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Author(s)	Rea, Kieran; Olango, Weredeselam M.; Okine, Bright N.; Madasu, Manish K.; McGuire, Iseult C.; Coyle, Kathleen; Harhen, Brendan; Roche, Michelle; Finn, David P.
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Summary

Impaired endocannabinoid signalling in the rostroventromedial medulla underpins genotype-dependent hyper-responsivity to noxious stimuli

Q3 Impaired endocannabinoid signalling in the ~~rostroventromedial~~ medulla underpins hyper-responsivity to a noxious inflammatory stimulus in the Wistar–Kyoto rat, a genetic background prone to heightened stress/affect.

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Impaired endocannabinoid signalling in the ~~rostroventromedial~~ medulla underpins genotype-dependent hyper-responsivity to noxious stimuli

Kieran Rea [a,c,1], Weredeselam M. Olango [a,c,1], Bright N. Okine [a,c], Manish K. Madasu [a,c], Iseult C. McGuire [a,c], Kathleen Coyle [a,c], Brendan Harhen [c], Michelle Roche [b,c], David P. Finn [a,b,*]

[a] Pharmacology and Therapeutics, National University of Ireland, Galway, Ireland
[b] Physiology, School of Medicine, National University of Ireland, Galway, Ireland
[c] NCBES ~~Neuroscience Cluster~~ and Centre for Pain Research, National University of Ireland, Galway, Ireland

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ABSTRACT

Pain is both a sensory and an emotional experience, and is subject to modulation by a number of factors including genetic background modulating stress/affect. The Wistar–Kyoto (WKY) rat exhibits a stress-hyperresponsive and depressive-like phenotype and increased sensitivity to noxious stimuli, compared with other rat strains. Here, we show that this genotype-dependent hyperalgesia is associated with impaired pain-related mobilisation of endocannabinoids and transcription of their synthesising enzymes in the ~~rostroventromedial~~ medulla (RVM). Pharmacological blockade of the CB_1 receptor potentiates the hyperalgesia in WKY rats, whereas inhibition of the endocannabinoid catabolising enzyme, fatty acid amide hydrolase, attenuates the hyperalgesia. The latter effect is mediated by CB_1 receptors in the RVM. Together, these behavioural, neurochemical, and molecular data indicate that impaired endocannabinoid signalling in the RVM underpins hyper-responsivity to noxious stimuli in a genetic background prone to heightened stress/affect.

1. Introduction

The ability to perceive pain and respond appropriately is essential for survival. However, excessive or persistent pain constitutes a major healthcare problem for those who experience it, and for society and economies. Pain is both a sensory and an emotional experience and is subject to modulation by a number of factors. A key factor is the contribution of genetic background and its influence on stress responding and affective processing. An increased understanding of how such factors can influence pain is important from a fundamental physiological perspective, and may also aid the identification of new therapeutic targets for the treatment of persistent pain and its exacerbation by, and/or co-morbidity with, stress-related affective disorders.

The influence of genetic background and stress/affect on pain can be examined by comparing behavioural, neurochemical, and molecular responses to noxious stimuli across different rodent strains. The Wistar–Kyoto (WKY) inbred rat strain exhibits a stress-hyperresponsive and depressive-like phenotype [5,29,63,64] and displays increased sensitivity to visceral and somatic noxious stimuli, compared with other rat strains [5,31,33,71,85,90]. As such, the WKY rat represents a useful model with which to study the impact of genetic background and negative affect on pain processing.

The endogenous cannabinoid (endocannabinoid) system plays a key role in the modulation of both pain processing and emotionality [23,27,38,39,48,87,88]. This system comprises at least 2 receptors, the CB_1 [14,52] and CB_2 [58] receptors, of which the CB_1 receptor is most abundant in the brain. *N*-arachidonoylethanolamide (anandamide: AEA [15]) and 2-arachidonoylglycerol (2-AG [53,83]) are the 2 most extensively studied endogenous ligands for the cannabinoid receptors. AEA and 2-AG are synthesized from phospholipid precursors by *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) [17,45] and diacylglycerol lipase (DAGL) [75], respectively, and are catablolised primarily by fatty acid amide hydrolase (FAAH) [10,62,76] and monoacylglycerol lipase [MAGL] [30], respectively. Both endocannabinoids have similar affinity for both CB_1 [47] and CB_2 [32] receptors; however, 2-AG usually has higher efficacy than AEA at both receptors [32,47]. Work from our laboratory and others has demonstrated

* Corresponding author. Address: Pharmacology and Therapeutics, School of Medicine, University Road, National University of Ireland, Galway, Ireland. Tel.: +353 91 495280; fax: +353 91 495586.
E-mail address: david.finn@nuigalway.ie (D.P. Finn).
[1] K.R. and W.M.O. contributed equally to this work.

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an important role for these endocannabinoids in stress–pain interactions, regulating both stress-induced analgesia [6–8,28,60,67,69] and stress-induced hyperalgesia [40,77]. To date, however, the contribution of the endocannabinoid system to altered nociceptive responding in genetic backgrounds predisposed to negative affect has not been investigated. Research has shown that the endocannabinoid system of WKY rats is dysfunctional, with altered expression of endocannabinoid catabolising enzymes likely contributing to their depressive phenotype [86]. However, the extent to which alterations in the endocannabinoid system may explain altered nociceptive responding in WKY rats is unknown, and was the focus of the current studies.

We have used behavioural, neurochemical, and molecular approaches to test the hypothesis that enhanced pain-related behavioural responding to the noxious inflammatory stimulus of intra-plantar formalin injection in WKY rats is mediated by impaired mobilisation of endocannabinoid-CB_1 receptor signalling. Our studies focused on the role of the endocannabinoid system in the ~~rostroventromedial~~ medulla (RVM), given its key role in top–down descending modulation of pain [21,22,36,91], and evidence that CB_1 receptors in the RVM [37,51] regulate nociceptive processing [50,55,84].

2. Methods

2.1. Animals

For all experiments, male Sprague–Dawley (SD) and/or Wistar–Kyoto (WKY) rats (Harlan, UK) were used. Animals were singly housed, and holding rooms were maintained at a constant temperature (21 ± 2°C) under standard lighting conditions (12:12-hour light/dark, lights on from 0800 to 2000 h). Experiments were carried out during the light phase between 0800 and 1700 h. Food and water were available ad libitum. The experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP [www.iasp-pain.org/AM/Template.cfm?Section=Animal_Research]. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609.

