



Provided by the author(s) and University of Galway in accordance with publisher policies. Please cite the published version when available.

Title	A role for the ventral hippocampal endocannabinoid system in fear-conditioned analgesia and fear responding in the presence of nociceptive tone in rats.
Author(s)	Ford, Gemma K.; Kieran, Siobhan; Dolan, Kenneth; Harhen, Brendan; Finn, David P.
Publication Date	2011-08
Publication Information	Ford GK, Kieran S, Dolan K, Harhen B, Finn DP (2011). A role for the ventral hippocampal endocannabinoid system in fear-conditioned analgesia and fear responding in the presence of nociceptive tone in rats. <i>Pain</i> , 152(11):2495-504
Publisher	Elsevier
Item record	http://hdl.handle.net/10379/2525

Downloaded 2024-03-13T09:12:21Z

Some rights reserved. For more information, please see the item record link above.



A role for the ventral hippocampal endocannabinoid system in fear-conditioned analgesia and fear responding in the presence of nociceptive tone in rats

Gemma K.Ford^{1,2,3}, Siobhan Kieran¹, Kenneth Dolan¹, Brendan Harhen^{2,3}, David P. Finn^{1,2,3,*}

¹ Pharmacology and Therapeutics, School of Medicine, ²NCBES Neuroscience Cluster and ³Centre for Pain Research, National University of Ireland, Galway, University Road, Galway, Ireland

*Correspondence to Dr. David P. Finn, Pharmacology and Therapeutics, School of Medicine, National University of Ireland, Galway, University Road, Galway, Ireland.

Tel. +353-91-495280. Fax. +353-91-525700.

Email: david.finn@nuigalway.ie.

URL: http://www.nuigalway.ie/pharmacology/Dr_David_Finn.html

Number of text pages: 32

Number of Figures: 5

Number of Tables: 1

Keywords: Pain; Fear; ventral hippocampus; cannabinoid type 1 (CB₁) receptor; fatty acid amide hydrolase (FAAH); URB597; endocannabinoids; *N*-acylethanolamines

1. Introduction

Fear-conditioned analgesia (FCA) is an important survival response expressed upon re-exposure to a context or cue, paired, through Pavlovian conditioning, with an aversive stimulus. Induction of FCA results in suppression of pain/nociception in both humans and rodents [9,27]. Elucidation of substrates underpinning FCA advances our understanding of endogenous analgesic mechanisms and may aid identification of new therapeutic targets for pain and fear-related anxiety disorders, or their co-morbidity.

Aside from its function in learning and memory [50,53], the hippocampus has an established role in pain and aversion [2,46]. Pharmacological [54,55], molecular [13,42], electrophysiological [41,81] and neuroimaging [73] studies have demonstrated that it is an important structure subserving formalin-evoked nociceptive behaviour in rodents. Human neuroimaging studies have demonstrated that the hippocampus is activated by noxious stimuli [7] and in post-traumatic stress disorder (PTSD) patients exhibiting reduced pain responding [28]. In addition, the hippocampus, particularly the ventral aspect, is anatomically connected to components of the pain neuromatrix including the amygdala and prefrontal cortex (PFC) [35,66]. These regions are key components of the descending pain pathway [5] and facilitate the expression of FCA [9,27,31]. With respect to hippocampal involvement in conditioned aversion, evidence suggests that this structure mediates conditioned fear when the conditioned stimulus is contextual [43,51]. Pharmacological inactivation or lesioning of the hippocampus impairs freezing in rats following re-exposure to contextual cues previously paired with footshock [4,6,43,49,64,68,75,77]. Furthermore, the hippocampus is critically involved in extinction of conditioned aversion, and deficits in fear extinction have been purported to underlie anxiety disorders such as PTSD [58]. The ventral hippocampus (vHip) has received increasing attention due to 1) its reciprocal connections with the PFC and amygdala, which integrate anxiety or fear with contextual information relevant to pain [35,66] and 2) structural or functional alterations in the anterior hippocampus (the human analog of the vHip) following

context conditioning and recall of fear extinction memory [3,58]. However, despite this evidence, no studies have investigated the role of the hippocampus in FCA.

The endocannabinoid system is a key regulator of pain [33] and fear [44]. The expression and extinction of conditioned fear and stress-induced analgesia (including FCA), can be altered by manipulating endocannabinoid tone [10,11,25,34,52,57,60,69,70]. The hippocampus contains high concentrations of the endocannabinoids, 2-arachidonoylglycerol (2-AG) and *N*-arachidonylethanolamide (anandamide; AEA), and the related *N*-acylethanolamines, *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA) [23,28]. There is high expression of cannabinoid₁ (CB₁) receptors and the enzyme fatty acid amide hydrolase (FAAH) in the hippocampus [17,40] and evidence supports a role for the hippocampal endocannabinoid system in modulating aversive behaviour and memory [1,71,72,78]. The present study investigated the hypothesis that the endocannabinoid system in the vHip plays a role in the expression of FCA and fear-pain interactions.

Specifically, we investigated the effects of intra-vHip administration of the FAAH inhibitor, URB597, the CB₁ receptor antagonist/inverse agonist, rimonabant, or co-administration of these drugs, on FCA and on the expression of conditioned aversive behaviour in the presence of formalin-evoked nociceptive tone in rats. We also determined whether FCA was associated with alterations in vHip endocannabinoid concentrations.

2. Materials and Methods

2.1. Animals

Male Lister-hooded rats (220-260 g 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 prior to surgery. The animals were maintained at a constant temperature ($22 \pm 2^\circ \text{C}$) under standard lighting conditions (12:12 h light:dark cycle, lights on from 08.00 to 20.00 h). Food and water were available *ad libitum*. Rats weighed between 250-310 g on the experimental days. The experimental protocol was carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee, National University of Ireland, Galway under license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609 and all efforts were made to minimise the number of animals used and their suffering. All *in vivo* experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP.

2.2. Intra-ventral hippocampus guide cannula implantation

Stainless steel guide cannulae (12mm length, Plastics One Inc., Roanoke, Virginia, USA) were stereotaxically implanted above the right and left vHip (AP -0.46cm, ML $\pm 0.5\text{cm}$ relative to bregma, DV -0.59cm from skull surface, [63] under isoflurane anaesthesia (1-3% in O_2 ; 0.5 L/min). A second cohort of rats, designated off-target controls, had cannulae stereotaxically implanted above the right and left medial geniculate nucleus (AP -0.46cm, ML $\pm 0.32\text{cm}$ relative to bregma, DV -0.5cm from skull surface). A stylet made from stainless steel tubing (Plastics One Inc., Roanoke, Virginia, USA) was inserted into the

guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (1.25mg/25µl s.c., Rimadyl, Pfizer, Kent, UK), was administered before the surgery to manage post-operative analgesia. Following cannulae implantation, the rats were housed singly. At least 6 days were allowed for recovery post-surgery. During this period, the rats were handled and their body weight and general health monitored once daily.

