Attenuation of fear#conditioned analgesia in rats by monoacylglycerol lipase inhibition in the anterior cingulate cortex: Potential role for CB2 receptors

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Attenuation of fear-conditioned analgesia in rats by monoacylglycerol lipase inhibition in the anterior cingulate cortex: Potential role for CB_2 receptors<<Query: AUTHOR: Running titles no longer required Ans: Ok>>

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(vermilion) have been identified correctly. Ans: Ok>> Corcoran[1,3], Darragh Mattimoe[1,3], Michelle Roche[2,3], David P. Finn*[1,3]

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Abstract

Background and Purpose

Improved understanding of brain mechanisms regulating endogenous analgesia is important from a fundamental physiological perspective and for identification of novel therapeutic strategies for pain. The endocannabinoid system plays a key role in stress-induced analgesia, including fear-conditioned analgesia (FCA), a potent form of endogenous analgesia. Here, we studied the role of the endocannabinoid 2-arachidonoyl glycerol (2-AG) within the anterior cingulate cortex (ACC; a brain region implicated in the affective component of pain) in FCA in rats.

Experimental Approach

FCA was modelled in male Lister-hooded rats by assessing formalin-evoked nociceptive behaviour in an arena previously paired with footshock. The effects of intra-ACC administration of MJN110 (inhibitor of

monoacylglycerol lipase [MGL], the primary enzyme catabolizing 2-AG), AM630 (CB_2 receptor antagonist), AM251 (CB_1 receptor antagonist) or MJN110 + AM630 on FCA were assessed.

Key Results

MJN110 attenuated FCA when microinjected into the ACC, an effect associated with increased levels of 2-AG in the ACC. This effect of MJN110 on FCA was unaltered by co-administration of AM251 but was blocked by AM630, which alone reduced nociceptive behaviour in non-fear-conditioned rats. RT-qPCR confirmed that mRNA encoding CB_1 and CB_2 receptors was detectable in the ACC of formalin-injected rats and unchanged in those expressing FCA.

Conclusion and Implications

These results suggest that an MGL substrate in the ACC, likely 2-AG, modulates FCA and that within the ACC, 2-AG-CB_2 receptor signalling may suppress this form of endogenous analgesia. These results may facilitate increased understanding and improved treatment of pain- and fear-related disorders and their co-morbidity.

Figure S1: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110 + AM251 directly into the infralimbic cortex (IL) on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. *p<0.05, FC vs. NFC; #p<0.05 vs FC VEH (Tukey's). All data are expressed as mean ± SEM (n=8-11 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Figure S2: Effects of fear-conditioning and bilateral administration of MJN110 and MJN110+AM251 directly into the prelimbic cortex (PrL) on formalin-evoked nociceptive behaviour in rats over the full 30-minute testing period subdivided into 10-minute time bins. *p<0.05, FC vs. NFC; #p<0.05 vs FC VEH (Tukey's). All data are expressed as mean ± SEM (n = 8–11 per group) NFC: Non-fear-conditioned; FC: Fear-conditioned; VEH: Vehicle.

Abbreviations

ACC	anterior cingulate cortex
AM251	(N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), MJN110 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate)
AM630	(6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone, AM630, 6-iodopravadoline)
CB_1	cannabinoid$_1$ receptor
CB_2	cannabinoid$_2$ receptor
CPS	composite pain score
2-AG	2-arachidonoylglycerol
FC	fear-conditioned
FCA	fear-conditioned analgesia
IL	infralimbic
MGL	monoacylglycerol lipase
NFC	non-fear-conditioned

mPFC	medial prefrontal cortex
PrL	prelimbic
VEH	vehicle

What is already known<<Query: Each bullet point should only be 15 words long also abbreviations should be used to a minimum Ans: Done>>

- The endocannabinoid system is a key regulator of stress-induced and fear-conditioned analgesia ~~(FCA)~~.
- Anterior cingulate cortex (implicated in affective component of pain) -anandamide/CB_1 signalling does not modulate ~~FCA~~fear-conditioned analgesia.

What does this study add

- 2-arachidonoylglycerol CB_2 receptor signalling within the anterior cingulate cortex attenuates fear-conditioned analgesia.

What it is clinical significance

- These results advance our understanding of endogenous analgesia<<Query: AUTHOR: "These results advance understanding of endogenous analgesia." This sentence has been modified for clarity. Please check and confirm that the intended meaning has been retained. Ans: Ok>>.
- These results suggest novel therapeutic approaches to treat pain- and fear-related disorders, and their co-morbidity<<Query: AUTHOR: "These results inform development … their comorbidity." This sentence has been modified for clarity. Please check and confirm that the intended meaning has been retained. Ans: Ok>>.

1 INTRODUCTION

Chronic, persistent pain imposes an enormous health and socio-economic burden and represents a major unmet clinical need. A deeper understanding of brain mechanisms regulating endogenous analgesia is of importance from a fundamental physiological perspective and for the identification and development of novel therapeutic strategies for improved treatment of pain. Fear-conditioned analgesia (FCA) is robust pain suppression expressed upon re-exposure to a context previously paired with an aversive stimulus (Ford et al., 2008<<Query: AUTHOR: Reference "Ford et al., 2008" has not been included in the Reference List; please supply full publication details. Ans: Please change the in-text citation to Ford and Finn, 2008.

that reference has already been supplied in reference list:

Ford, G. K., & Finn, D. P. (2008). Clinical correlates of stress-induced analgesia: evidence from pharmacological studies. Pain, 140(1), 3–7.

all of the references that only have two authors have the same problem with the in-text citation throughout the manuscript. In each case the full reference has already been supplied in the reference list so there is nothing new to add or change there but the in-text citations need to be changed from first author et al year to first author and second author year. I have provided the details for each other case under the relevant author query number below.>>; Butler et al., 2009<<Query: AUTHOR: Reference "Butler et al., 2009" has not been included in the Reference List; please supply full publication details. Ans: Please change citation to Butler and Finn, 2009.that reference has been supplied in reference list:

Butler, R. K., & Finn, D. P. (2009). Stress-induced analgesia. Progress in Neurobiology, 88(3), 184–202. https://doi.org/10.1016/j.pneurobio.2009.04.003>>). Fear-conditioned analgesia is a potent form of endogenous analgesia and a useful model with which to study the neurobiological mechanisms underpinning endogenous pain suppression and fear–pain interactions.

The endocannabinoid system consists of cannabinoid1 (CB1) and cannabinoid2 (CB2) receptors, their endogenous ligands, anandamide and 2-arachidonoylglycerol (2-AG), and their key catabolizing enzymes, fatty acid amide hydrolase and monoacylglycerol lipase (MGL). The endocannabinoid system has been shown to play a key role in the expression of fear-conditioned analgesia (Finn et al., 2004; Butler, Rea, Lang, Gavin, & Finn, 2008; Ford et al., 2008; Butler et al., 2009; Ford, Kieran, Dolan, Harhen, & Finn, 2011; Olango, Roche, Ford, Harhen, & Finn, 2012; Rea et al., 2013; Corcoran, Roche, & Finn, 2015) and other forms of stress-induced analgesia (SIA; Connell, Bolton, Olsen, Piomelli, & Hohmann, 2006; Hohmann et al., 2005; Sorge et al., 2014; Suplita, Farthing, Gutierrez, & Hohmann, 2005; Suplita, Gutierrez, Fegley, Piomelli, & Hohmann, 2006) in rodents, with key sites of action within the amygdala, periaqueductal grey, ventral hippocampus and rostral ventromedial medulla.