2.2. Experimental design

Three separate experiments were performed. In all experiments, animals were randomly assigned to treatment groups, and the sequence of treatments and testing was also randomised to control for the order of testing. Experiment 1 investigated whether enhanced formalin-evoked nociceptive behaviour in WKY rats vs their SD counterparts was associated with alterations in endocannabinoid levels in the RVM or genes coding for the enzymes and receptors of the endocannabinoid system. A total of 24 male Sprague-Dawley rats and 24 male WKY rats (285–320 g) received an intra-plantar injection of 50 μL formalin (2.5% in 0.9% saline, s.c.) or 0.9% saline (control group) into the right hindpaw immediately after a 10-minute habituation exposure to the formalin test arena. This design resulted in 4 experimental groups, as follows: SD-Saline (SD-Sal); SD-Formalin (SD-Form); WKY-Saline (WKY-Sal); and WKY-Formalin (WKY-Form) (n = 10–12 per group). At the peak of the second phase of the formalin test (30 minutes after formalin injection), rats were killed by decapitation. Brains were removed rapidly and were snap-frozen on dry ice and stored at −80°C before microdissection of the RVM and subsequent analysis of AEA and 2-AG levels using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Microdissected RVM tissue was also analysed by quantitative reverse transcription–polymerase chain reaction (RT-PCR) for expression of genes coding for the CB_1 receptor and for the endocannabinoid-related enzymes NAPE-PLD, DAGLα, FAAH, and MAGL. A separate cohort of rats (n = 6 per group) were treated exactly as described above to generate RVM tissue for western blot analysis of CB_1 receptor expression.

In experiment 2, we investigated the effects of pharmacological blockade of the CB_1 receptor or inhibition of FAAH on formalin-evoked nociceptive behaviour in WKY and SD rats. A total of 32 male Sprague–Dawley rats and 32 male Wistar–Kyoto rats (250–350 g) were assessed in the formalin test, with subjects receiving intraperitoneal (i.p.) injection of the CB_1 receptor antagonist/inverse agonist AM251 (3 mg/kg), the FAAH inhibitor URB597 (0.5 mg/kg) or vehicle (ethanol:cremaphor:saline vehicle in a ratio of 1:1:18; 3 mL/kg) before intra-plantar formalin injection. Rats were habituated to the formalin test arena for 10 minutes before formalin injection. URB597 and AM251 were administered 60 minutes and 30 minutes before formalin injection, respectively, based on previous studies in our laboratory and others demonstrating their in vivo efficacy at these doses and times of administration [1,7,8,34,35,41,44,49]. To control for the different times of injection of the 2 drugs, half of the vehicle-treated control rats received vehicle at 30 minutes and half at 60 minutes before intra-plantar formalin injection. These 2 vehicle-treated cohorts were subsequently combined as 1 group after statistical analysis confirmed that there were no differences between them for any of the experimental parameters examined. This design resulted in 6 experimental groups (n = 6–10 per group): SD-Vehicle [SD-Veh]; SD-AM251 (3 mg/kg) [SD-AM251]; SD-URB597 (0.5 mg/kg) [SD-URB]; WKY-Vehicle [WKY-Veh]; WKY-AM251 (3 mg/kg) [WKY-AM251]; and WKY-URB597 (0.5 mg/kg) [WKY-URB]. At the end of the formalin test (ie, 70 minutes after formalin injection), the rats were killed by decapitation.

Experiment 3 was conducted to investigate whether URB597-mediated suppression of formalin-induced hyperalgesia (result from experiment 2) in the WKY rats is mediated by AEA-induced activation of CB_1 receptors in the RVM. Male ~~Wistar–Kyoto~~ rats (280–350 g; Harlan, UK) were implanted with stainless steel guide cannulae targeting the RVM. On the test day, URB597 (0.5 mg/kg) or vehicle was administered by i.p. injection 60 minutes before formalin injection. Fifteen minutes before formalin injection, 0.3 μL of AM251 (1 μg/0.3 μL) or dimethylsulfoxide vehicle (DMSO, 100%) was microinjected over 1 minute through an injection needle that protruded 1 mm beyond the tip of the pre-implanted guide cannula, with the aid of a Hamilton microsyringe attached to polyethylene tubing and a Harvard PHD2000 infusion pump (Harvard Apparatus, Kent, UK) as described previously [28,60,67,69,72,73]. The injection needle was left in place for 1 minute more after infusion to allow for drug diffusion away from the injector tip. This dose of AM251 was chosen on the basis of previous studies demonstrating that microinjection of AM251 into different regions of the brain modulated behavioural responses to analgesic compounds in various animal models of pain [18,12,20]. After microinjection of AM251 or DMSO vehicle directly into the RVM, animals were immediately placed in a Perspex arena to habituate for 10 minutes. Animals were subsequently injected with formalin under brief anaesthesia and returned to the formalin test arena for behavioural analysis. They were killed by decapitation at 70 minutes after formalin administration. A 0.3-μL quantity of 1% fast green dye was microinjected via the guide cannula, and brains were rapidly removed, snap-frozen on dry ice, and stored at −80°C before injection site verification. Microdissection of the RVM was performed in conjunction with injection site verification, and the microdissected tissue was analysed for endocannabinoid levels using LC-MS/MS.

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2.3. Drug preparation

The FAAH inhibitor URB597 [(3-(3-carbomoylphenyl)phenyl)N-cyclohexylcarbamate)] and formalin were purchased from Sigma Aldrich (Dublin, Ireland). The CB_1 receptor antagonist/inverse agonist AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide was purchased from Abcam (Cambridge, UK). URB597 (0.5 mg/kg) or vehicle was administered by i.p. injection 60 minutes before formalin injection, whereas AM251 (3.0 mg/kg) was administered by i.p. injection 30 minutes before formalin injection. Both drugs were reconstituted as an emulsion in ethanol:cremaphor:saline vehicle in a ratio of 1:1:18 and administered at an injection volume of 3 mL/kg. For intra-RVM microinjections, AM251 was prepared to a concentration of 1 μg per 0.3 μL of DMSO vehicle (dimethylsulfoxide, 100%) and 0.3 μL was microinjected with the aid of a Hamilton microsyringe as described above.

2.4. Formalin test

Rats were placed in a Perspex observation chamber (30 × 30 × 40 cm; LxWxH) at 30 lux for a 10-minute habituation period, after which time they received an intra-plantar injection of 50 μL formalin (2.5% in 0.9% saline) or 0.9% saline into the right hindpaw under brief isoflurane anaesthesia as described previously [7,8,24–26,28,67,69,72,73]. Rats were returned to their home cage for another 3 minutes, at which point they were returned to the same Perspex observation chamber to which they had previously been exposed. A video camera located beneath the observation chamber was used to record animal behaviour onto DVD for subsequent analysis. Behaviour was analysed with the aid of EthoVision XT7 software by a rater blinded to treatments. Formalin-evoked nociceptive behaviour was 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 composite pain score [CPS = (C1 + 2(C2))/(total duration of analysis period)] according to the method of Watson et al. [89].

2.5. Punch microdissection of RVM tissue

In experiments 1 and 3, frozen coronal brain sections (300-μm in thickness) containing the RVM were cut on a cryostat (MICROM, Germany). A series of 300-μm-thick sections (from AP −9.16 to −11.6 mm relative to bregma) were punched using cylindrical brain punchers (Harvard Apparatus; internal diameter 2 mm), with the aid of the rat brain atlas of Paxinos and Watson [66]. Punched RVM samples encompassed the gigantocellular reticularis nucleus, raphe magnus nucleus, medial lemniscus, raphe pallidus nucleus, pyramidal tracts, ventral aspect of the pontine reticular nucleus, and trigeminothalamic tract. These samples were weighed and stored at −80°C before extraction for determination of the concentrations of the endocannabinoids by LC-MS/MS, gene expression analysis using quantitative RT-PCR or protein expression using western blotting.

