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J Psychopharmacol 2012 26: 83 originally published online 17 September 2011

DOI: 10.1177/0269881111413823

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

Ryan K Butler^{1,*}, Gemma K Ford^{1,*}, Michelle Hogan¹, Michelle Roche², Karen M Doyle², John P Kelly¹, David A Kendall³, Victoria Chapman³ and David P Finn¹

Journal of Psychopharmacology

26(1) 83–91

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DOI: 10.1177/0269881111413823

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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 grey (PAG) by immunoblotting. FCA was modelled 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 expression of fear-related behaviour 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.

Keywords

Akt (protein kinase B), endocannabinoids, fatty acid amide hydrolase (FAAH), fear, pain, periaqueductal grey (PAG), rats

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) signalling system plays a key role in the regulation of pain (Guindon and Hohmann, 2009; Moreira et al., 2009; Rea et al., 2007) and conditioned fear (Chhatwal and Ressler, 2007; Finn, 2010; Finn et al., 2004; Marsicano et al., 2002; Resstel et al., 2009; Roche et al., 2007). Furthermore, there is a significant body of evidence implicating the endocannabinoid system in mediating analgesia associated with

conditioned FCA or unconditioned aversive stimuli (Butler and Finn, 2009; Ford and Finn, 2008; Hohmann and Suplita, 2006). For example, we have previously demonstrated that pharmacological blockade of the cannabinoid type 1 (CB₁) receptor attenuates FCA in rats (Finn et al.,

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2004). Moreover, pharmacological inhibition of fatty acid amide hydrolase (FAAH) – the enzyme responsible for the catabolism of the endocannabinoid anandamide (AEA) and the related *N*-acylethanolamines viz. *N*-oleoyl ethanolamide (OEA) and *N*-palmitoyl ethanolamide (PEA) – enhances FCA via CB₁ and CB₂ 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 (for review see Butler and Finn, 2009; Connell et al., 2006; Hohmann et al., 2005; Suplita et al., 2005). 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 CB₁ receptor activates protein kinase B/Akt signalling (Gomez del Pulgar et al., 2000). In addition, Akt activation has been shown to play an important role in nociception (Sun et al., 2006) and conditioned fear (Dahlhoff et al., 2010; Sui et al., 2008). Furthermore, conditioned fear-induced increases in Akt phosphorylation in the basolateral amygdala and dorsal hippocampus are more pronounced in mice lacking the CB₁ receptor compared with wild-type controls, suggesting an inhibitory effect of endocannabinoid-CB₁ receptor signalling 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 three in plastic bottomed cages (45 × 20 × 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 intraperitoneal (i.p.) route at a dose of 0.3 mg/kg.

Experimental procedures

The procedure for inducing FCA was identical to that which we have described previously (Butler et al., 2008; Finn et al., 2004, 2006; Roche et al., 2010; Roche et al., 2007). It consisted of two phases, conditioning and testing, occurring 24 h apart. Subjects were randomly assigned to groups and the sequence of testing was randomized in order to minimize any confounding effects of the testing procedure.

On the conditioning day, rats were placed in a Perspex fear-conditioning/observation chamber (30 × 30 × 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 either 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 (Butler et al., 2008; Jayamanne et al., 2006; Kathuria et al., 2003). Thirty minutes 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 isoflurane anaesthesia. 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 vocalization 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 interval post-formalin was chosen on the basis of previous studies which demonstrated that nociceptive behaviours during the formalin test are relatively stable over this period and that FCA and conditioned fear is

CB₁-dependent in this time-frame (Finn et al., 2004, 2003; 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 Vernier 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 categorized 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: $\text{CPS} = (\text{pain } 1 + 2[\text{pain } 2]) / (\text{total duration of analysis period})$. 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 vocalizations, 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 0.75 mm for dorsal PAG, 1.0 mm for ventrolateral PAG, 2 mm for dorsal hippocampus). The length of tissue punched was approximately 1.8 mm for PAG (start: bregma -5.8 mm end: bregma -7.64 mm) and 0.9 mm for the dorsal hippocampus (start: bregma -2.80 mm end: bregma -3.70 mm (Palkovits, 1980; Paxinos and Watson, 1998). Punched tissue was stored at -80°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 $14,000 \times g$ for 15 min 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 min. The denatured protein solution was then loaded onto a Phastgel Homogenous 20 (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) using $8 \times 1 \mu\text{L}$ 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 min at 250 V, 10 mA, 3 W, 65 Vh at 15°C . The gel was then transferred for 1 h at 20 V, 25 mA, 1 W, at 15°C onto a Hybond-P PVDF membrane. Following the transfer, the membrane was rocked in blocking solution (5% BSA, 0.1% Tween20 in Tris-buffered saline [TBS (20 mM Trizma-base, 137 mM NaCl, pH to 7.6)]) for 1 h. The membrane was washed twice for 5 min 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, Massachusetts, 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 h 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 min. The film was developed for 30 s, briefly dipped in water, and fixed for 2 min (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 min. The membrane was washed four 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, Massachusetts, 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 AEA and 2-arachidonoyl glycerol (2-AG) and the related *N*-acylethanolamines viz. PEA and 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 homogenized for 3 s using an ultrasonic probe in ice-cold 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 $14,000 \times g$ 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 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 phase A (water with 0.1% formic acid) and phase B (acetonitrile with 0.1% formic acid), with a flow rate of 12 µL/min. 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 15 min to re-equilibrate the column. The total run time was 35 min. Under these conditions, AEA, 2-AG, PEA and OEA, eluted at the retention times 11.5 min, 12.9 min, 14.2 min and 15.2 min, respectively.

