative Analysis Software are initially expressed in ng per mg of tissue by dividing by the weight of the punched tissue. To express values as nmol or pmols per mg the corresponding values are then divided by the molar mass of each analyte expressed as ng/nmole or pg/pmole. Linearity 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 <2.0 pmol/g for all analytes except for 2-AG where it was 12.1 pmol/g.

Statistical analysis

SPSS statistical software was used to analyse all data. Two-way analysis of variance (ANOVA) was used to assess the effects of URB597 (factor 1) and fear conditioning (factor 2) on formalin-evoked nociceptive behaviour. Three-way ANOVA was used to assess the effects of URB597 (factor 1), fear conditioning (factor 2) and formalin (factor 3) on general exploratory behaviours, hindpaw oedema, and relative phospho-protein expression. *Post-hoc* pairwise comparisons were made using Fisher's LSD test when appropriate. Fear-related behavioural data were not normally distributed and so were analysed non-parametrically as changes over time were analysed using a Friedman Two-Way ANOVA by rank followed by Wilcoxon matched pairs test for within-subjects effects and Mann-Whitney-U tests for between-subjects effects. Student's unpaired, two-tailed t-test was used to compare levels of endocannabinoids and *N*-acylethanolamines in the two groups assayed by LC-MS-MS. Data were considered significant when $P < 0.05$. Results are expressed as group means ± standard error of the mean (± SEM).

Results

Effects of URB597 on fear-conditioned analgesia

Rats receiving intra-plantar injection of saline exhibited little or no paw-directed nociceptive behaviour (data not shown). In contrast, intra-plantar injection of formalin produced robust licking, biting, shaking, flinching and elevation of the injected paw as indicated by the composite pain score (CPS) (Fig. 1). Fear-conditioned, vehicle-treated rats displayed significantly less formalin-evoked nociceptive behaviour (i.e. a reduced CPS) compared with non-fear-conditioned counterparts (Fig. 1; FC-Veh-Form vs No FC-Veh-Form), confirming the expression of fear-conditioned analgesia (FCA). Administration of the FAAH inhibitor, URB597, did not alter formalin-induced nociceptive behaviour *per se* (NoFC-Veh-Form vs. NoFC-URB597-Form) but significantly enhanced the expression of FCA (Fig. 1; FC-URB597-Form vs FC-Veh-Form).

[Insert Figure 1 here]

Effects of URB597 on conditioned aversive behaviour

Fear-conditioned rats receiving systemic administration of vehicle and intraplantar injection of saline displayed the conditioned aversive behaviours of freezing, 22 kHz ultrasonic vocalisations and their co-occurrance (Fig. 2), compared with non-fear-conditioned counterparts which expressed negligible levels of these fear-related behaviours (data not shown). Compared with fear-conditioned rats receiving intra-

plantar saline, intra-plantar injection of formalin to fear-conditioned rats significantly reduced the duration of freezing-22 kHz ultrasonic vocalisation co-occurrence and freezing alone in the first 10 minutes of the trial and reduced the duration of 22 kHz ultrasonic vocalisations in the first 5 minutes of the trial (Fig. 2; FC-Veh-Form vs FC-Veh-Sal). The FAAH inhibitor URB597 significantly reduced the duration of freezing behaviour over the first 5 minutes of the trial and reduced the duration of freezing-22 kHz ultrasonic vocalisations co-occurrence from 3-7 min (Fig. 2; FC-URB597-Sal vs FC-Veh-Sal). In formalin-treated rats, URB597 significantly increased the duration of freezing-22 kHz ultrasonic vocalisation co-occurrence and 22 kHz ultrasonic vocalisations alone in the last 5 minutes of the trial and increased the duration of freezing behaviour from 6-15 minutes, compared with respective vehicle-treated controls (Fig. 2; FC-URB597-Form vs FC-Veh-Form).