2.3. Chemicals and drug preparation

The FAAH inhibitor URB597 [(3'-carbamoyl-biphenyl-3-yl)-cyclohexylcarbamate) Cayman Europe, Tallinn, Estonia] (0.1mM), the CB₁ receptor antagonist rimonabant (SR141716A) [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-H-pyrazole-3-carboxamide, NIMH Chemical Synthesis Programme: Batch no. 10937-163-1] (2mM), and a solution of both drugs (URB597 0.1mM + Rimonabant 2mM) were all prepared fresh on the day of use, dissolved in dimethyl sulfoxide vehicle (Sigma, Ireland). *N*-Arachidonyl ethanolamide (anandamide; AEA), 2-arachidonyl glycerol (2-AG), *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA), and their corresponding synthetic deuterated internal standards; *N*-arachidonyl ethanolamide-d8 (AEA-d8), 2-arachidonylglycerol-d8 (2AG-d8), *N*-palmitoyl ethanolamide-d4 (PEA-d4), *N*-oleoyl ethanolamide-d2 (OEA-d2) were all purchased from Cayman Europe (Tallinn, Estonia). Acetonitrile and formic acid were from Lennox Laboratory Supplies (Dublin, Ireland). All solvents and chemicals were of HPLC grade or higher.

2.4. Experimental procedures

The experimental procedure was identical to that described previously [10,11,25,60,70]. In brief, it consisted of two phases, conditioning and testing, occurring 24h apart. Subjects were randomly assigned to groups and the sequence of testing was randomized throughout the experiment in order to minimise any confounding effects associated with the order of testing.

On the conditioning day, rats were placed in a Perspex fear-conditioning/observation chamber (30 x 30 x 30 cm) and after 15 seconds they received the first of 10 footshocks (0.4mA, 1second duration; LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK) spaced 60 seconds apart. Fifteen seconds after the last footshock, rats were returned to their home cage. Controls not receiving footshock were exposed to the chamber for an equivalent 9.5 minute period. The Perspex arena and stainless steel bars were cleaned with 0.5% acetic acid between each conditioning session and between each animal during test day to remove any olfactory cues.

The test phase commenced 23.5h later when the subjects received an intra-plantar injection of 50 μ L formalin (2.5% in 0.9% saline, s.c.) into the right hindpaw under brief isoflurane anaesthesia. Rats were returned to their home cage for a further 15minutes, after which they received intra-vHip microinjections of either URB597 (0.1mM in 1 μ L), rimonabant (2mM in 1 μ L), combination of both drugs (URB597 0.1mM + rimonabant 2mM in 1 μ L) or vehicle (1 μ L DMSO) into the right and left vHip. Drugs or vehicle were microinjected bilaterally into the vHip in a volume of 1 μ L or deliberately off-target (medial geniculate nucleus) in a volume of 300 nL using an injector and Hamilton syringe attached to 50cm-long polyethylene tubing (0.75 mm OD, 0.28 mm ID, Harvard Apparatus, Kent UK) to minimize handling and enable injections to be carried out while the rats remained in the home cage. Microinjections were performed over a period of 1 minute and the needle was left in position for a further 1 minute to allow

diffusion of the drug before the cannula was withdrawn. The study design resulted in seven experimental groups: no-fear-conditioning+formalin+vehicle (NFC-Form-Veh; n =9), no-fear-conditioning+formalin+URB597 (NFC-Form-URB597; n = 7), no-fear-conditioning + formalin + rimonabant (NFC-Form-Rim; n= 8), fear-conditioning+formalin+vehicle (FC-Form-Veh; n=8), fear-conditioning+formalin+URB597 (FC-Form-URB597; n =8), fear-conditioning+formalin+rimonabant (FC-Form-Rim; n=8), and fear-conditioning+formalin+URB597+rimonabant (FC-Form-URB597+Rim; n=9). Six animals were used as off-target controls: fear-conditioning+formalin+vehicle (FC-Form-Veh; n = 3), fear-conditioning+formalin+URB597 (FC-Form-URB597; n = 3). Following intra-vHip injection, rats were returned to their home cage until 30 minutes post-formalin injection (i.e. 15 minutes post-drug or vehicle), at which point they were returned to the same perspex observation chamber to which they had been exposed during the footshock conditioning phase 24 h earlier. A bat detector (Batbox Duet, Batbox Ltd, West Sussex, UK) was positioned above the arena to detect ultrasonic vocalisation in the 22 kHz range and behaviours were recorded for 15 minutes from a video camera positioned under the observation chamber. The 30-45 minutes 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 are regulated by the endocannabinoid system [11,25,70]. Immediately following completion of the 15 minute test trial, rats were removed from the arena, decapitated, and 2% fast green dye (dissolved in DMSO) was immediately microinjected into the vHip (1µL) or off-target into the medial geniculate nucleus (300nL) to mark the injection sites. The brains were removed quickly, snap-frozen on dry ice and stored at -80 °C for subsequent histological verification of cannula positioning in the vHip and endocannabinoid measurements. All brains were frozen within three minutes post-decapitation.

2.5. Behavioural analysis

Behaviour was analysed using the Observer[®] software package (Noldus, Wageningen, Netherlands), which allowed for continuous event recording over each 15 minute trial. A rater blind to experimental conditions assessed fear-related behaviours (duration of freezing and 22 kHz ultrasonic vocalisation), formalin-evoked nociceptive behaviours and motor behaviours (rearing, grooming and walking) for the total duration of the trial as described previously [10,11,25,26,69,70]. Freezing was defined as cessation of all visible movement except that necessary for respiration. Formalin-evoked nociceptive behaviour, categorised as time spent raising the paw above the floor without contact with any other surface and holding, licking, biting, shaking or flinching the paw was scored and summated over the entire duration of the trial. Formalin-induced oedema was assessed by measuring the difference between the post-mortem diameter of the right hind paw and the diameter measured before formalin administration using Vernier callipers.

2.6. Histology

The sites of injection were determined prior to data analysis. Coronal sections of the vHip (30µm thickness) were collected on a cryostat and brain sections containing the fast green dye were mounted onto glass slides and counter-stained with cresyl violet in order to locate the precise position of the site of microinjection using a light microscope.

Ninety-five percent of the injections were placed within the borders of the right and left vHip in the CA1, CA3 and dentate gyrus regions (Figure 1) with the remaining 5% positioned outside the vHip on one or

both sides. Only the results of experiments in which both injections were correctly positioned in the vHip were included in the analyses.