Analyte detection was carried out in electrospray-positive ionization mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd., Cork, Ireland). Instrument conditions, in particular source parameters such as fragmentor voltage and collision energy, were optimized for each analyte by infusing standards separately. Quantitation of target endocannabinoids was achieved by positive ion electrospray ionization and multiple reaction monitoring 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's per mg of tissue by dividing by the weight of the punched tissue. To express values as nmol or pmol per mg the corresponding values are then divided by the molar mass of each analyte expressed as ng/nmol or pg/pmol. Linearity was determined over a range of 18.75 ng to 71.5 fg except for 2-AG which was 187.5 ng to 715 fg. 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 least squares difference (LSD) test where appropriate. Fear-related behavioural data were not normally distributed and so were analysed non-parametrically as changes over time using a Friedman Two-Way ANOVA by rank followed by Wilcoxon matched pairs test for within-subject effects and Mann-Whitney *U*-tests for between-subject 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 CPS (Figure 1). Fear-conditioned, vehicle-treated rats displayed significantly less formalin-evoked nociceptive behaviour (i.e. a reduced CPS) compared with their non-fear-conditioned counterparts (Figure 1; FC-Veh-Form vs. No FC-Veh-Form), confirming the expression of FCA. Administration of the FAAH inhibitor, URB597, did not alter formalin-induced nociceptive behaviour per se (No FC-Veh-Form vs. No FC-URB597-Form) but significantly enhanced the expression of FCA (Figure 1; FC-URB597-Form vs. FC-Veh-Form).

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 vocalizations, and their co-occurrence (Figure 2) compared with their 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 vocalization co-occurrence and freezing alone in the first 10 min of the trial, and reduced the duration of 22-kHz ultrasonic vocalizations in the first 5 min of the trial (Figure 2; FC-Veh-Form vs. FC-Veh-Sal). The FAAH inhibitor, URB597, significantly reduced the duration of freezing behaviour over the first 5 min of the trial and reduced the duration of freezing–22-kHz ultrasonic vocalization co-occurrence from 3 to 7 min (Figure 2;

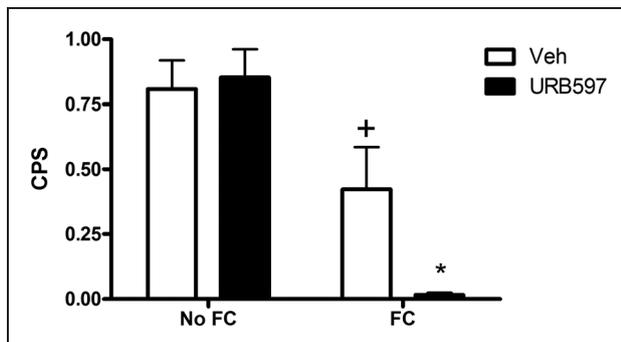


Figure 1. Effect of URB597 (0.3 mg/kg, i.p.) on the composite pain score (CPS) in non-fear-conditioned, formalin-treated and fear-conditioned formalin-treated rats. Data are means \pm SEM ($n = 7-9$). + $p < 0.05$ vs. No FC-Veh-Form, * $p < 0.05$ vs. FC-Veh-Form. Two-way ANOVA followed by Fisher's LSD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

