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Evidence for a role of GABAergic and glutamatergic signalling in the basolateral amygdala in endocannabinoid-mediated fear-conditioned analgesia in rats

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ABSTRACT

The basolateral amygdala (BLA) is a key substrate facilitating the expression of fear-conditioned analgesia (FCA). However, the neurochemical mechanisms in the BLA which mediate this potent suppression of pain responding during fear remain unknown. The present study investigated the role of cannabinoid$_1$ (CB_1) receptors and interactions with GABAergic ($GABA_A$ receptor) and glutamatergic (metabotropic glutamate receptor type 5; mGluR5) signalling in the BLA in formalin-evoked nociceptive behaviour and FCA in rats. Reexposure to a context previously paired with foot shock significantly reduced formalin-evoked nociceptive behaviour. Systemic or intra-BLA microinjection of the CB_1 receptor antagonist/inverse agonist AM251 prevented this expression of FCA, while injection of AM251 into the central nucleus of the amygdala did not. The suppression of FCA by systemic AM251 administration was partially attenuated by intra-BLA administration of either the $GABA_A$ receptor antagonist bicuculline or the mGluR5 antagonist 2-methyl-6-(phenylethynyl) pyridine, (MPEP). Bilateral microinjection of MPEP, but not bicuculline, alone into the BLA enhanced formalin-evoked nociceptive behaviour. Postmortem analyses revealed that FCA was associated with a significant increase in tissue levels of anandamide in the BLA side contralateral to intraplantar formalin injection. In addition, fear-conditioned rats exhibited a robust formalin-induced increase in levels of 2-arachidonyl glycerol and *N*-palmitoylethanolamide in the ipsilateral and contralateral BLA, respectively. These data suggest that CB_1 receptors in the BLA facilitate the expression of FCA, through a mechanism which is likely to involve the modulation of GABAergic and glutamatergic signalling.

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1. Introduction

The transmission of nociceptive information within the central nervous system is subject to modulation by complex, coordinated neural processes at a number of different anatomical loci. Neural substrates mediating the expression of pain and fear overlap, and determining the mechanisms by which fear can suppress pain responding may help us better understand the nature of these phenomena and their interaction. As a critical element of both the limbic system and the descending inhibitory pain pathway, the basolateral amygdala (BLA) is involved in emotional processing and coordination of appropriate responses to conditioned aversive stimuli [13] and also plays a key role in the expression of fear-conditioned analgesia (FCA) [27–29]. FCA is characterized by a robust suppression of nociceptive behaviour during or after expression of classical Pavlovian conditioned fear [5,16,20,25].

Studies of the supraspinal neurotransmitter systems involved in FCA have focussed predominantly on γ-aminobutyric acid (GABA)ergic [25,27,65] and opioidergic [8,15,23,26,30] mechanisms. Recent evidence supports a key role for the endogenous cannabinoid (endocannabinoid) system in mediating FCA [6,7,16,18,21,60,68,69], as well as unconditioned stress-induced analgesia [36,80] in rats. However, potential interactions of the endocannabinoid system with the classical neurotransmitter systems (e.g., GABAergic and glutamatergic) during FCA are lacking.

Immunohistochemical studies have confirmed a dense expression of cannabinoid$_1$ (CB_1) receptors on GABAergic interneurons [34,45,50,61,83,84], and on glutamatergic pyramidal projection

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neurons [50,52,59,61] in multiple brain regions involved in the expression of both conditioned fear and nociception [33,34], including the BLA. Presynaptically located CB_1 receptors are $G_{i/o}$-protein coupled receptors and are negatively coupled to adenylyl cyclase [38], positively coupled to mitogen-activated protein kinase [4] and coupled to a variety of ion channels, including potassium and calcium [37]; as such, they likely modulate the release of GABA and glutamate from neurons in the BLA.

Here, we investigated the involvement of GABAergic and glutamatergic signalling in the BLA in endocannabinoid-mediated FCA. Previous studies from our laboratory [67] and by others [25] have demonstrated that $GABA_A$ receptor activation in the BLA attenuates FCA. A role for glutamatergic neurotransmission in the expression of endocannabinoid-mediated FCA is also likely because on-demand synthesis and retrograde release of endocannabinoids has been demonstrated after the activation of group I metabotropic glutamate receptors [24,44,75] and plays a key role in the periaqueductal grey during unconditioned stress-induced analgesia [24]. We tested the hypothesis that endocannabinoid-mediated FCA is regulated by the ligand-gated chloride ion channel $GABA_A$ receptors or/and G_q-protein coupled mGluR5 in the BLA, by utilising the $GABA_A$ receptor antagonist bicuculline or the mGluR5 antagonist MPEP, in combination with the CB_1 receptor antagonist AM251. These pharmacological behavioural studies were supplemented by neurochemical analysis of levels of endocannabinoids (AEA, 2-AG) and related *N*-acylethanolamines (*N*-palmitoylethanolamide [PEA] and *N*-oleoylethanolamide [OEA]) in the BLA of rats after expression of FCA.

2. Materials and methods

2.1. Animals

Male Lister-hooded rats (280–350 g; Charles River, Margate, Kent, UK) were used. Animals were housed 3–4 per cage before surgery and were maintained at a constant temperature (21 ± 2°C) under standard lighting conditions (12:12 h light–dark, lights on from 0800 to 2000 h). Experiments were carried out during the light phase between 0800 and 1700 h. Food and water were available ad libitum. The experimental protocol was carried out after approval by 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.

2.2. Cannulae implantation

Stainless steel guide cannulae (Plastics One Inc., Roanoke, VA) were stereotaxically implanted 1 mm above the right and left BLA (anteroposterior: 0.25 cm, Mediolateral ± 0.48 cm relative to bregma; dorsoventral: 0.71 cm from skull surface) and 1 mm above the central nucleus of the amygdala (CeA; anteroposterior: 0.212 cm, ML ± 0.40 cm relative to bregma; dorsoventral: 0.65 cm from skull surface) [62] under isoflurane anaesthesia (2–3% in O_2; 0.5 L/min). Cannulations of and injections into the CeA were carried out to confirm the anatomical specificity of the effects of intra-BLA administration of AM251 on FCA. The cannulae were permanently fixed to the skull with stainless steel screws and carboxylate cement. A stylet made from stainless steel tubing (Plastics One Inc., Roanoke, VA, USA) was inserted into the guide cannula to prevent blockage by debris. The nonsteroidal anti-inflammatory agent carprofen (250 μL, 0.5% s.c.) (Rimadyl; Pfizer, Kent, UK), and the broad-spectrum antibiotic enrofloxacin (250 μL, 0.5% s.c.) (Baytril; Bayer Ltd., Dublin, Ireland), were administered before surgery to manage postoperative pain and to prevent infection, respectively. After cannulae implantation, the rats were housed singly and administered enrofloxacin (250 μL, 0.5% s.c.) for a further 3 days. Rats were allowed to recover for at least 6 days before experimentation. During this period, the rats were handled and their body weight and general health monitored on a daily basis.

2.3. Drug preparation

The $GABA_A$ receptor antagonist bicuculline methobromide ([R-(R*,S*)]-5-(6,8-dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-gisoquinolinium bromide), the mGlu5 receptor antagonist MPEP (2-methyl-6-(phenylethynyl) pyridine), and formalin were purchased from Sigma–Aldrich (Dublin, Ireland). AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide was purchased from Ascent Scientific, Bristol, UK.

AM251 was administered systemically on test days by intraperitoneal (i.p.) injection at a dose of 3 mg/kg (3 mL/kg dose volume). On test days, the drug was reconstituted as an emulsion in ethanol/Cremophor/saline vehicle in a ratio of 1:1:18. The dose of AM251 and time of administration were chosen on the basis of our previous work that used this CB_1 receptor antagonist/inverse agonist or its structural analogue, rimonabant (SR141716A) [7,16,85], and studies from other laboratories [35,40,78]. For bilateral intra-BLA and intra-CeA microinjections, AM251 was prepared to a concentration of 180 mM in DMSO vehicle (dimethylsulfoxide, 100%), and 0.5 μL was bilaterally injected based on our previous studies investigating the effects of intra-BLA administration of rimonabant [69].

Bicuculline was prepared in sterile saline (0.5 μL of a 136 μM solution) and microinjected bilaterally into the BLA on the test day. The chosen dose and time of administration of bicuculline into the BLA have been demonstrated to be efficacious in other behavioural studies without resulting in convulsions [19] or overt effects on locomotor activity [76].

MPEP was prepared in sterile saline (0.5 μL of a 200 μM solution) and microinjected bilaterally into the BLA on the test day. The dose and time of MPEP administration were chosen based on previous studies demonstrating its efficacy after intracerebral administration [1,63,71].