The medial prefrontal cortex (mPFC) is a limbic system-related region consisting of three distinct subregions in rodents, the infralimbic, prelimbic and anterior cingulate cortices (ACCs; Vertes, 2002; Vertes, 2004). These subregions can be distinguished by their anatomical connectivity, cytoarchitecture and function. The medial prefrontal cortex has numerous functional roles, including processing and modulation of pain and fear (Calejesan, Kim, & Zhuo, 2000; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006; Corcoran et al., 2007<<Query: AUTHOR: Reference "Corcoran et al., 2007" has not been included in the Reference List; please supply full publication details. Ans: Please change citation to Corcoran and Quirk, 2007

Corcoran, K. A., & Quirk, G. J. (2007). Activity in prelimbic cortex is necessary for the expression of learned, but not innate, fears. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 27(4), 840–844. https://doi.org/10.1523/JNEUROSCI.5327-06.2007>>; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; Sharpe et al., 2014<<Query: AUTHOR: Reference "Sharpe et al., 2014" has not been included in the Reference List; please supply full publication details. Ans: Please change citation to Sharpe and Killcross, 2014

Sharpe, M., & Killcross, S. (2014). The prelimbic cortex uses contextual cues to modulate responding towards predictive stimuli during fear renewal. Neurobiology of Learning and Memory, 118C, 20–29.>>; Almada, Coimbra, & Brandao, 2015). The anterior cingulate cortex is involved in the modulation of fear behaviour (Cullen, Gilman, Winiecki, Riccio, & Jasnow, 2015; Einarsson, Pors, & Nader, 2015; Falconi-Sobrinho, Anjos-Garcia, de Oliveira, & Coimbra, 2017), the cognitive-affective component of pain (Johansen, Fields, & Manning, 2001) and in the top-down descending modulation of pain (Calejesan et al., 2000; Fuchs, Peng, Boyette-Davis, & Uhelski, 2014). Recently, we have reported a role for the CB_1 receptor (antinociceptive) and fatty acid amide hydrolase substrates (antinociceptive or pronociceptive) in the infralimbic and prelimbic, but not in the anterior cingulate cortex, in fear-conditioned analgesia (Rea, McGowan, Corcoran, Roche, & Finn, 2019). However, the role of monoacylglycerol lipase/2-AG and CB_2 receptors in the anterior cingulate cortex in expression of fear-conditioned analgesia has not yet been investigated and is the focus of this current study.

Converging data have demonstrated that 2-AG may inhibit fear, anxiety and pain (Busquets-Garcia et al., 2011; Sciolino, Zhou, & Hohmann, 2011; Sumislawski, Ramikie, & Patel, 2011; Rea, Ford, et al., 2014; Llorente-Berzal et al., 2015; Hartley et al., 2016; Bluett et al., 2017; Wilkerson et al., 2017). Thus, the overall aim of the present study was to test the hypothesis that monoacylglycerol lipase inhibition within the anterior cingulate cortex, with consequent elevations in 2-AG levels, will facilitate/enhance the expression of fear-conditioned analgesia and reduce formalin-evoked nociceptive behaviour and conditioned fear in the presence of nociceptive tone, via CB_1 and/or CB_2 receptors. Elucidation of the neurobiological mechanisms underpinning of fear-conditioned analgesia will advance our understanding of the physiology of endogenous analgesia and may aid in the development of new potential therapeutic strategies for pain and co-morbid fear-related disorders.

2 METHODS

2.1 Animals

A total of 152 male Lister-Hooded rats (260–325 g [~9–10 weeks old] on day of behavioural testing; Charles River, Margate, Kent, UK) were used. This model has been in use successfully for numerous years (Butler et al., 2011; Finn et al., 2003; Finn et al., 2004; Rea et al., 2013; Rea et al., 2019; Rea, Lang, & Finn, 2009). Animals were housed two to three per cage before surgery and singly thereafter in plastic bottomed cages (L: 45 × H: 20 × W: 20 cm) with wood shavings as bedding. They were maintained at a constant temperature (22 ± 2°C) under standard lighting conditions (12:12-hr light–dark, lights on from 08:00 a.m. to 08:00 p.m.). Experiments were carried out during the light phase between 08:00 a.m. and 05:00 p.m. Food (14% Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, UK) and water were available *ad libitum*. Subjects were randomly assigned to experimental groups and the sequence of testing was randomized throughout the experiment. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway and the work carried out under license from the Irish Department of Health and Children, in compliance with the European Communities Council directives 86/609 and 2010/63. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*.

2.2 Cannulae implantation

Animals were left to acclimatize for 4–8 days after delivery before surgery. All surgery was performed aseptically. Stainless steel guide cannulae (5 mm length, 22G, Plastics One Inc., Roanoke, VA, USA) were stereotaxically implanted bilaterally 1 mm above the right and left anterior cingulate cortex (AP + 1.0 mm relative to bregma, ML ± 1.3 mm at a 12° angle, DV – 1.3 mm from dura, toothbar set at −3.0 mm; Paxinos & Watson, 1998<<Query: AUTHOR: The citation "Paxinos et al., 1998" has been changed to "Paxinos & Watson, 1998" to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary. Ans: Ok>>) under isoflurane anaesthesia (2–3% in O_2; 0.5 $L \cdot min^{-1}$). Rats were deemed to be surgically anaesthetised when the pedal withdrawal reflex was absent. 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 (5 $mg \cdot kg^{-1}$, s.c.; Rimadyl, Pfizer, Kent, UK), and the broad-spectrum antibiotic, enrofloxacin (2.5 $mg \cdot kg^{-1}$, s.c.; Baytril, Bayer Ltd., Dublin, Ireland), were administered before surgery to manage post-operative pain and to prevent infection. Following cannulae implantation, the rats were housed singly and administered enrofloxacin (2.5 $mg \cdot kg^{-1}$, s.c.) for a further 4 days. Rats were allowed to recover for at least 6 days prior to experimentation. During this period, the rats were handled daily, stylets checked and their body weight and general health monitored daily using animal welfare scoresheets.

2.3 Drug preparation

Formalin (37% formaldehyde solution), DMSO (100%), AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), MJN110 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate) and AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone, AM630, 6-iodopravadoline) were purchased from Sigma-Aldrich, Dublin, Ireland.

On test days, solutions of 1.3 nmols/0.3 µl MJN110 (monoacylglycerol lipase inhibitor), 1.5 nmols/0.3 µl AM630 (CB_2 antagonist), 1.3 nmols/0.3 µl MJN110 + 0.6 nmols/0.3 µl AM251 (CB_1 antagonist) or 1.3 nmols/0.3 µl MJN110 + 1.5 nmols/0.3 µl AM630 were prepared using stock solutions and 100% DMSO vehicle (VEH). A solution of 2.5% formalin (Sigma-Aldrich, Dublin, Ireland) was prepared from a 37% stock solution diluted with 0.9% sterile saline.

These doses of AM251, MJN110 and AM630 are based on previous work carried out by our laboratory and evidence from the literature (Almeida-Santos et al., 2013; Freitas, Salgado-Rohner, Hallak, Crippa, & Coimbra, 2013; Lisboa et al., 2010; Rea et al., 2019; Stern et al., 2017; Sticht et al., 2016; Wills et al., 2016).

2.4 Experimental procedures

The fear-conditioned analgesia procedure was essentially as described previously (Butler et al., 2008; Finn et al., 2004; Ford et al., 2011; Olango et al., 2012; Rea et al., 2009; Rea et al., 2019; Roche et al., 2010; Roche, O'Connor, Diskin, & Finn, 2007). The experiment consisted of two phases, conditioning and testing, occurring 24 hr apart. Testing occurred 6–7 days following surgery. The design and time course of the experiments was identical to that depicted in fig. 1 of Rea et al. (2019), with the exception of the drugs administered. The experimental procedures conformed to the BJP guidelines (Curtis et al., 2018).

The conditioning phase involved placing the rats in a Perspex fear-conditioning/observation box (30 × 30 × 40 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; 15 s following the final footshock, the rats were returned to their home cage. Control rats not receiving footshock were exposed to the arena for an equivalent 10 min. The arena was cleaned using 0.5% acetic acid after each rat.

The test phase commenced 23.5 hr later when the subjects received an intra-plantar injection of 50-µl formalin (2.5% formaldehyde solution prepared in sterile saline) into the right hind paw under brief isoflurane anaesthesia. 15 min later, rats received bilateral microinjections (0.3 µl per side) of either VEH (100% DMSO), MJN110 (1.3 nmols) or MJN110 + AM251 (1.3 nmols + 0.6 nmols) into the anterior cingulate cortex (Experiment 1) and in the follow-up study either VEH (100% DMSO), MJN110 (1.3 nmols), AM630 (1.5 nmols) or MJN110 + AM630 (1.3 nmols + 1.5 nmols) into the anterior cingulate cortex (Experiment 2). For each study, a volume of 0.3 µl was injected for 60 s using a 1-µl Hamilton microsyringe attached to polyethylene tubing and a Harvard PHD2000 infusion pump (Harvard Apparatus, Kent, UK). The injector was left for another 60 s before removal to allow adequate drug infusion.

