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Author(s)	Rea, Kieran; Olango, Weredeselam M.; Harhen, Brendan; Kerr, Daniel M.; Galligan, Rachel; Fitzgerald, Sean; Moore, Maeve; Roche, Michelle; Finn, David P.
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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

Kieran Rea [1, 3], Weredeselam M. Olango [1,3], Brendan Harhen [3], Daniel M. Kerr [1,2,3], Rachel Galligan [1], Sean Fitzgerald [1], Maeve Moore [1] , Michelle Roche [2,3] and David P. Finn [1,3]

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

Corresponding author

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

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

Email: david.finn@nuigalway.ie

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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. Re-exposure to a context previously paired with footshock 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. Post-mortem analyses revealed that FCA was associated with a significant increase in tissue levels of anandamide in the BLA side contralateral to intra-plantar 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.

Introduction

The transmission of nociceptive information within the CNS is subject to modulation by complex, co-ordinated 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 amygdaloid complex (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 characterised by a robust suppression of nociceptive behaviour during or following expression of classical Pavlovian conditioned fear [5,16,20,25].

Studies of the supra-spinal neurotransmitter systems involved in FCA have focused predominantly on γ-aminobutyric acid (GABA)ergic [25,27,65] and opioidergic [8,15,23,30] mechanisms. Recent evidence supports a key role for the endocannabinoid system in mediating FCA [6,7,16,21,60,68,69], as well as unconditioned stress-induced analgesia [36,80] in rats. However, potential interactions of the endogenous cannabinoid (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 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]; and, as such, 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 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 since 'on-demand' synthesis and retrograde release of endocannabinoids has been demonstrated following 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 following expression of FCA.

Materials and Methods

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^{o}C) under standard lighting conditions (12:12 hour light–dark, lights on from 0800-2000h). Experiments were carried out during the light phase between 0800 and 1700h. Food and water were available *ad libitum*. The experimental protocol was carried out following

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.

Cannulae Implantation

Stainless steel guide cannulae (Plastics One Inc., Roanoke, Virginia, USA) were stereotaxically implanted 1 mm above the right and left BLA (AP - 0.25 cm, ML ± 0.48 cm relative to bregma, DV - 0.71 cm from skull surface) and 1 mm above the central nucleus of the amygdala (CeA; AP - 0.212 cm, ML ± 0.40 cm relative to bregma, DV - 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 using stainless-steel screws and carboxylate cement. 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 (250µL, 0.5% s.c.) (Rimadyl, Pfizer, Kent, UK), and the broad spectrum antibiotic, enrofloxacin (250µL, 0.5% s.c.) (Baytril, Bayer Ltd., Dublin, Ireland), were administered before surgery to manage post-operative pain and to prevent infection, respectively. Following cannulae implantation, the rats were housed singly and administered enrofloxacin (250µL, 0.5% s.c.) for a further 3 days. Rats were allowed to recover for at least 6 days prior to experimentation. During this period, the rats were handled and their body weight and general health monitored on a daily basis.

Drug Preparation

The $GABA_A$ receptor antagonist bicuculline methbromide ([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 3mg/kg (3mL/kg dose volume). On test days, the drug was re-constituted as an emulsion in ethanol: cremaphor: saline vehicle in a ratio of 1:1:18. The dose of AM251 and time of administration were chosen based on our previous work using 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 180mM in DMSO vehicle (dimethylsulfoxide, 100%) and 0.5µL was bilaterally injected based on our previous studies investigating the effects of intra-BLA administration of rimonabant [69].

Bicuculline was prepared in sterile saline (0.5µL of a 136µM 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 shown 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 (0.5µL of a 200µM 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 following intracerebral administration [1,63,71].

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 (2-3min) isoflurane anaesthesia for injection of 50µL formalin injection 30 minutes prior to arena re-exposure.

Experimental Procedures

The FCA paradigm was essentially as described previously [7,16,17,21,60,66-69]. In brief, it consisted of two phases, conditioning and testing, occurring 24 h apart. Subjects were randomly assigned to groups and the sequence of testing was randomised. On the conditioning day, rats were placed in a Perspex fear-conditioning/ observation chamber (30 x 30 x 30 cm) and after 15 s they received the first of 10 footshocks (0.4 mA, 1 s duration; LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK) spaced 60 s apart. Fifteen seconds after the last footshock, rats were returned to their home cage. Controls not receiving footshock were exposed to the chamber for an equivalent 9.5 min period. Three experiments, each using a different cohort of rats were carried out (Experiments 1, 2 and 3 as described below). The test phase for all 3 experiments commenced 23.5 hours later when the subjects received an intra-plantar injection of 50 µL formalin (2.5% formalin solution prepared in sterile saline) into the right hindpaw under brief isoflurane anaesthesia. Formalin-induced oedema was assessed by measuring the change in the diameter of the right hindpaw immediately before, and 45 or 60 minutes after, formalin administration, using Vernier callipers.

