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Endocannabinoid-mediated enhancement of fear-conditioned analgesia in rats: Opioid receptor dependency and molecular correlates

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Abstract

The opioid and endocannabinoid systems mediate analgesia expressed upon re-exposure to a contextually aversive stimulus (fear-conditioned analgesia; FCA), and modulate the mitogen-activated protein kinase (MAPK) pathway. However, an interaction between the opioid and endocannabinoid systems during FCA has not been investigated at the behavioural or molecular level. FCA was modeled in male Lister-hooded rats by assessing formalin-evoked nociceptive behaviour in an arena previously paired with footshock. Administration of the fatty acid amide hydrolase and endocannabinoid catabolism inhibitor, URB597 (0.3 mg/kg, i.p.), enhanced expression of FCA. The opioid receptor antagonist, naloxone, attenuated FCA and attenuated the URB597-induced enhancement of FCA. SR141716A (CB_1 antagonist) and SR144528 (CB_2 antagonist) also attenuated the URB597-mediated enhancement of FCA. Expression of FCA was associated with increased relative phospho-ERK2 expression in the amygdala, an effect blocked by naloxone, SR141716A, and SR144528. Furthermore, URB597-mediated enhancement of FCA was associated with reduced phospho-ERK1 and phospho-ERK2 in the amygdala. Phospho-ERK1/2 expression in the hippocampus, prefrontal cortex, and thalamus was unchanged following FCA and drug treatment. None of the drugs affected formalin-evoked nociceptive behaviour or phospho-ERK1/2 expression in non-fear-conditioned rats. These data suggest that endocannabinoid-mediated enhancement of FCA is abolished by pharmacological blockade of opioid receptors as well as CB_1 or CB_2 receptors. Both pharmacological enhancement (with URB597) and attenuation (with naloxone) of this form of endogenous analgesia were associated with reduced expression of phospho-ERK1/2 in the amygdaloid complex arguing against a causal role for ERK1/2 signaling in the amygdala during expression of FCA or its modulation by opioids or cannabinoids.

Keywords: Pain; Fear; Anxiety; Cannabinoid; Opioid; Extracellular signal-regulated kinase; URB597; Rat; Amygdala

1. Introduction

Given the high incidence of co-morbidity of anxiety disorders with persistent pain conditions [41] and recent evidence for altered pain processing in patients with post-traumatic stress disorder (PTSD) [16], studies of the neurobiological mechanisms underpinning altered pain-related activity in the presence of conditioned aversive stimuli are of fundamental physiological and potential therapeutic significance. Animal models of fear-conditioned analgesia (FCA) provide a useful tool with which to study the marked reduction in nociception which is expressed upon re-exposure to a context previously paired with an aversive stimulus.

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Pharmacological blockade of mu-opioid receptors attenuates anti-nociception expressed upon exposure to unconditioned [27] and conditioned [18,19] stressors. However, non-opioid mediated mechanisms are also involved (for review see [1]). Several recent studies have implicated the endogenous cannabinoid (endocannabinoid) system in non-opioid dependent forms of endogenous analgesia associated with aversion [12,21,42]. Conditioned fear in rodents is associated with an increase in endocannabinoids in the basolateral amygdala [29] and pharmacological blockade of CB_1 receptors attenuates FCA [12]. These data suggest that endocannabinoids released in response to conditioned aversive stimuli may act at CB_1 receptors to mediate FCA in a manner similar to that demonstrated for unconditioned stress-induced analgesia [21]. The present study has tested this hypothesis.

The ability of either mu-opioid [19] or CB_1 receptor antagonists [12] to completely attenuate FCA suggests that signaling through both mu-opioid and CB_1 receptors is necessary for the expression of FCA. Indeed, interactions between the endocannabinoid and opioid systems have been suggested at molecular and behavioural levels. A recent study proposed the formation of μ-opioid/CB_1 heterodimers [34]. In addition, a synergistic anti-nociceptive effect upon activation of both the opioid and endocannabinoid systems has been demonstrated [33,45]. However, attenuation of cannabinoid/opioid-induced anti-nociception by pharmacological or genetic inhibition of opioid/CB_1 receptors appears to be dependent upon the type of noxious stimulus (for review see [28]). Here we tested the hypothesis that endocannabinoid-mediated FCA is attenuated or abolished following pharmacological blockade of opioid receptors.

Stimulation of mu-opioid and CB_1 receptors activates similar signal transduction mechanisms including the mitogen-activated protein kinase (MAPK) pathway via phosphorylation of extracellular signal-regulated kinase (ERK)1/2 [2,15]. Recent evidence demonstrates a role for ERK phosphorylation in the amygdala in regulation of nociceptive responses during persistent inflammatory pain [6]. In addition, conditioned fear is associated with increased phospho-ERK expression in the amygdala, an effect sensitive to genetic deletion of the CB_1 receptor [5]. Here, we tested the hypothesis that opioid- and endocannabinoid-mediated FCA may also be associated with MAPK activation in the amygdala and in other key supraspinal components involved in nociceptive and fear processing (periaqueductal grey [PAG], thalamus, prefrontal cortex, hippocampus).

The first aim of the present study was to investigate the effects of pharmacological inhibition of fatty acid amide hydrolase (FAAH), the enzyme responsible for the degradation of the endocannabinoid, anandamide, on FCA and associated alterations in brain regional MAPK activation. The second aim was to investigate the extent to which the effects of FAAH inhibition were dependent on signaling through CB_1, CB_2, or opioid receptors.

2. Materials and methods

2.1. Animals

Male Lister-hooded rats (220–260 g; Charles River, Margate, Kent, UK) were housed in groups of 3 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 between 250 and 310 g on the experimental days. The Lister-hooded strain was chosen for comparison with our previous work demonstrating robust expression of fear-conditioned analgesia in this strain and its mediation by the endocannabinoid system [12,13,36]. All *in vivo* experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP [46] and were carried out following approval from the Animal 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.