A 2.5% formalin solution was freshly prepared in sterile saline on test days. All of the compounds were administered on the test day while the animals were under brief (2–3 min) isoflurane anaesthesia for injection of 50 μL formalin injection 30 min before arena reexposure.

2.4. Experimental procedures

The FCA paradigm was essentially as described previously [7,16,17,21,60,66–69]. In brief, it consisted of 2 phases, conditioning and testing, occurring 24 h apart. Subjects were randomly assigned to groups and the sequence of testing was randomized. 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 foot shocks (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 foot shock, rats were returned to their home cage. Control animals not receiving foot shock were exposed to the chamber for an equivalent 9.5 min period. Three experiments, each using a different cohort of rats, were carried out. The test phase for all 3 experiments commenced 23.5 h later when the subjects received an intraplantar injection of 50 μL formalin (2.5% formalin solution prepared in sterile saline) into the right hind paw under brief isoflurane anaesthesia. Formalin-induced oedema was assessed by measuring the change in the diameter of the right hind

Table 1
Experimental groups in experiment 1.

Group No.	Conditioning	Formalin i.pl.	AM251/ vehicle i.p.	Saline/ bicuculline/ MPEP intra-BLA	No. per group
1	FC	Formalin	VEH	SAL	9
2	NO FC	Formalin	VEH	SAL	8
3	FC	Formalin	AM251	SAL	8
4	NO FC	Formalin	AM251	SAL	8
5	FC	Formalin	VEH	BIC	10
6	NO FC	Formalin	VEH	BIC	8
7	FC	Formalin	AM251	BIC	9
8	NO FC	Formalin	AM251	BIC	8
9	FC	Formalin	VEH	MPEP	9
10	NO FC	Formalin	VEH	MPEP	10
11	FC	Formalin	AM251	MPEP	9
12	NO FC	Formalin	AM251	MPEP	10

i.pl., intraplantar; i.p., intraperitoneal; FC, fear conditioned; NO FC, non – fear conditioned; VEH, vehicle; SAL, saline; BIC, bicuculline; MPEP, 2-methyl-6-(phenylethynyl) pyridine; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; BLA, basolateral amygdala; CeA, central nucleus of the amygdala.

Table 2
Experimental groups in experiment 2.

Group No.	Conditioning	Formalin i.pl.	AM251/vehicle	No. per group
Intra-BLA				
1	FC	Formalin	VEH	9
2	NO FC	Formalin	VEH	8
3	FC	Formalin	AM251	8
4	NO FC	Formalin	AM251	8
Intra-CeA				
5	FC	Formalin	VEH	5
6	FC	Formalin	AM251	6

i.pl., intraplantar; FC, fear conditioned; NO FC, non – fear conditioned; VEH, vehicle; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; BLA, basolateral amygdala; CeA, central nucleus of the amygdala.

paw immediately before, and 45 or 60 min after, formalin administration with Vernier callipers.

Experiment 1. At the time of formalin injection, rats also received intra-BLA microinjection of bicuculline (BIC 0.5 μL of a 136 μM solution), MPEP (0.5 μL of a 200 μM solution) or sterile saline (SAL 0.5 μL) into the right and left BLA; and an intraperitoneal injection of either AM251 (3 mg/kg) or vehicle (VEH). A full description of the intracerebral microinjection procedure has been published previously [69]. This design resulted in 12 experimental groups, as listed in Table 1.

Experiment 2. The test phase commenced 23.5 h after conditioning with the rats receiving an intraplantar injection of 50 μL formalin (2.5% formalin in sterile saline) into the right hind paw under brief isoflurane anaesthesia. Rats were returned to their home cage for a further 20 min, after which they received intra-BLA microinjection of either AM251 (180 mM) or DMSO vehicle (0.5 μL) into the right and left BLA or CeA. This design resulted in 6 experimental groups (n = 8–9 per group for BLA, and n = 5–6 for CeA groups) as illustrated in Table 2. In this study, animals were only reexposed to the arena for 15 min to correlate with previous studies [68,69].

Experiment 3. A third cohort of rats was used to investigate changes in endocannabinoid levels in the BLA associated with expression of conditioned fear, formalin-evoked nociception, and FCA (FC-FORM, NO FC-FORM, FC-SAL, NO FC-SAL; n = 5–7 per group). The experimental procedure was identical to that described above except that these animals did not undergo cannulae implantation so as to avoid any confounding as a result of damage to the BLA and surrounding area, and no drugs were administered.

In all 3 experiments, rats were returned to their home cage until 30 min after formalin injection, at which point they were returned to the same Perspex observation chamber to which they had been exposed during the conditioning phase. A video camera located beneath the observation chamber was used to monitor animal behaviour. The video feed from the camera was recorded onto a DVD for 15 or 30 min. The 30–60 min postformalin interval was chosen on the basis of previous studies demonstrating that formalin-evoked nociceptive behaviour is stable over this time period, is endocannabinoid mediated, and is subject to supraspinal modulation [7,16,17,21,60,66–69].

At the end of the test phase (45 or 60 min after formalin injection), rats were killed by decapitation. Brains were excised, then snap-frozen on dry ice and stored at −80°C. Rats in experiments 1 and 2 received intra-BLA or intra-CeA injection of fastgreen dye (0.5 μL of 1% solution) after decapitation for subsequent histological confirmation of the microinjection sites. In experiment 3, endocannabinoids and *N*-acylethanolamines were quantified from tissue punches of the BLA.

2.5. Behavioural analysis

Behaviour was analysed by the Ethovision XT 7.0 software package (Noldus, Wageningen, The Netherlands), which allowed for continuous event recording over each 30 min trial. A rater blinded to experimental conditions assessed nociceptive behaviour (composite pain score, CPS) as described previously [7,16,17,21,60,66–69]. Pain behaviours are categorized as time spent raising the formalin-injected paw above the floor without contact with any other surface (C1), and holding, licking, biting, shaking or flinching the injected paw (C2) to obtain a CPS [CPS = (C1 + 2(C2))/(total duration of analysis period)] as described by Watson and colleagues [86]. Total distance travelled (cm) was automatically tracked with this system and was used as an index of locomotor activity.

2.6. Histological verification of intracerebral microinjection sites

The sites of intracerebral microinjection were determined before data analysis. Brain sections with fastgreen dye mark were collected (30 μm thickness), mounted on gelatinized glass slides, and counterstained with cresyl violet to locate the precise position of microinjection sites under light microscopy.

2.7. Quantitation of endocannabinoids and N-acylethanolamines in BLA tissue by liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Frozen coronal brain sections (300 μm) containing the BLA from rats in experiment 3 were cut on a cryostat. Tissue from the left and right BLA was punched from the frozen sections (between bregma −2.56 mm and bregma −3.6 mm) [62] with cylindrical brain punchers (Harvard Apparatus, Whitehall, PA, USA; internal diameter, 2 mm). Each punched tissue sample was kept frozen throughout the collection procedure, weighed: (average weight of punched tissue = 4.3 mg) and stored at −80°C before extraction for, and determination of, the concentrations of the endocannabinoids AEA and 2-AG and the related *N*-acylethanolamines or so-called entourage compounds PEA and OEA by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) as described previously [6,21,60]. Tissue extraction was carried out via a lipid extraction method as follows: Each brain tissue sample was first homogenized in 400 μL 100% acetonitrile containing known fixed amounts of deuterated internal standards (0.014 nmol AEA-d8, 0.48 nmol 2-AG-d8, 0.016 nmol PEA-d4, 0.015 nmol OEA-d2). Homogenates were centrifuged at 14,000*g* for 15 min at 4°C and the supernatant was collected and evaporated to dryness in a centrifugal evaporator. Lyophilized samples were resuspended in 40 μL 65% acetonitrile and 2 μL were injected onto a Zorbax C-18

column (150 × 0.5 mm internal diameter) from a cooled autosampler maintained at 4°C (Agilent Technologies Ltd., Cork, Ireland). Mobile phases consisted of A (high-performance liquid chromatography[HPLC] grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 12 μL/min. Reversed-phase gradient elution began initially at 65% B and over 10 min was ramped linearly up to 100% B. At 10 min, the gradient was held at 100% B up to 20 min. At 20.1 min, the gradient returned to initial conditions for a further 10 min to reequilibrate the column. The total run time was 30 min. Under these conditions, AEA, 2-AG, PEA, and OEA eluted at the after retention times: 11.36, 12.8, 14.48, and 15.21 min, respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd). Instrument conditions and source parameters including fragmentor voltage and collision energy were optimized for each analyte of interest before assay of samples. Quantitation of target endocannabinoids was achieved by positive ion electrospray ionisation and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [$M^+ \cdot H^+$] of the analytes of interest and the deuterated forms of the internal standards. Precursor and product ion mass-to-charge (m/z) ratios for all analytes and their corresponding deuterated forms were as follows: PEA (m/z = 300.3–62.1); PEA-d4 (m/z = 304.3–62.1); OEA (m/z = 326.3–62.1); OEA-d2 (m/z = 328.3–62.1); AEA (m/z = 348.3–62.1); AEA-d8 (m/z = 356.3–63.1); 2-AG (m/z = 379.3–287.2); 2-AG-d8 (m/z = 387.3–294.2). Quantitation of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was performed by Masshunter Quantitative Analysis Software (Agilent Technologies Ltd). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio with a 10-point calibration curve constructed from a range of concentrations of the nondeuterated form of each analyte and a fixed amount of deuterated internal standard. The values obtained from the Masshunter Quantitative Analysis Software are initially expressed in ng per mg of tissue by dividing by the weight of the punched tissue. To express values as nanomoles or picomoles per milligram of tissue, the corresponding values are then divided by the molar mass of each analyte (expressed as ng/nmol or pg/pmol). Linearity (regression analysis determined R^2 values of 0.99 or greater for each analyte) was determined over a range of 18.75 ng to 71.5 fg except for 2-AG, which was 187.5 ng to 715 fg. The limit of quantification was 1.32 pmol/g, 12.1 pmol/g, 1.5 pmol/g, and 1.41 pmol/g for AEA, 2-AG, PEA, and OEA respectively.