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

Table 1. Summary of experimental groups in Experiment 1. 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.

Experiment 2: The test phase commenced 23.5 h following conditioning with the rats receiving an intra-plantar injection of 50 µL formalin (2.5% formalin in sterile saline) into the right hindpaw 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 (180mM) 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 re-exposed to the arena for 15 minutes to correlate with previous studies [68,69].

Group No:	Conditioning	Formalin *i.pl.*	AM251/Vehicle *Intra-BLA*	No. Per group
1	FC	Formalin	VEH	9
2	No FC	Formalin	VEH	8
3	FC	Formalin	AM251	8
4	No FC	Formalin	AM251	8
Group No:	Conditioning	Formalin *i.pl.*	AM251/Vehicle *Intra-CeA*	No. Per group
5	FC	Formalin	VEH	5
6	FC	Formalin	AM251	6

Table 2. Summary of experimental groups in Experiment 2. 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.

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 confounds due to 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 post-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 DVD for 15 or 30 minutes. The 30–60 min post-formalin 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 subject to supraspinal modulation [7,16,17,21,60,66-69].

At the end of the test phase, (45 or 60 min post-formalin injection), rats were sacrificed by decapitation, brains were excised, snap-frozen on dry ice and stored at -80^{o}C. Rats in Experiments 1 and 2 received intra-BLA or intra-CeA injection of fast-green dye (0.5µL of 1% solution) following decapitation for subsequent histological confirmation of the microinjection sites. In experiment 3, endocannabinoids and *N*-acylethanolamines were quantified from tissue punches of the BLA as described below.

Behavioural Analysis

Behaviour was analysed using the Ethovision XT 7.0 software package (Noldus, Netherlands), which allowed for continuous event recording over each 30 min trial. A rater blind 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)]. Total distance travelled (cm) was automatically tracked using this system and was used as an index of locomotor activity.

Histological verification of intra-cerebral microinjection sites

The sites of intra-cerebral microinjection were determined prior to data analysis. Brain sections with fast-green dye mark were collected (30µm thickness), mounted on gelatinised glass slides and counterstained with cresyl violet to locate the precise position of microinjection sites under light microscopy.

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

Frozen coronal brain sections (300µm) 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 -2.56mm and bregma -3.6mm) [62] using cylindrical brain punchers (Harvard Apparatus, internal diameter 2mm). Each punched tissue sample was kept frozen throughout the collection procedure, weighed: (average weight of punched tissue = 4.3mg) and stored at -80^{o}C prior to 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 using a lipid extraction method as follows: Each brain tissue sample was first homogenised in 400µL 100% acetonitrile containing known fixed amounts of deuterated internal standards (0.014 nmol AEA-d8, 0.48nmol 2-AG-d8, 0.016nmol 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 re-suspended in 40µL 65% acetonitrile and 2µL were injected onto a Zorbax® C18 column (150 × 0.5mm internal diameter) from a cooled autosampler maintained at 4°C (Agilent Technologies Ltd, Cork, Ireland). Mobile phases consisted of A (HPLC grade water

with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 12µL/min. Reversed-phase gradient elution began initially at 65% B and over 10min was ramped linearly up to 100% B. At 10min, the gradient was held at 100% B up to 20min. At 20.1min, the gradient returned to initial conditions for a further 10mins to re-equilibrate the column. The total run time was 30min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 11.36min, 12.8min, 14.48min and 15.21min respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Instrument conditions and source parameters including fragmentor voltage and collision energy were optimised for each analyte of interest prior to assay of samples. Quantitation of target endocannabinoids was achieved by positive ion electrospray ionization and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [M + H+] of the analytes of interest and the deuterated 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 using Masshunter Quantitative Analysis Software (Agilent Technologies Ltd, Cork, Ireland). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard. The values obtained from the Masshunter Quantitative Analysis Software

are initially expressed in ng per mg of tissue by dividing by the weight of the punched tissue. To express values as nmol or pmols per mg of tissue, the corresponding values are then divided by the molar mass of each analyte expressed as ng/nmole or pg/pmole. Linearity (regression analysis determined R^2 values of 0.99 or greater for each analyte) was determined over a range of 18.75ng to 71.5fg except for 2-AG which was 187.5ng-715fg. The limit of quantification was 1.32pmol/g, 12.1pmol/g, 1.5pmol/g, 1.41pmol/g for AEA, 2-AG, PEA and OEA respectively.