2.6. Quantitative RT-PCR analysis of the expression of endocannabinoid-related genes

Total RNA was extracted from post-mortem tissue using a Machery–Nagel extraction kit (Nucleospin RNA II; Technopath, Ireland) according to the manufacturer's instructions and as previously described [42,43]. RNA quality (1.8-2 260/280 ratio) and quantity was assessed using a Nanodrop spectrophotometer (ND-1000; Nanodrop, Labtech International, UK) and normalised to a concentration of 5 ng/μL. A 50-ng quantity of RNA from each sample 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 gene of interest using an Applied Biosystems 'stepOne plus' instrument (Bio-Sciences, Dun Laoghaire, Ireland). Assay IDs for the genes examined were as follows for rat *CB_1* (Rn00562880_m1), *FAAH* (Rn00577086_m1), *MAGL* (Rn00593297_m1), *NAPE-PLD* (Rn01786262_m1), and *DAGLα* (Rn01454304_m1). VIC-labelled *GAPDH* (4308313) was used as the house-keeping gene and endogenous control. A no-template control (NTC) reaction was included in all assays. The relative expression of target genes to endogenous control was calculated using the formula 2-ΔCt, where ΔCt represents the magnitude of the difference between cycle threshold (Ct) values of the target and endogenous control, and the result was expressed as a percentage of the mean value of the control group.

2.7. Western blot analysis of CB_1 receptor expression in the RVM

Western blotting was performed according to methods described previously [59], with minor modifications. Briefly, frozen punches of the RVM weighing approximately 10 mg were lysed by brief (3-second) sonication in radio-immunoprecipitation assay (RIPA) lysis buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCl, pH 7.6, 0.5% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mmol/L Na_3VO_4, 10 mmol/L NaF containing 1% protease inhibitor cocktail [Sigma-Aldrich, Dublin, Ireland]) at a ratio of 1:10 (w/v) in a 1.5-mL microcentrifuge tube. Homogenate was placed on a shaker for 45 minutes at 4° with gentle agitation to allow for complete dissociation of nucleo-protein complexes and then centrifuged at 13,200 rpm (Eppendorf Centrifuge 5415R Stevenage, UK) for 20 minutes at 4°C. The supernatant was collected and protein content determined by Bradford assay [4]. A 36-μg quantity of protein sample in loading buffer (4X sample loading buffer: 25% v/v 1 mol/L Tris HCl, pH 6.8, 5% w/v sodium dodecyl sulfate (SDS), 20% v/v glycerol, 2.5% Bromophenol blue (0.2% w/v in 100% ethanol), and 20% v/v of 2-mercaptoethanol, made up to a total volume of 20 mL in distilled water), was boiled at 100°C for 5 minutes, briefly centrifuged, and subjected to 9% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 120 mV for 2 hours. The separated protein samples were electroblotted onto a nitrocellulose membrane (Nitrocellulose membrane, CAS# 9004-70-0; Bio-Rad, Dublin, Ireland) at 100 mV for 1 hour. Protein transfer efficiency was verified by ponceau (0.1% ponceau dye in 5% acetic acid) staining. Membranes were blocked in 5% non-fat dry milk in 0.05% Tris-buffered saline/Tween 20 (TBST) solution for 1 hour at room temperature and incubated with rabbit polyclonal antibody to the CB_1 receptor (C-term) (1:200, catalog no. 10006590; Cayman Chemical, MI) and mouse monoclonal antibody to β-Actin (1:10,000, A5441; Sigma-Aldrich, Dublin, Ireland) diluted in 5% milk/0.05% TBST overnight at 4°C. Membranes were subjected to 3 10-minute washes in 0.05% TBST and incubated with secondary antibody solution containing IRDye conjugated goat anti-rabbit (λ800) and goat anti-mouse (λ700) (LI-COR Biosciences Abingdon Park, Oxford, UK) diluted 1:10,000 in 1% milk/0.05% TBST for 1 hour. Three 5-minute washing steps were then performed with washing solution and 1 final 5-minute wash in distilled H_2O. Blots were scanned on a LI-COR Odyssey imager. IR band intensities for glycosylated (~62-kDa), and non-glycosylated (~53-kDa) CB_1 receptor protein expression [19] and β-actin (~42-kDa) for each sample were generated automatically using the background subtraction method of the LI-COR Image Studio Ver. 2.0 imaging software. The ratio of CB_1 receptor intensity to β-actin intensity was then calculated for each sample, and expressed as a percentage of mean SD saline values.

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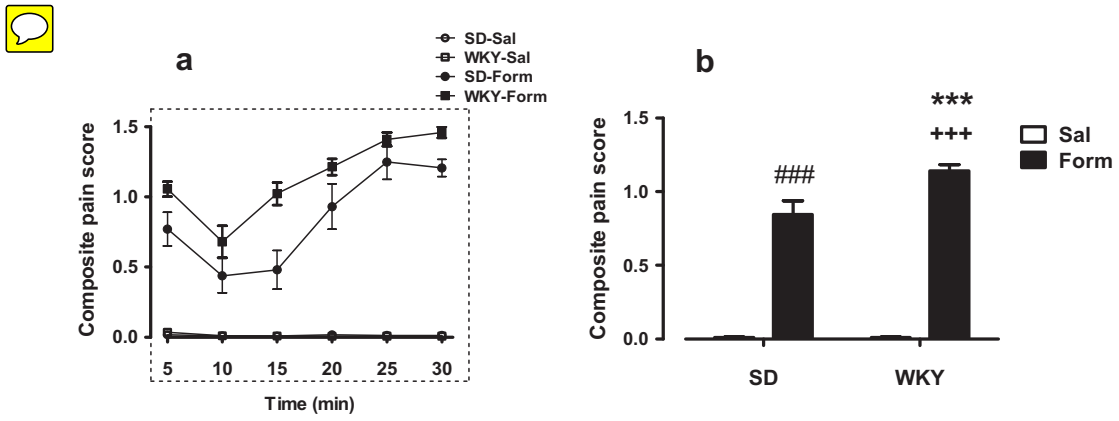

Fig. 1. WKY rats display a greater nociceptive response to intra-plantar formalin administration compared with their SD counterparts over the total duration of the 30-minute trial. (a) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats. (b) Graphic representation of formalin-evoked nociceptive behaviour in SD and WKY rats over entire 30-minute trial. Two-way ANOVA (effects of strain: $F_{1,45} = 10.436$, $P = .002$; formalin: $F_{1,45} = 462.314$, $P < .001$ and strain × formalin interaction: $F_{1,45} = 10.347$, $P = .002$) followed by Fisher's LSD post-hoc test ($^{###}P < .001$ vs SD-Sal, $^{+++}P < .001$ vs WKY-Sal, $^{***}P < 0.001$ vs SD-Form). Data are expressed as mean ± SEM (n = 10–12 rats per group). Form, formalin; Sal, saline solution; SD, Sprague–Dawley; WKY, Wistar–Kyoto.