[Insert Figure 2 here]

Effects of formalin, fear-conditioning and URB597 on general exploratory/locomotor behaviours and hindpaw oedema

Intra-plantar injection of formalin to non-fear-conditioned, vehicle-treated rats significantly reduced general exploratory/locomotor behaviour including sniffing, walking, grooming, rearing, and total number of rears, compared with saline-treated counterparts (Table 1; No FC-Veh-Form vs No FC-Veh-Sal). Similarly, fear conditioning in vehicle-treated rats receiving intra-plantar injection of saline was also associated with significant reductions in all of these general exploratory/locomotor behaviours compared with non-fear-conditioned counterparts (Table 1; FC-Veh-Sal vs

No FC-Veh-Sal). Systemic administration of URB597 had no significant effect on these general exploratory/locomotor behaviours *per se* (Table 1; No FC-Veh-Sal vs No FC-URB597-Sal) or on the alterations in these behaviours induced by formalin (Table 1; No FC-Veh-Form vs No FC-URB597-Form) or conditioned fear (Table 1; FC-Veh-Sal vs FC-URB597-Sal). Fear-conditioned, formalin-treated rats displayed significantly less walking following systemic injection of URB597, compared to vehicle-treated counterparts (Table 1; FC-URB597-Form vs FC-Veh-Form). URB597 had no significant effect on any of the other behaviours assessed.

Intra-plantar injection of formalin produced comparable oedema of the treated right hindpaw in fear-conditioned and non-fear-conditioned rats as indicated by an increase in paw diameter following injection, compared with saline-treated rats (Table 1; No FC-Veh-Form vs No FC-Veh-Sal or FC-Veh-Form vs FC-Veh-Sal). Systemic administration of URB597 had no significant effect on the magnitude of these formalin-evoked increases in paw diameter.

[Insert Table 1 here]

Effects of formalin, fear-conditioning and URB597 on relative expression of phospho-Akt in the PAG

Relative levels of phospho-Akt were significantly higher in the PAG of fear-conditioned, saline-treated rats which received vehicle compared to their non-fear-conditioned counterparts (Fig. 3; FC-Veh-Sal vs No FC-Veh-Sal). Systemic injection of URB597 significantly attenuated this fear-induced increase in relative phosho-Akt

expression in the PAG (Fig. 3; FC-URB597-Sal vs FC-Veh-Sal). The fear-induced increase in phospho-Akt was not observed in rats that had received intra-plantar injection of formalin (Fig. 3; FC-Veh-Form vs No FC-Veh-Form and FC-Veh-Sal vs FC-Veh-Form).

[Insert Figure 3 here]

Effects of URB597 on levels of endocannabinoids and related N-acylethanolamines in the dorsal hippocampus

To confirm the ability of URB597 (at the dose and time point administered here) to elevate FAAH substrates in the brain, concentrations of the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) and the related *N*-acylethanolamines *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) were measured in dorsal hippocampal tissue from non-fear-conditioned, non-formalin treated rats receiving either URB597 or vehicle injection (i.e. No FC-Veh-Sal vs No FC-URB597-Sal). URB597 significantly increased concentrations of the *N*-acylethanolamines and FAAH substrates PEA (No FC-Veh-Sal: 247 ± 45 pmol/g vs No FC-URB597-Sal: 599 ± 99 pmol/g, $P < 0.01$ Student's t-test) and OEA (No FC-Veh-Sal: 185 ± 40 pmol/g vs No FC-URB597-Sal: 396 ± 85 pmol/g, $P < 0.05$ Student's t-test) in the dorsal hippocampus. Levels of anandamide and 2-AG were also higher in URB597-treated rats but effects on these endocannabinoids did not reach statistical significance (Anandamide: No FC-Veh-Sal: 127 ± 16 pmol/g vs No FC-URB597-Sal: 172 ± 29 pmol/g; 2-AG: No FC-Veh-Sal: 7.96 ± 2.35 nmol/g vs No

FC-URB597-Sal: 17.02 ± 4.35 nmol/g).

Discussion

In this study, acute systemic administration of the FAAH inhibitor, URB597, enhanced antinociception expressed upon exposure to an aversively conditioned context (i.e. enhanced FCA). URB597 increased conditioned aversive behaviour in formalin-treated rats but reduced conditioned aversive behaviour in rats not receiving formalin. Moreover, expression of conditioned fear was associated with an increase in relative expression of phospho-Akt in the PAG in the absence but not in the presence of nociceptive tone and this fear-induced increase in phospho-Akt was attenuated by URB597.