2.8. Statistical analysis

The SPSS statistical software package (SPSS v15.0 for Microsoft Windows; IBM, Armonk, NY, USA) was used to analyse all data. Paw oedema was analysed by a paired Student's *t* test, while the effects of AM251 microinjection in the CeA were analysed by an unpaired Student's *t* test. Mass spectrometry analysis (after log transformation) and behavioural data were analysed by 2- or 3-way analysis of variance (ANOVA) followed by Fisher's LSD posthoc test where appropriate. Data were considered significant when $P < .05$. Results are expressed as group means ± standard error of the mean (SEM).

3. Results

3.1. Histological verification of injector placement

For experiment 1, 85% and for experiment 2, 80% of the intracerebral microinjections were placed within the borders of the left and right BLA, with the remaining injections positioned proximal to, but outside the borders of, this region (Figs. 1 and 2). For off-site control experiments targeting the CeA, all cannulae were within the borders of the CeA (Fig. 2b). Only the results of experiments in which both microinjections were accurately positioned within the borders of the left and right BLA or CeA were included in the analyses.

3.2. Systemic or intra-BLA administration of AM251 attenuates FCA

Intraplantar injection of formalin-increased oedema ($T_{98} = 33.023$, $P < .001$) and produced robust licking, biting, shaking, flinching, and elevation of the injected right hind paw. Vehicle-treated fear-conditioned rats displayed significantly less formalin-evoked nociceptive behaviour as indicated by the composite pain score (CPS) compared with non-fear-conditioned counterparts (Fig. 3, FC-VEH-SAL vs NO FC-VEH-SAL; Fig. 4, FC-VEH vs NO FC-VEH), confirming the expression of FCA. Systemic administration of AM251 (3 mg/kg, i.p.) significantly attenuated the expression of FCA (Fig. 3, FC-VEH-SAL vs FC-AM251-SAL), while having no effect on formalin-evoked nociceptive behaviour per se (Fig. 3, NO FC-VEH-SAL vs NO FC-AM251-SAL).

Similar to the effects observed after i.p. administration of AM251, microinjection of AM251 bilaterally into the BLA significantly attenuated FCA (Fig. 4, FC-VEH vs FC-AM251) while having no effect on formalin-evoked nociceptive behaviour per se (Fig. 4, NO FC-VEH vs NO FC-AM251) over the 15 min trial.

The microinjection of AM251 bilaterally into the CeA had no effect on FCA as indicated by the comparison of CPS values in fear-conditioned, formalin-treated animals receiving intra-CeA injection of vehicle and their AM251-treated counterparts (FC-VEH vs FC-AM251, 0.36 ± 0.13 vs 0.31 ± 0.17).

3.3. Bilateral intra-BLA microinjection of bicuculline partially attenuates the reversal of FCA by systemic AM251 administration

There were no effects of bilateral microinjection of bicuculline into the BLA on formalin-evoked nociceptive behaviour per se (Fig. 5, NO FC-VEH-BIC vs NO FC-VEH-SAL) or on FCA (Fig. 5, FC-VEH-BIC vs FC-VEH-VEH). However, intra-BLA bicuculline microinjection partially attenuated the reversal of FCA by systemic AM251 administration (Fig. 5, FC-AM251-SAL vs FC-AM251-BIC).

3.4. Effects of intra-BLA microinjection of MPEP on formalin-evoked nociceptive behaviour per se and on FCA in the absence or presence of AM251

Bilateral microinjection of MPEP into the BLA significantly increased formalin-evoked nociceptive behaviour per se (Fig. 6, NO FC-VEH-MPEP vs NO FC-VEH-SAL) and tended to reverse FCA, albeit nonsignificantly (Fig. 6, FC-VEH-MPEP vs FC-VEH-SAL). Intra-BLA MPEP resulted in a partial but significant attenuation of the reversal of FCA by AM251 (Fig. 6, FC-AM251-SAL vs FC-AM251-MPEP).

3.5. Lack of effect of intra-BLA and systemic drug administration on locomotor activity

There was no effect of foot shock on locomotor activity over the 30 min trial ($F(1,109) = 0.342$, $P = .560$) as determined by automated tracking of total distance travelled (data not shown). AM251 did not affect locomotor activity in saline- or formalin-treated rats when administered systemically ($F(1,109) = 2.303$, $P = .132$) or bilaterally into the BLA ($F(1,24) = 0.382$, $P = .543$). There was no effect of intra-BLA bicuculline or MPEP administration on locomotor activity ($F(2,109) = 0.159$, $P = .853$), compared with vehicle-treated controls (data not shown).

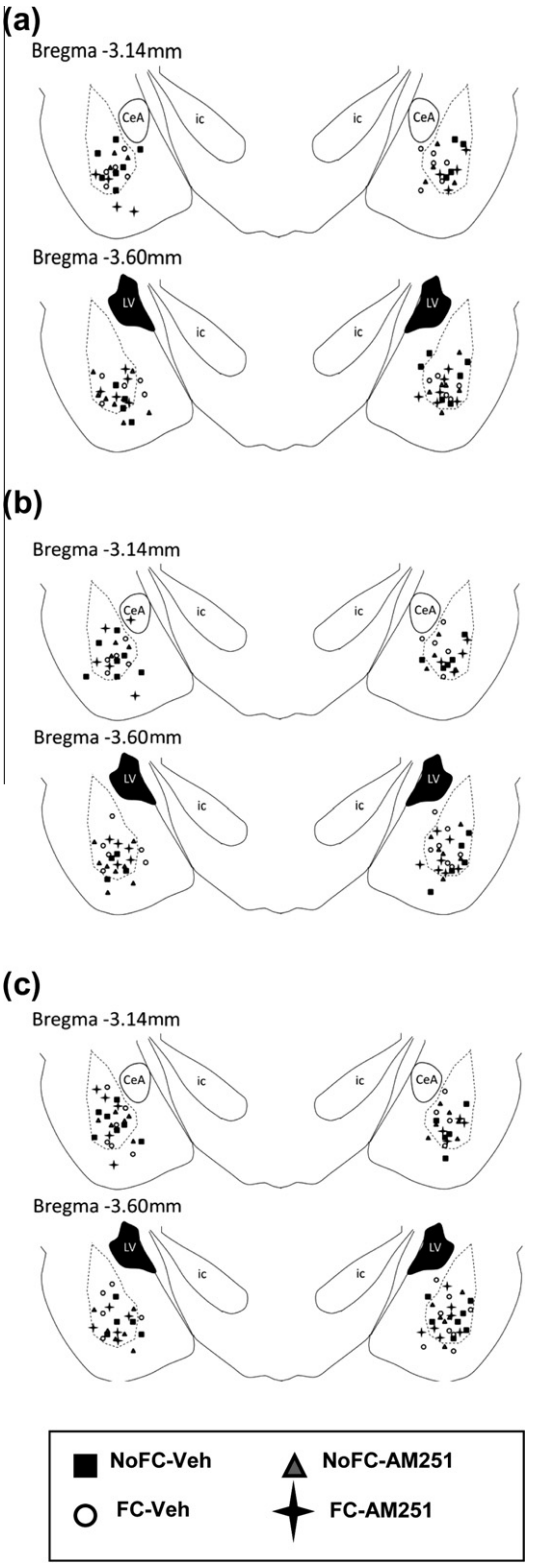

Fig. 1. Schematic depicting the sites of microinjection of (A) vehicle (saline), (B) bicuculline, or (C) MPEP into the left and right BLA in experiment 1. FC, fear conditioning; NO FC, no fear conditioning; SAL, saline; CeA, central nucleus of the amygdala; ic, internal capsule; LV, lateral ventricle.