Statistical analysis

SPSS statistical package (SPSS v15.0 for Microsoft Windows; SPSS, Inc., Chicago, IL) was used to analyse all data. Paw oedema was analysed using a paired Student's t-test, while the effects of AM251 microinjection in the CeA were analysed using an unpaired Student's t-test. Mass spectrometry analysis (following log transformation) and behavioural data were analysed using 2- or 3-way analysis of variance (ANOVA) followed by Fisher's LSD post-hoc test where appropriate. Data were considered significant when $P < 0.05$. Results are expressed as group means ± standard error of the mean (±SEM).

Results

Histological verification of injector placement

For experiment 1, eighty-five percent and for experiment 2, eighty percent 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 (Figures 1 and 2). For off-site control experiments targeting the CeA, all cannulae were

within the borders of the CeA (Figure 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.

Systemic or intra-BLA administration of AM251 attenuates FCA

Intra-plantar injection of formalin increased oedema (T_{98} = 33.023, p<0.001) and produced robust licking, biting, shaking, flinching and elevation of the injected right hindpaw. 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. I.p. administration of AM251 (3 mg/kg) 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 following 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 minute 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).

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).

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 non-significantly (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).

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

There was no effect of footshock on locomotor activity over the 30 minute trial ($F(1,109) = 0.342$, $p = 0.560$) 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 ($F(1,109) = 2.303$, $p = 0.132$) or bilaterally into the BLA ($F(1,24) = 0.382$, $p = 0.543$). There was no effect of intra-BLA bicuculline or MPEP administration on

locomotor activity ($F(2,109) = 0.159$, $p = 0.853$), compared with vehicle-treated controls (data not shown).

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

In rats receiving intra-plantar injection of saline, there were no significant differences between contralateral and ipsilateral BLA 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 intra-plantar 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 ($p = 0.08$) and OEA ($p = 0.06$).

Discussion

This study shows that systemic administration of the CB_1 receptor antagonist/inverse agonist, AM251, and its bilateral 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 following 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 (FC), 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 shown to attenuate the suppression of nociceptive behaviour elicited by exposure of rats to unconditioned footshock stress [10,36]. Moreover, we have previously shown 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 show 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 (FC), 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 occur 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 following 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 (for review see [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 two 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 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 post-synaptic localisation on glutamatergic neurons in the hippocampus. However, Maione *et al.* (2006) reported

opposing roles of TRPV1 and CB_1 receptor activation in pain behaviours in rats and that TRPV1 receptors are often co-expressed with CB_1 receptors in the PAG [51]. Similarly, Micale *et al.* (2009) demonstrated opposing roles of these receptors in anxiety tests in mice and also reported a co-expression 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; while 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 shown 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 hypothesised 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 localised 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 re-exposure to the arena, that 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.

While 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 showed that in mice, DHPG-induced peripheral hypersensitivity is reduced following 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 post-synaptic 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.

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.

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Figures

Fig. 1. Schematic depicting the sites of microinjection of (A) Vehicle (saline) or (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.

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 (3mg/kg, i.p.) attenuates FCA in rats. Data are means ± SEM (n = 8-10). A 2-way ANOVA revealed a significant effect of fear-conditioning ($F(1,38) = 17.901$, $p < 0.001$) and of i.p. drug administration ($F(1,38) = 26.845$, $p < 0.001$) and an interaction effect ($F(1,38) = 7.367$, $p = 0.010$) on nociceptive behaviour. *$P < 0.05$ vs. NO FC-VEH-SAL. $^{+}P < 0.05$ 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 (180nM) microinjection into the BLA attenuates FCA in rats. Data are means ± SEM (n = [A] 8-10; [B] 5-6). 2-way ANOVA determined that AM251 bilaterally microinjected into the BLA significantly attenuated the reversal of formalin-evoked nociceptive behaviour by fear-conditioning ($F(1,24) = 25.768$, $p < 0.001$). * $P < 0.05$

vs. NO FC-VEH. $ P < 0.05 for FC-AM251 vs. NO FC-AM251. + P < 0.05 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 means ± SEM (n = 8-10). A 3-way ANOVA revealed a significant effect of fear-conditioning ($F(1,66) = 39.716$, $p < 0.001$) and of i.p. AM251 administration ($F(1,66) = 48.531$, $p < 0.001$) on nociceptive behaviour. *P < 0.05 vs. corresponding NO FC-VEH treatment groups. $^{+}P < 0.05$ vs. corresponding FC-VEH treated groups. $^{\$}P < 0.05$ 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.