2.7. Measurement of endocannabinoids and related N-acylethanolamines from Palkovits punched vHip tissue using liquid chromatography- tandem mass spectrometry (LC-MS-MS)

Quantitation of endocannabinoids and *N*-acylethanolamines was essentially as described previously with minor modifications [10]. Frozen coronal brain sections (300 μ m) containing the vHip from vehicle-treated rats (i.e. NFC-Form-Veh and FC-Form-Veh) were cut on a cryostat. vHip tissue was punched from the frozen sections using cylindrical brain punchers (Harvard Apparatus, internal diameter 2mm). vHip punches (average weights of punched tissue: 9.5 mg) were taken between bregma -4.30 mm and bregma -6.72mm) [61,63]. Each punched tissue sample was kept frozen on dry ice throughout the collection procedure, weighed and stored at -80°C prior to extraction for liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS). During extraction, punched tissue was first homogenised in 400 μ L of 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-d4, 0.015nmol OEA-d2). Homogenates were centrifuged at 14,000 g for 15 minutes at 4°C and the supernatant was collected and evaporated to dryness in a centrifugal evaporator. Lyophilised samples were resuspended in 40 μ L 65% acetonitrile and 2 μ L injected onto a Zorbax® C18 column (150 \times 0.5 mm internal diameter; Agilent Technologies Ltd, Cork, Ireland) 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 12 μ L/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 10 mins to re-equilibrate the column. The total run time was 30 min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 9.1 min, 9.8 min, 10.4 min and 10.8 min, respectively (Figure 5). Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6410 mass spectrometer (Agilent Technologies Ltd., Cork, Ireland). Instrument conditions and in particular source parameters such as fragmentor voltage and collision energy were optimised for each analyte by injecting standards separately. Quantitation of target analytes was achieved by positive ion electrospray ionization and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions of the analytes of interest and the deuterated form of the internal standard. MRM spectra and mass-to-charge (m/z) ratios of each analyte of interest and its corresponding internal standard are displayed in Figure 5. Quantitation of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was performed using Masshunter Quantitative Analysis Software (Agilent Technologies Ltd., Cork, Ireland). The amount of analyte in samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard. Linearity was determined over a range of 18.75 ng to 71.5 fg except for 2-AG which was 187.5 ng- 715 fg. The limit of quantification was 6.1 pmol/g for all analytes except for 2-AG where it was 48.3 pmol/g.

2.8. Statistical analysis

SPSS 17.0 statistical package was used to analyse all data. All data were tested for normality using Shapiro-Wilk test and for equality of variance using Levene's test. Non-parametric statistics were necessary to analyse behavioural data since these data were not normally distributed ($P < 0.05$ for Shapiro-Wilk and Levene's test for homogeneity of variance test). Behavioural (nociceptive) data were analysed using Kruskal Wallis ANOVA by rank followed by Mann-Whitney-U test. Behavioural changes over time (fear data) were analysed using a Friedman Two-Way ANOVA by rank followed by Wilcoxon matched pairs test for within-subjects effects (time and treatment) and Mann-Whitney-U test for between-subjects effects (treatment). Parametric statistics were employed to analyse the neurochemical data since these data were normally distributed ($P > 0.05$ for Shapiro-Wilk and Levene's test for homogeneity of variance test). Student's independent t-test was used to compare endocannabinoid concentrations within the vHip of non-fear-conditioned rats receiving intra-vHip vehicle and intra-plantar formalin injection versus their fear-conditioned counterparts. Student's independent t-test was also used to compare URB597 effects on behaviour in off-site control rats as these data followed a normal Gaussian distribution ($P < 0.05$ for Shapiro-Wilk and Levene's test for homogeneity of variance test). The level of statistical significance was set at $P < 0.05$. Results are expressed as group medians \pm interquartile range with min/max values for behavioural data and means \pm standard error of the mean (SEM) for neurochemical data and off-target behavioural data.

3. Results

3.1. Effects of intra-vHip administration of URB597 and/or rimonabant on fear-conditioned analgesia

Intra-plantar injection of formalin produced robust licking, biting, shaking, flinching and elevation of the injected paw (Figure 2). Fear-conditioned rats receiving intra-vHip vehicle displayed significantly less formalin-evoked nociceptive behaviour throughout the entire 15-minute trial period, compared with non-fear-conditioned rats treated with vehicle (Figure 2, Kruskal Wallis ANOVA by rank followed by Mann-Whitney-U test; significant effect of treatment: $X^2 = 33.487$, $P < 0.001$; NFC-Form-Veh vs. FC-Form-Veh, $U=0$, $p<0.001$), confirming the expression of fear-conditioned analgesia (FCA). The FAAH inhibitor, URB597, administered alone into the vHip, significantly enhanced the expression of FCA over the entire test trial (Figure 2, FC-Form-URB597 vs. FC-Form-Veh; $U = 12$, $P<0.05$). Off-target administration of the same dose of URB597 had no significant effect on the expression of FCA over the entire test trial (FC-Form-Veh vs. FC-Form-URB597, Mean \pm SEM = $25.0 \pm 8.34s$ vs. $31.0 \pm 1.55s$; $t_{(4)} = 0.708$, $p=0.518$). Intra-vHip administration of URB597 had no significant effect on formalin-induced nociceptive behaviour *per se* in non-fear-conditioned rats (Figure 2, NFC-Form-Veh vs. NFC-Form-URB597; $U=15$, $P=0.081$). The URB597-induced enhancement of the expression of FCA was significantly blocked by co-administration of the CB₁ receptor antagonist/inverse agonist rimonabant (Figure 2, FC-Form-URB597 vs. FC-Form-URB597+ Rim; $U = 13$, $P<0.05$). Intra-vHip administration of rimonabant alone, had no significant effects on formalin-induced nociceptive behaviour in fear-conditioned rats, nor did this drug have any significant effects on formalin-induced nociceptive behaviour

per se in non-fear-conditioned rats (Figure 2, NFC-Form-Rim vs. NFC-Form-Veh; U=25, P=0.29 and FC-Form-Rim vs. FC-Form-Veh; U= 23, P=3.45).

Intra-plantar injection of formalin also resulted in hind paw oedema (change in paw diameter in NFC-Form-Veh group = 0.18 ± 0.02 mm) which was unaffected by fear conditioning or intra-vHip drug administration (data not shown).

3.2. Effects of intra-vHip administration of URB597 and/or rimonabant on expression of contextual fear-related behaviour in the presence of formalin-evoked nociceptive tone

Fear-conditioned, formalin-injected rats receiving intra-vHip vehicle expressed significant contextually-induced freezing behaviour, 22 kHz ultrasound emission, and co-occurrence of these two behaviours throughout the test trial, compared with non-fear-conditioned counterparts in which these fear-related behaviours were not detectable (Figure 3, Kruskal Wallis ANOVA by rank followed by Mann-Whitney-U test; A significant effect of treatment: $X^2 = 42.78$, $P < 0.001$ NFC-Form-Veh vs. FC-Form-Veh U=0, $p < 0.0001$; B significant effect of treatment: $X^2 = 38.4$, $P < 0.001$; NFC-Form-Veh vs. FC-Form-Veh U=4.5, $p < 0.001$; C significant effect of treatment: $X^2 = 23.2$, $P < 0.001$, NFC-Form-Veh vs. FC-Form-Veh; U=4.5, $P < 0.001$, NFC data represented as dotted line). Temporal analysis of fear-related behaviour revealed that intra-vHip URB597 significantly increased the duration of freezing + 22 kHz ultrasonic vocalisation co-occurrence only in the first 5 minutes of the trial (Figure 3C, Friedman two-way ANOVA by rank followed by Mann-Whitney-U test; significant effect of treatment: $X^2 = 31.045$, $P < 0.001$; FC-Form-Veh 0-5min vs. FC-Form-URB597 0-5mins; U= 13, $P < 0.05$). This URB597-induced increase in conditioned aversive behaviour during tonic persistent pain tended to be blocked by co-administration of rimonabant although this effect failed to reach statistical significance (Figure 3C, FC-Form-URB597 0-5