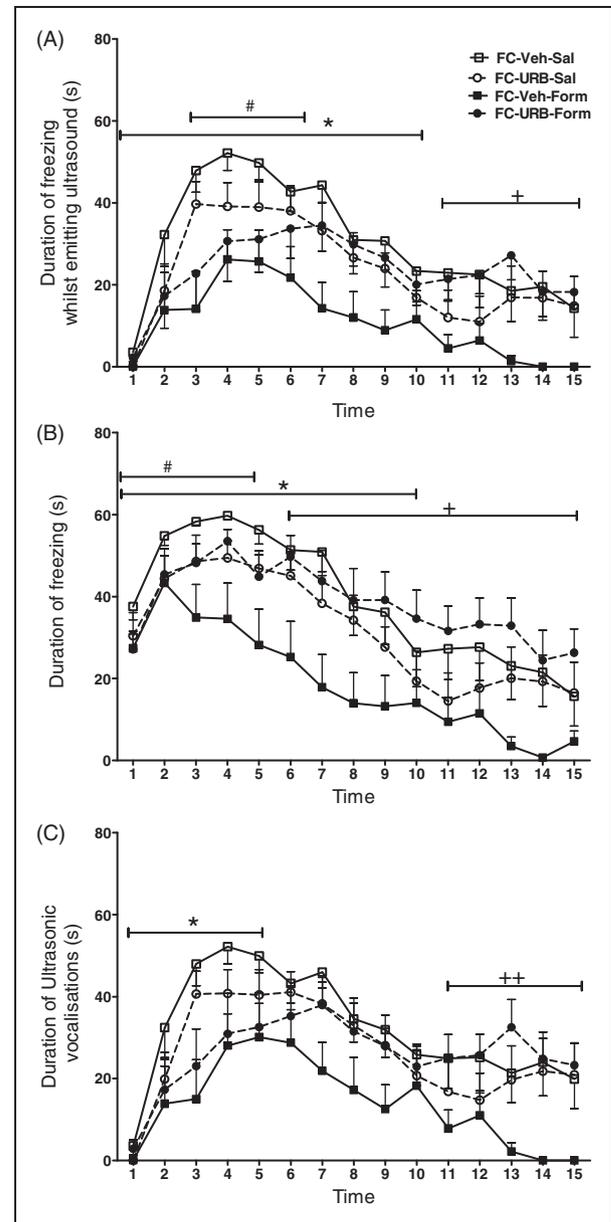


Figure 2. Effect of URB597 on the expression of contextually-induced fear behaviour in fear-conditioned saline-treated rats and fear-conditioned formalin-treated rats. Data are means \pm SEM. ($n = 8-10$). (A) Freezing behaviour whilst emitting 22-kHz ultrasonic vocalizations (significant effect of treatment $\chi^2 = 29.302$, $p < 0.001$; * $p < 0.05$ for FC-Veh-Sal vs. FC-Veh-Form at 0-10 min; # $p < 0.05$ for FC-Veh-Sal vs. FC-URB-Sal 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 $\chi^2 = 39.176$, $p < 0.001$; * $p < 0.05$ for FC-Veh-Sal vs. FC-Veh-Form at 0-10 min; # $p < 0.05$ for FC-Veh-Sal vs. FC-URB-Sal at 0-5 min; + $p < 0.05$ for FC-Veh-Form vs. FC-URB-Form at 6-15 min). (C) 22-kHz ultrasonic vocalizations (significant effect of treatment $\chi^2 = 25.220$, $p < 0.01$; * $p < 0.05$ 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.

FC-URB597-Sal vs. FC-Veh-Sal). In formalin-treated rats, URB597 significantly increased the duration of freezing–22-kHz ultrasonic vocalization co-occurrence and 22 kHz ultrasonic vocalizations alone in the last 5 min of the trial and increased the duration of freezing behaviour from 6 to 15 min compared with the respective vehicle-treated controls (Figure 2; FC-URB597-Form vs FC-Veh-Form).

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 their 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 their 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 with their 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.

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 with their non-fear-conditioned counterparts (Figure 3; FC-Veh-Sal vs. No FC-Veh-Sal). Systemic injection of URB597 significantly attenuated this fear-induced increase in relative phospho-Akt expression in the PAG (Figure 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 (Figure 3; FC-Veh-Form vs. No FC-Veh-Form and FC-Veh-Sal vs. FC-Veh-Form).

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 AEA and 2-arachidonoyl glycerol (2-AG) and the related *N*-acylethanolamines viz. PEA and 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)

Table 1. Effects of fear conditioning, formalin, and URB597 on general exploratory behaviours and hindpaw oedema in rats^a