Fig. 6 Bilateral MPEP microinjection into the BLA attenuates the reversal of FCA by systemic AM251 administration in rats. Data are means ± SEM (n = 8-10). A 3-way ANOVA revealed a significant effect of fear-conditioning ($F(1,74) = 38.825$, $p < 0.001$) and of i.p. AM251 administration ($F(1,74) = 13.702$, $p < 0.001$) on nociceptive behaviour. Furthermore these analyses revealed a significant interaction effect between fear-conditioning and i.p. AM251 administration ($F(1,74) = 9.426$, $p < 0.05$), and between MPEP and i.p. AM251 administration ($F(1,74) = 8.082$, $p < 0.05$) $^{\#}P < 0.05$ NO FC-VEH-MPEP vs. NO FC-VEH-SAL. *P < 0.05 vs. corresponding NO FC-VEH treated groups. $^{+}P < 0.05$ for FC-AM251-SAL vs. FC-VEH-SAL. $^{\$}P < 0.05$ 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.

Fig. 7 Alterations in levels of AEA, 2-AG, PEA and OEA associated with conditioned fear, formalin-evoked nociceptive behaviour and FCA. Data are means ± SEM (n = 5-7). 3-way ANOVA revealed a significant effect of formalin administration on 2-AG and PEA levels ($F(1,44) = 9.334$, $p = 0.004$ and $F(1,44) = 4.348$, $p = 0.044$ respectively), and a significant difference between levels of AEA ($F(1,44) = 7.200$, $p = 0.011$), PEA ($F(1,44) = 6.692$, $p = 0.014$) and OEA ($F(1,44) = 7.143$, $p = 0.011$) between contralateral and ipsilateral BLA. There was a significant interaction between fear-conditioning and formalin administration for AEA ($F(1,44) = 8.729$, $p = 0.005$), 2-AG ($F(1,44) = 6.689$, $p = 0.014$), and OEA ($F(1,44) = 4.294$, $p = 0.045$) with a strong trend towards an interaction in PEA ($F(1,44) = 4.065$, $p = 0.051$). * denotes where groups are significantly different ($p < 0.05$). 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.

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 since intra-BLA administration of mGlu5 receptor antagonist, MPEP, increased formalin-evoked nociceptive behaviour. Conditioned fear may facilitate the synthesis of endocannabinoids in the post-synaptic 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.

Anandamide (AEA); 2-arachidonylglycerol (2-AG); cannabinoid$_1$ receptor (CB_1R); chloride ion (Cl^-); cytosolic calcium (Ca^{++}); diacylglycerol (DAG); diacylglycerol lipase (DGL); G-protein coupled receptor (GPCR); inositol triphosphate (IP_3); metabotropic glutamate receptor (mGluR); N-arachidonoyl-phosphatidylethanolamine (NAPE); N-acyltransferase (NAT); phosphatidylcholine (PhosC); phosphatidylethanolamine (PhosEA); phosphoinositol (PIP_2); phospholipase C enzyme (PLC); phospholipase D enzyme (PLD); voltage-gated ion channels (VGIC).

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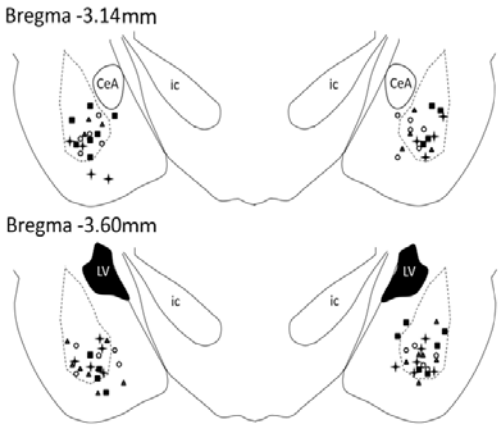
Fig. 1a



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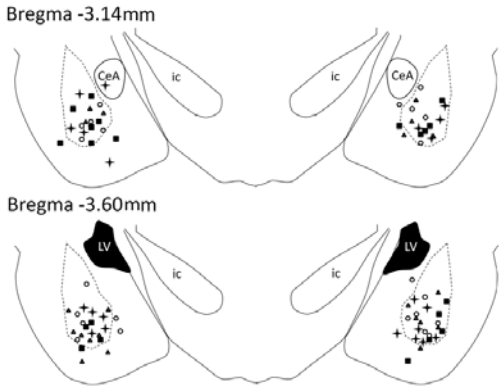