3.6. Alterations in levels of AEA, 2-AG, PEA and OEA associated with conditioned fear, formalin-evoked nociceptive behaviour and FCA

In rats receiving intraplantar injection of saline, there were no significant differences between contralateral and ipsilateral BLA

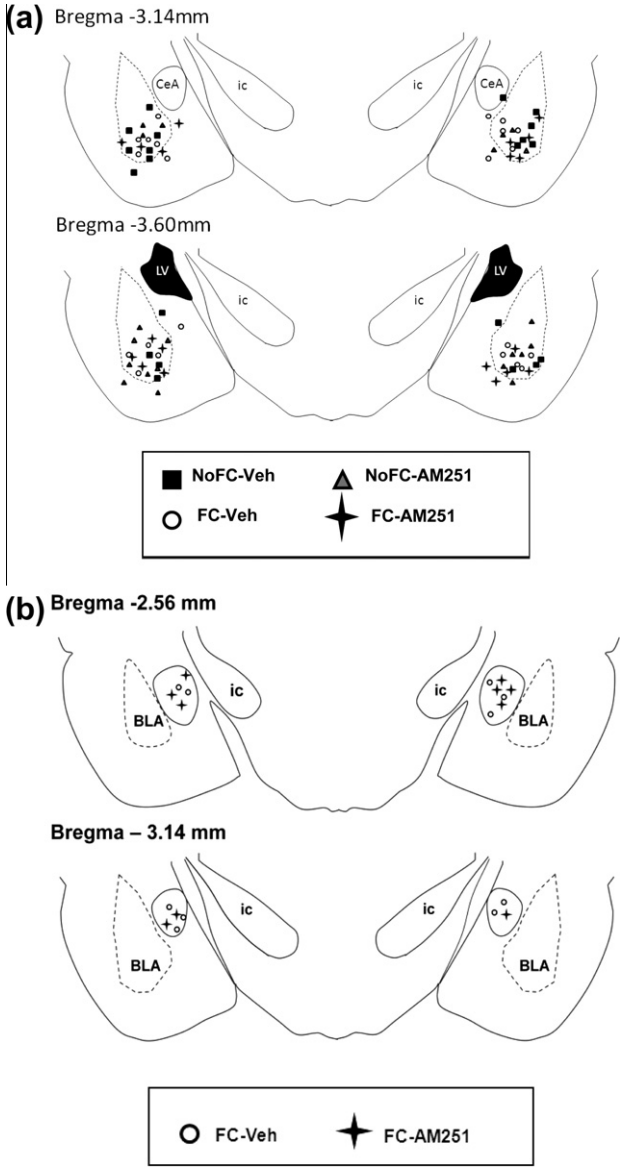

Fig. 2. Schematic diagram depicting the sites of microinjection of vehicle (DMSO) or AM251 into the left and right (A) BLA or (B) CeA in experiment 2. FC, fear conditioning; NO FC, no fear conditioning; CeA, central nucleus of the amygdala; BLA, basolateral amygdala; ic, internal capsule; LV, lateral ventricle.

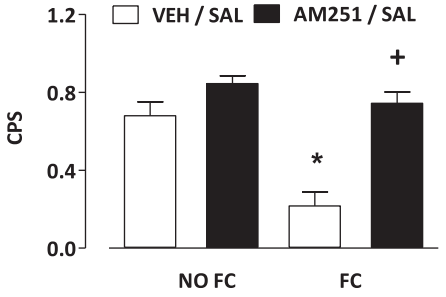

Fig. 3. Systemic administration of AM251 (3 mg/kg, i.p.) attenuates FCA in rats. Data are presented as mean ± SEM (n = 8–10). A 2-way ANOVA revealed a significant effect of fear conditioning ($F(1,38) = 17.901$, $P < .001$) and of i.p. drug administration ($F(1,38) = 26.845$, $P < .001$) and an interaction effect ($F(1,38) = 7.367$, $P = .010$) on nociceptive behaviour. $^{*}P < .05$ vs NO FC-VEH-SAL. $^{+}P < .05$ for FC-AM251-SAL vs FC-VEH-SAL. CPS, composite pain score; FC, fear conditioning; NO FC, no fear conditioning; SAL, saline; VEH, vehicle.

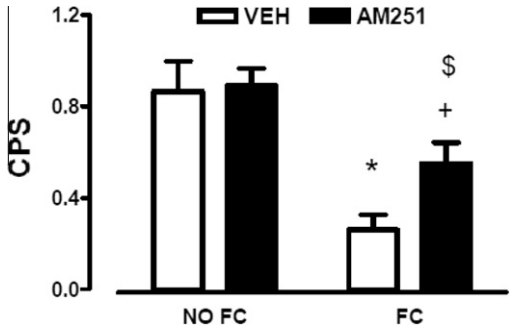

Fig. 4. Bilateral AM251 (180 nM) microinjection into the BLA attenuates FCA in rats. Data are presented as mean ± SEM (n = 8–10 in A, 5–6 in B). A 2-way ANOVA determined that AM251 bilaterally microinjected into the BLA significantly attenuated the reversal of formalin-evoked nociceptive behaviour by fear conditioning ($F(1,24) = 25.768$, $P < .001$). $^{*}P < .05$ vs NO FC-VEH. $^{\$}P < .05$ for FC-AM251 vs NO FC-AM251. $^{+}P < .05$ for FC-AM251 vs FC-VEH. FC, fear conditioning; NO FC, no fear conditioning; VEH, vehicle.

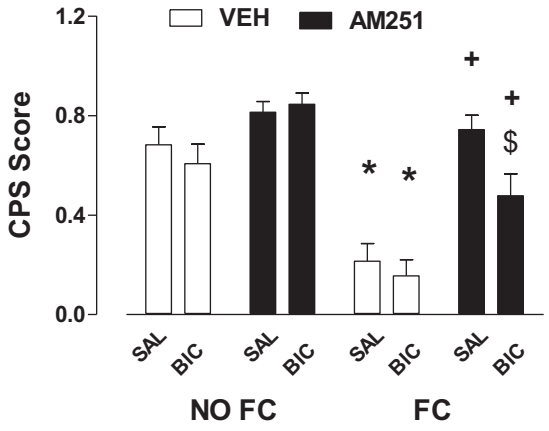

Fig. 5. Bilateral bicuculline microinjection into the BLA attenuates the reversal of FCA by systemic AM251 administration in rats. Data are presented as mean ± SEM (n = 8–10). A 3-way ANOVA revealed a significant effect of fear-conditioning ($F(1,66) = 39.716$, $P < .001$) and of i.p. AM251 administration ($F(1,66) = 48.531$, $P < .001$) on nociceptive behaviour. $^{*}P < .05$ vs corresponding NO FC-VEH treatment groups. $^{+}P < .05$ vs corresponding FC-VEH treated groups. $^{\$}P < .05$ FC-AM251-SAL vs FC-AM251-BIC. CPS, composite pain score; BIC, bicuculline; FC, fear conditioning; NO FC, no fear conditioning; SAL, saline; VEH, vehicle.

levels of AEA, OEA, PEA or 2-AG, irrespective of fear conditioning (Fig. 7). Despite some trends, there were no significant effects of fear conditioning on levels of AEA, 2-AG, OEA, or PEA in the contralateral or ipsilateral BLA of rats receiving intraplantar injection of saline (NO FC-SAL vs FC-SAL; Fig. 7). Comparing levels of analytes in the BLA of saline-treated rats vs formalin-treated counterparts, we found that formalin injection had no significant effect on levels of AEA, 2-AG or PEA in the BLA of non-fear-conditioned rats but did reduce levels of OEA in the contralateral BLA only (NO FC-SAL vs NO FC-FORM; Fig. 7). In contrast, in fear-conditioned rats, formalin treatment resulted in increased levels of 2-AG in the ipsilateral BLA and increased levels of PEA in the contralateral BLA (FC-SAL vs FC-FORM; Fig. 7). FCA was associated with significant increases in tissue levels of AEA but not 2-AG, in the contralateral BLA only (NO FC-FORM vs FC-FORM; Fig. 7), and trends for a similar effect on PEA (P = .08) and OEA (P = .06).