Immediately following the intracerebral microinjections, rats were returned to their home cages for 15 min prior to being returned to the same Perspex arenas in which they had been conditioned. A video camera located beneath the observation chamber was used to monitor animal behaviour. The video feed was recorded for 30 min. 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 (Butler et al., 2008; Finn et al., 2004; Finn et al., 2006; Ford et al., 2011; Olango et al., 2012; Rea et al., 2009; Rea et al., 2019; Rea, Roche, & Finn, 2011; Roche et al., 2007; Roche et al., 2010).

At the end of the test phase (60 min post-formalin injection), rats were killed by decapitation, and an intracerebral microinjection of fast-green dye (0.3 µl of 1% solution) was performed following decapitation for subsequent histological confirmation of the microinjection sites. The brains were subsequently excised, snap-frozen on dry ice and stored at −80°C.

This design resulted in six or eight experimental groups (Starting n = 10–12 per group for surgery; final n after removal of outliers where the cannula were placed in the wrong position or where formalin injection was unsatisfactory are demonstrated in Tables 1 and 2). A group receiving AM251 alone was not included because we have recently shown, using an identical experimental design and methodology, that intra- anterior cingulate cortex administration of this dose of AM251 alone has no effect on fear-conditioned analgesia or conditioned fear (Rea et al. 2018).

Table 1 Summary of experimental<<Query: AUTHOR: Please check and confirm that tables are presented correctly. Ans: Ok>> groups in Experiment 1

Group	Conditioning	Formalin *i.pl.*	Drug/Vehicle	n
1	FC	Formalin	100% DMSO	9
2	NFC	Formalin	100% DMSO	8
3	FC	Formalin	MJN110	10
4	NFC	Formalin	MJN110	9
5	FC	Formalin	MJN110 + AM251	11
6	NFC	Formalin	MJN110 + AM251	10

Abbreviations: FC, fear-conditioned; NFC, non-fear-conditioned; MJN110, (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate); AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide.

Table 2 Summary of experimental groups in Experiment 2

Group	Conditioning	Formalin *i.pl.*	Drug/Vehicle	n
1	FC	Formalin	100% DMSO	9
2	NFC	Formalin	100% DMSO	8
3	FC	Formalin	MJN110	8
4	NFC	Formalin	MJN110	8
5	FC	Formalin	AM630	10
6	NFC	Formalin	AM630	10
7	FC	Formalin	MJN110 + AM630	10
8	NFC	Formalin	MJN110 + AM630	8

Abbreviations: FC, fear-conditioned; NFC, non-fear-conditioned; MJN110, (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate); AM630, (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone, 6-iodopravadoline).

"Off-target" control experiments that employed identical methodology to the anterior cingulate cortex experiments were performed and targeted the infralimbic (AP + 2 mm relative to bregma, ML ± 1.5 mm relative to bregma, and at a 12° angle, DV – 3.6 mm from dura, toothbar set at −3 mm) and prelimbic (AP + 2.4 mm relative to bregma, ML ± 1.5 mm relative to bregma, and at a 12° angle and DV – 2.3 mm from dura, toothbar set at −3.0 mm) cortex regions. Composite pain score (CPS) data for these experiments are presented in Figures S1 and S2 (n = 8–11 per group).

2.5 Behavioural analysis

Ethovision XT 11.5 software package (Noldus, Wageningen, the Netherlands; RRID:SCR_000441) was used to analyse behaviour, allowing for continuous event recording over each 30-min trial. The behaviours assessed (by an experimenter blind to treatment) were fear behaviour scored as the duration of freezing (defined as the cessation of all visible movement except that necessary for breathing) and nociceptive behaviours (CPS). Nociceptive behaviours were measured using the weighted composite pain scoring technique (Watson, Sufka, & Coderre, 1997). Nociceptive behaviours can be divided into two types according to this method; the first is time spent elevating the formalin injected paw without contact with any other surface (Pain 1). The second is the spent holding, licking, biting, shaking, or flinching the formalin injected paw (Pain 2). The composite pain scoring equation is given as composite pain scoring [CPS = (Pain 1 + 2(Pain 2))/(total duration of analysis period)] (Watson et al., 1997). Distance moved was also measured using the animal tracking function in Ethovision and the number of faecal pellets excreted during the trials were also counted (defecation).

2.6 Histological verification of intracerebral injection sites

Microinjection sites were refined using two animals before the start of each experiment. Brain sections with fast-green 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.

For each rat in each experiment, frozen coronal brain sections were cut at 150 μm thickness on a cryostat at −20°C from the start of the medial prefrontal cortex (bregma 3.7 mm) through to the end (bregma −0.26 mm) with reference to the rat brain atlas (Paxinos & Watson, 1998). Sections were collected on superfrost glass slides and microinjection site positions were determined and noted on photocopied images from rat brain atlas.

2.7 Tissue isolation by Palkovits punch

After histological verification was carried out, tissue from the left and right anterior cingulate cortex (bregma 3.70 to 2.20 mm, 0.5-mm puncher, and bregma 1.70 to −0.26 mm, 2-mm puncher) was punched from frozen 150-μm coronal sections using cylindrical brain punchers (Harvard Apparatus, internal diameters 0.5 and 2 mm). Punched regions were kept frozen throughout the collection procedure. After collection punches were placed in labelled 1.5-ml microfuge tubes, tissue weight recorded and stored at −80°C.

2.8 Measurement of levels of 2-AG in the anterior cingulate cortex

Quantification of 2-AG levels in the anterior cingulate cortex tissue punches was carried out following a lipid extraction method described previously (Ford et al., 2011; Kerr et al., 2012; Olango et al., 2012; Rea et al., 2013; Rea, Olango, et al., 2014). This method was modified slightly to take into account low tissue weights and endocannabinoid levels. Punched tissue was homogenized for ~3 s using an ultrasonic homogeniser/sonicater (Mason, Dublin, Ireland) and placed immediately on ice in 200 µl of 100% acetonitrile containing a fixed amount of deuterated internal standard (50-ng d8-2-AG) and 75 µl of 100% pure acetonitrile (to ensure volume consistency between samples and standard; see below). Following homogenization, samples were centrifuged at 4°C for 15 min at 14,000× *g* (Hettich® centrifuge Mikro 22R, Hettich, Germany). Once the centrifugation step was complete, the supernatant was collected from each sample and placed in newly labelled 1.5-ml microfuge tubes.

A 10-point standard curve was prepared. Ten 1.5-ml microfuge tubes were labelled 10 to 1, and 75 µl of 100% acetonitrile were added to each. The highest standard (Standard 10) was then created by adding 25 µl of 100% acetonitrile containing known fixed amount of undeuterated internal standard (125-ng 2-AG). Samples were vortexed for 20 s, and 25 µl of this mixture was removed and placed in next microfuge tube in sequence (moving from highest: Standard 10 to lowest: Standard 1). This process of serial dilution was repeated for each of the standards until the lowest standard, Standard 1, was reached; 25 µl of mixture was also removed from Standard 1 and discarded to ensure all standards contain the same volume of liquid; 200 µl of 100% acetonitrile containing known fixed amount of deuterated internal standard (50-ng d8-2-AG) was added to each standard before vortexing each microfuge tube again.

LC–tandem mass spectrometry was then performed to allow for measurement of 2-AG; 8 µl of each sample was injected onto a Zorbex SB C18 column (Agilent Santa Clara, California, USA) having length, internal diameter, and particle size dimensions of 50 mm, 2.1 mm, and 1.8 µm, respectively, for chromatographic separation. Solution A (HPLC-grade water with 0.1% formic acid) and Solution B (100% acetonitrile with 0.1% formic acid) comprised the mobile phases which had a flow rate of 300 $\mu l \cdot min^{-1}$. A reversed phase gradient elution was used, comprising of 45% Solution B for the first minute, then linearly increased to 100% until 5 min into the run and maintained at 100% Solution B until the assay run finished at 12 min. A further 4.1 min was required to re-equilibrate the column at 45% Solution B before the next injection. Analyte detection was carried out in electrospray positive ionization mode on an Agilent 1200 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies, Cork, Ireland).