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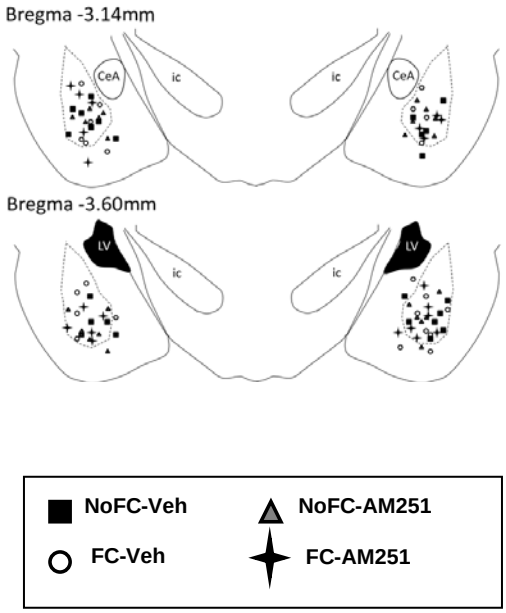

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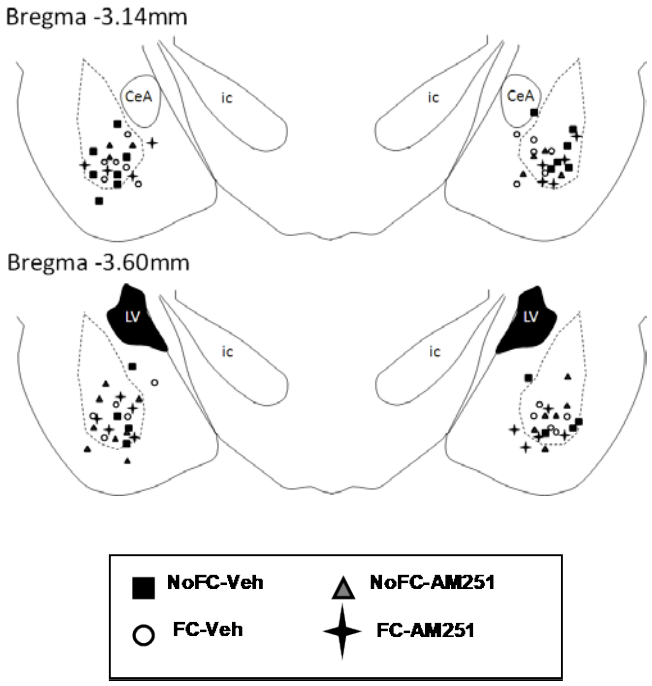
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