2.2. Drug preparation

The FAAH inhibitor URB597 [(3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) Cayman Chemical, Ann Arbor, Michigan, USA] (0.3 mg/kg), opioid receptor antagonist naloxone hydrochloride dihydrate (Sigma–Aldrich Ireland, Ltd., Dublin, Ireland) (8.56 mg/kg naloxone salt or 7 mg/kg free base naloxone), CB_1 receptor antagonist SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-H-pyrazole-3-carboxamide, NIMH Chemical Synthesis Programme: Batch No. 10937-163-1] (1 mg/kg), and CB_2 receptor antagonist SR144528 [N-[(1s)-endo-1,3,3-timethylbicyclo[2.2.1]heptan-2-yl]5-(4-choro-3-methylpanyl)-1-(4-methlbenzyl)pyrazole-3-carboxamide, NIMH Chemical Synthesis Programme: Batch No. 11183-173-2] (1 mg/kg) were all dissolved in ethanol:cremaphor:saline (1:1:18) vehicle on the day of use. The drugs were administered at a volume of 1 ml/kg via the intra-peritoneal (i.p.) route.

2.3. Experimental procedures

The procedure for inducing fear-conditioned analgesia (FCA) was identical to that which we have described

previously [12,13,36]. 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 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% acetic acid between each conditioning treatment. There were two separate experiments in this study (Experiments 1 and 2). Experiment 1 investigated the effects of the compounds administered on fear-conditioned analgesia while Experiment 2 investigated their effects on pain *per se*. There were 9 experimental groups in Experiment 1 (full details below), 8 of which were exposed to the arena and received footshocks while a control group was exposed to the arena for an equivalent 9.5-min period without receiving footshocks. In Experiment 2, there were 5 experimental groups, all of which were exposed to the arena without receiving footshocks.

The test phase of the behavioural experiments began 23 h later. In Experiment 1, rats received an i.p. injection of URB597 (0.3 mg/kg) or vehicle and a second i.p. injection of either naloxone (7 mg/kg), SR141716A (1 mg/kg), SR144528 (1 mg/kg), or vehicle. In Experiment 2, rats received an i.p. injection of naloxone (7 mg/kg), SR141716A (1 mg/kg), SR144528 (1 mg/kg) or URB597 (0.3 mg/kg) all co-administered with a second i.p. vehicle injection. The dose of URB597 and time of administration were chosen on the basis of previous studies demonstrating its efficacy in rats [22,25] and pilot work in our laboratory demonstrating that this dosing regime enhanced FCA without affecting formalin-evoked nociceptive behaviour *per se*. Antagonist doses were based on previous studies demonstrating their ability to attenuate FCA without affecting formalin-evoked nociceptive behaviour *per se* [12,20]; the time of antagonist administration was based on our work demonstrating that co-administration of CB_1 or CB_2 receptor antagonists with URB597 attenuates the effects of this FAAH inhibitor [35]. Thirty minutes after i.p. injections, rats were briefly anaesthetised with isofluorane and the diameter of the right hindpaw was measured with Verniers calipers followed by an intra-plantar injection of 50 μl formalin (2.5% formaldehyde). Exactly 24 h following conditioning, (i.e. 30 min after intra-plantar injection of formalin), rats were returned to the Perspex arena previously paired with footshock. Before re-exposure, and between animals, the stainless steel bars and the arena were cleaned with 0.5% acetic acid. The study design resulted in 9 groups in Experiments 1 and 5 groups in Experiment 2: Experiment 1: no fear-conditioning + Vehicle + Vehicle (No FC-Veh-Veh), Fear-conditioning + Vehicle + Vehicle (FC-Veh-Veh), Fear-conditioning + Vehicle + URB597 (FC-Veh-URB597), Fear-conditioning + Naloxone + URB597 (FC-Nal-URB597), Fear-conditioning + SR141716A + URB597 (FC-SR141-URB597), Fear-conditioning + SR144528 + URB597 (FC-SR144-URB597), Fear-conditioning + Naloxone + Vehicle (FC-Nal-Veh), Fear-conditioning + SR141716A + Vehicle (FC-SR141-Veh), Fear-conditioning + SR144528 + Vehicle (FC-SR144-Veh);

Experiment 2: No fear-conditioning + Vehicle + Vehicle (No FC-Veh-Veh), No fear-conditioning + naloxone + vehicle (No FC-Nal-Veh), No fear-conditioning + SR141716A + vehicle (No FC-SR141-Veh), No fear-conditioning + SR144528 + vehicle (No FC-SR144-Veh), No fear-conditioning + URB597 + vehicle (No FC-URB597-Veh). 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 from 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 formalin-evoked nociceptive behaviour is stable over this time period and that fear-conditioned analgesia and conditioned fear expressed during this period is CB_1-dependent [12,14,36]. Following the 15-min re-exposure, rats were removed from the arena and rapidly decapitated. The brains were quickly removed and discrete brain areas (amygdaloid complex, hippocampus, periaqueductal grey, prefrontal cortex, and thalamus) were dissected on an ice-cold plate, snap frozen on dry ice and stored at −80 °C prior to Western Immunoblotting. 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.

2.3.1. 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 rater blind to the experimental conditions assessed behaviour. Formalin-evoked nociceptive behaviour was scored according to the weighted composite pain scoring technique ($CPS\text{-}WST_{0,1,2}$) described by Watson et al. [44]. 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). Formalin-induced oedema was assessed by

measuring the difference between the post-mortem diameter of the right hind paw and that measured before formalin administration. Aversive behaviour assessed included duration of freezing (cessation of all visible movement except that necessary for respiration) while emitting 22 kHz ultrasonic vocalisations. General exploratory and locomotor behaviours assessed included duration of sniffing, walking, grooming, rearing and rearing frequency.