4. Discussion

This study demonstrates that systemic administration of the CB_1 receptor antagonist/inverse agonist, AM251, and its bilateral

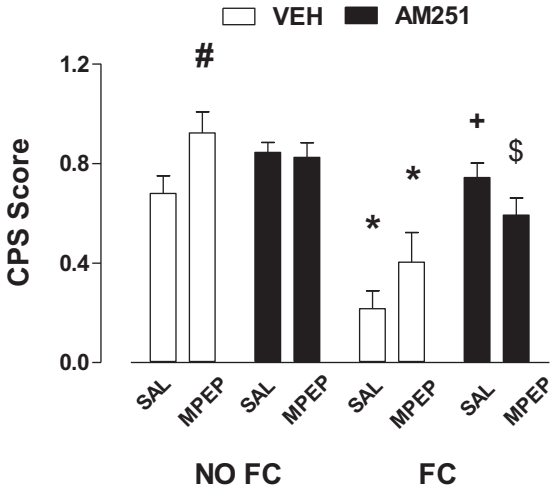

Fig. 6. Bilateral MPEP microinjection into the BLA attenuates the reversal of FCA by systemic AM251 administration in rats. Data are presented as mean ± SEM (n = 8–10). A 3-way ANOVA revealed a significant effect of fear conditioning ($F(1,74) = 38.825$, $P < .001$) and of i.p. AM251 administration ($F(1,74) = 13.702$, $P < .001$) on nociceptive behaviour. Furthermore, these analyses revealed a significant interaction effect between fear conditioning and i.p. AM251 administration ($F(1,74) = 9.426$, $P < .05$), and between MPEP and i.p. AM251 administration ($F(1,74) = 8.082$, $P < .05$) $^{\#}P < .05$ NO FC-VEH-MPEP vs NO FC-VEH-SAL. $^{*}P < .05$ vs corresponding NO FC-VEH treated groups. $^{+}P < .05$ for FC-AM251-SAL vs FC-VEH-SAL. $^{\$}P < .05$ FC-AM251-SAL vs FC-AM251-MPEP. CPS, composite pain score; FC, fear conditioning; NO FC, no fear conditioning; MPEP, 2-methyl-6-(phenylethynyl) pyridine; SAL, saline; VEH, vehicle.

microinjection into the BLA but not off-site into the CeA, attenuate antinociception expressed upon exposure to an aversively conditioned context (i.e., attenuated FCA). The blockade of FCA by systemically administered AM251 was partially attenuated by microinjection of the $GABA_A$ receptor antagonist, bicuculline, or the mGlu5 receptor antagonist, MPEP, bilaterally into the BLA. While the expression of formalin-evoked nociceptive behaviour was enhanced after bilateral microinjection of MPEP into the BLA, FCA was still expressed in MPEP-treated animals. FCA was associated with increased tissue levels of the endocannabinoid AEA, and strong trends for increased tissue levels of the related *N*-acylethanolamines PEA and OEA, in the BLA side contralateral to the formalin-injected paw. In fear-conditioned rats, formalin treatment resulted in increased levels of 2-AG in the ipsilateral BLA and increased levels of PEA in the contralateral BLA.

Our finding that systemic injection of AM251 attenuates FCA in rats is comparable to that previously reported for the CB_1 receptor antagonist/inverse agonist, rimonabant [16]. Systemic or intra-BLA administration of rimonabant have also been demonstrated to attenuate the suppression of nociceptive behaviour elicited by exposure of rats to unconditioned foot shock stress [10,36]. Moreover, we have previously demonstrated that pharmacological inhibition of the AEA-degrading enzyme fatty acid amide hydrolase (FAAH) significantly enhances FCA via a CB_1 receptor-dependent mechanism [6,7]. The present study builds upon and extends these earlier studies by demonstrating a key role for the BLA in endocannabinoid-mediated FCA. Thus, we demonstrate that FCA is associated with increased tissue levels of AEA and strong trends for similar increases in PEA and OEA, in the BLA side contralateral to formalin injection and that direct intra-BLA administration of AM251 attenuates FCA, presumably via blockade of the actions of AEA on the CB_1 receptor. Moreover, in fear-conditioned rats, formalin treatment resulted in increased levels of 2-AG in the ipsilateral BLA and increased levels of PEA in the contralateral BLA. Thus, fear conditioning may prime the BLA to mount a robust formalin-induced increase in 2-AG and PEA in a manner which does not oc-

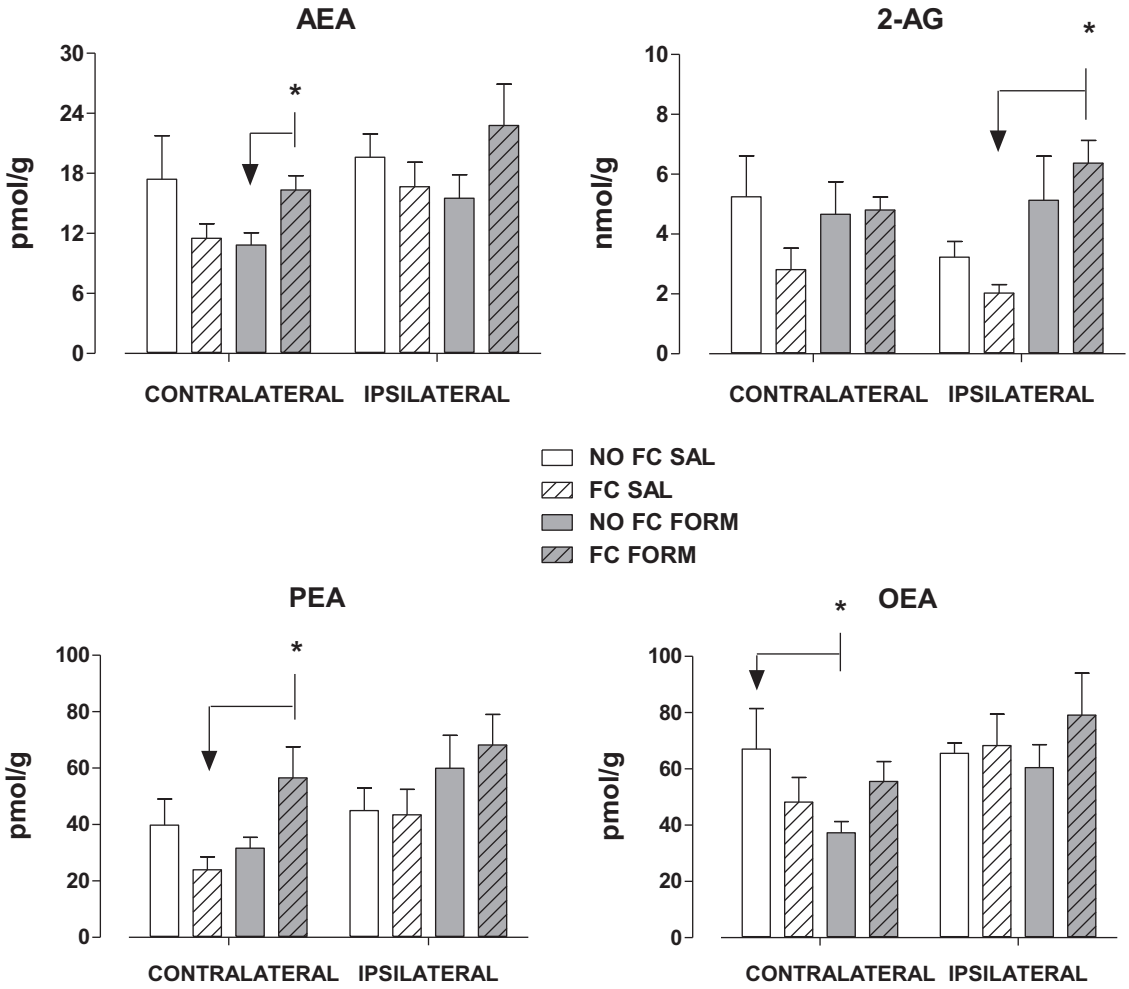

Fig. 7. Alterations in levels of AEA, 2-AG, PEA, and OEA associated with conditioned fear, formalin-evoked nociceptive behaviour, and FCA. Data are presented as mean ± SEM (n = 5–7). A 3-way ANOVA revealed a significant effect of formalin administration on 2-AG and PEA levels ($F(1,44) = 9.334$, $P = .004$ and $F(1,44) = 4.348$, $P = .044$ respectively), and a significant difference between levels of AEA ($F(1,44) = 7.200$, $P = .011$), PEA ($F(1,44) = 6.692$, $P = .014$), and OEA ($F(1,44) = 7.143$, $P = .011$) between contralateral and ipsilateral BLA. There was a significant interaction between fear conditioning and formalin administration for AEA ($F(1,44) = 8.729$, $P = .005$), 2-AG ($F(1,44) = 6.689$, $P = .014$), and OEA ($F(1,44) = 4.294$, $P = .045$) with a strong trend towards an interaction in PEA ($F(1,44) = 4.065$, $P = .051$). *Groups significantly different ($P < .05$). AEA, anandamide; 2-AG, 2-arachidonyl glycerol; FC, fear conditioning; NO FC, no fear conditioning; FORM, formalin; *N*-oleoylethanolamide, OEA; *N*-palmitoylethanolamide, PEA; SAL, saline; VEH, vehicle.

cur in non-fear-conditioned rats. Though not themselves active at CB_1 receptors [47,74], it is possible that the increased levels of OEA and PEA may further enhance AEA-CB_1 signalling via an 'entourage' effect, whereby they compete with AEA for catabolism by FAAH [3,43,54]. Alternatively, OEA and PEA may themselves modulate FCA via non-CB_1 receptor mechanisms [11,41,49,79]; however, this seems unlikely, given that the pharmacological blockade of CB_1 with systemic AM251 administration completely attenuated FCA. Furthermore, FCA was not associated with any change in AEA, PEA, and OEA levels in the ipsilateral BLA, while levels were enhanced in the contralateral BLA. Recent evidence suggests a hemispheric lateralisation of pain processing and modulation in amygdalar neurons which may account for some of these effects [9,42]. However, further work would be needed to investigate the effect of fear conditioning on differential responses of endocannabinoid levels in the left and right BLA in response to formalin administration in either paw.