2.8. Quantitation of endocannabinoids in RVM tissue using LC-MS/MS

Quantitation of endocannabinoids was essentially as described previously [7,28,42,43,60,68]. In brief, each sample was first homogenized in 400 µL 100% acetonitrile containing known fixed amounts of deuterated internal standards (0.014 nmol AEA-d8 and 0.48 nmol 2-AG-d8). Homogenates were centrifuged at 14,000*g* for 15 minutes at 4°C, and the supernatant was collected and evaporated to dryness. Lyophilized samples were resuspended in 40 µL 65% acetonitrile, and a 2-µL quantity was injected onto a Zorbax C18 column (150 × 0.5 mm internal diameter) from a cooled autosampler maintained at 4°C (Agilent Technologies, Cork, Ireland). Mobile phases consisted of A (high-performance liquid chromatography [HPLC]–grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 12 µL/min. Reverse-phase gradient elution began initially at 65% B and over 10 min was ramped linearly up to 100% B. At 10 minutes, the gradient was held at 100% B up to 20 minutes. At 20.1 minutes, the gradient returned to initial conditions for another 10 minutes to re-equilibrate the column. Analyte detection was carried out in electrospray-positive ionization mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies, Cork, Ireland). Quantitation of each analyte was performed using MassHunter Quantitative Analysis Software (Agilent Technologies, Cork, Ireland). The limit of quantification was 1.32 pmol/g and 12.1 pmol/g, for AEA and 2-AG, respectively.

2.9. Stereotactic implantation of Guide Cannulae into the RVM

For experiment 3, stainless steel guide cannulae (Plastics One Inc., Roanoke, VA, USA) were stereotactically implanted 1 mm above the RVM (AP, 1.10 cm, ML ± 0.00 cm relative to bregma; DV, 0.83 cm from dura) [66] under isoflurane anaesthesia (2%–3% in O_2; 0.5 L/min). 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, VA) was inserted into the guide cannulae to prevent blockage by debris. The non-steroidal anti-inflammatory agent carprofen (5 mg/kg s.c.) (Rimadyl; Pfizer, Kent, UK), and the broad-spectrum antibiotic enrofloxacin (2.5 mg/kg s.c.) (Baytril; Bayer, Dublin, Ireland), were administered before surgery to manage post-operative pain and to prevent infection, respectively. After cannulae implantation, the rats were housed singly and administered enrofloxacin (2.5 mg/kg s.c.) for another 3 days. Rats were allowed to recover for at least 6 days before experimentation. During this period, the rats were handled and their body weight and general health monitored on a daily basis.

2.10. Histological verification of microinjection sites

For experiment 3, the sites of intra-cerebral microinjection were determined before data analysis. Brain sections with fast-green dye mark were collected on a cryostat (30-µm thickness), mounted on gelatinised glass slides, and counterstained with

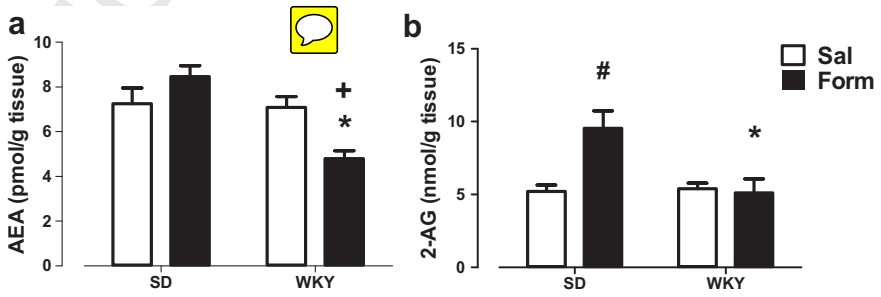

Fig. 2. AEA (a) and 2-AG (b) levels in the RVM after intra-plantar saline or formalin administration in SD and WKY rats. Two-way analysis of variance for AEA (strain × formalin interaction: $F_{1,19} = 5.149$, $P = .037$) and 2-AG (strain × formalin interaction: $F_{1,23} = 7.148$, $P = .015$) followed by Fisher's least squares difference post-hoc test ($^{\#}P = .023$ vs SD-Sal, $^{*}P = .047$ vs WKY-Sal, $^{*}P = .016$ and 0.023 vs SD-Form for AEA and 2-AG respectively). Data are expressed as mean ± SEM (n = 5 or 6). AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; Form, formalin; Sal, saline solution; SD, Sprague–Dawley; WKY, Wistar–Kyoto.

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cresyl violet to locate the precise position of microinjection sites under light microscopy.

2.11. Data analysis

The SPSS statistical package (IBM SPSS v20.0 for Windows; SPSS, Inc., Chicago, IL) was used to analyse all data. All data passed normality testing (Shapiro–Wilk test). The time course of formalin-evoked nociceptive behaviour is presented in 5-minute bins for each study. Further analysis of data collapsed over extended periods of the formalin trials or analysis of mRNA, neurochemical, or protein expression data was carried out using 2-way analysis of variance (ANOVA) followed by Fisher's least squares difference (LSD) post hoc test where appropriate. Two-tailed unpaired Student *t* tests were performed to analyse neurochemical data from experiment 3. Data were considered significant at $P < .05$. Results are expressed as group mean ± standard error of the mean (SEM).

3. Results

3.1. Formalin-evoked nociceptive responding is increased in WKY compared with SD counterparts

In Experiment 1, intra-plantar injection of formalin produced robust licking, biting, shaking, flinching and elevation of the injected right hindpaw of both WKY and SD rats, expressed as the composite pain score (CPS). Formalin-treated rats of both strains displayed significantly greater nociceptive behaviour compared with saline-treated controls, in which composite pain scores were negligible (Fig. 1a SD/WKY-FORM vs SD/WKY-SAL, $P < .001$). However, WKY rats exhibited significantly greater formalin-evoked nociceptive behaviour compared with SD rats (Fig. 1b WKY-FORM vs SD-FORM, $P < .001$) throughout the 30-minute trial.

3.2. WKY rats exhibit a deficit in formalin-evoked mobilisation of AEA and 2-AG in the RVM

Data from mass spectrometry analysis of punch-dissected RVM tissue are presented in Fig 2.

Intra-plantar formalin administration to WKY rats was associated with decreased levels of AEA, with no change in 2-AG levels (Fig. 2a WKY-Sal vs WKY-Form, $P < .05$). In contrast, intra-plantar formalin administration to SD rats had no significant effect on AEA levels, but was associated with increased levels of 2-AG in the RVM (Fig. 2b SD-Sal vs SDForm, $P < .05$). Formalin-treated WKY rats had significantly lower levels of AEA and 2-AG when compared with SD counterparts (Fig. 2 SD-Form vs WKY-Form, $P < .05$).