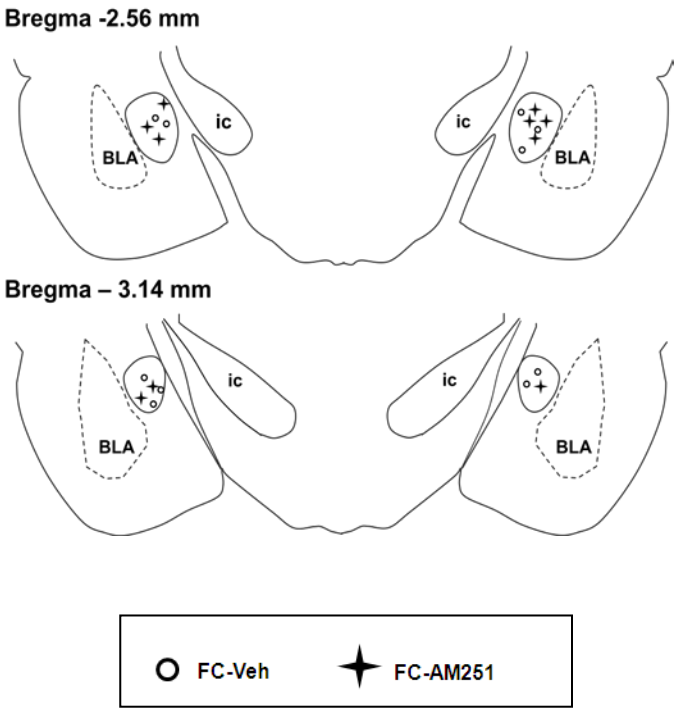
Fig. 2b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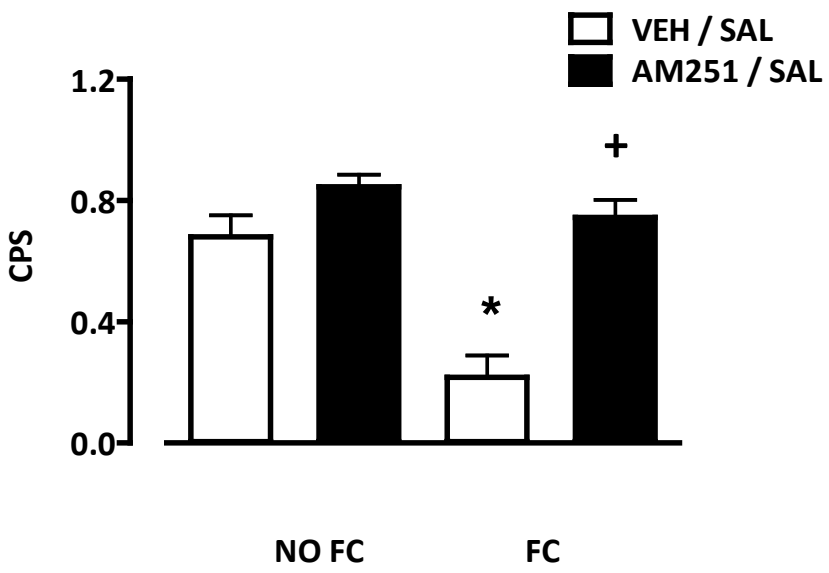
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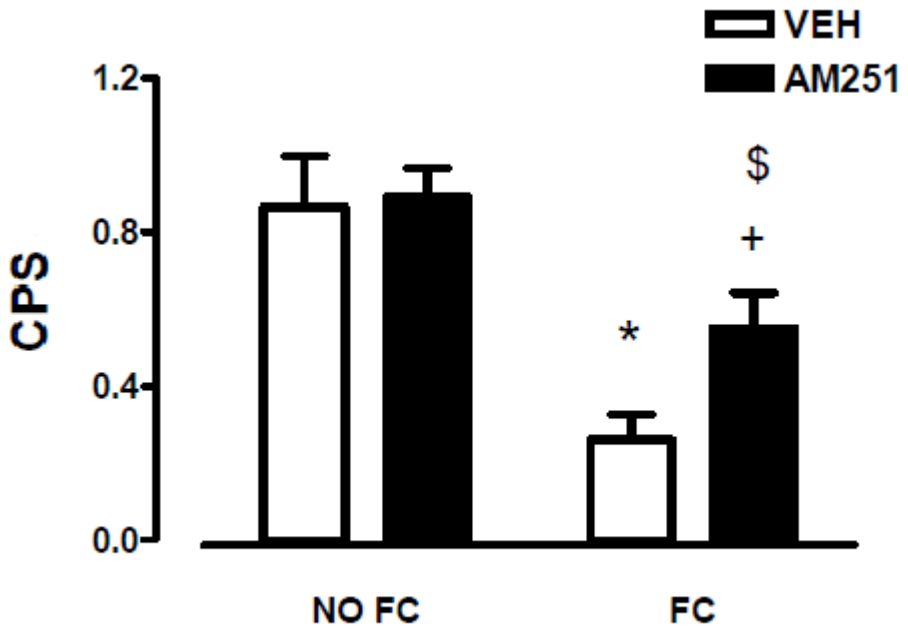