			Sniffing (s)	Walking (s)	Grooming (s)	Rearing (s)	Total rears (number)	Δpaw diameter (cm)
No FC	Veh	Sal	452.3 ± 27.9	23.5 ± 2.6	142.5 ± 21.3	80.8 ± 10.1	34.3 ± 3.4	0.028 ± 0.013
No FC	URB597	Sal	439.0 ± 26.0	23.2 ± 2.3	152.2 ± 25.7	78.3 ± 8.6	33.2 ± 4.0	0.022 ± 0.011
No FC	Veh	Form	244.3 ± 29.4 [‡]	16.3 ± 2.0 [†]	83.5 ± 18.3 [†]	16.8 ± 4.0 [‡]	6.1 ± 1.5 [‡]	0.140 ± 0.011 [‡]
No FC	URB597	Form	277.6 ± 51.9	16.6 ± 1.8	69.6 ± 9.8	15.3 ± 4.0	8.4 ± 2.2	0.148 ± 0.022
FC	Veh	Sal	140.1 ± 35.9 [‡]	8.9 ± 2.8 [‡]	38.4 ± 9.8 [‡]	41.9 ± 15.8 [†]	17.7 ± 6.2 [‡]	0.046 ± 0.015
FC	URB597	Sal	198.8 ± 44.8	10.3 ± 3.4	46.7 ± 11.3	66.7 ± 17.6	26.1 ± 6.3	0.035 ± 0.007
FC	Veh	Form	166.2 ± 45.7	12.0 ± 3.0	56.0 ± 15.3	28.3 ± 14.0	11.3 ± 4.8	0.149 ± 0.015 ^{##}
FC	URB597	Form	123.2 ± 23.7	3.3 ± 0.7 ^{**}	35.4 ± 9.7	20.2 ± 6.3	7.2 ± 1.7	0.133 ± 0.012

^aData are means ± SEM ($n = 8-10$). [‡] $p < 0.01$, [†] $p < 0.05$ 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 × formalin $F_{1,72} = 10.26$, $p = 0.002$. Rearing: main effect of formalin $F_{1,72} = 34.15$, $p < 0.001$; conditioning × formalin $F_{1,72} = 4.35$, $p = 0.041$. Total rears: main effect of formalin $F_{1,72} = 42.98$, $p < 0.001$; conditioning × 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-conditioned; Veh, vehicle; Sal, saline; Form, formalin.

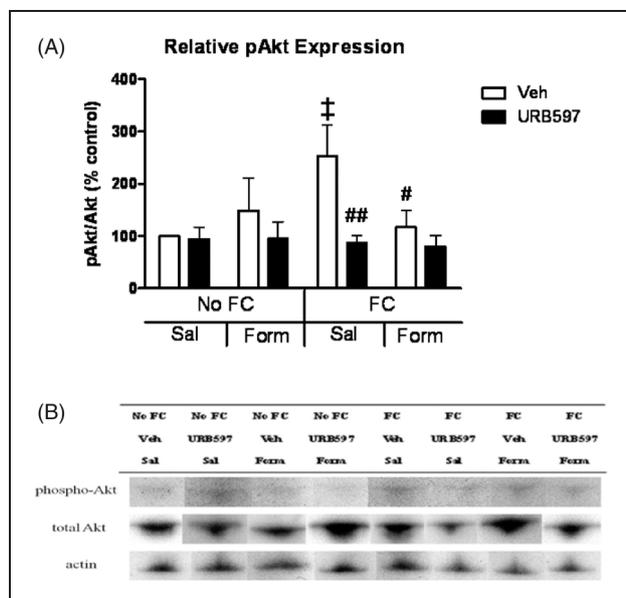


Figure 3. Relative phospho-Akt expression in the periaqueductal grey (PAG) following persistent pain, fear-conditioning, and fear-conditioned analgesia with and without URB597. (A) Data are means \pm SEM ($n = 5-8$). [‡] $p < 0.01$ vs. No FC-Veh-Sal, ^{##} $p < 0.01$, [#] $p < 0.05$ vs. FC-Veh-Sal. Three-way ANOVA followed by Fisher's LSD post-hoc test (conditioning \times URB597 $F_{1,53} = 6.17$, $p = 0.017$). FC, fear-conditioned; Form, formalin; Veh, vehicle. (B) Representative photomicrograph depicts bands for total Akt, phospho-Akt, and pan-Actin in the PAG from immunoblotting experiments.

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 a FAAH inhibitor, URB597, to rats 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 (Butler et al., 2008; Calcagnetti et al., 1987; Finn et al., 2004,

2006; Helmstetter and Fanselow, 1987a, 1987b; Roche et al., 2007). Furthermore, the results confirm our previous finding that systemic administration of the 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 CB₁ 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 CB₂ receptor antagonist. Studies by Hohmann and colleagues have demonstrated that a form of unconditioned stress-induced analgesia, which combines footshock stress with the tail-flick test, is enhanced by systemic administration of URB597 in a CB₁ 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-CB₁/non-CB₂ 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 (de Novellis 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 URB597 given i.p. 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 characterize 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 vocalization). 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 CB₁ 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 CB₁ 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 CB₁-null^{-/-} mice compared with wild-type littermates, suggesting, perhaps, that tonic endocannabinoid signalling through the CB₁ 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, which is the first to investigate the effects of formalin injection on Akt activation in the PAG, 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.

Funding

This work was supported by research grants from Science Foundation Ireland [grant number 05/Y12/B686] and the Health Research Board of Ireland [grant number RP/2005/92].

Conflict of interest

None declared.

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