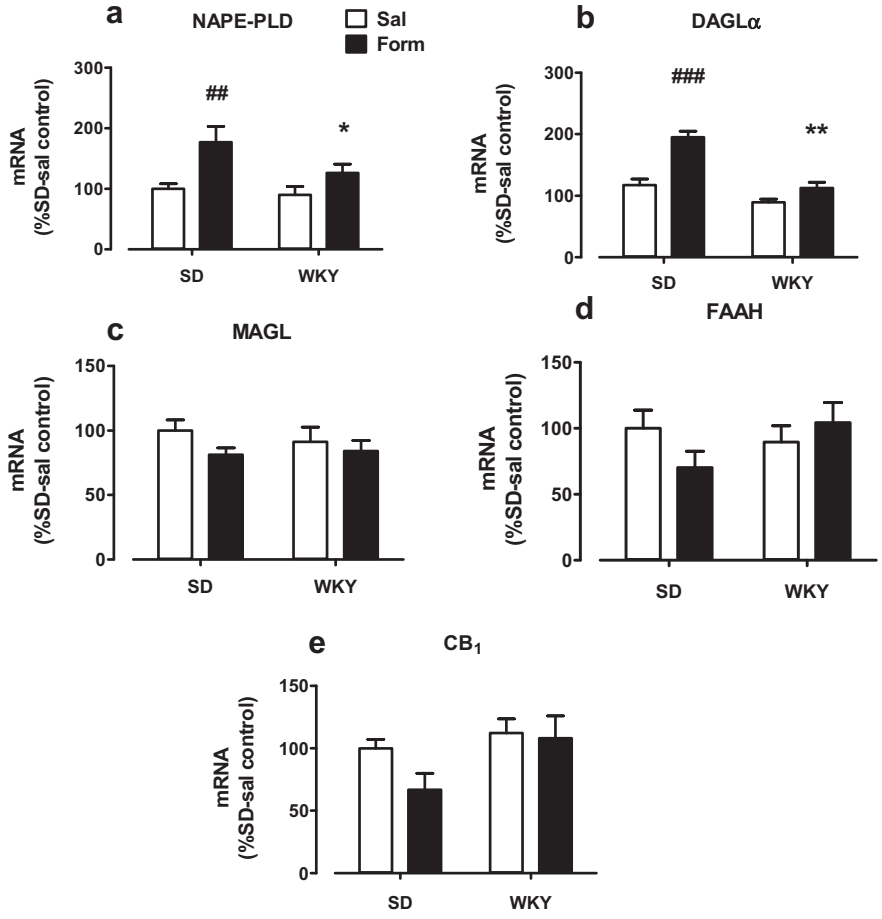

Fig. 3. Intra-plantar formalin administration is associated with increased expression of NAPE-PLD and DAGLα mRNA in the RVM of SD, but not WKY, rats. Two-way analysis of variance for NAPE-PLD (formalin: $F_{1,20} = 9.040$, $P = .008$) and DAGL-a (formalin: $F_{1,20} = 18.968$, $P < .001$, strain: $F_{1,20} = 8.357$, $P = .034$ and strain × formalin interaction: $F_{(1,20)} = 8.357$, $P = .010$) mRNA levels followed by Fisher's least squares difference post-hoc test ($^{\#\#}P = .003$, $^{\#\#\#}P < 0.001$ SD-Sal vs SD-Form; $^{*}P = 0.04$, $^{**}P = 0.002$ SD-Form vs WKY-Form). All data are expressed as mean percentage of SD-saline control levels as mean ± SEM (n = 5 or 6). WKY, Wistar-Kyoto; DAGLα, diacylglycerol lipase α; FAAH, fatty acid amide hydrolase; Form, formalin; MAGL, monoacylglygerol lipase; NAPE-PLD, *N*-arachidonoyl-phosphatidylethanolamine phospholipase-D; Sal, saline solution; SD, Sprague-Dawley.

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3.3. Intra-plantar formalin administration is associated with increased levels of NAPE-PLD and DAGLα mRNA in the RVM of SD, but not WKY, rats

There were no significant differences in levels of mRNA coding for the enzymes responsible for the synthesis (NAPE-PLD or DAGLα) or catabolism (FAAH or MAGL) of AEA or 2- AG, or in levels of mRNA or protein for the CB_1 receptor, between saline-treated SD and WKY rats (Figs. 3 and 4, SD-Sal vs WKY-Sal). Intra-plantar formalin administration had no effect on levels of mRNA for MAGL, FAAH, or CB_1 receptor in either strain or on CB_1 receptor protein expression (Figs. 3 and 4, SD/WKY-Sal vs SD/WKY-Form). However, intra-plantar formalin administration was associated with increased levels of mRNA for NAPE-PLD (Fig. 3a, SD-Form vs SD-Sal, $P = .01$) and DAGLα (Fig. 3b, SD-Form vs SD-Sal, $P < .001$) in SD rats but not in WKY counterparts.

3.3.1. Increased formalin-evoked nociceptive responding in WKY rats is subject to modulation by pharmacological manipulation of the endocannabinoid system

In experiment 2, we repeated the finding of increased formalin-evoked nociceptive responding in WKY rats compared with SD rats, evident here over the first 40 minutes after formalin administration (Fig. 5b and c WKY-Veh vs SD-Veh, $P < .001$). We focussed our efforts on pharmacological modulation of AEA rather than 2-AG because we observed a formalin-evoked reduction in the former, but not the latter, in the RVM of WKY rats. Systemic administration of the FAAH inhibitor URB597 (0.5 mg/kg i.p.), 60 minutes before formalin, significantly attenuated nociceptive behaviour in WKY rats over the first 40 minutes of the trial (Fig. 5b and c WKY-Veh vs WKY-URB597, $P < .001$), whereas it had no effect in their SD counterparts (Fig. 5a and c SD-Veh vs SD-URB597). Conversely, systemic administration of the CB_1 receptor antagonist/inverse agonist AM251 (3 mg/kg), 30 minutes before formalin injection, significantly potentiated nociceptive responding in WKY rats 35–70 minutes after formalin administration (Fig. 5b and d, WKY-Veh vs WKY-AM251, $P < 0.05$), while having no effect on formalin-evoked nociceptive behaviour in SD rats (Fig. 5a and d, SD-Veh vs SD-AM251).

3.4. Increased formalin-evoked nociceptive responding in WKY rats is subject to modulation by the endocannabinoid system in the RVM

Experiment 3 was carried out to evaluate whether the URB597-induced suppression of formalin-evoked hyperalgesia in the WKY rats, as observed in experiment 2, is mediated by AEA-induced activation of CB_1 receptors in the RVM. The reduction in formalin-evoked nociceptive behaviour after systemic administration of URB597 (0.5 mg/kg i.p.) to WKYrats that we observed in the first 35 minutes of the formalin trial in experiment 2 was apparent over the first 15 minutes in experiment 3 (Fig. 6a and b VEH-DMSO vs URB597-DMSO, $P < .05$), and was prevented by microinjection of the CB_1 receptor antagonist AM251 (1.0 μg/0.3 μL) directly into the RVM (Fig. 6a and b, URB597-DMSO vs URB597-AM251, $P < .05$). In all, 80% of the intracerebral microinjections were placed within the borders of the RVM, with the remaining injections positioned proximal to, but outside the borders of, this region (Fig. 8). Only the results of experiments in which microinjections were accurately positioned within the borders of the RVM were included in the analyses.