min vs. FC-Form-URB597 + Rim 0-5 min: $U = 24$, $P = 0.248$). Intra-vHip rimonabant, administered either alone or in combination with URB597, had no significant effect on fear responding in the presence of formalin-evoked nociceptive tone throughout the entire trial. Contextually-induced freezing behaviour decreased significantly over the course of the test trial in rats that had received intra-vHip vehicle, rimonabant alone, and rimonabant in combination with URB597 indicating short-term within-trial extinction of conditioned fear responding in these rats (Figure 3A, Friedman two-way ANOVA by rank followed by Wilcoxon Matched pairs test; FC-Form 0-5 min vs. FC-Form 11-15 min; Vehicle: $Z = 2.20$, $P < 0.05$; Rimonabant: $Z = 2.20$, $P < 0.05$; URB597 + Rimonabant: $Z = 2.52$, $P < 0.05$). However, intra-vHip administration of URB597 disrupted this short-term extinction of fear-related behaviour by prolonging the duration of freezing in fear-conditioned, formalin-treated rats (Figure 3A, FC-Form-URB597 0-5min vs. FC-Form-URB597 11-15min, $Z = 1.86$, $P = 0.063$), an effect that was significantly attenuated by co-administration of rimonabant (FC-Form-URB597 11-15min vs. FC-Form-URB597 + Rim 11-15min: $U = 10$, $P < 0.05$). Contextually-induced 22 kHz aversive vocalisations decreased significantly over the course of the test trial in rats that had received either intra-vHip vehicle or rimonabant alone (Figure 3B, FC-Form 0-5 min vs. FC-Form 11-15 min; Vehicle: $Z = 1.99$, $P < 0.05$; Rimonabant: $Z = 2.37$, $P < 0.05$; URB597 + Rimonabant: $Z = 2.52$, $P < 0.05$). Intra-vHip administration of URB597, administered either alone, or in combination with rimonabant, disrupted this short-term extinction of fear-related aversive vocalisations (Figure 3B, FC-Form 0-5 min vs. FC-Form 11-15 min; URB597: $Z = 0.70$, $P = 0.484$; URB597 + Rimonabant: $Z = 0.845$, $P = 0.398$).

3.3. Effects of fear-conditioning and intra-vHip administration of URB597 and/or rimonabant on motor activity

There was no significant effect of fear conditioning or intra-vHip URB597 or rimonabant, alone or in combination, on rat motor activity measured as the sum duration of rearing, grooming and walking (Figure 4).

3.4. Expression of FCA is associated with increases in levels of endocannabinoids and PEA in the vHip

Fear-related alterations in levels of the endocannabinoids, AEA and 2-AG, and the related *N*-acylethanolamines, PEA and OEA, in the vHip of vehicle-treated formalin-injected rats killed immediately after completion of the 15 minute behavioural trial were examined. FCA was still expressed robustly at this time-point (Kruskal Wallis ANOVA by rank followed by Mann-Whitney-U test on formalin-evoked nociceptive data from the last minute of the trial; significant effect of treatment: $X^2 = 29.495$, $P < 0.001$; NFC-Form-Veh vs. FC-Form-Veh $U = 3$, $P = 0.001$). All analytes were detectable in tissue punches of the vHip and were above the limit of quantitation for the assay (Table 1, Figure 5). The concentrations of all analytes in punched vHip tissue were similar to the range of concentrations reported in the literature for gross dissected rat hippocampal tissue [19,22,23,67]. Levels of AEA, 2-AG and PEA were all significantly higher in the vHip of fear-conditioned, formalin-treated rats which received intra-vHip vehicle, compared with their non-fear-conditioned counterparts (Table 1, NFC-Form-Veh vs. FC-Form-Veh; AEA: $t_{(14)} = 3.42$, $P < 0.01$; 2-AG: $t_{(14)} = 2.79$, $P < 0.05$; PEA: $t_{(15)} = 2.23$, $P < 0.05$). Thus, expression of FCA was accompanied by increases in vHip levels of these analytes. An increase in levels of OEA was also observed but this failed to reach statistical significance ($t_{(15)} = 1.539$, $P = 0.145$; Table1).

4. Discussion

The results of the present study revealed that pharmacological inhibition of the enzyme FAAH within the rat vHip enhanced antinociception expressed upon exposure to an aversively conditioned context (i.e. FCA). This effect is likely to be CB₁ receptor-mediated as it was blocked by the CB₁ receptor antagonist/inverse agonist rimonabant. Inhibition of vHip FAAH also selectively increased the expression of conditioned aversive behaviour in the presence of formalin-evoked nociceptive tone. Moreover, expression of FCA was associated with elevated levels of the endocannabinoids, AEA and 2-AG, and the related fatty acid amide, PEA, in the vHip. Together, these data suggest an important role for the endocannabinoid system in the vHip in endogenous pain suppression during conditioned aversion and extend the current literature to include the ventral pole of the hippocampus as another neural substrate mediating this important survival response.

The antinociception observed here following re-exposure to an aversively conditioned context corroborates previous studies demonstrating robust expression of FCA in rats following Pavlovian fear conditioning [11,20,25,31,69,70,80]. Our previous work has demonstrated that this form of potent endogenous analgesia is mediated by the endocannabinoid system [11,25]. Thus, systemic administration of the CB₁ receptor antagonist/inverse agonist, rimonabant, prevents expression of FCA in rats [25] while systemic administration of the FAAH inhibitor, URB597, enhances FCA via CB₁ and CB₂ receptor-dependent mechanisms [11]. These findings corroborate the work of Hohmann and colleagues who have demonstrated involvement of the endocannabinoid system in a form of unconditioned stress-induced analgesia in rats [34]. Stress-induced analgesia is thought to be mediated by activation of the descending inhibitory pain pathway, of which the amygdala, PAG and RVM are key components, and indeed a key role for the endocannabinoid system in these brain regions during expression of stress-induced analgesia