Ratiometric quantification was carried out using the Agilent MASSHUNTER Quantitative Analysis Software (Agilent Technologies, Cork, Ireland). This software allows for the creation of a standard curve from the peak area of the undeuterated standard against its corresponding deuterated internal standard. The amount of analyte in each unknown sample was then calculated from this standard curve of Relative response versus Relative concentration, that is, (Peak Area $analyte_{(undeuterated)}$/Peak area $analyte_{(deuterated)}$) versus (Conc $analyte_{(undeuterated)}$/Conc $analyte_{(deuterated)}$). The limit of quantification was 12.1 $pmol \cdot g^{-1}$ of 2-AG.

2.9 Quantitation of levels of mRNA encoding CB_1 and CB_2 receptors within the anterior cingulate cortex by RT-qPCR

Quantitative real-time PCR (qRT-PCR) was carried out as described previously (Burke, Kerr, Moriarty, Finn, & Roche, 2014; Kerr et al., 2012; Rea, Olango, et al., 2014).

Total RNA was extracted from the pellets collected from anterior cingulate cortex tissue (2 mg ± 0.3 mg) following processing for mass spectrometry (MS) using a Machery-Nagal extraction kit (Nucleospin RNA II; Technopath, Ireland) according to the manufacturer's instructions. RNA quality (1.8–2 260/280 ratio) and quantity was assessed using a Nanodrop spectrophotometer (ND-1000; Nanodrop, Labtech International, UK) and normalized to a concentration of 10 $ng \cdot \mu l^{-1}$. The RNA was reverse transcribed to cDNA using an Invitrogen Superscript III reverse transcriptase custom kit (Bio-Sciences, Dun Laoghaire, Ireland). Taqman gene expression assays (Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probe were used to quantify the genes of interest using an Applied Biosystems “stepOne plus”

instrument (Bio-Sciences, Dun Laoghaire, Ireland). The assay ID for the CNR1 (CB_1) gene was Rn00562880 and for CNR2 (CB_2), it was Rn03993699. VIC-labelled GAPDH (4308313) was used as the house-keeping gene. A no-template control (NTC) reaction was included in all assays.

2.10 Statistical analysis

Previous published studies and power analysis suggested that when using ANOVA, the sample sizes used would yield sufficient power to reliably detect differences in the means between groups with sufficient power (i.e., >90%). The IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA; RRID:SCR_002865) was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro–Wilk's and Levene's test respectively. All data except the duration of freezing and defecation were found to be parametric. Mass spectrometry data were analysed by two-way ANOVA, with fearconditioning and drug treatment as factors. RT-qPCR data were analysed by Student's unpaired, two-tailed t-test. Time course behavioural data were analysed by two-way repeated measures ANOVA with time as the within-subjects factor and fear conditioning and drug treatment as the between-subjects factors. Sphericity was tested using Mauchly's test for sphericity. If sphericity was violated, a Greenhouse–Geisser correction was used. Post hoc pairwise comparisons were made with Tukey's test when appropriate. For non-parametric data, Kruskal–Wallis was performed followed by Dunn–Bonferroni post hoc where appropriate. Data were considered significant when $P < .05$. Results are expressed as group means ± SEM or median with interquartile. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.11 Materials

Rat food (14% Harlan-Teklad-2014 Maintenance<<Query: AUTHOR: Bold emphases in the text have been deleted. Please check. Ans: Ok>> Diet, Harlan Laboratories, Belton, Loughborough, UK); Stainless steel guide cannulae (5 mm length, 22G, Plastics One Inc., Roanoke, VA, USA); Stainless steel stylets (Plastics One Inc., Roanoke, Virginia, USA); Rimadyl (Pfizer, Kent, UK); Baytril (Bayer Ltd., Dublin, Ireland); Formalin (37% formaldehyde solution; Sigma-Aldrich, Dublin, Ireland); DMSO (100%; Sigma-Aldrich, Dublin, Ireland); AM251 (Sigma-Aldrich, Dublin, Ireland); MJN110 (Sigma-Aldrich, Dublin, Ireland); AM630 (Sigma-Aldrich, Dublin, Ireland); Footshock grid (LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK); Hamilton microsyringes and Harvard PHD2000 infusion pump (Harvard Apparatus, Kent, UK); Ethovision XT 11.5 software package (Noldus, Wageningen, the Netherlands); Cylindrical brain punchers (Harvard Apparatus); Ultrasonic homogeniser/sonicater (Mason, Dublin, Ireland); Centrifuge (Hettich® centrifuge Mikro 22R, Hettich, Germany); Zorbex SB C18 column (Agilent Santa Clara, California, USA); Agilent 1200 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies, Cork, Ireland); Agilent MASSHUNTER Quantitative Analysis Software (Agilent Technologies, Cork, Ireland); Machery-Nagal extraction kit (Nucleospin RNA II; Technopath, Ireland); Nanodrop spectrophotometer (ND-1000; Nanodrop, Labtech International, UK); Invitrogen Superscript III reverse transcriptase custom kit (Bio-Sciences, Dun Laoghaire, Ireland); Taqman gene expression assays (Applied Biosystems, UK); Applied Biosystems "stepOne plus" instrument (Bio-Sciences, Dun Laoghaire, Ireland); IBM SPSS statistical software package (SPSS v23.0 for Microsoft Windows; Chicago, IL, USA).

2.12 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 RESULTS

3.1 Histological verification of microinjection sites

3.1.1 Experiments 1 and 2— anterior cingulate cortex

86% (experiment 1) or 89% (experiment 2) of the injections were within the borders of the anterior cingulate cortex (Figures 1 and 2) with the remaining 14% or 11%, respectively, were placed just above the anterior cingulate cortex in the motor cortex (M2) or lateral to the anterior cingulate cortex in the corpus callosum. The data analysed were derived only from rats in which bilateral injections were correctly positioned in the anterior cingulate cortex.

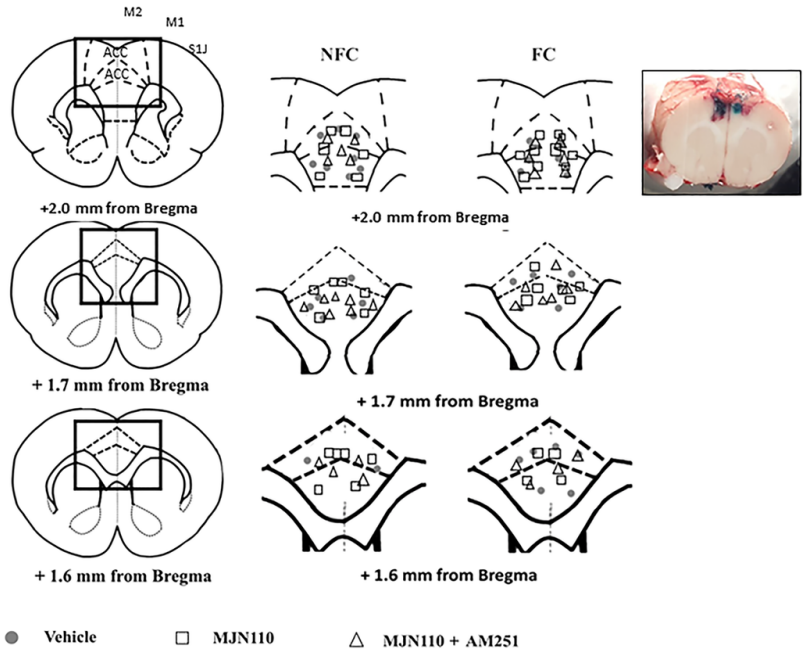

Figure 1 Schematic<<Query: AUTHOR: Please check and confirm if the set of figure captions and corresponding figure artworks are presented correctly. Ans: Ok>> depicting the sites of cannula implantation for the anterior cingulate cortex (ACC) in Experiment 1. Adapted from Paxinos and Watson (1998). NFC, non-fear-conditioned; FC, fear conditioned