3.5. Pharmacological effect of URB597 administration on AEA and 2-AG levels in the RVM of saline- or formalin-treated SD and WKY rats

Measurement of endocannabinoid levels in the RVM of WKY rats at the end of experiment 3revealed that URB597 significantly increased levels of AEA, but not 2-AG, in the RVM of WKY rats (Fig. 7 WKY-Veh vs WKY-URB597; t8 = 2.464, $P < .05$ for AEA and t8 = 0.980, $P = 0.36$ for 2-AG).

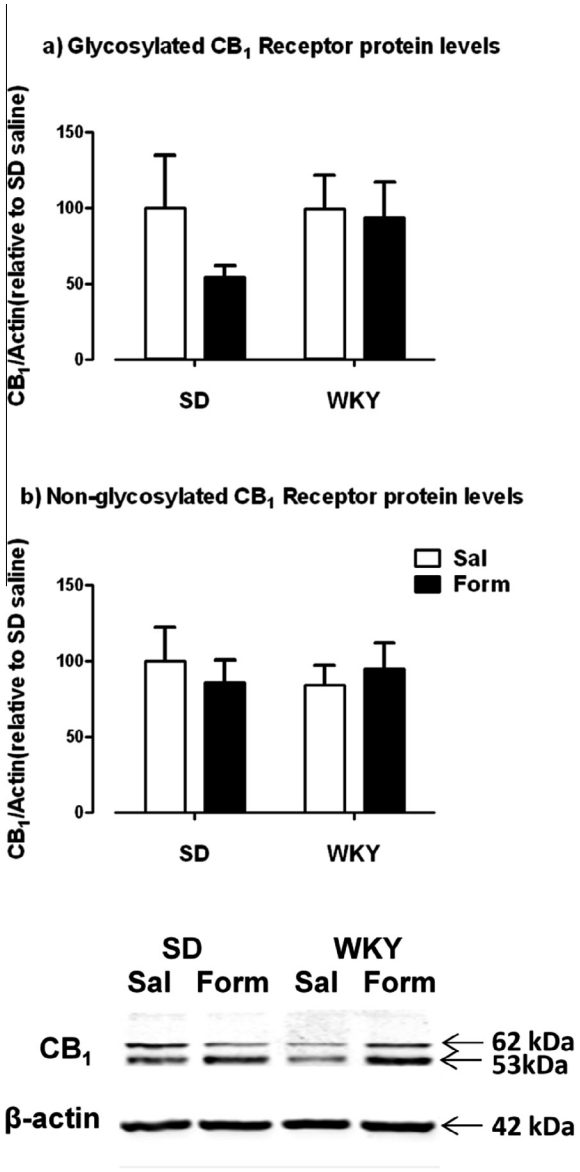

Fig. 4. Expression of (a) glycosylated (~62 kDa) or (b) non-glycosylated (~53 kDa) CB_1 receptor in RVM tissue from saline- or formalin-injected SD and WKY rats. Two-way ANOVA revealed no significant effect of strain, formalin administration or their interaction. Data are expressed as mean percentage of SD-saline control ± SEM (n = 6). Form, formalin; RVM, rostroventromedial medulla; SD, Sal, saline solution; Sprague–Dawley; WKY, Wistar–Kyoto.

4. Discussion

The data presented here suggest that impaired endocannabinoid-CB_1 receptor signalling in the RVM underpins the hyperalgesic response of stress-hyperresponsive WKY rats to intra-plantar formalin injection, compared with stress-normoresponsive SD rats. The magnitude of formalin-evoked nociceptive behaviour in WKY rats was greater than in SD rats, and this hyperalgesic phenotype was attenuated by inhibition of AEA catabolism and exacerbated by CB_1 receptor blockade. Moreover, the increased formalin-evoked nociceptive response in WKY rats was associated with lower tissue levels of AEA and 2-AG, and blunted formalin-evoked

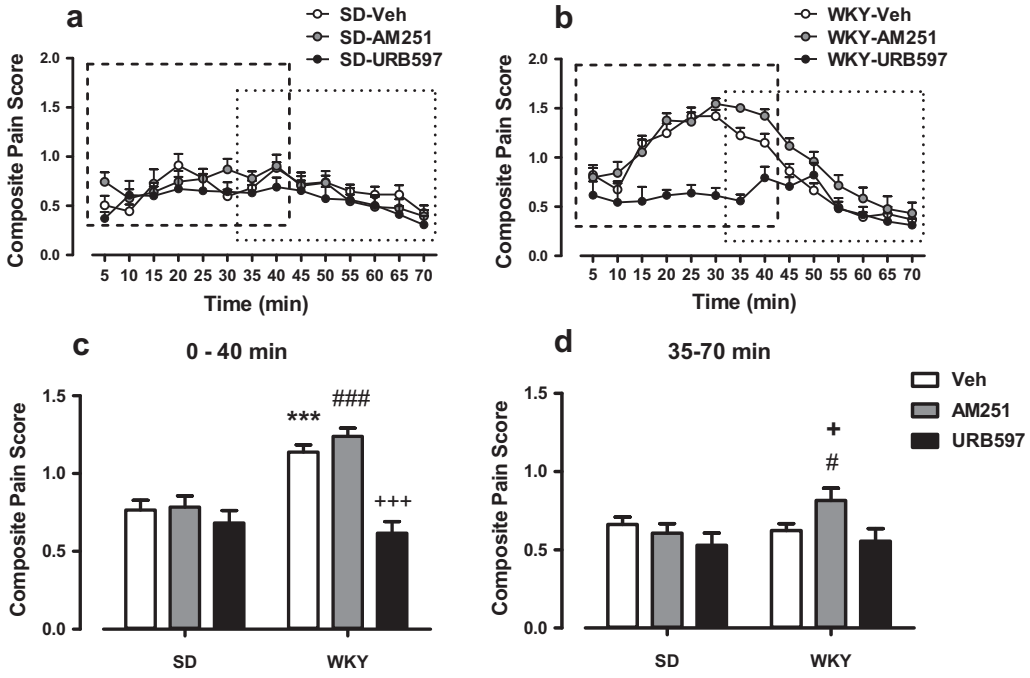

Fig. 5. AM251 (3 mg/kg i.p.) potentiates, whereas URB597 (0.5 mg/kg i.p.) attenuates, the enhanced nociceptive response of WKY rats to formalin administration. (a and b) Temporal profile of formalin-evoked nociceptive behaviour in SD and WKY rats after AM251 and URB597 administration. (c and d) Graphic representations of collapsed data analysed over periods indicated by boxed areas in panels a and b. Two-way analysis of variance (a) 0 to 40 minutes after formalin administration (strain: $F_{1,62} = 14.650$, $P < 0.001$; drug: $F_{2,62} = 16.899$, $P < .001$ and strain × drug interaction: $F_{2,62} = 8.685$, $P = .001$) and (b) 35 to 70 minutes after formalin (drug: $F_{2,62} = 3.324$, $P = .043$), followed by Fisher's least squares difference post-hoc test ($^{***}P < .001$ vs SD-Veh, $^{+}P = .039$, $^{+++}P < .001$ vs WKY-Veh and $^{\#}P = .032$, $^{\#\#\#}P < .001$ vs SD-AM251). Data are expressed as mean ± SEM (n = 10–12); SD, Sprague–Dawley; Veh, vehicle; WKY, Wistar–Kyoto;.