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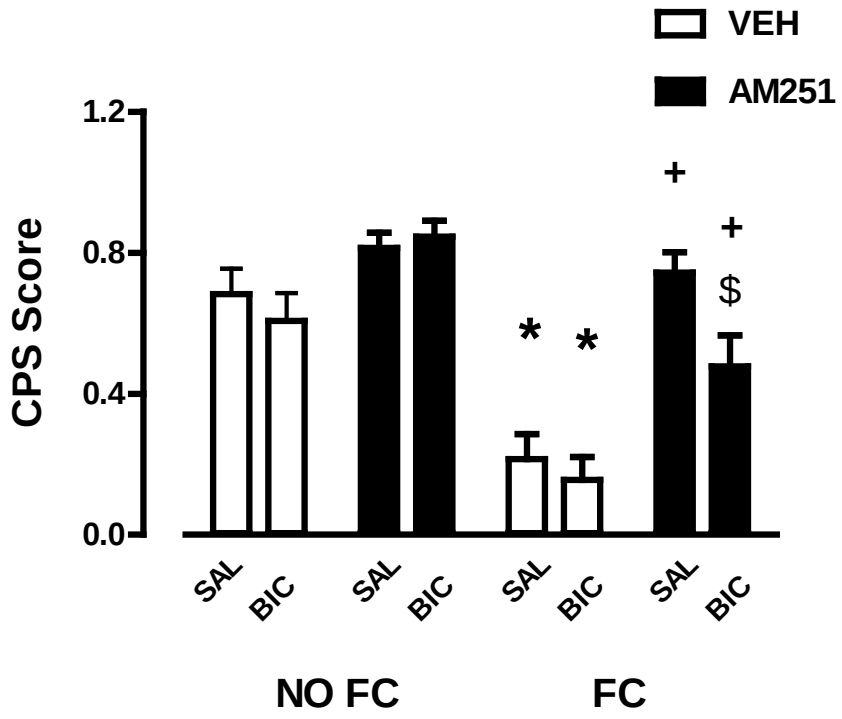
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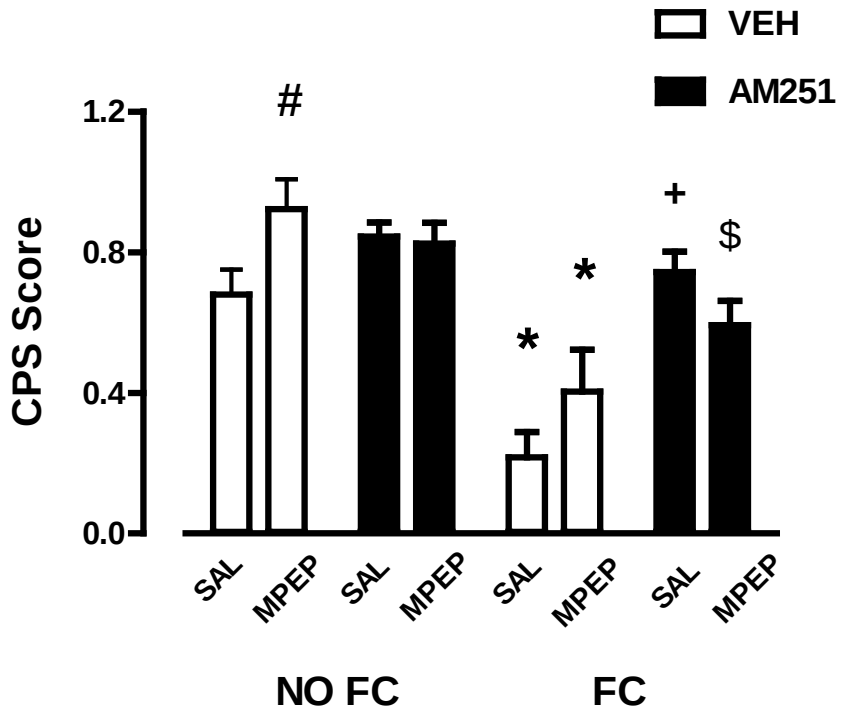
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