The reduction in nociceptive behaviour observed here upon re-exposure to a context previously paired with footshock confirms previous reports of the phenomenon of FCA (Calcagnetti et al., 1987; Helmstetter and Fanselow, 1987a, b, Finn et al., 2004, Finn et al., 2006, Roche et al., 2007, Butler et al., 2008). Furthermore, the results confirm our previous finding that systemic administration of the FAAH inhibitor URB597 enhances FCA in rats (Butler et al., 2008) and add to the body of evidence indicating a role for the endocannabinoid system in mediating analgesia expressed in response to both conditioned and unconditioned stress. For example, Finn *et al*. (2004) showed that systemic administration of the CB_1 receptor antagonist/inverse agonist, SR141716A (rimonabant), attenuated FCA in rats, while Butler *et al*. (2008) reported that URB597-induced enhancement of FCA was blocked by either rimonabant or by a selective CB_2 receptor antagonist. Studies by Hohmann and colleagues have demonstrated that a form of unconditioned stress-induced analgesia (SIA) which combines footshock stress with the tail-flick test is enhanced by systemic

administration of URB597 in a CB_1 receptor-dependent manner (Hohmann et al., 2005) and is mediated by the endocannabinoid system in brain regions including the PAG (Hohmann et al., 2005; Suplita et al., 2005), rostral ventromedial medulla (Suplita et al., 2005) and basolateral amygdala (Connell et al., 2006). It is possible that some of the effects of URB597 observed in the present study may arise as a consequence of FAAH substrate activity at non-CB_1/non-CB_2 receptors. More specifically, there is good evidence for an important role of AEA activity at the transient receptor potential vanilloid 1 channel (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). Though its potential role in FCA has not yet been investigated, Suplita et al. (2005) showed that TRPV1 was not involved in a form of unconditioned stress-induced analgesia in rats. While confirming a role for FAAH in FCA, we found that administration of URB597 had no effect on formalin-evoked nociceptive behaviour *per se* or on general exploratory/locomotor behaviour at the dose used and under these conditions, indicating a specific effect on FCA. The first publication to describe URB597 showed that a 0.5 mg/kg i.p., but not a 0.1 mg/kg i.p, injection of URB597 produced antinociceptive effects in the mouse hot-plate test (Kathuria et al., 2003). Another study has shown that 0.3 mg/kg i.p. URB597 can reduce mechanical allodynia and thermal hyperalgesia induced by a hindpaw injection of Complete Freund's Adjuvant (Jayamanne et al., 2006). Thus, the dose of URB597 needed to produce antinociception in rodents is probably dependent on the model, strain and time-points investigated. The present study was designed with the specific aim of investigating the effects of URB597 on pain-fear interactions over a specific 15 minute period during the second phase of the formalin response and further studies would be needed to fully characterise the effects of systemically administered

URB597 in the rat formalin test. Our LC-MS-MS results confirmed that under the present conditions and time-frame, URB597 was capable of inducing significant elevations in concentrations of the FAAH substrates PEA and OEA in brain tissue (dorsal hippocampus), indicating that the drug was pharmacologically active at the dose administered and over the time-course studied here. Smaller URB597-induced elevations in concentrations of anandamide and 2-AG were also observed but did not reach statistical significance at the time-point sampled.

Valuable insights into the molecular correlates of animal behaviour and its pharmacological modulation can be gained through assessment of intracellular signalling pathways. Here, we have found that phospho-Akt expression in the PAG is increased in rats expressing contextually induced conditioned fear in the absence, but not in the presence, of formalin-evoked nociceptive tone. These data suggest that fear engages the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway in the PAG in a state-dependent manner. Interestingly, the formalin-evoked attenuation of the fear-related increase in phospho-Akt expression was associated with a concomitant formalin-evoked attenuation of the fear-related behavioural response (i.e. freezing and 22 kHz ultrasonic vocalisation). Moreover, our results revealed that systemic administration of the FAAH inhibitor URB597 attenuates the fear-induced increase in phospho-Akt expression in the PAG, an effect which was accompanied by a reduction in fear-related behaviour in the early part of the trial. Together, these data suggest a tight correlation between fear behaviour, its modulation during nociception or FAAH inhibition, and PAG phospho-Akt expression. Previous work has demonstrated coupling of the PI3K/Akt pathway to CB_1 receptor activation (Gomez del Pulgar et al., 2000). To date, no published studies have investigated fear-related