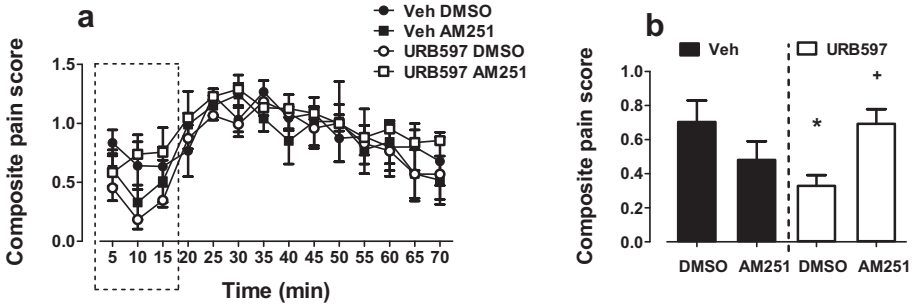

Fig. 6. Microinjection of AM251 (1.0 μg/0.3 μL) directly into the RVM of WKY rats prevented the systemic URB597 (0.5 mg/kg i.p.)–induced reduction in formalin-evoked nociceptive behaviour over the first 15 minutes of the 70-minute trial. (a) Temporal profile of formalin-evoked nociceptive behaviour in cannulated WKY rats. (b) Graphic representations of collapsed data analysed over the period indicated by the boxed area in panel a. Two-way analysis of variance (systemic drug × intra-RVM drug interaction: $F_{1,23} = 9.214$, $P = .007$) followed by Fisher's LSD post-hoc test ($^{*}P = .012$ vs VEH-DMSO and $^{+}P = 0.014$ vs URB597-DMSO). Data are expressed as mean ± SEM (n = 5–8).

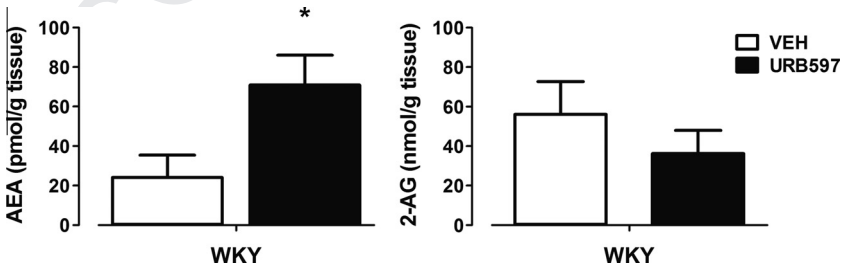

Fig. 7. Systemic URB597 (0.5 mg/kg i.p.) administration significantly increases AEA levels in the RVM of formalin-treated WKY rats receiving DMSO microinjection into the RVM. Two-tailed *t* test for AEA ($t_8 = 2.464$, $P = .039$) and 2-AG ($t_8 = 0.980$, $P = .356$) levels. Data are expressed as mean ± SEM (n = 5). AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; WKY, Wistar–Kyoto; DMSO, dimethyl sulfoxide; RVM, rostroventromedial medulla.

induction of NAPE-PLD and DAGLα mRNA, in the RVM, compared with those in SD controls. Furthermore, pharmacological blockade of CB_1 receptors in the RVM attenuated the antinociceptive effect of the FAAH inhibitor URB597 in WKY rats.

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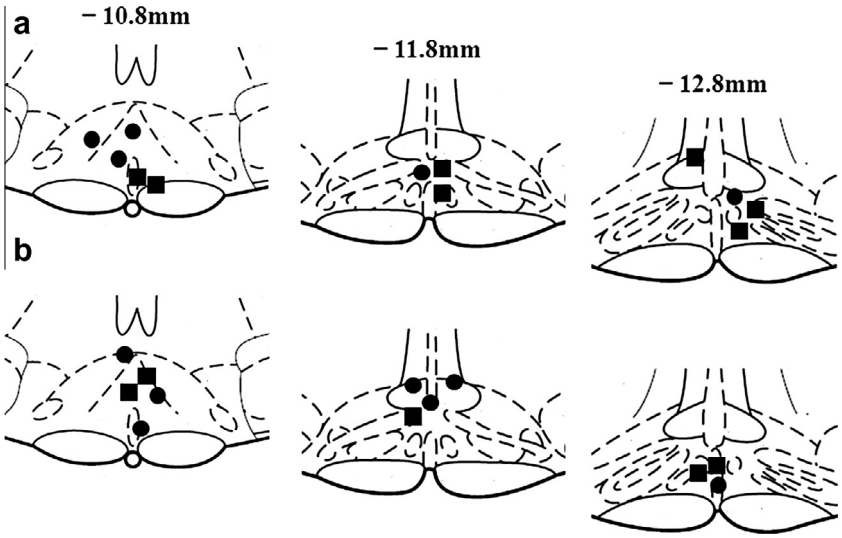

Fig. 8. Diagrammatic representation of the confirmed sites of microinjection of dimethyl sulfoxide (DMSO) (●) or AM251 (■) into the ~~rostroventromedial~~ ~~medulla~~ of Wistar–Kyoto rats receiving systemic administration of (a) vehicle or (b) URB597. Distances are relative to bregma. (Based on Paxinos and Watson [66]).

In this study, we replicated our earlier finding of increased nociceptive behaviour in WKY rats as compared to the SD comparator strain over the first 35 minutes of the formalin test [5]. We sought to extend these findings by investigating potential neurochemical and receptor mechanisms underlying the hyperalgesic phenotype, with a focus on the endocannabinoid system in the RVM given its key role in top–down descending modulation of pain [21,22,91] and evidence that CB_1 receptors in the RVM regulate nociceptive processing [36,50,55,84]. Levels of AEA and 2-AG, mRNA coding for catabolizing or synthesizing enzymes or CB_1 receptor mRNA or protein expression were similar in the RVM of SD and WKY rats receiving an intra-plantar injection of saline, suggesting that the resting endocannabinoid tone in the RVM is similar between the 2 strains. Formalin-evoked nociceptive behaviour was associated with increased levels of 2-AG in SD rats, and decreased AEA in the RVM of WKY rats. When compared with formalin-treated SD counterparts, RVM tissue levels of both AEA and 2-AG were lower in formalin-treated WKY rats. In addition, formalin-evoked nociceptive behaviour in SD rats was associated with increased RVM tissue levels of mRNA coding for NAPE-PLD and DAGL-α, the enzymes responsible for the synthesis of AEA and 2-AG, respectively. No such formalin-evoked increases were observed in WKY rats. Formalin injection had no effects on mRNA levels for the endocannabinoid catabolizing enzymes or on CB_1 receptor mRNA or protein expression in the RVM of either strain. These data suggest differential formalin-evoked recruitment of the endocannabinoid system in the RVM in 2 rat strains differing in their responsivity to an inflammatory noxious stimulus. Overall, our findings indicate impaired mobilization of endocannabinoids, and suppressed mRNA expression of genes coding for enzymes that synthesise the endocannabinoids, in the RVM of WKY rats in response to intra-plantar formalin injection. These alterations suggest reduced endocannabinoid tone in the RVM of WKY rats in response to a noxious insult, and we hypothesised that these alterations may play an important role in the hyperalgesic phenotype of WKY rats.