[16,34,74] and FCA [60,69] has been demonstrated. However, surprisingly, given its key role in contextual memory and limbic system function, there is a paucity of studies investigating the role of the vHip in stress- or fear-induced analgesia. Here, we demonstrate for the first time that the endocannabinoid system in the vHip plays an important role in regulating the expression of FCA in rats. Direct injection of the FAAH inhibitor URB597 into the vHip enhanced FCA. The lack of a significant effect of intra-vHip URB597 on formalin-evoked nociceptive behaviour in non-fear-conditioned rats suggests specific involvement of FAAH substrates in this region in the suppression of pain responding during fear rather than involvement in the regulation of pain responding *per se*. The URB597-induced enhancement of FCA was blocked by co-administration of rimonabant into the vHip and is therefore likely to be CB₁ receptor-mediated. Analysis of endocannabinoid tissue content in the vHip revealed that the expression of FCA was associated with an increase in levels of AEA and 2-AG in this brain region. Considering this result together with the pharmacological data, it seems reasonable to suggest that the URB597-induced enhancement of FCA may be a consequence of the drug's inhibitory effect on the catabolism of fear-mobilised AEA (and to a lesser extent 2-AG) in the vHip, with the enhanced levels of endocannabinoids then activating CB₁ receptors in this region to effect a change in behaviour. More work would be necessary, however, to determine whether local injection of URB597 selectively elevates AEA levels within the vHip. Moreover, we also observed that another FAAH substrate, PEA, was elevated in the vHip of rats expressing FCA. By competing with AEA as a substrate for FAAH, elevations in PEA may further enhance the levels and actions of AEA in the vHip via the so-called 'entourage effect' [45]. Alternatively, or in addition, PEA may exert effects directly through its actions at non-CB₁ receptor targets including peroxisome-proliferator-activated receptors (PPAR α ; [37,47]) and GPR55 [30]. It is worth noting that while rimonabant blocked the URB597-induced enhancement of FCA, it did not by itself have any effect on FCA. This result suggests that while CB₁ receptors in the vHip are involved in

the modulation of FCA following FAAH inhibition, their tonic activation by endocannabinoids does not appear to be critical for expression of FCA.

FCA is mediated by the activation of emotionally responsive brain regions, including those of the limbic system and brainstem, which recruit and modulate the activity of the descending inhibitory pain pathway via endocannabinergic, opioidergic, GABAergic and monoaminergic signalling, to dampen nociceptive transmission in the dorsal horn and reduce pain perception [9]. The vHip is a key component of the limbic system and it contains neurons that express cannabinoid, opioid, and GABA receptors [32,38,40,56]. There is a paucity of pharmacological studies investigating the involvement of the vHip in nociception, but microinjection of morphine into the dorsal hippocampus produces antinociception by inhibiting GABAergic interneurons resulting in the disinhibition of hippocampal pyramidal cells [8,21]. It has been suggested by these authors [21] that the observed antinociceptive response following injection of morphine into this region may be the result of activation and/or recruitment of a neural circuit similar to or perhaps identical to that described for the descending inhibitory pain pathway [5]. However, at the present time the specific neural substrates and circuitry mediating opioid-induced hippocampal antinociception are unknown. Similarly, the precise neural circuitry and neurochemical mechanisms mediating the enhancement of FCA by intra-vHip administration of URB597 observed here remain to be elucidated. However, one possibility is that the increased availability of fear-mobilised FAAH substrates following intra-vHip URB597 results in the activation of CB₁ receptors on GABAergic basket cells within this region [40], resulting in the inhibition of these GABAergic interneurons with consequent disinhibition of hippocampal pyramidal neurons, activation of anatomically connected downstream structures such as the amygdala [12,35,36,65], and ultimately enhanced antinociception through hyperactivation of the descending inhibitory pain pathway [5]. The results of the present study provide a solid foundation upon

which to design further studies aimed at elucidating the precise neurochemical mechanisms and neural circuitry involved in the modulation of FCA by endocannabinoid signalling in the vHip.

Our experimental design also enabled assessment of the role of the vHip endocannabinoid system in the expression of conditioned fear responding in the presence of formalin-evoked nociceptive tone. Our results demonstrated that intra-vHip administration of URB597 increased the co-occurrence of freezing and 22 kHz ultrasonic vocalisation, in the early part of the trial, and disrupted the short-term within-trial extinction of fear-related behaviour. These data indicate that during persistent pain state, FAAH inhibition within the vHip potentiates short-term expression of conditioned aversive behaviour. The initial URB597-induced enhancement of fear responding at the start of the trial was not significantly blocked by rimonabant; however, by the end of the trial, fear responding in rimonabant co-treated rats was minimal while rats which had received URB597 alone continued to express robust fear-related behaviour. Administration of rimonabant alone into the vHip had no significant effect on fear responding, suggesting that endocannabinoid signalling in this region may serve to modulate rather than mediate fear-related behaviour in the presence of nociceptive tone. This is the first report on the role of the vHip endocannabinoid system in fear responding in the presence of nociceptive tone. The role of the endocannabinoid system in fear responding *per se* (i.e. in the absence of nociceptive tone) has been examined extensively and the weight of evidence suggests that endocannabinoid-CB₁ receptor signalling serves to facilitate and enhance the extinction of conditioned fear responding (for review see [15,48,79]), while genetic deletion or pharmacological blockade of the CB₁ receptor attenuates short- and long-term extinction of conditioned fear responding [14,18,25,39,44,48,52,59,62,70,76]. The results of the present study, however, suggest that in the presence of formalin-evoked nociceptive tone, increased endocannabinoid signalling in the vHip (as a consequence of URB597 administration) in fact serves to

inhibit rather than enhance the short-term, within-trial extinction of fear responding. This result supports our previous studies which have provided additional evidence for differential modulation of fear responding by the endocannabinoid system in the presence versus absence of pain [25,69,70]. In addition, and in agreement with the present findings, there are also reports of anxiogenic effects (dependent on dose) of URB597 or the phytocannabinoid and CB₁ receptor agonist Δ^9 -tetrahydrocannabinol (THC) following their direct administration into the vHip [71,72]. Overall then, it is likely that the net effect of endocannabinoid activity on anxiety/fear-related behaviour will depend both on the specific region/sub-region and the environmental or aversive context under investigation.

Psychiatric disorders related to both maladaptation to stress and the extinction of aversive memories such as panic, phobia and PTSD can present with altered pain processing [29]. The aetiology of these disorders has been suggested to involve dysfunction of the endocannabinoid system (for review see [24]). Increased understanding of the neuroanatomy, neurochemistry and neuropharmacology of endogenous analgesic and aversive systems with models such as FCA, may aid in the development of new endocannabinoid-based therapies for these debilitating disorders. Here we propose the vHip as a candidate substrate mediating FCA and provide evidence that endocannabinoid signalling within this region may serve to modulate both FCA and fear responding in the presence of persistent pain state.

Acknowledgements

This work was supported by a grant from Science Foundation Ireland. The authors have no conflicts of interest to report.