2.4. Western immunoblotting

Frozen brain tissue was lysed at a ratio of 10 μl lysis buffer (80 mM sodium β-glycerophosphate, 1 mM dithiothreitol, 1 mM sodium fluoride, pH to 7.6) containing protease inhibitor cocktail (Sigma–Aldrich Ireland, Ltd., Dublin, Ireland) per 1 μg tissue weight. Tissue was homogenised 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 14,240*g* for 15 min at 4 °C. The supernatant was collected and Bradford assay was performed [3] to determine protein levels. Samples were diluted in ice-cold lysis buffer to give equal protein concentrations followed by addition of sample buffer (50 mM Tris–HCl, 1.84% SDS, 8% Glycerol, 2% bromophenol blue, and 5% 2-mercaptoethanol). Lysates were heated at 95 °C for 5 min. The proteins (40 μg in 20 μl of each sample) were then separated under reducing conditions by SDS–PAGE using 12% polyacrylamide gels and electroblotted onto a nitrocellulose protran membrane (0.2 μm; VWR International, UK). Membranes were rocked in blocking solution (5% milk, 0.5% Tween 20 in TBS) for two hours. Separate membranes were incubated in primary antibody diluent (2.5% milk, 0.05% Tween 20 in TBS) overnight at 4 °C containing anti-ERK1/2 (1:5000) or anti-phospho-ERK1/2 (1:2000) (Cell Signaling Technologies, Boston, MA, USA). Membranes were then rocked at room temperature in secondary antibody solution (2.5% BSA, 0.05% Tween 20 in TBS) containing secondary antibodies (1:10,000; peroxidase-conjugated AffiniPure mouse anti-rabbit IgG heavy and light antibodies; Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK). Chemiluminescence and film development were performed under safe light conditions. Membranes were exposed to chemiluminescent reagents (Thermo Fisher Scientific Inc., Rockford, IL, USA) for 5 min followed by exposure to film (Hyperfilm ECL; GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) for 1–10 s depending on the protein. The bands on all films were quantified using densitometric analysis on ImageJ (http://www.rsb.info.nih.gov/ij/) with an inverted lookup table. 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-ERK bands by the corrected integrated density value for the total ERK bands. The normalized values were divided by the control group (No FC-Veh-Veh) to obtain a percentage control value.

2.5. Statistical analysis

SPSS statistical package was used to analyse all data. All data were tested for equality of variance using Levene's test. Student's paired *t*-test was used to analyse the change in paw diameter following formalin administration and an unpaired *t*-test was used to compare behaviour and phospho-ERK1/2 expression in non-fear-conditioned vehicle-treated rats versus fear-conditioned vehicle-treated rats. Two-way analysis of variance was used to assess the effects of URB597 (factor 1) and antagonist treatment (factor 2) on behavioural and protein expression data in fear-conditioned rats. Post hoc pairwise comparisons were made using Fisher's LSD test when appropriate. Data were considered significant when $P < 0.05$. Results are expressed as group means ± standard error of the mean (±SEM).

3. Results

3.1. Effects of drugs on fear-conditioned analgesia

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. 1A and B). Fear-conditioned rats treated with vehicle displayed significantly less formalin-evoked nociceptive behaviour (i.e. CPS) compared with the non-fear-conditioned rats treated with vehicle (Fig. 1A, FC-Veh-Veh vs. No FC-Veh-Veh), confirming the expression of fear-conditioned analgesia (FCA). Systemic administration of naloxone, but not SR141716A or SR144528, significantly attenuated FCA (Fig. 1A, FC-Nal-Veh vs. FC-Veh-Veh). Administration of the FAAH inhibitor, URB597, significantly enhanced the expression of FCA (Fig. 1A, FC-Veh-URB597 vs. FC-Veh-Veh). This URB597-induced enhancement of FCA was blocked by co-administration of naloxone, SR141716A, or SR144528 (Fig. 1A, FC-Nal/SR141/SR144-URB597 vs. FC-Veh-URB597).

Importantly, none of the drugs investigated (naloxone, SR141716A, SR144528, or URB597) had any significant effect on formalin-evoked nociceptive behaviour in non-fear-conditioned rats (Fig. 1B, No FC-Nal/SR141/SR144/URB597-Veh vs. No FC-Veh-Veh).

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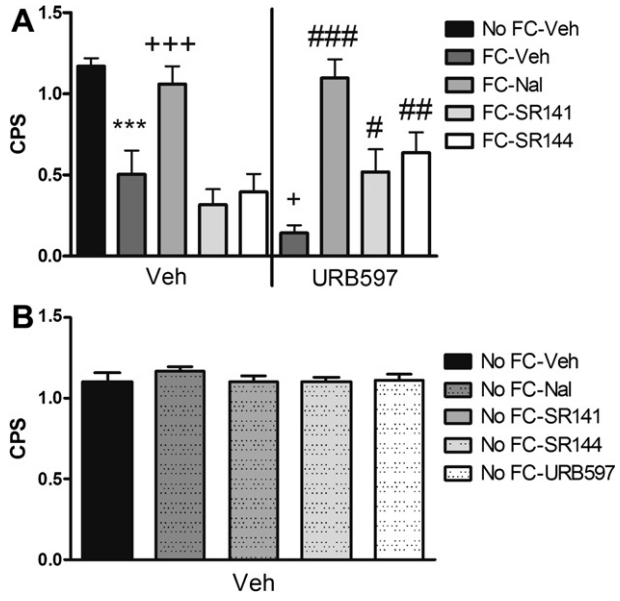