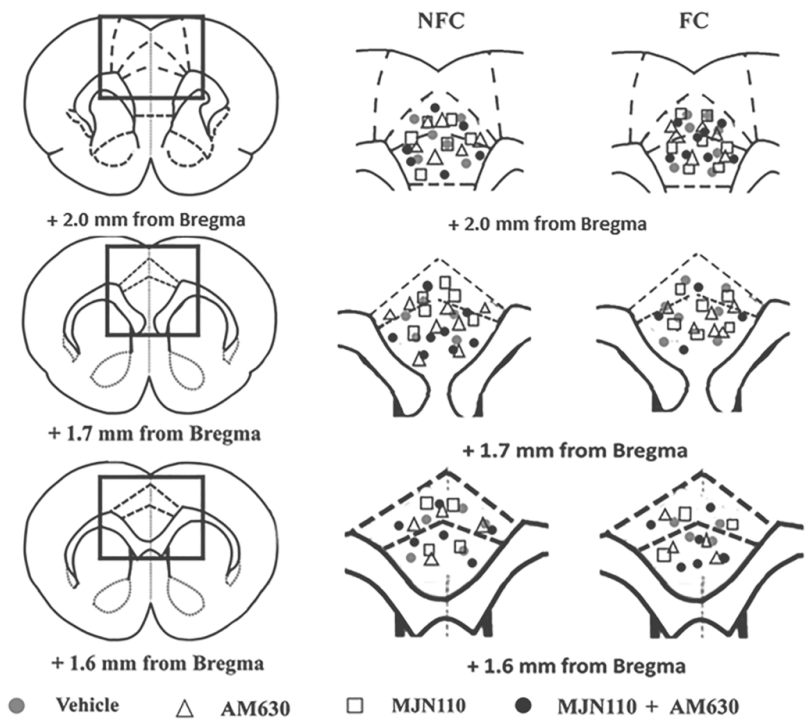

Figure 2 Schematic depicting the sites of cannula implantation for the anterior cingulate cortex (ACC) in experiment 2. Adapted from Paxinos and Watson (1998). NFC, non-fear-conditioned; FC, fear-conditioned

3.2 Intra-anterior cingulate cortex administration of an monoacylglycerol lipase inhibitor (MJN110) attenuates fear-conditioned analgesia via a CB_1 receptor-independent mechanism

Intra-plantar injection of formalin induced a robust nociceptive response in the form of elevation, licking, biting and shaking of the injected right hind paw.

Analysis of the formalin-evoked nociceptive data subdivided into 10-min time bins and using two-way repeated measures ANOVA revealed a significant main effect of fear conditioning, <<Query: Dear Authors, It has been agreed for some time that F values and P values for individual comparisons, especially in two-way ANOVA e.g. vs. time, vs treatment . vs sex etc should not be presented in the Results main text. Accordingly, I have removed them in this MS If you wish to have them in the MS, a Table containing these details has been allowed on previous occasions. This Table can be in the Supplementary files Ans: Ok as it is, no need for additional table, thank you.>>treatment and fear conditioning ∗ treatment on composite pain score. Mauchly's test of sphericity indicated that the assumption of sphericity had been violated and therefore, a Greenhouse–Geisser correction was used. There was a significant main effect of time, time ∗ fear conditioning and time ∗ fear conditioning ∗ treatment on composite pain score over the course of the testing period.

Further post hoc analysis revealed a significant reduction in formalin-evoked nociceptive behaviour in fear-conditioned VEH-treated rats for the entire 30-min testing period, confirming the expression of fear-conditioned analgesia throughout the trial. Intra-anterior cingulate cortex administration of MJN110 (monoacylglycerol lipase inhibitor) alone or in combination with AM251 (CB_1 antagonist/inverse agonist) significantly attenuated the expression of fear-conditioned analgesia in the final 20 min of the trial. MJN110 + AM251 also attenuated the expression of fear-conditioned analgesia in the first 10 min of the testing

period with similar trends in the first 10 min of the trial for MJN110 alone. There was no significant drug effect in non-fear-conditioned animals (Figure 3).

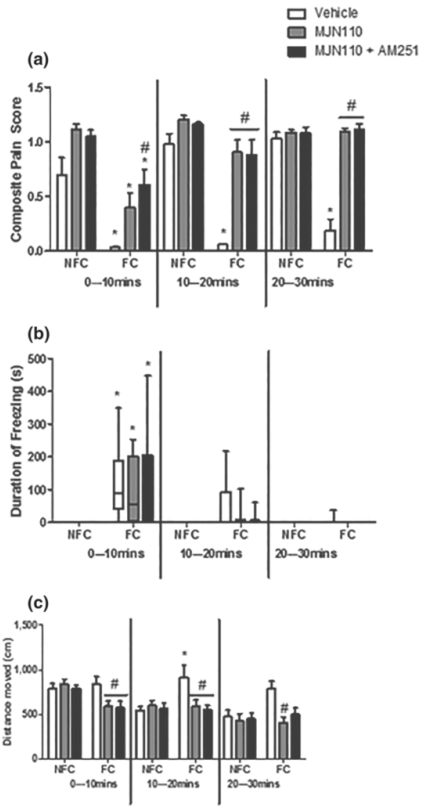

Figure 3 Effects of fear conditioning and bilateral administration of MJN110 and MJN110 + AM251 directly into the anterior cingulate cortex (ACC) on formalin-evoked nociceptive behaviour (a), the duration of freezing (b), and distance moved (c) in rats over the full 30-min testing period subdivided into 10-min time bins. $^{*}P < .05$, FC versus NFC; $^{\#}P < .05$ versus FC VEH (Tukey's). All data are expressed as mean ± SEM (n = 8–11 per group). NFC, non-fear-conditioned; FC, fear conditioned; VEH, vehicle

3.3 Intra-anterior cingulate cortex administration of an monoacylglycerol lipase inhibitor (MJN110) has no effect on expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

Analysis of the data subdivided into 10-min time bins using Kruskal–Wallis revealed a significant between-group effect on the duration of freezing at 0–10 min and 10–20 min, but not 20–30 min. Post hoc analysis using the Dunn–Bonferroni test revealed that fear-conditioned rats receiving VEH displayed significantly increased freezing behaviour in the first 10 min of the trial, compared with non-fear-conditioned VEH-treated counterparts. Neither MJN110 nor a combination of MJN110 + AM251 had any effect on the duration of freezing when microinjected into the anterior cingulate cortex (Figure 3).

3.4 Effect of fear conditioning on locomotor activity and defecation in formalin-treated rats and effects of pretreatment with <<Query: AUTHOR: I think this

makes the title clearer. please check Ans: OK>>MJN110 or MJN110 + AM251 on these changes

Analysis of the distance moved data subdivided into 10-min time bins and using two-way repeated measures ANOVA revealed a significant main effect of treatment and fear conditioning * treatment, but not fear conditioning. Mauchly's test of sphericity indicated that the assumption of sphericity had been violated and therefore, a Greenhouse–Geisser correction was used. There was a significant main effect of time and time * fear conditioning on distance moved over the course of the testing period.

Post hoc analysis revealed a significant increase in distance moved in fear-conditioned (FC) VEH-treated rats for the 10- to 20-min time bin (FC VEH vs. NFC (non-fear-conditioned) VEH, 10–20 min), an effect attenuated by MJN110 alone or in combination with AM251. Post hoc analysis also revealed a significant reduction in distance moved in fear-conditioned rats receiving MJN110 alone or in combination with AM251, for the first 10-min testing period, compared with VEH-treated counterparts (FC MJN110 or FC MJN110 + AM251 vs. FC VEH, 0–10 min); and fear-conditioned MJN110-treated rats showed significantly reduced distance moved during the final 10-min testing period compared with fear-conditioned VEH-treated rats (FC MJN110 vs. FC VEH, 20–30 min, Figure 3).