The behavioural results after intra-BLA AM251 microinjection are in contrast to earlier work from our laboratory where intra-BLA rimonabant had no significant effect on FCA [68,69] or formalin-evoked nociceptive behaviour [68] in rats. These compounds have differential affinity for cannabinoid targets such as GPR55 [31,32,48,72,73], transient receptor potential vanilloid type I (TRPV1) channel [14,22], and peroxisome proliferator activated receptors [64]. Because we have observed similar behavioural effects on FCA when either compound is administered systemically, it is likely that the discrepancy relates either to dose–response differences between the 2 compounds when administered intra-BLA or to differential activity at targets expressed in the BLA. TRPV1 immunoreactivity is high in the BLA [12,55,56,82,87], but to our knowledge, no studies have investigated the localisation of TRPV1 on GABAergic or glutamatergic neurons in the BLA despite reports of a presynaptic localisation of TRPV1 on GABAergic neurons and postsynaptic localisation on glutamatergic neurons in the hippocampus. However, one study reported opposing roles of TRPV1 and CB_1 receptor activation in pain behaviours in rats and that TRPV1 receptors are often coexpressed with CB_1 receptors in the periaqueductal grey [51]. Similarly, another study demonstrated opposing roles of these receptors in anxiety tests in mice and also reported a coexpression of TRPV1 and CB_1 receptors on neurons in the amygdala [56]. These findings may explain the differential effects of AM251 and rimonabant microinjection into the BLA on formalin-evoked nociceptive behaviour and FCA in rats.

The partial attenuation of AM251's suppression of FCA by the microinjection of bicuculline into the amygdala supports the contention that GABAergic transmission in the BLA plays a role in

endocannabinoid-mediated FCA (for review see [65]). A number of studies implicate a role for $GABA_A$ receptors in the BLA in the expression of fear [29,53,57] and in the suppression of pain-related behaviour elicited by unconditioned [2,58] and conditioned [25,27,67] fear in rats. However, the majority of these studies used the $GABA_A$ receptor agonists muscimol ($GABA_A$ receptor agonist) or benzodiazepines (allosteric modulators of the $GABA_A$ receptor) to suppress these behaviours; investigations into the blockade of $GABA_A$ receptors in the BLA on these behaviours are lacking. To our knowledge, no studies have previously investigated the effects of bicuculline administration into the BLA on formalin-evoked nociceptive behaviour. In vivo microdialysis studies from our laboratory and others have previously demonstrated that expression of conditioned fear and FCA are associated with (and may be facilitated by) a suppression of GABA release in the BLA [67,77]. In the present study, we hypothesized that AM251 would attenuate FCA by blocking CB_1 receptors on GABAergic neurons, thereby disinhibiting GABA release in the BLA leading to inhibition of the descending inhibitory pain pathway. CB_1 receptors have been localized to GABAergic neurons in the BLA [34,45,50,61,83], and would likely be activated as a consequence of the increased AEA levels associated with expression of FCA. We noted that intra-BLA microinjection of bicuculline alone had no effect on formalin-evoked nociception or FCA, suggesting that under these physiological circumstances, GABA release in the BLA is already suppressed and further blockade of the receptors has no effect. However, our finding that bilateral microinjection of bicuculline into the BLA partially suppressed the reversal of FCA by systemic AM251 administration suggests that GABAergic mechanisms in the BLA are disinhibited by CB_1 receptor blockade and are involved in the behavioural expression of FCA. It is possible that with a higher concentration of bicuculline, or at a timepoint closer to the reexposure to the arena, a stronger suppression of AM251's reversal of FCA would be observed. However, we did not wish to introduce confounding effects such as additional handling for microinjections or risk convulsive-like behaviour [19] or overt effects on locomotor activity [76], which might be expected with higher concentrations of bicuculline. Indeed, our analysis confirmed that our chosen dose and time of administration of intra-BLA bicuculline did not affect locomotor activity while having pharmacological activity on nociceptive behaviours.

Although there is evidence for a role of amygdalar mGluR5 in the acquisition and expression of fear [70,71], to our knowledge, only one study has investigated the role of these receptors in the amygdala in animal models of pain, and that was in the CeA [46]. These authors demonstrated that in mice, 3,5-dihydroxyphenylglycine-induced peripheral hypersensitivity is reduced after blockade or genetic disruption of mGluR5. In our hands, intra-BLA MPEP enhanced formalin-evoked nociception, irrespective of fear conditioning, yet FCA was still expressed. The magnitude of AM251's reversal of FCA was more than halved by MPEP. However, we cannot exclude the possibility that a greater formalin-evoked nociceptive response or a more pronounced attenuation of the AM251-induced reversal of FCA may have been observed with a higher dose of MPEP. The increase in formalin-evoked nociceptive behaviour irrespective of fear conditioning may be accounted for by the presence of mGluR5 on postsynaptic output neurons in the BLA, and their blockade may reduce descending inhibitory pain pathway activity. However, the blockade of CB_1R-mediated events with AM251 may reverse FCA by disinhibiting neuronal drive. Our observation that the reversal of FCA by AM251 was subject to modulation by intra-BLA MPEP administration may be explained by the presence of mGluR5 on GABAergic interneurons, as seen in other brain areas [39,81]. Fig. 8 provides a diagrammatic representation of the proposed circuitry and mechanisms in the BLA during endocannabinoid-mediated FCA based on the findings herein.

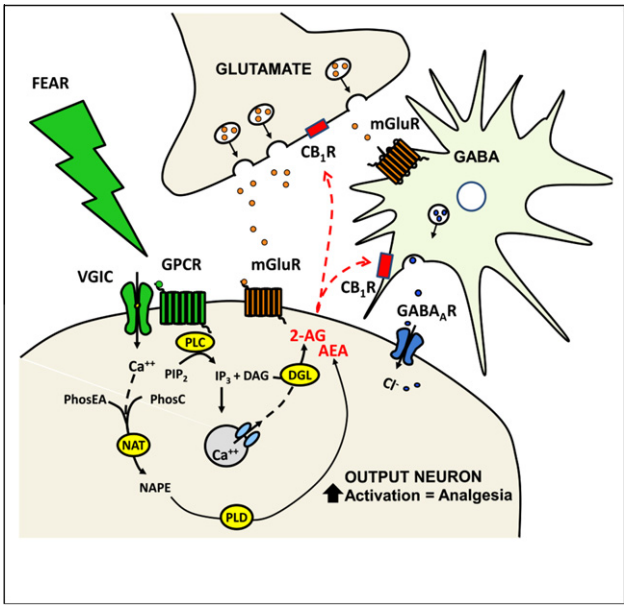

Fig. 8. Diagrammatic representation of proposed events in the BLA during endocannabinoid-mediated FCA. In non-fear-conditioned rats, formalin-evoked glutamate release and subsequent activation of mGluR5 on BLA output neurons may function to suppress nociceptive behaviour because intra-BLA administration of mGlu5 receptor antagonist, MPEP, increased formalin-evoked nociceptive behaviour. Conditioned fear may facilitate the synthesis of endocannabinoids in the postsynaptic output neurons of formalin-treated rats (as evidenced by the increase in BLA AEA and 2-AG levels observed in rats expressing FCA). These endocannabinoids then signal retrogradely and activate CB_1 receptors expressed on glutamatergic neurons and GABAergic interneurons. Activation of CB_1 receptors on glutamatergic neurons would reduce glutamate-mediated drive to GABAergic neurons, while activation of CB_1R on GABAergic neurons would reduce GABA release and disinhibit the output neurons which would in turn lead to activation of the descending inhibitory pain pathway and suppression of nociceptive behaviour (FCA). Blockade of CB_1 receptor–mediated events with AM251 would reverse FCA by disinhibiting neuronal drive. This reversal of FCA would be subject to modulation by blockade of mGluR5 on GABAergic interneurons and/or output neurons by MPEP or bicuculline. The net effect of excitatory and inhibitory impulses from GABAergic and glutamatergic neurons in this BLA network will ultimately determine the tone of the output neurons and effects on behaviour.