Fig. 1. (A) Effects of naloxone, SR141716A, and SR144528 on fear-conditioned analgesia in formalin-treated rats and its enhancement by the FAAH inhibitor URB597. $^{***}P < 0.001$ vs. No FC-Veh-Veh (Student's *t*-test, $P < 0.0005$), $^{+++}P < 0.001$, $^{+}P < 0.05$ vs. FC-Veh-Veh, $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ vs. FC-Veh-URB597 (two-way ANOVA followed by Fisher's post hoc test) (main effect of antagonist: $F_{3,102} = 14.825$, $P < 0.0005$). (B) Effects of naloxone, SR141716A, SR144528, and URB597 on formalin-evoked nociceptive behaviour in non-fear-conditioned rats. Data are means ± SEM ($n = 10$–14). CPS (composite pain score of formalin-evoked nociceptive behaviour). FC (fear conditioning), Veh (vehicle), Nal (Naloxone, 7 mg/kg, i.p.), SR141 (SR141716A, 1 mg/kg, i.p.), SR144 (SR144528, 1 mg/kg, i.p.).

3.2. Effects of drugs on conditioned aversive behaviour

Fear-conditioned rats receiving systemic administration of vehicle displayed the conditioned aversive behaviour of freezing while emitting 22 kHz ultrasonic vocalisations compared with non-fear-conditioned vehicle-treated rats which did not exhibit any freezing while ultrasounding (Fig. 2, FC-Veh-Veh vs. No FC-Veh-Veh). Systemic administration of the opioid receptor antagonist naloxone significantly reduced the duration of freezing while ultrasounding compared to fear-conditioned rats which received vehicle (Fig. 2, FC-Nal-Veh vs. FC-Veh-Veh). In contrast, the duration of freezing while ultrasounding was significantly increased in fear-conditioned rats receiving the FAAH inhibitor URB597 compared to fear-conditioned rats receiving vehicle injections (Fig. 2, FC-Veh-URB597 compared to FC-Veh-Veh). This URB597-induced enhancement of conditioned aversive behaviour was significantly attenuated by co-administration of naloxone, SR141716A, or SR144528 (Fig. 2, FC-Nal/SR141/SR144-URB597 vs. FC-Veh-URB597). Indeed, co-administration of naloxone with URB597 reduced the duration of conditioned aversive behaviour to levels comparable with rats receiving naloxone alone.

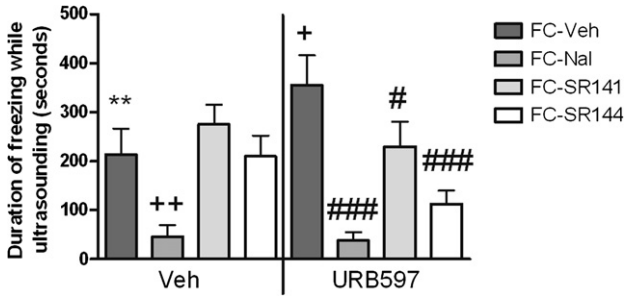

Fig. 2. Effects of naloxone, SR141716A, and SR144528 on conditioned aversive behaviour (duration of freezing while emitting 22 kHz ultrasonic vocalizations) in formalin-treated rats and its enhancement by the FAAH inhibitor URB597. Data are means ± SEM ($n = 11$–15). $^{**}P < 0.01$ vs. No FC-Veh-Veh (Student's *t*-test $P = 0.01$). Negligible value for No FC-Veh-Veh (not displayed). $^{++}P < 0.01$, $^{+}P < 0.05$ vs. FC-Veh-Veh, $^{###}P < 0.001$, $^{#}P < 0.05$ vs. FC-Veh-URB597 (two-way ANOVA followed by Fisher's post hoc test (main effect of antagonist $F_{3,103} = 11.87$, $P < 0.0005$; antagonist * URB597 $F_{3,103} = 2.942$, $P = 0.037$)). FC (fear conditioning), Veh (vehicle), Nal (Naloxone, 7 mg/kg, i.p.), SR141 (SR141716A, 1 mg/kg, i.p.), SR144 (SR144528, 1 mg/kg, i.p.).

3.3. Effects of fear-conditioning and drugs on general exploratory/locomotor behaviours and hindpaw oedema

Fear-conditioned rats which received a systemic injection of vehicle exhibited significantly less walking and grooming behaviours compared with their non-fear-conditioned counterparts (Table 1, FC-Veh-Veh vs. No FC-Veh-Veh). With the exception of a reduction in sniffing behaviour in rats which received URB597 co-administered with naloxone, SR141716A, or SR144528, compared with rats receiving URB597 co-administered with vehicle (Table 1, FC-Nal/SR141/SR144-URB597 vs. FC-Veh-URB597), there were no overt effects of antagonists on the general exploratory/locomotor behaviours analysed in fear-conditioned (Table 1) or non-fear-conditioned (data not shown) rats.

Intra-plantar injection of formalin produced oedema of the treated right hindpaw as indicated by an increase in paw diameter following injection (Table 1). The magnitude of the increase in paw diameter did not differ significantly between treatment groups.

3.4. Effects of fear-conditioning and drugs on relative phosphorylation of ERK1/2 expression in the amygdala

Levels of relative phospho-ERK2 expression were significantly higher in the amygdala of fear-conditioned, formalin-treated rats which received vehicle, compared to their non-fear-conditioned counterparts (Fig. 3B; No FC-Veh-Veh vs. FC-Veh-Veh). A similar fear-induced increase in levels of relative phospho-ERK1 expression was observed but just failed to reach statistical significance ($P = 0.071$; Fig. 3A). Fear-conditioned rats which received naloxone displayed lower levels of relative phospho-ERK1 and

Table 1
Effects of fear conditioning and drugs on general exploratory behaviours and hindpaw oedema in formalin-treated rats.