alterations in the expression of phospho-Akt in the PAG or its modulation by FAAH. There is, however, evidence for altered fear-induced expression of phospho-Akt in the prefrontal cortex, amygdala and dorsal hippocampus of mice lacking the CB_1 receptor (Cannich et al., 2004). Interestingly, this latter paper reported significantly higher fear-related expression of phospho-Akt in the basolateral amygdala and dorsal hippocampus of CB_1- null $^{-/-}$ mice compared with wild-type littermates, suggesting, perhaps, that tonic endocannabinoid signalling through the CB_1 receptor may act to suppress fear-related activation of phospho-Akt. The results of the present study, demonstrating attenuation of the fear-related increase in phospho-Akt in the PAG following administration of an inhibitor of endocannabinoid catabolism, support this hypothesis. The present study, the first to investigate the effects of formalin injection on PAG Akt activation, found no pain-related alterations in this kinase in this brain region. However, it remains possible that formalin-induced alterations in the expression of phospho-Akt in the PAG may have been evident at a different time-point or in discrete sub-columns of the PAG and future studies could address these aspects.

In conclusion, these data provide evidence for a role of FAAH in the suppression of pain responding by conditioned fear (i.e. FCA), in pain-related modulation of fear responding, and in fear-related increases in aversive behaviour and PAG phospho-Akt expression. In addition, the results suggest that fear-evoked activation of Akt in the PAG is abolished in the presence of nociceptive tone. Together, these findings advance our understanding of the biochemical and molecular substrates which may regulate the reciprocal relationship shared by pain and aversion.

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Abbreviations:

FAAH, fatty acid amide hydrolase; FCA, fear-conditioned analgesia, PAG, periaqueductal grey; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; OEA, *N*-oleoyl ethanolamide; PEA, *N*-palmitoyl ethanolamide; LC-MS-MS, liquid chromatography-tandem mass spectrometry; CB_1, cannabinoid receptor type I; FC, fear-conditioned; Veh, vehicle; Sal, saline; Form, formalin; CPS, composite pain score.

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Figure legends:

FIG. 1. Effect of URB597 (0.3 mg/kg, i.p.) on the composite pain score (CPS) in non fear-conditioned, formalin-treated and fear-conditioned formalin-treated rats. Data are means ± S.E.M. (n = 7-9). +P < 0.05 vs. No FC-Veh-Form, *P < 0.05 vs. FC-Veh-Form (two-way ANOVA followed by Fisher's LSD *post-hoc* test (conditioning $F_{1,32} = 27.86$, $P < 0.001$; Conditioning x URB597 $F_{1,32} = 3.77$, $P = 0.06$). FC (fear conditioning), Form (formalin), Veh (vehicle).