In conclusion, these data demonstrate a key role for the endocannabinoid system in the suppression of pain behaviour by conditioned fear and identify the BLA as a key neural substrate. Our data suggest that the reversal of FCA by systemic AM251 administration is subject to regulation by GABAergic and glutamatergic mechanisms at the level of the BLA. These findings advance our understanding of the role of the endocannabinoid system in the potent suppression of pain-related behaviour by conditioned fear and the neurochemical and receptor mechanisms involved.

Conflict of interest statement

The authors report no conflict of interest.

Acknowledgements

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References

[1] Ansah OB, Goncalves L, Almeida A, Pertovaara A. Enhanced pronociception by amygdaloid group I metabotropic glutamate receptors in nerve-injured animals. Exp Neurol 2009;216:66–74.

[2] Baptista D, Bussadori K, Nunes-de-Souza RL, Canto-de-Souza A. Blockade of fear-induced antinociception with intra-amygdala infusion of midazolam: influence of prior test experience. Brain Res 2009;1294:29–37.
[3] Bisogno T, Maurelli S, Melck D, De Petrocellis L, Di Marzo V. Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. J Biol Chem 1997;272:3315–23.
[4] Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G, Casellas P. Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. Biochem J 1995;312:637–41.
[5] Butler RK, Finn DP. Stress-induced analgesia. Prog Neurobiol 2009;88:184–202.
[6] Butler RK, Ford GK, Hogan M, Roche M, Doyle KM, Kelly JP, 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. J Psychopharmacol 2011;26:83–91.
[7] 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;140:491–500.
[8] Calcagnetti DJ, Helmstetter FJ, Fanselow MS. Quaternary naltrexone reveals the central mediation of conditional opioid analgesia. Pharmacol Biochem Behav 1987;27:529–31.
[9] Carrasquillo Y, Gereau 4th RW. Hemispheric lateralization of a molecular signal for pain modulation in the amygdala. Mol Pain 2008;4:24.
[10] 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;397:180–4.
[11] Costa B, Comelli F, Bettoni I, Colleoni M, Giagnoni G. The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain: involvement of CB(1), TRPV1 and PPAR gamma receptors and neurotrophic factors. PAIN® 2008;139:541–50.
[12] Cristino L, de Petrocellis L, Pryce G, Baker D, Guglielmotti V, Di Marzo V. Immunohistochemical localization of cannabinoid type 1 and vanilloid transient receptor potential vanilloid type 1 receptors in the mouse brain. Neuroscience 2006;139:1405–15.
[13] Davis M, Whalen PJ. The amygdala: vigilance and emotion. Mol Psychiatry 2001;6:13–34.
[14] De Petrocellis L, Bisogno T, Maccarrone M, Davis JB, Finazzi-Agro A, Di Marzo V. The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. J Biol Chem 2001;276:12856–63.
[15] Fanselow MS, Calcagnetti DJ, Helmstetter FJ. Role of mu and kappa opioid receptors in conditional fear-induced analgesia: the antagonistic actions of nor-binaltorphimine and the cyclic somatostatin octapeptide, Cys2Tyr3Orn5Pen7-amide. J Pharmacol Exp Ther 1989;250:825–30.
[16] 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;20:848–52.
[17] 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;138:1309–17.
[18] Finn DP. Endocannabinoid-mediated modulation of stress responses: physiological and pathophysiological significance. Immunobiology 2010;215:629–46.
[19] Flores-Gracia C, Nuche-Bricaire A, Crespo-Ramirez M, Miledi R, Fuxe K, Perez de la Mora M. GABA(A) rho receptor mechanisms in the rat amygdala and its role in the modulation of fear and anxiety. Psychopharmacology 2010;212:475–84.
[20] Ford GK, Finn DP. Clinical correlates of stress-induced analgesia: evidence from pharmacological studies. PAIN® 2008;140:3–7.
[21] Ford GK, Kieran S, Dolan K, Harhen B, Finn DP. A role for the ventral hippocampal endocannabinoid system in fear-conditioned analgesia and fear responding in the presence of nociceptive tone in rats. PAIN® 2011;152:2495–504.
[22] Gibson HE, Edwards JG, Page RS, Van Hook MJ, Kauer JA. TRPV1 channels mediate long-term depression at synapses on hippocampal interneurons. Neuron 2008;57:746–59.
[23] Good AJ, Westbrook RF. Effects of a microinjection of morphine into the amygdala on the acquisition and expression of conditioned fear and hypoalgesia in rats. Behav Neurosci 1995;109:631–41.
[24] Gregg LC, Jung KM, Spradley JM, Nyilas R, Suplita II RL, Zimmer A, Watanabe M, Mackie K, Katona I, Piomelli D, Hohmann AG. Activation of type 5 metabotropic glutamate receptors and diacylglycerol lipase-alpha initiates 2-arachidonoylglycerol formation and endocannabinoid-mediated analgesia. J Neurosci 2012;32:9457–68.
[25] Harris JA, Westbrook RF. Effects of benzodiazepine microinjection into the amygdala or periaqueductal gray on the expression of conditioned fear and hypoalgesia in rats. Behav Neurosci 1995;109:295–304.
[26] Harris JA, Westbrook RF, Duffield TQ, Bentivoglio M. Fos expression in the spinal cord is suppressed in rats displaying conditioned hypoalgesia. Behav Neurosci 1995;109:320–8.
[27] Helmstetter FJ. Stress-induced hypoalgesia and defensive freezing are attenuated by application of diazepam to the amygdala. Pharmacol Biochem Behav 1993;44:433–8.
[28] Helmstetter FJ, Bellgowan PS. Lesions of the amygdala block conditional hypoalgesia on the tail flick test. Brain Res 1993;612:253–7.
[29] Helmstetter FJ, Bellgowan PS. Effects of muscimol applied to the basolateral amygdala on acquisition and expression of contextual fear conditioning in rats. Behav Neurosci 1994;108:1005–9.
[30] Helmstetter FJ, Landeira-Fernandez J. Conditional hypoalgesia is attenuated by naltrexone applied to the periaqueductal gray. Brain Res 1990;537:88–92.
[31] Henstridge CM, Balenga NA, Ford LA, Ross RA, Waldhoer M, Irving AJ. The GPR55 ligand L-alpha-lysophosphatidylinositol promotes RhoA-dependent Ca^{2+} signaling and NFAT activation. FASEB J 2009;23:183–93.
[32] Henstridge CM, Balenga NA, Schroder R, Kargl JK, Platzer W, Martini L, Arthur S, Penman J, Whistler JL, Kostenis E, Waldhoer M, Irving AJ. GPR55 ligands promote receptor coupling to multiple signalling pathways. Br J Pharmacol 2010;160:604–14.
[33] Herkenham M. Characterization and localization of cannabinoid receptors in brain: an in vitro technique using slide-mounted tissue sections. NIDA Res Monogr 1991;112:129–45.
[34] 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;11:563–83.
[35] Hillard CJ, Manna S, Greenberg MJ, DiCamelli R, Ross RA, Stevenson LA, Murphy V, Pertwee RG, Campbell WB. Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). J Pharmacol Exp Ther 1999;289:1427–33.
[36] 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;435:1108–12.
[37] Howlett AC. The cannabinoid receptors. Prostaglandins Other Lipid Mediat 2002;68–9:619–31.
[38] Howlett AC, Qualy JM, Khachatrian LL. Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. Mol Pharmacol 1986;29:307–13.
[39] Hubert GW, Paquet M, Smith Y. Differential subcellular localization of mGluR1a and mGluR5 in the rat and monkey substantia nigra. J Neurosci 2001;21:1838–47.
[40] Jarbe TU, Andrzejewski ME, DiPatrizio NV. Interactions between the CB1 receptor agonist Delta 9-THC and the CB1 receptor antagonist SR-141716 in rats: open-field revisited. Pharmacol Biochem Behav 2002;73:911–9.
[41] Jhaveri MD, Richardson D, Chapman V. Endocannabinoid metabolism and uptake: novel targets for neuropathic and inflammatory pain. Br J Pharmacol 2007;152:624–32.
[42] Ji G, Neugebauer V. Hemispheric lateralization of pain processing by amygdala neurons. J Neurophysiol 2009;102:2253–64.
[43] Jonsson KO, Vandevoorde S, Lambert DM, Tiger G, Fowler CJ. Effects of homologues and analogues of palmitoylethanolamide upon the inactivation of the endocannabinoid anandamide. Br J Pharmacol 2001;133:1263–75.
[44] Jung KM, Mangieri R, Stapleton C, Kim J, Fegley D, Wallace M, Mackie K, Piomelli D. Stimulation of endocannabinoid formation in brain slice cultures through activation of group I metabotropic glutamate receptors. Mol Pharmacol 2005;68:1196–202.
[45] Katona I, Rancz EA, Acsady L, Ledent C, Mackie K, Hajos N, Freund TF. Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. J Neurosci 2001;21:9506–18.
[46] Kolber BJ, Montana MC, Carrasquillo Y, Xu J, Heinemann SF, Muglia LJ, Gereau IV RW. Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like behavior. J Neurosci 2010;30:8203–13.
[47] Lambert DM, DiPaolo FG, Sonveaux P, Kanyonyo M, Govaerts SJ, Hermans E, Bueb J, Delzenne NM, Tschirhart EJ. Analogues and homologues of *N*-palmitoylethanolamide, a putative endogenous CB(2) cannabinoid, as potential ligands for the cannabinoid receptors. Biochim Biophys Acta 1999;1440:266–74.
[48] Lauckner JE, Jensen JB, Chen HY, Lu HC, Hille B, Mackie K. GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. Proc Natl Acad Sci USA 2008;105:2699–704.
[49] Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D. The nuclear receptor peroxisome proliferator-activated receptor–alpha mediates the anti-inflammatory actions of palmitoylethanolamide. Mol Pharmacol 2005;67:9–15.
[50] Lu XR, Ong WY, Mackie K. A light and electron microscopic study of the CB1 cannabinoid receptor in monkey basal forebrain. J Neurocytol 1999;28:1045–51.
[51] Maione S, Bisogno T, de Novellis V, Palazzo E, Cristino L, Valenti M, Petrosino S, Guglielmotti V, Rossi F, Di Marzo V. Elevation of endocannabinoid levels in the ventrolateral periaqueductal grey through inhibition of fatty acid amide hydrolase affects descending nociceptive pathways via both cannabinoid receptor type 1 and transient receptor potential vanilloid type-1 receptors. J Pharmacol Exp Ther 2006;316:969–82.
[52] Marsicano G, Lutz B. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur J Neurosci 1999;11:4213–25.
[53] Martinez RC, de Oliveira AR, Brandao ML. Conditioned and unconditioned fear organized in the periaqueductal gray are differentially sensitive to injections of muscimol into amygdaloid nuclei. Neurobiol Learn Mem 2006;85:58–65.
[54] Maurelli S, Bisogno T, De Petrocellis L, Di Luccia A, Marino G, Di Marzo V. Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'. FEBS Lett 1995;377:82–6.