	No FC Veh Veh	FC Veh Veh	FC Nal Veh	FC SR141 Veh	FC SR144 Veh	FC Veh URB597	FC Nal URB597	FC SR141 URB597	FC SR144 URB597
Sniffing (seconds)	49.3 ± 6.3	42.2 ± 12.6	26.7 ± 6.0	24.0 ± 4.1	38.4 ± 6.9	65.7 ± 20.7	22.6 ± 4.7$^{\#\#}$	27.4 ± 5.6$^{\#\#}$	34.4 ± 6.1$^{\#}$
Walking (seconds)	35.6 ± 4.3	14.2 ± 3.5**	20.0 ± 2.8	13.0 ± 3.0	15.0 ± 2.7	13.2 ± 3.4	16.2 ± 3.5	13.9 ± 2.1	15.9 ± 2.9
Grooming (seconds)	42.9 ± 7.4	20.4 ± 2.9**	15.5 ± 4.8	33.9 ± 6.6	30.2 ± 9.0	24.2 ± 6.4	15.6 ± 2.5	28.2 ± 8.8	20.6 ± 4.9
Rearing (seconds)	20.9 ± 5.5	30.7 ± 8.7	18.2 ± 6.1	25.3 ± 9.0	30.6 ± 5.4	16.3 ± 3.2	11.1 ± 3.7	9.7 ± 3.2	20.8 ± 5.9
Total rears (number)	10.5 ± 2.2	11.2 ± 2.4	10.6 ± 2.6	9.9 ± 2.1	14.7 ± 2.4	10.0 ± 2.1	5.5 ± 1.2	7.1 ± 1.7	10.3 ± 2.3
Δpaw diameter (cm)	0.096 ± 0.018	0.088 ± 0.01	0.095 ± 0.01	0.103 ± 0.016	0.097 ± 0.02	0.093 ± 0.017	0.082 ± 0.013	0.072 ± 0.012	0.098 ± 0.014

Data are means ± SEM ($n = 10$–15).
Rearing: main effect of URB597 $F_{1,98} = 7.168$, $P = 0.009$. Total rears: main effect of URB597 $F_{1,100} = 4.765$, $P = 0.032$)). FC (fear conditioning), Veh (vehicle), Nal (Naloxone, 7 mg/kg, i.p), SR141 (SR141716A, 1 mg/kg, i.p.), SR144 (SR144528, 1 mg/kg i.p.).
$^{\#}$ $P < 0.05$ vs. FC-Veh-URB597 (two-way ANOVA followed by Fisher's post hoc test) (sniffing: main effect of antagonist $F_{3,100} = 3.779$, $P = 0.013$).
$^{\#\#}$ $P < 0.01$.
** $P < 0.01$ vs. No FC-Veh-Veh (Student's t-test: Walking $P = 0.001$; Grooming $P = 0.009$).

phospho-ERK2 expression in the amygdala, compared to fear-conditioned rats which received vehicle (Fig. 3A and B; FC-Nal-Veh vs. FC-Veh-Veh). Administration of SR141716A or SR144528 also significantly attenuated the fear-induced increase in levels of relative phospho-ERK2 expression in the amygdala, compared to fear-conditioned rats which received vehicle (Fig. 3B; FC-SR141/SR144-Veh vs. FC-Veh-Veh). Systemic administration of URB597 significantly reduced levels of relative phospho-ERK1 and phospho-ERK2 expression in the amygdala of fear-conditioned rats compared to fear-conditioned rats receiving vehicle (Fig. 3A and B; FC-veh-URB597 compared to FC-Veh-Veh). Co-administration of the CB_2 receptor antagonist SR144528 significantly attenuated the inhibitory effects of URB597 on relative phospho-ERK1 and phospho-ERK2 expression in the amygdala (Fig. 3A and B; FC-SR144-URB597 compared to FC-Veh-URB597) while co-administration of the CB_1 receptor antagonist SR141716A attenuated the effect of URB597 on phospho-ERK2 only (Fig. 3B; FC-SR141-URB597 compared to FC-Veh-URB597). Co-administration of naloxone had no significant effect on URB597-induced alterations in relative phospho-ERK1/2 expression in the amygdala.

None of the drugs investigated (naloxone, SR141716A, SR144528, or URB597) had any significant effect on relative phospho-ERK1 or phospho-ERK2 expression in the amygdala of non-fear-conditioned rats (Fig. 3C and D, No FC-Nal/SR141/SR144/URB597-Veh vs. No FC-Veh-Veh).

3.5. Effects of fear-conditioning and drugs on relative phosphorylation of ERK1/2 expression in the PAG

Fear-conditioning had no significant effect on levels of relative phospho-ERK1 and phospho-ERK2 expression in the PAG of formalin-treated rats (Fig. 4). However, administration of the CB_2 receptor antagonist, SR144528, or the FAAH inhibitor, URB597, significantly increased levels of relative phospho-ERK1 and phospho-ERK2 expression in the PAG of fear-conditioned, formalin-treated rats compared to their vehicle-treated counterparts (Fig. 4A and B, FC-SR144 or FC-Veh-URB597 vs. FC-Veh-Veh). Administration of naloxone or SR141716A had no significant effect on phospho-ERK1 and phospho-ERK2 expression in the PAG.

None of the drugs investigated (naloxone, SR141716A, SR144528, or URB597) had any significant effect on relative phospho-ERK1 or phospho-ERK2 expression in the PAG of non-fear-conditioned rats (Fig. 4C and D, No FC-Nal/SR141/SR144/URB597-Veh vs. No FC-Veh-Veh).

3.6. Effects of fear-conditioning and drugs on relative phosphorylation of ERK1/2 expression in the hippocampus, prefrontal cortex, and thalamus

There were no significant effects of fear-conditioning or drug treatment on relative levels of phospho-ERK1 or phospho-ERK2 expression in the hippocampus, prefrontal cortex, or thalamus of formalin-treated rats (data not shown).