References

- [1] Abush H, Akirav I. Cannabinoids modulate hippocampal memory and plasticity. *Hippocampus* 2009.
- [2] Aloisi AM, Zimmermann M, Herdegen T. Sex-dependent effects of formalin and restraint on c-Fos expression in the septum and hippocampus of the rat. *Neuroscience* 1997;81(4):951-958.
- [3] Alvarez RP, Biggs A, Chen G, Pine DS, Grillon C. Contextual fear conditioning in humans: cortical-hippocampal and amygdala contributions. *J Neurosci* 2008;28(24):6211-6219.
- [4] Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J. Regional dissociations within the hippocampus--memory and anxiety. *Neurosci Biobehav Rev* 2004;28(3):273-283.
- [5] Basbaum AI, Fields HL. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. *Annu Rev Neurosci* 1984;7:309-338.
- [6] Bast T, Zhang WN, Feldon J. The ventral hippocampus and fear conditioning in rats. Different anterograde amnesias of fear after tetrodotoxin inactivation and infusion of the GABA(A) agonist muscimol. *Exp Brain Res* 2001;139(1):39-52.
- [7] Bécerra L, Breiter HC, Wise R, Gonzalez RG, Borsook D. Reward circuitry activation by noxious thermal stimuli. *Neuron* 2001;32(5):927-946.
- [8] Blasco-Ibanez JM, Martinez-Guijarro FJ, Freund TF. Enkephalin-containing interneurons are specialized to innervate other interneurons in the hippocampal CA1 region of the rat and guinea-pig. *Eur J Neurosci* 1998;10(5):1784-1795.
- [9] Butler RK, Finn DP. Stress-induced analgesia. *Prog Neurobiol* 2009;88(3):184-202.
- [10] Butler RK, Ford GK, Hogan M, Roche M, Doyle KM, Kelly JK, Kendall DA, Chapman V, Finn DP. Fear-induced suppression of nociceptive behaviour and activation of Akt signalling in the rat periaqueductal grey: Role of fatty acid amide hydrolase. *Psychopharmacology* 2011;In Press.

- [11] Butler RK, Rea K, Lang Y, Gavin AM, Finn DP. Endocannabinoid-mediated enhancement of fear-conditioned analgesia in rats: opioid receptor dependency and molecular correlates. *Pain* 2008;140(3):491-500.
- [12] Canteras NS, Swanson LW. Projections of the ventral subiculum to the amygdala, septum, and hypothalamus: a PHAL anterograde tract-tracing study in the rat. *J Comp Neurol* 1992;324(2):180-194.
- [13] Ceccarelli I, Scaramuzzino A, Aloisi AM. Effects of formalin pain on hippocampal c-Fos expression in male and female rats. *Pharmacol Biochem Behav* 1999;64(4):797-802.
- [14] Chhatwal JP, Davis M, Maguschak KA, Ressler KJ. Enhancing cannabinoid neurotransmission augments the extinction of conditioned fear. *Neuropsychopharmacology* 2005;30(3):516-524.
- [15] Chhatwal JP, Ressler KJ. Modulation of fear and anxiety by the endogenous cannabinoid system. *CNS Spectr* 2007;12(3):211-220.
- [16] Connell K, Bolton N, Olsen D, Piomelli D, Hohmann AG. Role of the basolateral nucleus of the amygdala in endocannabinoid-mediated stress-induced analgesia. *Neurosci Lett* 2006;397(3):180-184.
- [17] Cristino L, Starowicz K, De Petrocellis L, Morishita J, Ueda N, Guglielmotti V, Di Marzo V. Immunohistochemical localization of anabolic and catabolic enzymes for anandamide and other putative endovanilloids in the hippocampus and cerebellar cortex of the mouse brain. *Neuroscience* 2008;151(4):955-968.
- [18] de Oliveira Alvares L, de Oliveira LF, Camboim C, Diehl F, Genro BP, Lanziotti VB, Quillfeldt JA. Amnestic effect of intrahippocampal AM251, a CB1-selective blocker, in the inhibitory avoidance, but not in the open field habituation task, in rats. *Neurobiol Learn Mem* 2005;83(2):119-124.

- [19] Di Marzo V, Berrendero F, Bisogno T, Gonzalez S, Cavaliere P, Romero J, Cebeira M, Ramos JA, Fernandez-Ruiz JJ. Enhancement of anandamide formation in the limbic forebrain and reduction of endocannabinoid contents in the striatum of delta9-tetrahydrocannabinol-tolerant rats. *J Neurochem* 2000;74(4):1627-1635.
- [20] Fanselow MS, Baackes MP. Conditioned fear-induced opiate analgesia on the Formalin test: Evidence for two aversive motivational systems. *Learning and Motivation* 1982;13(2):200-221.
- [21] Favaroni Mendes LA, Menescal-de-Oliveira L. Role of cholinergic, opioidergic and GABAergic neurotransmission of the dorsal hippocampus in the modulation of nociception in guinea pigs. *Life Sci* 2008;83(19-20):644-650.
- [22] Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D. Characterization of the fatty acid amide hydrolase inhibitor cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleoylethanolamide deactivation. *J Pharmacol Exp Ther* 2005;313(1):352-358.
- [23] Felder CC, Nielsen A, Briley EM, Palkovits M, Priller J, Axelrod J, Nguyen DN, Richardson JM, Riggin RM, Koppel GA, Paul SM, Becker GW. Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat. *FEBS Lett* 1996;393(2-3):231-235.
- [24] Finn DP. Endocannabinoid-mediated modulation of stress responses: Physiological and pathophysiological significance. *Immunobiology* 2009.
- [25] Finn DP, Beckett SR, Richardson D, Kendall DA, Marsden CA, Chapman V. Evidence for differential modulation of conditioned aversion and fear-conditioned analgesia by CB1 receptors. *Eur J Neurosci* 2004;20(3):848-852.

- [26] Finn DP, Jhaveri MD, Beckett SR, Madjd A, Kendall DA, Marsden CA, Chapman V. Behavioral, central monoaminergic and hypothalamo-pituitary-adrenal axis correlates of fear-conditioned analgesia in rats. *Neuroscience* 2006;138(4):1309-1317.
- [27] Ford GK, Finn DP. Clinical correlates of stress-induced analgesia: evidence from pharmacological studies. *Pain* 2008;140(1):3-7.
- [28] Freund TF, Katona I, Piomelli D. Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev* 2003;83(3):1017-1066.
- [29] Geuze E, Westenberg HG, Jochims A, de Kloet CS, Bohus M, Vermetten E, Schmahl C. Altered pain processing in veterans with posttraumatic stress disorder. *Arch Gen Psychiatry* 2007;64(1):76-85.
- [30] Godlewski G, Offertaler L, Wagner JA, Kunos G. Receptors for acylethanolamides-GPR55 and GPR119. *Prostaglandins Other Lipid Mediat* 2009;89(3-4):105-111.
- [31] Helmstetter FJ. The amygdala is essential for the expression of conditional hypoalgesia. *Behav Neurosci* 1992;106(3):518-528.
- [32] Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J Neurosci* 1991;11(2):563-583.
- [33] Hohmann AG, Suplita RL, 2nd. Endocannabinoid mechanisms of pain modulation. *Aaps J* 2006;8(4):E693-708.
- [34] Hohmann AG, Suplita RL, Bolton NM, Neely MH, Fegley D, Mangieri R, Krey JF, Walker JM, Holmes PV, Crystal JD, Duranti A, Tontini A, Mor M, Tarzia G, Piomelli D. An endocannabinoid mechanism for stress-induced analgesia. *Nature* 2005;435(7045):1108-1112.
- [35] Ishikawa A, Nakamura S. Ventral hippocampal neurons project axons simultaneously to the medial prefrontal cortex and amygdala in the rat. *J Neurophysiol* 2006;96(4):2134-2138.