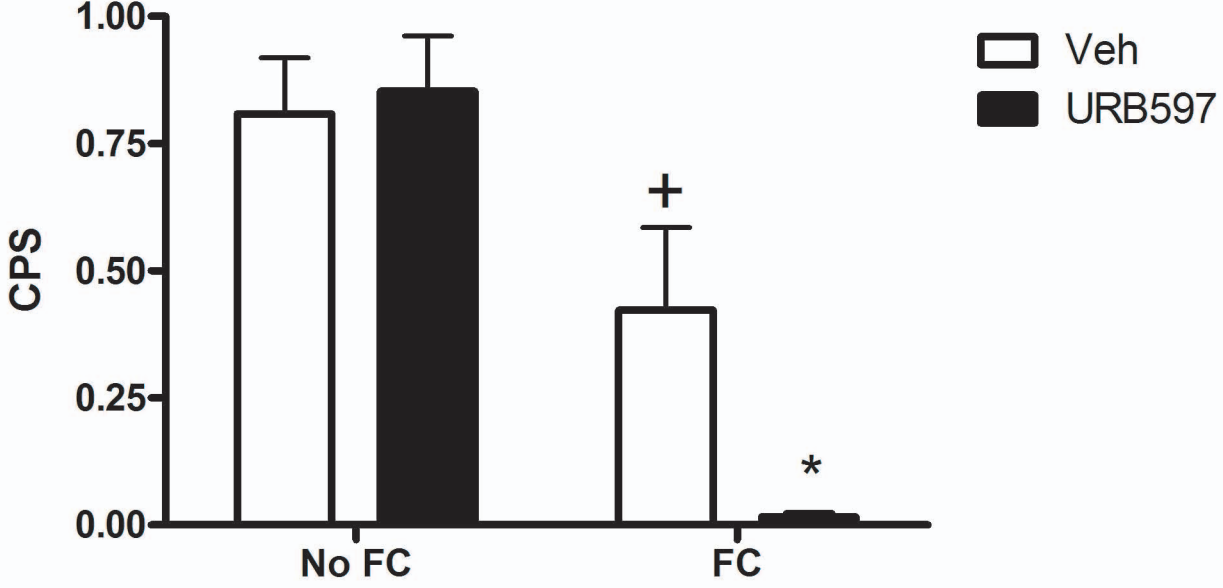
FIG. 2. Effect of URB597 on the expression of contextually-induced fear behaviour in fear-conditioned saline-treated and fear-conditioned formalin-treated rats. Data are means ± S.E.M. (n = 8-10). A. Freezing behaviour whilst emitting 22kHz ultrasonic vocalisations (Significant effect of treatment X^2 =29.302, P<0.001; *P < 0.05 for FC-Veh-Sal vs. FC-Veh-Form at 0-10 min; $^{\#}$P<0.05 for FC-Veh-Sal vs. FC-Veh-URB at 3-7 min; $^{+}$P<0.05 for FC-Veh-Form vs. FC-URB-Form at 11-15 min). B. Freezing behaviour (Significant effect of treatment X^2 =39.176, P < 0.001; *P < 0.05 for FC-Veh-Sal vs. FC-Veh-Form at 0-10 min; $^{\#}$P<0.05 for FC-Veh-Sal vs. FC-Veh-URB at 0-5 min; $^{+}$P<0.05 for FC-Veh-Form vs. FC-URB-Form at 6-15 min). C. 22kHz ultrasonic vocalisations (Significant effect of treatment X^2 =25.220, P < 0.01; *P < 0.05 for FC-Veh-Sal vs. FC-Veh-Form at 0-5 min; $^{++}$P<0.01 for FC-Veh-Form vs. FC-URB-Form at 6-15 min). FC (fear-conditioned), Form (formalin), Veh (vehicle).

FIG. 3. Relative phospho-Akt expression in the PAG following persistent pain, fear-conditioning, and fear-conditioned analgesia with and without URB597. (A) Data are means ± S.E.M. (n = 5-8). ‡P < 0.01 vs. No FC-Veh-Sal, $^{\#\#}$P < 0.01, $^{\#}$P < 0.05 vs. FC-

Veh-Sal (three-way ANOVA followed by Fisher's LSD *post-hoc* test (conditioning x URB597 $F_{1,53} = 6.17$, $P = 0.017$). FC (fear conditioning), Form (formalin), Veh (vehicle). (B) Representative photomicrograph depicts bands for total Akt, phospho-Akt and pan-Actin in the PAG from immunoblotting experiments.

Table legend:

Data are means ± S.E.M. (n = 8-10). ‡$P < 0.01$, †$P < 0.05$ vs. No FC-Veh-Sal, ##$P <$ 0.01 vs. FC-Veh-Sal, **$P < 0.01$ vs. FC-Veh-Form (three-way ANOVA followed by Fisher's *post-hoc* test (Sniffing: main effect of conditioning $F_{1,72} = 57.33$, $P < 0.001$; main effect of formalin $F_{1,72} = 16.32$, $P < 0.001$. Walking: main effect of conditioning $F_{1,72} = 41.32$, $P < 0.001$; main effect of formalin $F_{1,72} = 6.34$, $P = 0.014$. Grooming: main effect of conditioning $F_{1,72} = 34.59$, $P < 0.001$; main effect of formalin $F_{1,72} = 8.61$, $P = 0.005$; conditioning x formalin $F_{1,72} = 10.26$, $P = 0.002$. Rearing: main effect of formalin $F_{1,72} = 34.15$, $P < 0.001$; conditioning x formalin $F_{1,72} = 4.35$, $P = 0.041$. Total rears: main effect of formalin $F_{1,72} = 42.98$, $P < 0.001$; conditioning x formalin $F_{1,72} = 5.35$, $P = 0.024$. Δpaw diameter: main effect of formalin $F_{1,72} = 47.62$, $P < 0.001$)). FC (fear conditioning), Veh (vehicle), Sal (saline), Form (formalin).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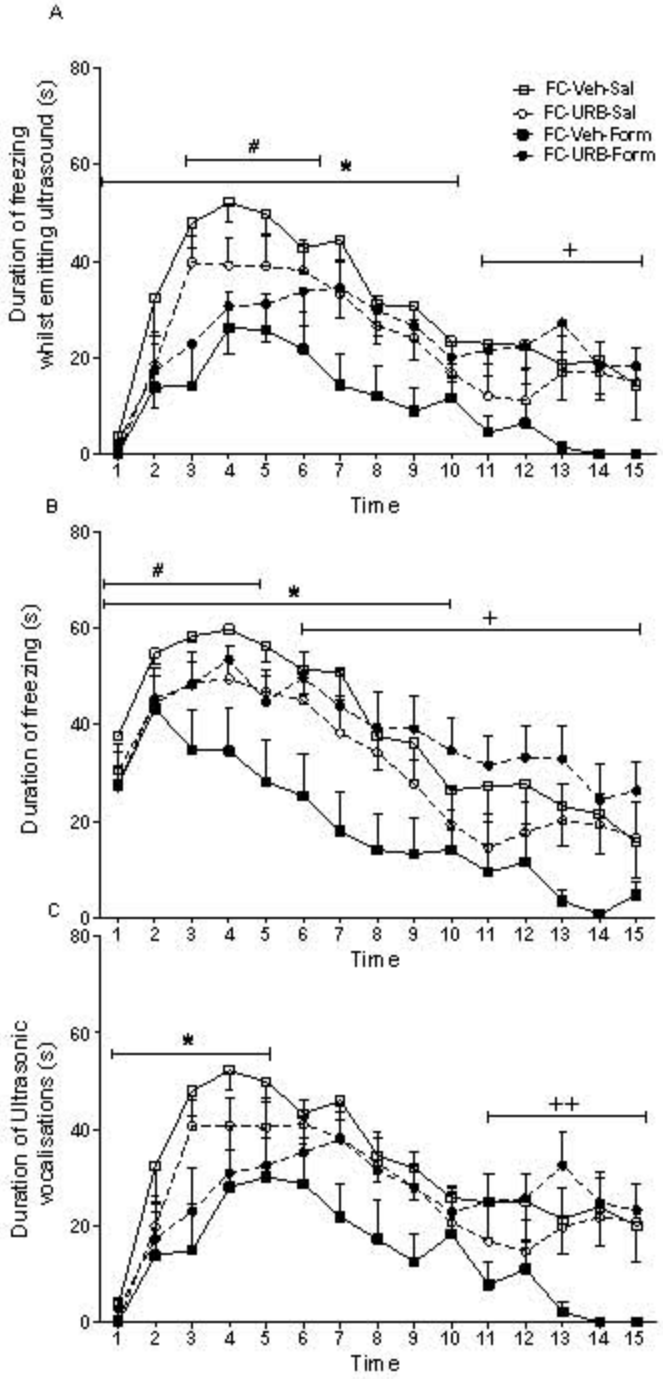
A
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FC-Veh-Sal
FC-URB-Sal
FC-Veh-Form
FC-URB-Form
#
*
+
Time
B
Duration of freezing (s)
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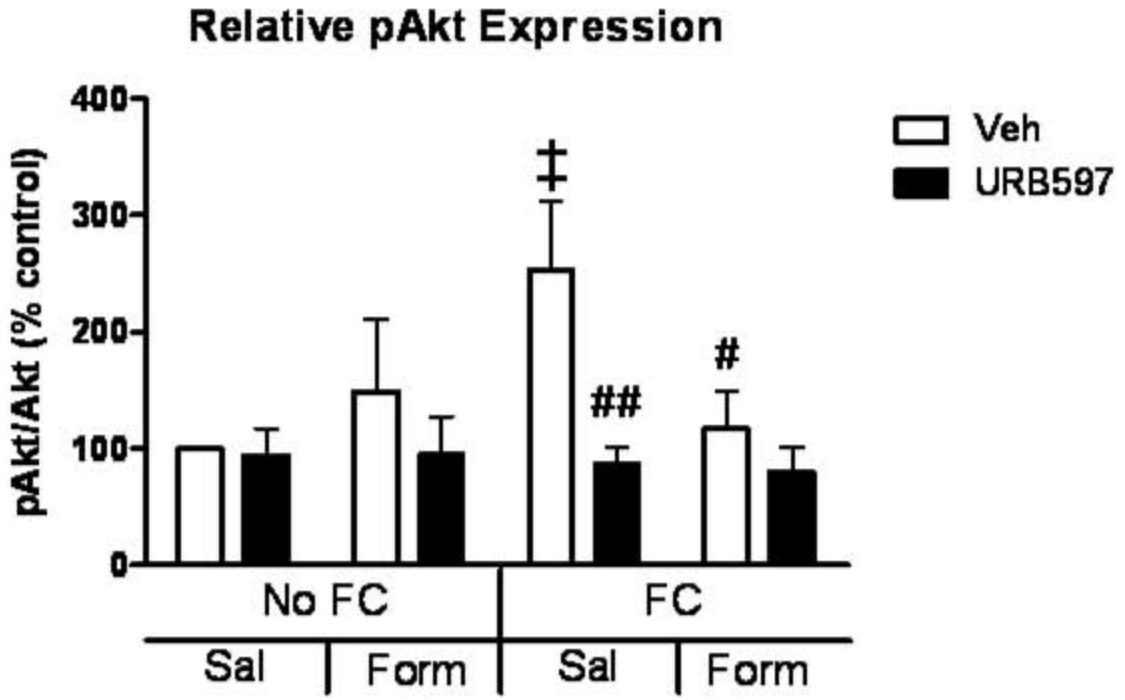
*
+
Time
C
Duration of Ultrasonic vocalisations (s)
*
++
Time