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4. Discussion

In this study, we demonstrate that the FAAH and endocannabinoid catabolism inhibitor, URB597, enhances analgesia expressed upon exposure to an aversively conditioned context (FCA). We also demonstrate that this URB597-mediated enhancement of FCA is abolished by pharmacological blockade of opioid receptors with naloxone. Furthermore, administration of CB_1 or CB_2 receptor antagonists independently attenuated the URB597-induced enhancement of FCA, suggesting that endocannabinoid-mediated FCA is also dependent on CB_1 and CB_2 receptors. Additional results demonstrated an inverse relationship between pain- and fear-related behaviours and their pharmacological modulation. Moreover, FCA was associated with increased relative expression of phospho-ERK in the amygdala, an effect prevented by drugs which either enhanced or attenuated FCA.

The nature and magnitude of FCA observed here corroborates a number of studies demonstrating reduced nociceptive behaviour in rats re-exposed to a context previously paired with footshock [4,12,13,19,20,36]. Finn et al. [12] showed that systemic administration of the CB_1 receptor antagonist, SR141716A, attenuated FCA in rats, suggesting that endocannabinoids, levels of which are known to be increased during fear expression [29], may act at the CB_1 receptor to mediate FCA. The results of the present study, demonstrating enhancement of FCA following systemic injection of URB597, an inhibitor of endocannabinoid catabolism, and blockade of this URB597-induced enhancement by the CB_1 receptor antagonist SR141716A, provide further support for this hypothesis. The lack of effect of URB597 on formalin-evoked nociceptive behaviour *per se* in the absence of conditioned fear confirms that this compound is specifically modulating FCA under the present experimental conditions and supports the idea that an increase in endocannabinoid release during the expression of conditioned fear underlies the expression of FCA. Studies by Hohmann and colleagues have demonstrated that unconditioned stress-induced analgesia (SIA) is also mediated by the endocannabinoid system [8,21,39,40]. Thus, systemic administration of URB597 enhances the increase in tail-flick latency typically observed following exposure to footshock, an effect blocked by the CB_1 receptor antagonist SR141716A [21]. These workers have also provided evidence that the endocannabinoid system in brain regions including the PAG [21], rostral ventromedial medulla [39] and basolateral amygdala [8] mediates this form of endogenous analgesia. Clearly then, there is evidence for a role of the endocannabinoid system in mediating analgesia in response to both conditioned and unconditioned stress. It remains to be determined whether the mechanisms and neural substrates involved

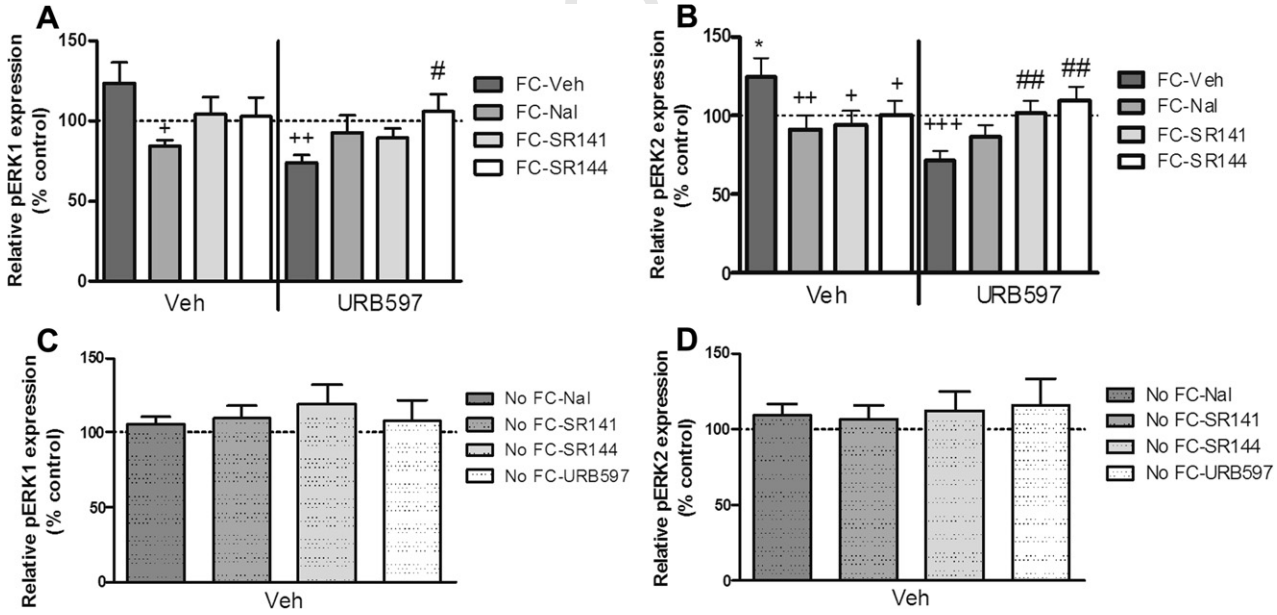

Fig. 3. Quantitative analysis of the effects of fear-conditioning on relative phospho-ERK1 (A) and phospho-ERK2 (B) expression in the amygdala of formalin-treated rats and modulation by URB597 and/or naloxone, SR141716A, and SR144528. $^{*}P < 0.05$ vs. No FC-Veh-Veh (Student's *t*-test (B: $P = 0.048$)), $^{+++}P < 0.001$, $^{++}P < 0.01$, $^{+}P < 0.05$ vs. FC-Veh-Veh, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. FC-Veh-URB597 (two-way ANOVA followed by Fisher's post hoc test (A: antagonist $*$ URB597 $F_{3,80} = 3.556$, $P = 0.018$; B: antagonist $*$ URB597 $F_{3,79} = 5.672$, $P = 0.002$). Quantitative analysis of the effects of naloxone, SR141716A, SR144528, and URB597 on relative phospho-ERK1 (C) and phospho-ERK2 (D) expression in the amygdala of formalin-treated rats which were not fear-conditioned. Data are means ± SEM ($n = 9$–11). The dotted line represents control value (No FC-Veh-Veh) normalized to 100%. FC (fear conditioning), Veh (vehicle), Nal (Naloxone, 7 mg/kg, i.p.), SR141 (SR141716A, 1 mg/kg, i.p.), SR144 (SR144528, 1 mg/kg, i.p.).