Kruskal–Wallis comparisons from k-independent samples revealed a significant effect on defecation (no. of faecal pellets excreted) over the 30-min testing period. Further post hoc analysis by Dunn–Bonferroni revealed that all fear-conditioned groups exhibited a significant increase in defecation over the course of the 30-min testing period compared to their non-fear-conditioned counterparts—data reported as [median, IQR] (FC VEH [5, 3] vs. NFC VEH [1, 1]); FC MJN110 [6, 3] vs. NFC MJN110 [2, 2]; FC MJN110 + AM251 [5, 3] vs. NFC MJN110 + AM251 [2, 2]). Treatment with MJN110 alone or in combination with AM251 had no effect on defecation in either fear-conditioned or non-fear-conditioned rats.

3.5 Elevation of 2-AG levels following microinjection of an monoacylglycerol lipase inhibitor (MJN110)

Two-way ANOVA revealed a significant effect of fear conditioning and treatment on the levels of 2-AG in the anterior cingulate cortex. Further post hoc analysis revealed a significant increase in the levels of 2-AG in the anterior cingulate cortex of MJN110-treated fear-conditioned rats, compared to fear-conditioned VEH-treated rats. In contrast, MJN110 had no significant effect on levels of 2-AG in the anterior cingulate cortex of non-fear-conditioned rats (Figure 4).

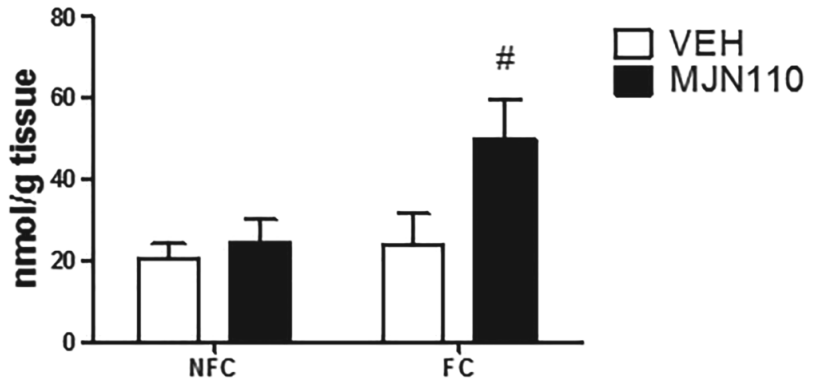

Figure 4 Effect of fear conditioning and MJN110 on the levels of 2-AG in the anterior cingulate cortex (ACC). $^{\#}P < .05$ versus FC VEH (Tukey's). All data are expressed as mean ± SEM (n = 20 rats per group). NFC, non-fear-conditioned; FC, fear-conditioned; VEH, vehicle

3.6 Attenuation of fear-conditioned analgesia by intra-anterior cingulate cortex administration of an monoacylglycerol lipase inhibitor (MJN110) is blocked by co-administration of a CB_2 antagonist (AM630)

Since we had shown that the attenuation of fear-conditioned analgesia by intra- anterior cingulate cortex administration of the monoacylglycerol lipase inhibitor was not blocked by a CB_1 antagonist, we next sought to determine the potential involvement of CB_2 receptors.

Intra-plantar injection of formalin induced a robust nociceptive response in the form of elevation, licking, biting and shaking of the injected right hind paw.

Analysis of the data subdivided into 10-min time bins and using two-way repeated measures ANOVA revealed a significant main effect of fear conditioning, treatment and fear conditioning ∗ treatment on CPS. Mauchly's test of sphericity indicated that the assumption of sphericity had been violated and therefore a Greenhouse–Geisser correction was used. There was a significant main effect of time, time ∗ fear conditioning, time ∗ treatment and time ∗ fear conditioning ∗ treatment.

Further post hoc analysis revealed a significant reduction in formalin-evoked nociceptive behaviour in fear-conditioned VEH-treated rats for the entire 30-min trial confirming the expression of fear-conditioned analgesia . Treatment with the monoacylglycerol lipase inhibitor MJN110 attenuated the expression of fear-conditioned analgesia for the entire 30-min testing period, an effect blocked by co-administration of the CB_2 antagonist AM630. Treatment with AM630 alone reduced formalin-evoked nociceptive behaviour in non-fear-conditioned (NFC) rats for the entire 30-min trial, an effect blocked by co-administration of MJN110 (Figure 5).

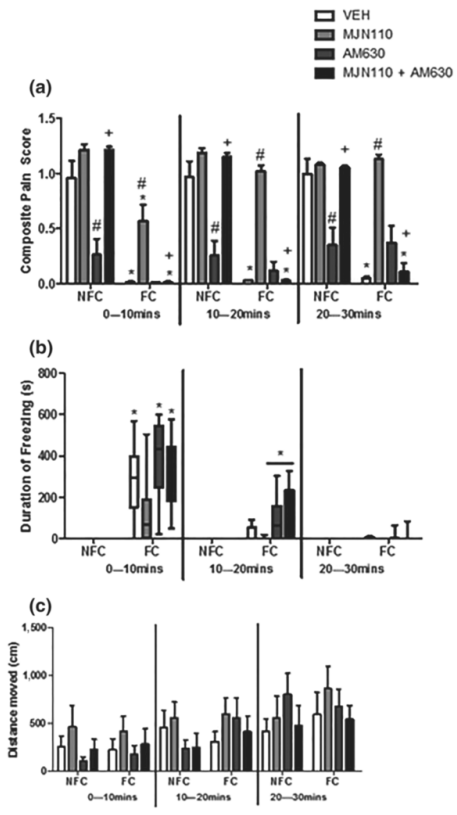

Figure 5 Effects of fear conditioning and bilateral administration of MJN110, AM630 or MJN110 + AM630 directly into the ACC on formalin-evoked nociceptive behaviour (a), the duration of

freezing (b), and distance moved (c) in rats over the full 30-min testing period subdivided into 10-min time bins. $^{*}P < .05$ FC versus NFC; $^{\#}P < .05$ versus VEH counterpart, $^{+}P < .05$ versus MJN110 (Tukey's). All data are expressed as mean ± SEM ($n = 8–10$ per group). NFC, non-fear-conditioned; FC, fear-conditioned; VEH, vehicle

3.7 No effects of intra-anterior cingulate cortex administration of an monoacylglycerol lipase inhibitor and/or CB_2 antagonist on expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone

Analysis of the data subdivided into 10-min time bins using Kruskal–Wallis revealed a significant between-group effect on the expression of freezing at 0–10 min, 10–20 min and 20–30 min. Post hoc analysis using the Dunn–Bonferroni test revealed that fear-conditioned rats receiving intra-anterior cingulate cortex VEH displayed significantly increased freezing behaviour in the first 10 min of the trial, compared with non-fear-conditioned VEH-treated counterparts. Fear-conditioned MJN110-treated rats did not display an increase in the duration of freezing. Rats that received intra-anterior cingulate cortex AM630 alone or in combination with MJN110 exhibited significant contextually induced freezing behaviour for a longer period of time (first 20 min) than VEH-treated fear-conditioned rats (first 10 min only; Figure 5).

3.8 Effect of fear conditioning, on locomotor activity and defecation in formalin-treated rats and effects of pretreatment with <<Query: AUTHOR: I think this makes the title clearer. please check Ans: Ok>>MJN110 or MJN110 + AM251 on these changes

Analysis of the distance moved data subdivided into 10-min time bins and using two-way repeated measures ANOVA revealed no main effect of fear conditioning, treatment or fear conditioning ∗ treatment . Mauchly's test of sphericity indicated that the assumption of sphericity had not been violated . There was a significant main effect of time on distance moved over the course of the testing period. Post hoc analysis revealed no significant difference in distance moved between groups during the 30-min trial period (Figure 5).

Kruskal–Wallis comparisons from k-independent samples revealed a significant effect on defecation (no. of faecal pellets excreted) over the 30-min testing period. Further post hoc analysis by Dunn–Bonferroni revealed that all fear-conditioned groups exhibited a significant increase in defecation over the course of the 30-min testing period compared to their non-fear-conditioned counterparts—data reported as [median, IQR] (FC VEH [6, 2] vs. NFC VEH [3, 3]); FC MJN110 [6, 4] vs. NFC MJN110 [3, 2]; FC AM630 [6, 2] vs. NFC AM630 [2, 1]; FC MJN110 + AM630 [5, 3] vs. NFC MJN110 + AM630 [2, 1]). Treatment with MJN110 alone or in combination with AM630 had no effect on defecation in either fear-conditioned or non-fear-conditioned rats.