- [36] Jasmin L, Ohara PT. Anatomical identification of neurons responsive to nociceptive stimuli. *Methods Mol Med* 2004;99:167-188.
- [37] Jhaveri MD, Richardson D, Robinson I, Garle MJ, Patel A, Sun Y, Sagar DR, Bennett AJ, Alexander SP, Kendall DA, Barrett DA, Chapman V. Inhibition of fatty acid amide hydrolase and cyclooxygenase-2 increases levels of endocannabinoid related molecules and produces analgesia via peroxisome proliferator-activated receptor-alpha in a model of inflammatory pain. *Neuropharmacology* 2008;55(1):85-93.
- [38] Kalyuzhny AE, Wessendorf MW. Relationship of mu- and delta-opioid receptors to GABAergic neurons in the central nervous system, including antinociceptive brainstem circuits. *J Comp Neurol* 1998;392(4):528-547.
- [39] Kamprath K, Marsicano G, Tang J, Monory K, Bisogno T, Di Marzo V, Lutz B, Wotjak CT. Cannabinoid CB1 receptor mediates fear extinction via habituation-like processes. *J Neurosci* 2006;26(25):6677-6686.
- [40] Katona I, Sperlagh B, Sik A, Kafalvi A, Vizi ES, Mackie K, Freund TF. Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J Neurosci* 1999;19(11):4544-4558.
- [41] Khanna S. Dorsal hippocampus field CA1 pyramidal cell responses to a persistent versus an acute nociceptive stimulus and their septal modulation. *Neuroscience* 1997;77(3):713-721.
- [42] Khanna S, Chang LS, Jiang F, Koh HC. Nociception-driven decreased induction of Fos protein in ventral hippocampus field CA1 of the rat. *Brain Res* 2004;1004(1-2):167-176.
- [43] Kim JJ, Fanselow MS. Modality-specific retrograde amnesia of fear. *Science* 1992;256(5057):675-677.

- [44] Lafenetre P, Chaouloff F, Marsicano G. The endocannabinoid system in the processing of anxiety and fear and how CB1 receptors may modulate fear extinction. *Pharmacol Res* 2007;56(5):367-381.
- [45] Lambert DM, Di Marzo V. The palmitoylethanolamide and oleamide enigmas : are these two fatty acid amides cannabimimetic? *Curr Med Chem* 1999;6(8):757-773.
- [46] Liu MG, Chen J. Roles of the hippocampal formation in pain information processing. *Neurosci Bull* 2009;25(5):237-266.
- [47] Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D. The nuclear receptor peroxisome proliferator-activated receptor- α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol* 2005;67(1):15-19.
- [48] Lutz B. The endocannabinoid system and extinction learning. *Mol Neurobiol* 2007;36(1):92-101.
- [49] Maren S. Neurotoxic or electrolytic lesions of the ventral subiculum produce deficits in the acquisition and expression of Pavlovian fear conditioning in rats. *Behav Neurosci* 1999;113(2):283-290.
- [50] Maren S. Neurobiology of Pavlovian fear conditioning. *Annu Rev Neurosci* 2001;24:897-931.
- [51] Maren S, Fanselow MS. Synaptic plasticity in the basolateral amygdala induced by hippocampal formation stimulation in vivo. *J Neurosci* 1995;15(11):7548-7564.
- [52] Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgansberger W, Di Marzo V, Lutz B. The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 2002;418(6897):530-534.
- [53] McEwen BS. Plasticity of the hippocampus: adaptation to chronic stress and allostatic load. *Ann N Y Acad Sci* 2001;933:265-277.

- [54] McKenna JE, Melzack R. Analgesia produced by lidocaine microinjection into the dentate gyrus. *Pain* 1992;49(1):105-112.
- [55] McKenna JE, Melzack R. Blocking NMDA receptors in the hippocampal dentate gyrus with AP5 produces analgesia in the formalin pain test. *Exp Neurol* 2001;172(1):92-99.
- [56] McLean S, Rothman RB, Jacobson AE, Rice KC, Herkenham M. Distribution of opiate receptor subtypes and enkephalin and dynorphin immunoreactivity in the hippocampus of squirrel, guinea pig, rat, and hamster. *J Comp Neurol* 1987;255(4):497-510.
- [57] Mikics E, Dombi T, Barsvari B, Varga B, Ledent C, Freund TF, Haller J. The effects of cannabinoids on contextual conditioned fear in CB1 knockout and CD1 mice. *Behav Pharmacol* 2006;17(3):223-230.
- [58] Milad MR, Rauch SL, Pitman RK, Quirk GJ. Fear extinction in rats: implications for human brain imaging and anxiety disorders. *Biol Psychol* 2006;73(1):61-71.
- [59] Niyuhire F, Varvel SA, Thorpe AJ, Stokes RJ, Wiley JL, Lichtman AH. The disruptive effects of the CB1 receptor antagonist rimonabant on extinction learning in mice are task-specific. *Psychopharmacology (Berl)* 2007;191(2):223-231.
- [60] Olango WM, Roche M, Ford GK, Harhen B, Finn DP. The endocannabinoid system in the rat dorsolateral periaqueductal grey mediates fear-conditioned analgesia and controls fear expression in the presence of nociceptive tone. *Br J Pharmacol* 2011. DOI: 10.1111/j.1476-5381.2011.01478.x
- [61] Palkovits M. Punch sampling biopsy technique. *Methods Enzymol* 1983;103:368-376.
- [62] Pamplona FA, Bitencourt RM, Takahashi RN. Short- and long-term effects of cannabinoids on the extinction of contextual fear memory in rats. *Neurobiol Learn Mem* 2008.
- [63] Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. New York: Academic Press, 1998.

- [64] Pentkowski NS, Blanchard DC, Lever C, Litvin Y, Blanchard RJ. Effects of lesions to the dorsal and ventral hippocampus on defensive behaviors in rats. *Eur J Neurosci* 2006;23(8):2185-2196.
- [65] Petrovich GD, Canteras NS, Swanson LW. Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. *Brain Res Brain Res Rev* 2001;38(1-2):247-289.
- [66] Pitkanen A, Pikkarainen M, Nurminen N, Ylinen A. Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. *Ann N Y Acad Sci* 2000;911:369-391.
- [67] Richardson D, Ortori CA, Chapman V, Kendall DA, Barrett DA. Quantitative profiling of endocannabinoids and related compounds in rat brain using liquid chromatography-tandem electrospray ionization mass spectrometry. *Anal Biochem* 2007;360(2):216-226.
- [68] Richmond MA, Yee BK, Pouzet B, Veenman L, Rawlins JN, Feldon J, Bannerman DM. Dissociating context and space within the hippocampus: effects of complete, dorsal, and ventral excitotoxic hippocampal lesions on conditioned freezing and spatial learning. *Behav Neurosci* 1999;113(6):1189-1203.
- [69] Roche M, Johnston P, Mhuirheartaigh ON, Olango WM, Mackie K, Finn DP. Effects of intra-basolateral amygdala administration of rimonabant on nociceptive behaviour and neuronal activity in the presence or absence of contextual fear. *Eur J Pain* 2010;14(5):487-495.
- [70] Roche M, O'Connor E, Diskin C, Finn DP. The effect of CB(1) receptor antagonism in the right basolateral amygdala on conditioned fear and associated analgesia in rats. *Eur J Neurosci* 2007;26(9):2643-2653.
- [71] Roohbakhsh A, Keshavarz S, Hasanein P, Rezvani ME, Moghaddam AH. Role of endocannabinoid system in the ventral hippocampus of rats in the modulation of anxiety-like behaviours. *Basic Clin Pharmacol Toxicol* 2009;105(5):333-338.