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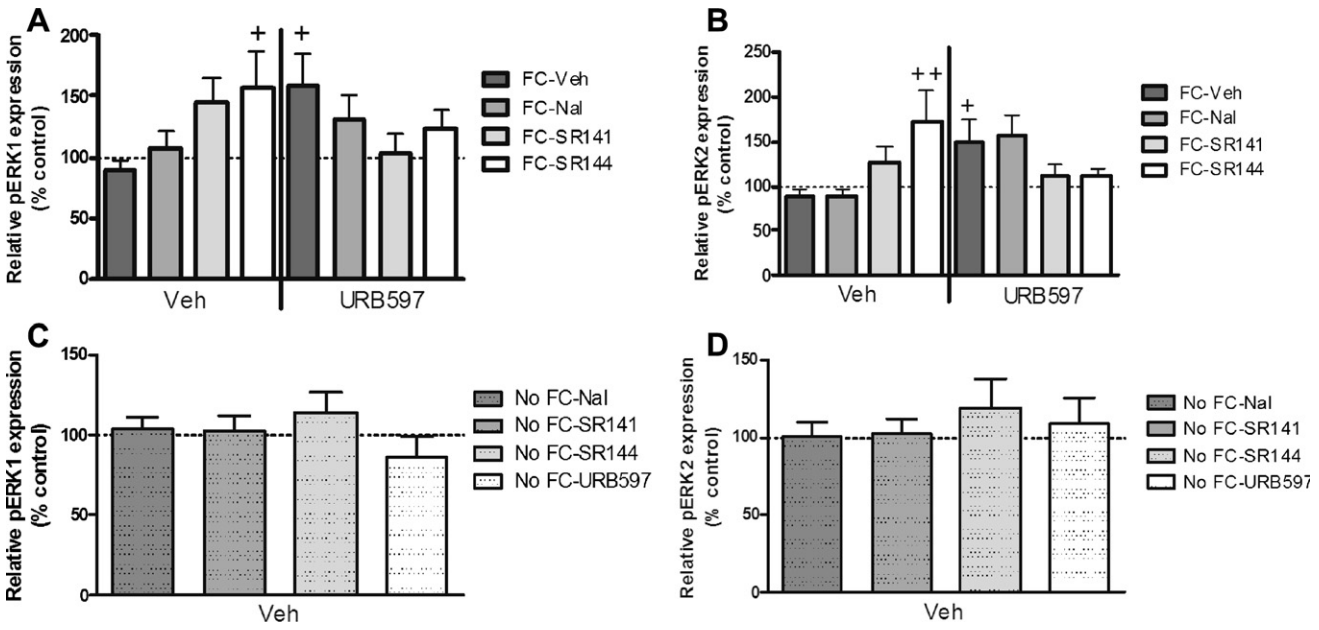

Fig. 4. Quantitative analysis of the effects of fear-conditioning on relative phospho-ERK1 (A) and phospho-ERK2 (B) expression in the periaqueductal grey (PAG) of formalin-treated rats and modulation by URB597 and/or naloxone, SR141716A, and SR144528. $^{++}P < 0.01$, $^{+}P < 0.05$ vs. FC-Veh-Veh (two-way ANOVA followed by Fisher's post hoc test (A: antagonist * URB597 $F_{3,80} = 3.185$, $P = 0.029$; B: antagonist * URB597 $F_{3,76} = 4.644$, $P = 0.005$)). Quantitative analysis of the effects of naloxone, SR141716A, SR144528, and URB597 on relative phospho-ERK1 (C) and phospho-ERK2 (D) expression in the PAG of formalin-treated rats which were not fear-conditioned. Data are means ± SEM ($n = 9$–11). The dotted line represents control value (No FC-Veh-Veh) normalized to 100%. FC (fear conditioning), Veh (vehicle), Nal (Naloxone, 7 mg/kg, i.p.), SR141 (SR141716A, 1 mg/kg, i.p.), SR144 (SR144528, 1 mg/kg, i.p.).

are common to both forms, but recent evidence from our laboratory suggests this may not be the case [36]. In addition, the present study revealed that the URB597-mediated enhancement of FCA was also blocked by the selective CB_2 receptor antagonist SR144528. There is a body of evidence supporting a role for the CB_2 receptor in analgesia (for review see [24]). The possibility that this cannabinoid receptor subtype may also be involved in mediating analgesia expressed following exposure to psychological, conditioned stress is intriguing, particularly in light of recent evidence suggesting that the CB_2 receptor may also be expressed supraspinally [17,32,43]. In the present study, neither the CB_1 nor the CB_2 ~~receptors~~ antagonists, administered alone, attenuated FCA. The lack of effect of the CB_2 antagonist is in agreement with Hohmann et al. [21] and suggests that activation of this subtype in response to conditioned or unconditioned stress may be insufficient to mediate this form of endogenous analgesia in the absence of FAAH inhibition. Systemic administration of the CB_1 receptor antagonist has, however, been shown previously to attenuate FCA [12] and the lack of effect in the present study may be due to the difference in time of administration (60 min prior to context re-exposure here versus 30 min in Finn et al. [12]) and/or increased injection-related stress in the present study which employed two i.p. injections to awake rats versus Finn et al. [12] which employed just a single i.p. injection to anaesthetised rats. Nevertheless, the CB_1 receptor dependency of the URB597-mediated enhancement of FCA highlights the importance of this cannabinoid receptor subtype in analgesia associated with aversion. A final point of consideration with respect to the mechanism of action of URB597 relates to evidence demonstrating that this compound may also activate non-cannabinoid receptor targets, including members of the transient receptor potential (TRP) ion channel superfamily. URB597 may indirectly activate TRPV1 via the increase in anandamide which results from FAAH inhibition [9]. Furthermore, it has recently been proposed that URB597 is a direct agonist at TRPA1 [31]. TRPV1 does not appear to mediate SIA [39], but the role of TRPA1 in endogenous analgesia remains to be determined.