[55] Mezey E, Toth ZE, Cortright DN, Arzubi MK, Krause JE, Elde R, Guo A, Blumberg PM, Szallasi A. Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. Proc Natl Acad Sci USA 2000;97:3655–60.
[56] Micale V, Cristino L, Tamburella A, Petrosino S, Leggio GM, Drago F, Di Marzo V. Anxiolytic effects in mice of a dual blocker of fatty acid amide hydrolase and transient receptor potential vanilloid type-1 channels. Neuropsychopharmacology 2009;34:593–606.
[57] Muller J, Corodimas KP, Fridel Z, LeDoux JE. Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli. Behav Neurosci 1997;111:683–91.
[58] Nunes-de-Souza RL, Canto-de-Souza A, Da-Costa M, Fornari RV, Graeff FG, Pela IR. Anxiety-induced antinociception in mice. Effects of systemic and intra-amygdala administration of 8-OH-DPAT and midazolam. Psychopharmacology (Berl) 2000;150:300–10.
[59] Nyilas R, Dudok B, Urbán GM, Mackie K, Watanabe M, Cravatt BF, Freund TF, Katona I. Enzymatic machinery for endocannabinoid biosynthesis associated with calcium stores in glutamatergic axon terminals. J Neurosci 2008;28:1058–63.
[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 nocicpetive tone. Br J Pharmacol 2012;165:2549–60.
[61] Ong WY, Mackie K. A light and electron microscopic study of the CB1 cannabinoid receptor in primate brain. Neuroscience 1999;92:1177–91.
[62] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. San Diego, CA: Academic Press; 1997.
[63] Perez de la Mora M, Lara-Garcia D, Jacobsen KX, Vazquez-Garcia M, Crespo-Ramirez M, Flores-Gracia C, Escamilla-Marvan E, Fuxe K. Anxiolytic-like effects of the selective metabotropic glutamate receptor 5 antagonist MPEP after its intra-amygdaloid microinjection in three different non-conditioned rat models of anxiety. Eur J Neurosci 2006;23:2749–59.
[64] Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, Mechoulam R, Ross RA. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. Pharmacol Rev 2010;62:588–631.
[65] Rea K, Finn DP. The role of supraspinal GABA and glutamate in the mediation and modulation of pain. In: D'Alonso S, Grasso KL, editors. Acute pain: causes, effects and treatment. Hauppauge, NY: Nova Science Publishers; 2009. p. 169–212.
[66] Rea K, Lang Y, Finn DP. Alterations in extracellular levels of gamma-aminobutyric acid in the rat basolateral amygdala and periaqueductal gray during conditioned fear, persistent pain and fear-conditioned analgesia. J Pain 2009;10:1088–98.
[67] Rea K, Roche M, Finn DP. Modulation of conditioned fear, fear-conditioned analgesia, and brain regional c-Fos expression following administration of muscimol into the rat basolateral amygdala. J Pain 2011;12:712–21.
[68] Roche M, Johnston P, Mhuircheartaigh 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;14:487–95.
[69] 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;26:2643–53.
[70] Rodrigues SM, Bauer EP, Farb CR, Schafe GE, LeDoux JE. The group I metabotropic glutamate receptor mGluR5 is required for fear memory formation and long-term potentiation in the lateral amygdala. J Neurosci 2002;22:5219–29.
[71] Rudy JW, Matus-Amat P. DHPG activation of group 1 mGluRs in BLA enhances fear conditioning. Learn Mem 2009;16:421–5.
[72] Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ. The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol 2007;152:1092–101.
[73] Sharir H, Abood ME. Pharmacological characterization of GPR55, a putative cannabinoid receptor. Pharmacol Ther 2010;126:301–13.
[74] Sheskin T, Hanus L, Slager J, Vogel Z, Mechoulam R. Structural requirements for binding of anandamide-type compounds to the brain cannabinoid receptor. J Med Chem 1997;40:659–67.
[75] Shin RM, Tully K, Li Y, Cho JH, Higuchi M, Suhara T, Bolshakov VY. Hierarchical order of coexisting pre- and postsynaptic forms of long-term potentiation at synapses in amygdala. Proc Natl Acad Sci USA 2010;107:19073–8.
[76] Soltis RP, Cook JC, Gregg AE, Sanders BJ. Interaction of GABA and excitatory amino acids in the basolateral amygdala: role in cardiovascular regulation. J Neurosci 1997;17:9367–74.
[77] Stork O, Ji FY, Obata K. Reduction of extracellular GABA in the mouse amygdala during and following confrontation with a conditioned fear stimulus. Neurosci Lett 2002;327:138–42.
[78] Strangman NM, Patrick SL, Hohmann AG, Tsou K, Walker JM. Evidence for a role of endogenous cannabinoids in the modulation of acute and tonic pain sensitivity. Brain Res 1998;813:323–8.
[79] Suardiaz M, Estivill-Torrus G, Goicoechea C, Bilbao A, Rodriguez de Fonseca F. Analgesic properties of oleoylethanolamide (OEA) in visceral and inflammatory pain. PAIN® 2007;133:99–110.
[80] Suplita 2nd RL, 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;49:1201–9.
[81] Tallaksen-Greene SJ, Kaatz KW, Romano C, Albin RL. Localization of mGluR1a-like immunoreactivity and mGluR5-like immunoreactivity in identified populations of striatal neurons. Brain Res 1998;780:210–7.
[82] Toth A, Boczan J, Kedei N, Lizanecz E, Bagi Z, Papp Z, Edes I, Csiba L, Blumberg PM. Expression and distribution of vanilloid receptor 1 (TRPV1) in the adult rat brain. Brain Res Mol Brain Res 2005;135:162–8.
[83] Tsou K, Mackie K, Sanudo-Pena MC, Walker JM. Cannabinoid CB1 receptors are localized primarily on cholecystokinin-containing gabaergic interneurons in the rat hippocampal formation. Neuroscience 1999;93:969–75.
[84] Tsou K, Brown S, Sañudo-Peña MC, Mackie K, Walker JM. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 1998;83:393–411.
[85] Walsh S, Gorman AM, Finn DP, Dowd E. The effects of cannabinoid drugs on abnormal involuntary movements in dyskinetic and non-dyskinetic 6-hydroxydopamine lesioned rats. Brain Res 2010;1363:40–8.
[86] Watson GS, Sufka KJ, Coderre TJ. Optimal scoring strategies and weights for the formalin test in rats. PAIN® 1997;70:53–8.
[87] Zschenderlein C, Gebhardt C, Von Bohlen Und Halbach O, Kulisch C, Albrecht D. Capsaicin-induced changes in LTP in the lateral amygdala are mediated by TRPV1. PLoS One 2011;6:e16116.