A.


Relative pAkt Expression
pAkt/Akt (% control)
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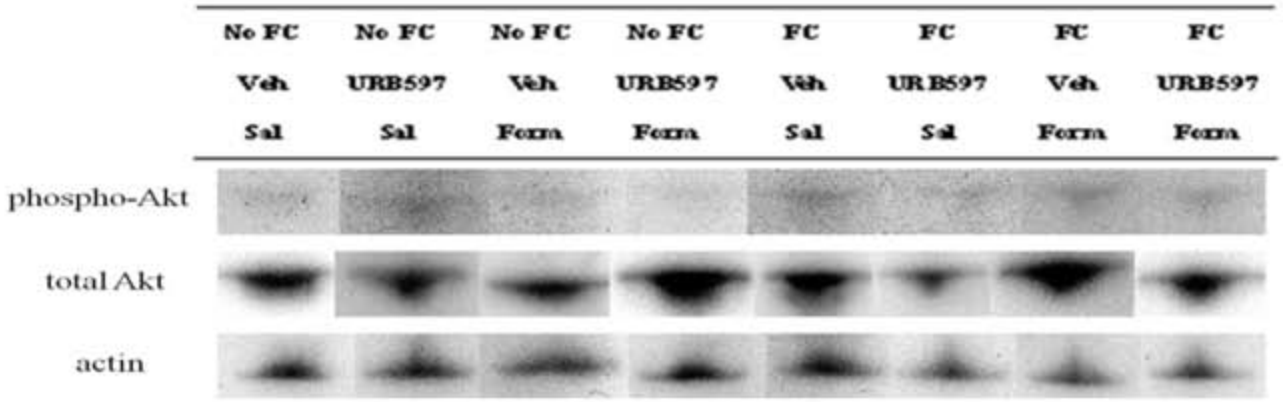
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