3.9 Expression of CB_1 and CB_2 in the anterior cingulate cortex

Using RT-qPCR, we confirmed that mRNA encoding both CB_1 and CB_2 receptors was detectable within the anterior cingulate cortex. Student's unpaired *t*-tests <<Query: AUTHOR: Is this the test? Ans: Yes>>confirmed that fear conditioning had no effect on levels of CB_1 or CB_2 mRNA, measured at the end of the 30-min test trial (Figure 6).

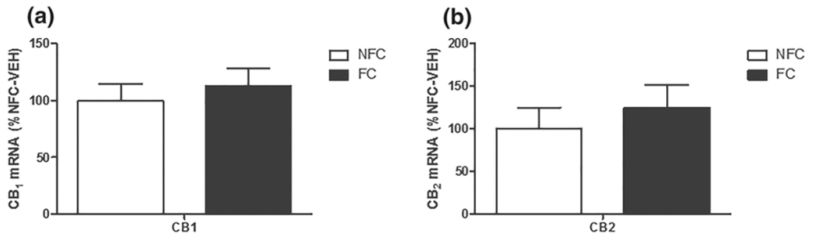

Figure 6 Levels of (a) CB_1 and (b) CB_2 mRNA in the anterior cingulate cortex (ACC) of vehicle (VEH)-treated fear-conditioned (FC) and non-fear-conditioned (NFC) rats that received intra-plantar injection of formalin. RT-qPCR on tissue harvested at the end of the 30-min behavioural trial. Data are expressed as mean ± SEM (n = 8–9 per group)

4 DISCUSSION

The data presented herein indicate for the first time that monoacylglycerol lipase inhibition in the anterior cingulate cortex modulates fear–pain interactions. Microinjection of the monoacylglycerol lipase inhibitor MJN110 into the anterior cingulate cortex attenuated the expression of fear-conditioned analgesia, an effect unopposed by co-administration of the CB_1 receptor antagonist AM251. The attenuation of fear-conditioned analgesia by intra-anterior cingulate cortex administration of MJN110 was associated with an increase in levels of 2-AG within the anterior cingulate cortex and was blocked upon co-administration of the CB_2 antagonist AM630. AM630 alone or in combination with MJN110 prolonged the expression of contextually induced freezing in fear-conditioned rats. In the absence of contextual fear conditioning, intra-AM630 alone decreased formalin-evoked nociceptive behaviour, an effect attenuated upon co-administration with MJN110. These findings suggest an important role for an monoacylglycerol lipase substrate, likely 2-AG, in the anterior cingulate cortex in the modulation of fear-conditioned analgesia. The data also suggest that in the anterior cingulate cortex and under these experimental conditions, fear-conditioned analgesia may be attenuated by 2-AG-CB_2 receptor signalling. Moreover, CB_2 receptors in this region may facilitate formalin-evoked nociceptive behaviour.

Monoacylglycerol lipase inhibition in the anterior cingulate cortex attenuated the expression of fear-conditioned analgesia, while having no effect on the expression of contextually induced freezing, locomotor activity (distance moved) or defecation. These findings together indicate that the expression of fear-conditioned analgesia specifically, and not locomotor activity, fear-related freezing or defecation, is suppressed by an monoacylglycerol lipase substrate in the anterior cingulate cortex and extend our previous work demonstrating that the medial prefrontal cortex is an important neural substrate for fatty acid amide hydrolase substrate- and/or CB_1 receptor-mediated regulation of fear-conditioned analgesia (Rea et al., 2019), alongside the ventral hippocampus (Ford et al., 2011), dorsolateral periaqueductal grey (Olango et al., 2012) and the basolateral amygdala (Rea et al., 2013; Roche et al., 2007). Moreover, these data are the first to suggest that the anterior cingulate cortex is an important neural substrate for fear-conditioned analgesia and its modulation by the endocannabinoid system.

The effect of MJN110 on fear-conditioned analgesia was not blocked by co-administration with the CB_1 antagonist AM251, suggesting that monoacylglycerol lipase inhibition in the anterior cingulate cortex modulates the expression of fear-conditioned analgesia via a non-CB_1 receptor target. Other non-CB_1 targets of monoacylglycerol lipase substrates/2-AG could include CB_2 (Gonsiorek et al., 2000; Sugiura et al., 2000), GABAA (Sigel et al., 2011), PPARs (Bouaboula et al., 2005), adenosine A3 receptors (Lane, Beukers, Mulder-Krieger, & Ijzerman, 2010), TRPV1 (Yusaku et al., 2008), and GPR55 (Ryberg et al., 2007). In our follow-up experiment, the attenuation of fear-conditioned analgesia by the monoacylglycerol lipase inhibitor was blocked by co-administration with the CB_2 antagonist AM630. This result, coupled with the MJN110-induced increase in 2-AG within the anterior cingulate cortex and demonstration that mRNA encoding CB_2 receptors was detectable in the anterior cingulate cortex, suggests that 2-AG may act via CB_2 within this sub-region

to attenuate fear-conditioned analgesia. We acknowledge that a limitation of our study is that it utilized only one CB_2 antagonist at one dose, and therefore caution is required in drawing strong conclusions with respect to potential role of CB_2. CB_2 receptor expression is often very low or undetectable in the brain of naïve animals. However, under the present experimental conditions of formalin injection, fear conditioning and intra-cerebral guide cannulae implantation, it may be up-regulated. Previous studies have found that unconditioned stress-induced analgesia is mediated by the mobilization of 2-AG and CB_1 receptor activation in the rat dorsal periaqueductal grey (Gregg et al., 2012; Hohmann et al., 2005). Our data here however, showing that 2-AG signalling in the anterior cingulate cortex attenuates rather than mediates fear-conditioned analgesia, provide evidence for brain regional specificity in both the direction of effect and the receptor mechanisms by which 2-AG modulates fear/stress-induced pain suppression. Fear-conditioned per se did not alter levels of 2-AG in the anterior cingulate cortex and this result is in line with those of Marsicano et al. (2002) who previously showed that fear conditioning was not associated with any change in 2-AG levels in the medial prefrontal cortex of mice. Moreover, the MJN110-induced attenuation of fear-conditioned analgesia suggests a pro-nociceptive or facilitatory role for 2-AG on pain-related behaviour, and so therefore, it is perhaps not surprising that fear-conditioned-formalin treated rats (which express very robust fear-conditioned analgesia) would not exhibit any increase in anterior cingulate cortex 2-AG levels (i.e. low levels of anterior cingulate cortex 2-AG are likely facilitating expression of fear-conditioned analgesia). Previous studies have demonstrated footshock (Hohmann et al., 2005; Olango et al., 2012) or fear conditioning (Marsicano et al., 2002) induced increases in endocannabinoid levels in regions such as the PAG and amygdala, where the role of the endocannabinoid system in fear-conditioned analgesia/stress-induced analgesia is the opposite to that observed here for the anterior cingulate cortex (i.e. in the amygdala and PAG, elevated levels of endocannabinoids facilitate and/or enhance fear-conditioned analgesia/stress-induced analgesia, rather than abolish it). Again, this points to the region-specific nature of the anterior cingulate cortex results reported herein and is an intriguing aspect of our study. The specificity of the observed effects to the anterior cingulate cortex is also suggested by the results of our off-target control experiments which revealed some differences in the temporal profile and magnitude of the effects of MJN110 (+/− AM251) on fear-conditioned analgesia following administration into either the infralimbic or prelimbic, compared with intra-anterior cingulate cortex administration.