Subsequent pharmacological manipulation studies confirmed a role for the endocannabinoid system in the hyperalgesic phenotype expressed in WKY rats. We focussed our efforts on pharmacological modulation of AEA rather than 2-AG because we observed a formalin-evoked reduction in the former, but not the latter, in the RVM of WKY rats. Systemic administration of the FAAH inhibitor URB597 reduced, whereas the CB_1 receptor antagonist AM251 potentiated, formalin-evoked nociceptive behaviour in WKY rats. In contrast, neither drug had any effect on formalin-evoked nociceptive behaviour in SD rats at the doses administered here. We propose that a deficit in formalin-evoked endocannabinoid tone in the CNS of WKY rats may explain the efficacy of these 2 endocannabinoid system modulators in WKY, but not SD, rats; that is, against a background of reduced endocannabinoid tone in discrete brain regions such as the RVM, as indicated from our reported tissue levels of AEA and 2-AG from experiment 1, pharmacological inhibition of FAAH and blockade of CB_1 may be more effective in modulating pain-related behaviour in WKY rats than in the SD strain, in which endocannabinoid tone was greater. Thus, in WKY rats, FAAH inhibition would be expected to restore endocannabinoid tone and reduce hyperalgesia, whereas CB_1 receptor blockade would further exacerbate the deficit in endocannabinoid tone, thereby potentiating hyperalgesia, as was observed. Our study is the first to investigate the effects of these endocannabinoid system modulators on nociceptive behaviour in WKY rats. Furthermore, our data support earlier findings in which URB597 [34] and AM251 [1,9] failed to alter formalin-evoked nociceptive responses in SD rats. In comparison, the CB_1 receptor antagonist rimonabant has been reported to modestly increase formalin-evoked nociceptive behaviour in SD rats when administered 5 minutes after formalin [82].

Further direct evidence for a role of the endocannabinoid system in the RVM in the modulation of pain is provided by drug microinjection and electrophysiological studies. Microinjection of cannabinoid compounds directly into the RVM have been shown to modulate neuronal firing in cells of the RVM under conditions of acute [50,54,55,57] and persistent [57] inflammatory pain, as determined by in vivo electrophysiological studies. The RVM has a dense population of serotonergic [3,11,46,61,78], GABAergic [2,56], and glutamatergic neurons [81]; however, the expression of CB_1 receptors on GABAergic, glutamatergic, or serotonergic neurons in the RVM has yet to be confirmed anatomically (for review, see Rea et al. [70]). Moreover, to our knowledge, no studies to date have investigated serotonergic, GABAergic, or glutamatergic tone in the RVM of WKY vs SD rats. As such, the precise neurochemical mechanisms by which endocannabinoids in the RVM influence hyperalgesia in the WKY rat requires further scrutiny and should be the subject of future studies. In an animal model of unconditioned

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stress-induced analgesia (SIA) involving exposure to footshock with subsequent assessment of rat tail-flick responses, microinjection of the CB_1 receptor antagonist rimonabant into the RVM suppressed SIA, whereas intra-RVM administration of URB597 enhanced SIA [84]. In the present study, we used site-specific drug microinjection methodology to further investigate the role of CB_1 receptors in the RVM in mediating the antinociceptive effects of systemically administered URB597 in WKY rats, and investigated changes in endocannabinoid levels associated with URB597 administration. In RVM-cannulated WKY rats, systemic administration of URB597 reduced formalin-evoked nociceptive behaviour, albeit with an onset and duration of action that were earlier and shorter, respectively, than was observed in the WKY rats in experiment 2 that were not cannulated and did not receive intra-RVM DMSO. The suppression of formalin-evoked nociceptive behaviour in WKY rats by the FAAH inhibitor URB597 was associated with increased levels of AEA, but not 2-AG, in the RVM. Intra-RVM administration of AM251 prevented the URB597-induced reduction in formalin-evoked nociceptive behaviour, whereas it had no effect on nociceptive behaviour when administered alone. Taken with the data discussed above, these results together strongly suggest that the URB597-mediated reduction in formalin-evoked nociceptive behaviour in WKY rats is mediated through the activation of CB_1 receptors in the RVM by elevated levels of AEA arising from FAAH inhibition. In addition to activating CB_1 receptors, AEA is also an agonist at the transient receptor potential vanilloid subtype 1 (TRPV1) channel [13,16,74,79], the expression of which has been reported in the RVM [80]. However, to our knowledge, there are no studies investigating the effects of administration of TRPV1 agonists or antagonists directly into the RVM on nociceptive behaviour, and so it is uncertain whether TRPV1 in the RVM plays a direct role in the regulation of nociception. However, given that the antinociceptive effects of systemically administered URB597 were blocked completely by intra-RVM administration of the selective CB_1 receptor antagonist AM251 in the present study, and given that AM251 does not have any activity directly at TRPV1 [65], it seems very likely that the antinociceptive effects of URB597 in the present study are mediated exclusively by CB_1 receptors, without any direct involvement of TRPV1. Although we cannot definitively rule out the possibility that blockade of the CB_1 receptor activates non-CB_1 targets (eg, GPR55) or shunts AEA towards activation of other receptors in the RVM, it also seems unlikely that such mechanisms can explain the effects observed herein, because (1) intra-RVM administration of AM251 alone had no effect on formalin-evoked nociceptive behaviour, and (2) URB597 (and the resulting AEA elevation) had no effect on formalin-evoked nociceptive behaviour in animals receiving intra-RVM AM251. Overall, these data support the contention that hyperalgesia in the WKY rats is mediated, at least in part, via a deficit in endocannabinoid-CB_1 tone in the RVM.

4.1. Conclusions

In conclusion, our data provide evidence for an altered nociceptive response in a genetic strain predisposed to negative affect. The results indicate a compromised endocannabinoid system in the RVM of WKY rats compared with SD controls, a key neuroanatomical brain region involved in descending pain modulation. Dysfunction of the endocannabinoid system in this top–down control system may be maladaptive, contributing to exacerbated responsivity to noxious stimuli. Pharmacological normalisation of this endocannabinoid system dysfunction attenuated this genotype-dependent hyperalgesia in rodents and may represent a useful and novel therapeutic approach for the treatment of patients with pain that is exacerbated by negative affect or co-morbid with stress-related psychiatric disorders.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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