- [72] Rubino T, Guidali C, Vigano D, Realini N, Valenti M, Massi P, Parolaro D. CB1 receptor stimulation in specific brain areas differently modulate anxiety-related behaviour. *Neuropharmacology* 2008;54(1):151-160.
- [73] Shih YY, Chen YY, Chen CC, Chen JC, Chang C, Jaw FS. Whole-brain functional magnetic resonance imaging mapping of acute nociceptive responses induced by formalin in rats using atlas registration-based event-related analysis. *J Neurosci Res* 2008;86(8):1801-1811.
- [74] Suplita RL, 2nd, Farthing JN, Gutierrez T, Hohmann AG. Inhibition of fatty-acid amide hydrolase enhances cannabinoid stress-induced analgesia: sites of action in the dorsolateral periaqueductal gray and rostral ventromedial medulla. *Neuropharmacology* 2005;49(8):1201-1209.
- [75] Sutherland RJ, O'Brien J, Lehmann H. Absence of systems consolidation of fear memories after dorsal, ventral, or complete hippocampal damage. *Hippocampus* 2008;18(7):710-718.
- [76] Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ, Kida S. Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* 2004;24(20):4787-4795.
- [77] Trivedi MA, Coover GD. Lesions of the ventral hippocampus, but not the dorsal hippocampus, impair conditioned fear expression and inhibitory avoidance on the elevated T-maze. *Neurobiol Learn Mem* 2004;81(3):172-184.
- [78] Viveros MP, Marco EM, Llorente R, Lamota L. The role of the hippocampus in mediating emotional responses to nicotine and cannabinoids: a possible neural substrate for functional interactions. *Behav Pharmacol* 2007;18(5-6):375-389.
- [79] Viveros MP, Marco EM, Llorente R, Lopez-Gallardo M. Endocannabinoid system and synaptic plasticity: implications for emotional responses. *Neural Plast* 2007;2007:52908.

- [80] Westbrook RF, Greeley JD, Nabke CP, Swinbourne AL. Aversive conditioning in the rat: effects of a benzodiazepine and of an opioid agonist and antagonist on conditioned hypoalgesia and fear. *J Exp Psychol Anim Behav Process* 1991;17(3):219-230.
- [81] Zheng F, Khanna S. Hippocampal field CA1 interneuronal nociceptive responses: modulation by medial septal region and morphine. *Neuroscience* 1999;93(1):45-55.

Figure Legends

Figure 1. Schematic depicting the sites of injector placement in rats that received Vehicle (open squares), URB597 (open triangles), rimonabant (closed circles) or URB597 + rimonabant (grey triangles) bilaterally into the ventral hippocampus. Adapted from [63].

Figure 2. Effects of URB597 and rimonabant, administered alone or in combination directly into the ventral hippocampus, on expression of formalin-evoked nociceptive behaviour and fear-conditioned analgesia (FCA) in rats throughout the entire 15 min trial. ^{**}P < 0.01 vs. NFC-Form counterpart; [#]P < 0.05, vs. FC-Form-Veh; ⁺P < 0.05 vs. FC-Form-URB597 (Kruskal Wallis ANOVA by rank followed by Mann-Whitney-U test) (Significant effect of treatment: $X^2 = 33.487$, $P < 0.001$). Data are median \pm interquartile range with min/max values (n = 7–9). FC (fear conditioned), NFC (non-fear conditioned), Form (formalin), Veh (vehicle), Rim (rimonabant).

Figure 3. Effects of URB597 and rimonabant, administered alone or in combination directly into the ventral hippocampus, on expression of contextually induced freezing behaviour (**A**), duration of 22kHz ultrasonic vocalisations (**B**) and the co-occurrence of contextually induced freezing behaviour whilst emitting 22kHz ultrasonic vocalisations (**C**) in rats. [#]P < 0.05 for FC-Form-Veh vs. FC-Form-URB597 at 0-5 min; ⁺P < 0.05 for FC-Form-URB597 vs. FC-Form-URB597+Rim at 11-15 min; ^ψP < 0.05 all FC-groups 0-5 min vs all FC-groups 11-15 min (Friedman two-way ANOVA by rank followed by Wilcoxon Matched pairs test for within-subjects effects and Mann-Whitney-U test for between subjects effects) (**A**. Significant effect of Treatment $X^2 = 30.401$, $P < 0.001$; **B**. Significant effect of Treatment $X^2 = 22.311$, $P < 0.05$; **C**. Significant effect of Treatment $X^2 = 31.045$, $P < 0.001$). Fear-related behaviour in non-fear-

conditioned rats receiving either vehicle, URB597 or rimonabant was not detected in the present study. It is represented here as a dotted line on the figure. Data are median \pm interquartile range with min/max values ($n = 7-9$). FC (fear conditioned), NFC (non-fear conditioned), Form (formalin), Veh (vehicle), Rim (rimonabant).

Figure 4. Effects of fear-conditioning and intra-vHip URB597 and rimonabant, administered alone or in combination directly into the ventral hippocampus, on motor activity measured as the sum duration of rearing, grooming and walking. Data are median \pm interquartile range with min/max values ($n = 7-9$). FC (fear conditioned), NFC (non-fear conditioned), Form (formalin), Veh (vehicle), Rim (rimonabant).

Figure 5. Representative chromatograms and precursor and product ion mass-to-charge (m/z) ratios for all analytes detected in rat ventral hippocampus and their corresponding deuterated synthetic analytes used as internal standards. 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 ($m/z = 387.3-294.2$).

Table 1. Endocannabinoid concentrations measured within the ventral hippocampus of both non-fear conditioned and fear-conditioned formalin-treated rats receiving intra-vHip vehicle.

	NFC-Form-Veh	FC-Form-Veh
AEA (pmol/g)	61.9 ± 5.51	102.0 ± 10.54 **
2AG (nmol/g)	7.61 ± 1.47	18.42 ± 3.97 *
PEA (nmol/g)	0.230 ± 0.043	0.382 ± 0.054 *
OEA (nmol/g)	0.221 ± 0.031	0.298 ± 0.040

Data are means ± SEM (n = 7-9). ** P < 0.01 , * P < 0.05 vs. NFC- Veh-Form (Student's *t*-test). FC (fear conditioning), NFC (Non-fear conditioned).

Figure 1.