Intra-anterior cingulate cortex administration of MJN110 alone or in combination with AM251 had no effect on the expression of contextually induced freezing behaviours in fear conditioned rats, despite attenuating fear-conditioned analgesia. These findings corroborate previous studies from our laboratory (Finn et al., 2004; Rea et al., 2013; Roche et al., 2007; Roche et al., 2010) and those of others (Kinscheck, Watkins, & Mayer, 1984; Helmstetter et al., 1987<<Query: AUTHOR: Reference “Helmstetter et al., 1987” has not been included in the Reference List; please supply full publication details. Ans: Please change the in-text citation to Helmstetter and Fanselow, 1987.

that reference has already been provided in the Reference List:

Helmstetter, F. J., & Fanselow, M. S. (1987). Effects of naltrexone on learning and performance of conditional fear-induced freezing and opioid analgesia. Physiology & Behavior, 39(4), 501–505. https://doi.org/10.1016/0031-9384(87)90380-5>>), demonstrating that fear-conditioned analgesia can be altered independently of the expression of fear-related freezing in the presence of nociceptive tone. Indeed, Rea<<Query: AUTHOR: “Rea et al. (2018)” is cited in text but not provided in the reference list. Please provide details in the list or delete the citation from the text. Ans: In-text citation should read Rea et al. (2019). Now changed.>> et al. (20189) found that either URB597 or AM251 alone or in combination had no effect on the expression of fear-related behaviour in the anterior cingulate cortex.

The data presented in Experiment 2 suggest that elevated levels of 2-AG arising from monoacylglycerol lipase inhibition may act via CB_2 in the anterior cingulate cortex to attenuate fear-conditioned analgesia. den Boon et al. have found that CB_2 receptors are expressed intracellularly and postsynaptically in the medial prefrontal cortex and decrease neuronal excitability (den Boon et al., 2012; den Boon et al., 2014). In the anterior cingulate cortex, it is possible that elevated levels of 2-AG arising from monoacylglycerol lipase inhibition act via postsynaptic CB_2 receptors to decrease neuronal excitability and attenuate fear-conditioned

analgesia. In line with this theory, a study by de Freitas et al. (2014) indicated a role for glutamatergic signalling in the prelimbic in mediating innate fear-induced antinociception. They found that administration of the NMDA receptor antagonist LY235959 directly into the prelimbic reduced innate fear-induced antinociception and panic-like behaviours elicited by $GABA_A$ receptor blockade in the medial hypothalamus while administration of the AMPA/kainite receptor antagonist NBQX directly into the prelimbic reduced only innate fear-induced antinociception (de Freitas et al., 2014). Another study demonstrated that pretreatment of the anterior cingulate cortex with the NMDA receptor antagonist LY235959 reduced fear-induced antinociception in a similar paradigm (Falconi-Sobrinho et al., 2017).

AM630 alone had no effect on the expression of fear-conditioned analgesia, but rats administered intra-anterior cingulate cortex AM630 expressed significant contextually induced freezing behaviour for a longer period of time than in VEH-treated counterparts, an effect unopposed by co-administration of MJN110, suggesting a possible role for CB_2 receptors independent of 2-AG levels in the anterior cingulate cortex in the termination of fear-related behaviours. It is possible that CB_2 in the anterior cingulate cortex is involved in short-term within-trial extinction of fear. However, to date, there are a paucity of studies investigating the role of CB_2 receptors in fear extinction (Ruehle, Rey, Remmers, & Lutz, 2012) and systemic administration of AM630 had no effect on freezing behaviour in mice exposed to cued fear-conditioning procedure (Li & Kim, 2016<<Query: AUTHOR: The citation “Li et al., 2016” has been changed to “Li & Kim, 2016” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary. Ans: Ok, thanks.>>). A prolongation of fear following CB_2 antagonism suggests a potential anxiolytic effect of CB_2 receptor signalling in the anterior cingulate cortex. Systemic administration of the CB_2 receptor agonist β-caryophyllene produced anxiolytic effects in the open field, elevated plus and the marble burying test in mice (Bahi et al., 2014). Moreover, systemic administration of AM630 attenuated the anxiolytic phenotype of mutant Fmr1-knockout mice (Busquets-Garcia et al., 2013). Systemic administration of the monoacylglycerol lipase inhibitor JZL184 produced anxiolytic effects in the elevated zero and plus maze tests, an effect abolished by pretreatment with either SR144528 or AM630, indicating an anxiolytic effect of 2-AG at CB_2 receptors (Busquets-Garcia et al., 2011). However, we found the effect of CB_2 receptor blockade to be unaltered by monoacylglycerol lipase inhibition. Similarly, increasing the levels of 2-AG via monoacylglycerol lipase inhibition alone did not produce an anxiolytic effect in our experiments. It should be noted that the studies described above looked mostly at innate anxiety-related behaviours in the absence of pain, while we looked at fear-related behaviours in the presence of pain and thus a direct comparison is difficult. Further studies are warranted to fully elucidate the role of CB_2 and 2-AG in the anterior cingulate cortex on fear responding in the absence of pain.

Intra-anterior cingulate cortex administration of AM630 decreased formalin-evoked nociceptive behaviour in non-fear-conditioned rats, an effect blocked by co-administration of MJN110, suggesting that CB_2 receptors in the anterior cingulate cortex may facilitate the expression of formalin-evoked nociceptive behaviour in rats, an effect dependent on an monoacylglycerol lipase substrate. The lack of effect of MJN110 on levels of 2-AG in non-fear-conditioned rats suggests either (a) levels of 2-AG were elevated at an earlier timepoint or (b) involvement of another monoacylglycerol lipase substrate. It is possible also that fear conditioned may in some way prime the anterior cingulate cortex such that it is more sensitive or responsive to the effects of monoacylglycerol lipase inhibition than the non-fear-condition. It is worth noting that peripheral and spinal activation of CB_2 receptors has been shown to be anti-nociceptive in various animal models of pain (Clayton, Marshall, Bountra, & O'Shaughnessy, 2002; Ibrahim et al., 2003; Malan et al., 2001; Nackley, Makriyannis, & Hohmann, 2003; Quartilho et al., 2003) and systemically administered AM630 has been shown to be pro-nociceptive (Wang, Wang, Xu, Ma, & Wang, 2015). However, few studies have investigated the role of CB_2 receptors supraspinally. Jhaveri et al. found that the CB_2 agonist JWH-133 injected directly into the central posterior nucleus of the thalamus reduced both non-noxious and noxious mechanically evoked responses of thalamic neurons in a rat model of neuropathic pain, an effect blocked by CB_2 antagonism. Alone, CB_2 antagonism increased the burst activity of noxious but not non-noxious neuronal activity in the thalamus of spinal nerve ligated rats (Jhaveri et al., 2008). However, differences in the brain region targeted and pain models used make it difficult to compare our results directly with those of Jhaveri et al. Our data suggest that CB_2 receptors in the anterior cingulate cortex may play a pro-nociceptive role, facilitating formalin-evoked nociceptive behaviour, accepting that the data are based on utilization of one CB_2 antagonist at one dose and

that confirmation with a different CB_2 antagonist or CB_2 receptor knockdown would be valuable. The precise expression and subcellular localization of CB_2 in subregions of the medial prefrontal cortex requires further investigation. den Boon et al. state that CB_2 is located postsynaptically and intracellularly in layer II/III of pyramidal cells of the rodent medial prefrontal cortex, acting to inhibit neuronal excitability (den Boon et al., 2012; den Boon et al., 2014). It is possible that postsynaptic CB_2 receptors in the medial prefrontal cortex act to reduce neuronal excitability and facilitate pain-related behaviour. It is also possible that microglial CB_2 receptors could be involved due to (a) up-regulation as a result of prior fear conditioning or formalin-induced pain and/or (b) local neuroinflammatory response due to guide cannula implantation in the anterior cingulate cortex.

In conclusion, the present study provides new evidence to support a role for an monoacylglycerol lipase substrate, likely 2-AG, in the anterior cingulate cortex, in the modulation of fear-conditioned analgesia. The data also suggest that fear-conditioned analgesia may be attenuated by 2-AG-CB_2 receptor signalling in the anterior cingulate cortex and that CB_2 receptors in the anterior cingulate cortex may facilitate formalin-evoked nociceptive behaviour and terminate fear-related behaviours. Elucidation of the role of the endocannabinoid system in pain–fear interactions may facilitate increased understanding of, and development of new therapeutic approaches for, pain- and fear-related disorders and their co-morbidity.

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AUTHOR CONTRIBUTIONS

L.C., M.R., and D.P.F. all contributed to study design and preparation of the manuscript. L.C. and D.M. collected the data. L.C. analysed the data. M.R. and D.P.F. supervised the work, and all authors contributed to data interpretation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organisations engaged with supporting research.

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