The effects of naloxone here corroborate previous reports demonstrating attenuation of FCA following pharmacological blockade of opioid receptors [18,19]. We also demonstrate that the enhancement of FCA by URB597 is prevented by naloxone, suggesting a role for opioid receptors in endocannabinoid-mediated FCA. Previous studies have shown that (a) cannabinoid and mu-opioid receptor agonists act synergistically to induce anti-nociception [33,45] and (b) anti-nociceptive effects of the FAAH inhibitors URB597 [23] and OL135 [7] are blocked by naloxone. Recent evidence at the molecular level suggests that the CB_1 and mu-opi-

oid receptors may heterodimerize [34]. While the extent to which this putative molecular mechanism of crosstalk functions *in vivo* remains unknown, our data suggest that cannabinoid/opioid receptor interactions may extend to modulation of endogenous analgesia expressed during fear. Indeed, the abolition by naloxone of FCA in both vehicle- and URB597-treated rats may indicate that endogenous opioids are a final common step in the neurotransmitter pathways involved in this model of FCA.

The reduction in nociceptive behaviour during FCA was associated with an increase in conditioned aversive freezing while ultrasounding. Furthermore, the enhancement of FCA with URB597 occurred with a concomitant increase in conditioned aversive behaviours. In contrast, the attenuation by opioid, CB_1, or CB_2 receptor antagonists of both FCA, and its enhancement by URB597, was associated with a decrease in conditioned aversive behaviours. These data support a number of studies demonstrating an inverse relationship between the pain- and fear-related behaviours and suggesting that conditioned aversive behaviour and analgesia are mediated by common, overlapping neural substrates [11]. However, there is also evidence suggesting that it is possible to pharmacologically modulate FCA without altering fear responding [19] and vice versa [26,36]. On this point, we concur with Harris and Westbrook [18] who concluded that fear is sufficient but not necessary for analgesic responses to environmental stimuli [18].

Assessment of intracellular signaling can provide valuable insight into molecular correlates of animal behaviour and its pharmacological modulation. Previous work has demonstrated coupling of the MAPK pathway to both mu-opioid [15] and cannabinoid receptor [2] activation. Moreover, evidence suggests amygdala ERK activation plays a functional role in peripheral hypersensitivity during the formalin test [6] and in the consolidation [38] and reconsolidation [10] of conditioned fear. There is also evidence for altered fear-induced expression of phospho-ERKs in the amygdala of mice lacking the CB_1 receptor [5]. To date, however, no studies have investigated alterations in supraspinal MAPK signaling associated with FCA. Here, we demonstrate for the first time that expression of FCA is associated with increased expression of phospho-ERK2 in the amygdaloid complex. This increase was attenuated by administration of the antagonists naloxone, SR141716A, or SR144528, suggesting that opioid or endocannabinoid signaling through opioid, CB_1 or CB_2 receptors may mediate phosphorylation of ERK2 during FCA. However, we also observed decreased phospho-ERK2 and phospho-ERK1 expression in the amygdaloid complex following endocannabinoid-mediated enhancement of FCA by URB597. That is, attenuation of fear-induced phospho-ERK expression in the amygdaloid complex accompanied both the hyperalgesic effect of naloxone and the ~~hyopalgesic~~ effect Q2 of URB597 in this FCA model. This apparent dichotomy suggests that alterations in ERK1/2 phosphorylation in this brain region may not be causally related to expression of FCA or its modulation by cannabinoids or opioids. In the PAG, expression of FCA was not associated with altered expression of phospho-ERK1/2. The URB597-mediated enhancement of FCA was, however, associated with increased expression of phospho-ERK1/2 in the PAG. Here again though, the finding that the CB_2 receptor antagonist, SR144528 also increased phospho-ERK1/2 expression in the PAG but had no effect on FCA suggests that ERK phosphorylation in the PAG, as in the amygdala, may not be directly coupled to FCA. Activation of the CB_1 receptor, which is increased after administration of URB597, has been shown to increase or decrease phosphorylation of ERK. No fear- or drug-induced alterations in phospho-ERK1/2 expression were detected in the hippocampus, prefrontal cortex, and thalamus but one cannot exclude the possibility that the temporal profile of ERK1/2 phosphorylation in these regions differs from that in the amygdaloid complex and PAG.

In conclusion, this study has demonstrated that pharmacological inhibition of endocannabinoid degradation enhances FCA and that this endocannabinoid-mediated response is abolished following pharmacological blockade of opioid, CB_1 and CB_2 receptors. Discrete fear- and drug-induced alterations in phospho-ERK1/2 expression were observed in the amygdaloid complex and PAG but these were not consistent with a causal role for MAPK signaling in FCA. These data represent an advancement in our understanding of the pharmacology and molecular neurobiology of this form of potent endogenous analgesia.

Conflict of interest statement

The authors declare no conflict of interest.

Uncited reference

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