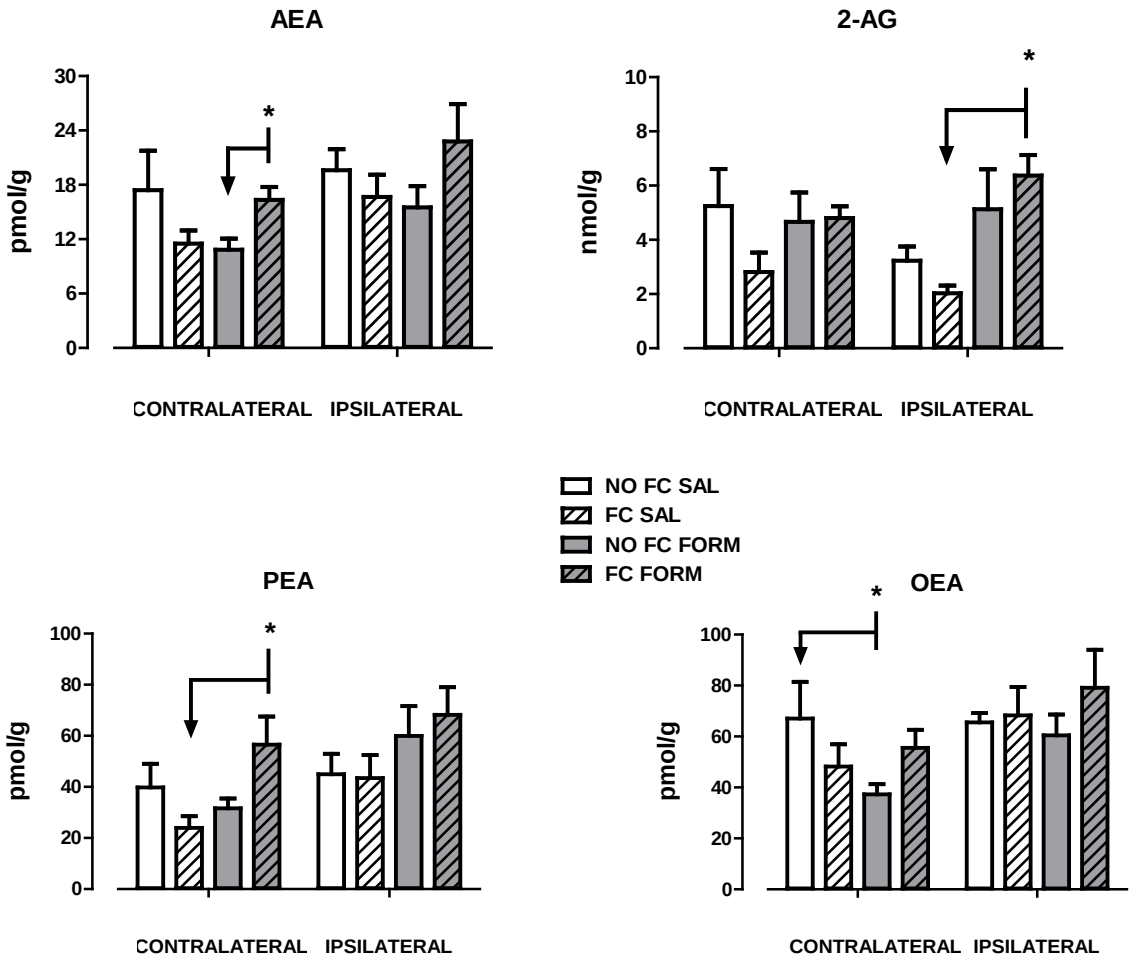
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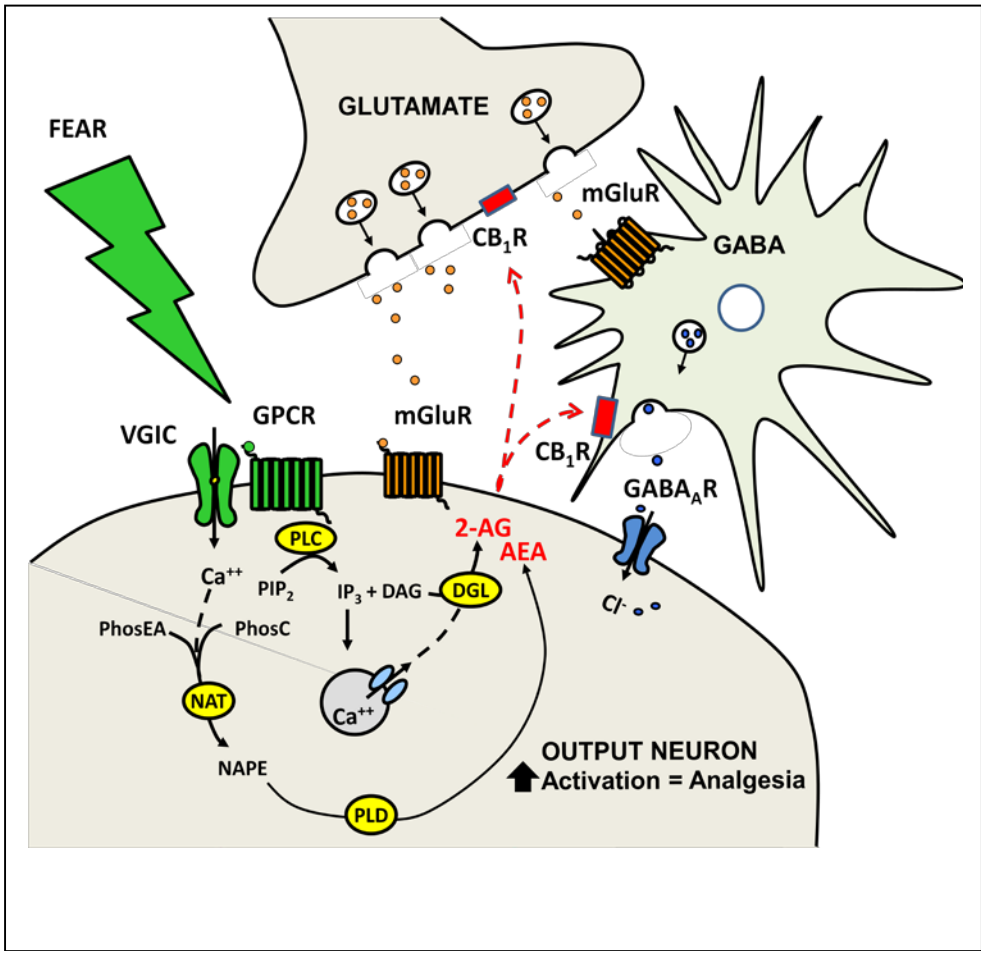
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NAT
Ca++
NAPE
PLD
OUTPUT NEURON
Activation = Analgesia