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Characterisation of peroxisome proliferator-activated receptor signalling in the midbrain periaqueductal grey of rats genetically prone to heightened stress, negative affect and hyperalgesia

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Abstract

The stress-hyperresponsive Wistar-Kyoto (WKY) rat strain exhibits a hyperalgesic phenotype and is a useful genetic model for studying stress-pain interactions. Peroxisome proliferator-activated receptor (PPAR) signalling in the midbrain periaqueductal grey (PAG) modulates pain. This study characterised PPAR signalling in the PAG of WKY rats exposed to the formalin test of inflammatory pain, versus Sprague-Dawley (SD) controls.

Formalin injection reduced levels of the endogenous PPAR ligands *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA) in the lateral(l) PAG of SD rats, but not WKY rats which exhibited higher levels of these analytes compared with formalin-injected SD counterparts. Levels of mRNA coding for fatty acid amide hydrolase (FAAH; catabolises PEA and OEA) were lower in the lPAG of WKY versus SD rats. PPARγ mRNA and protein levels in the lPAG were higher in saline-treated WKY rats, with PPARγ protein levels reduced by formalin treatment in WKY rats only. In the dorsolateral(dl) or ventrolateral(vl) PAG, there were no effects of formalin injection on PEA or OEA levels but there were some differences in levels of these analytes between saline-treated WKY and SD rats and some formalin-evoked alterations in levels of PPARα, PPARγ or FAAH mRNA in WKY and/or SD rats. Pharmacological blockade of PPARγ in the lPAG enhanced formalin-evoked nociceptive behaviour in WKY, but not SD, rats.

These data indicate differences in the PPAR signalling system in the PAG of WKY versus SD rats and suggest that enhanced PEA/OEA-mediated tone at PPARγ in the lPAG may represent an adaptive mechanism to lower hyperalgesia in WKY rats.

Keywords

Wistar-Kyoto (WKY); peroxisome proliferator-activated receptor (PPAR); descending pain pathway; formalin; *N*-palmitoylethanolamide (PEA); *N*-oleoylethanolamide (OEA)

1 Introduction

The Wistar-Kyoto (WKY) rat is a stress-hyperresponsive strain that exhibits an anxiogenic and depressive phenotype [37; 42] and increased sensitivity to visceral [28], thermal and inflammatory [7; 35; 43] noxious stimuli, compared with other commonly used rat strains. This behavioural phenotype makes the WKY rat a suitable genetic model for studying interactions between stress/affect and pain, the clinical manifestations of which represent a major challenge [6; 19; 30]. Although the mechanisms underpinning hyperalgesia in WKY rats remain poorly understood, it is reasonable to suggest that molecular and/or neurochemical alterations within endogenous pain modulatory systems, in particular within the midbrain periaqueductal grey (PAG), a key component of the descending pain pathway [3], may play a role.

The PAG is comprised of 3 columns, namely the dorsolateral(dl), lateral(l) and ventrolateral(vl) columns, and is widely recognised as an orchestrator of descending pain pathway activation, subject to the modulatory influences of endogenous bioactive lipids including *N*-palmitoylethanolamide (PEA), an endogenous agonist of the peroxisome proliferator-activated receptor (PPAR) signalling system. Intra-PAG administration of PEA increases response latency of rats in the tail-flick test, and reduces ongoing activity of ON and OFF cells in the RVM [13]. Thus, in parallel with dysfunctional endocannabinoid signalling in the rostral ventromedial medulla (RVM), a key component of the descending pain pathway, which we have previously reported in WKY rats [34], alterations in the PPAR signalling system, in particular within the PAG, could also influence or underlie increased pain responding in this rat strain. We therefore hypothesised that differences in mobilisation of endogenous components of PPAR signalling within the PAG may contribute to increased pain responding in WKY rats, compared with SD counterparts.

The PPAR signalling system consists of a family of ligand-dependent nuclear transcription factors, PPARα, β, and γ, widely expressed in the peripheral and central nervous systems [5], and their endogenous ligands including, PEA [22] and the related N-acylethanolamine *N*-oleoylethanolamide (OEA) [17]. Evidence from pharmacological and genetic studies has demonstrated a role for both PPARα and PPARγ in peripheral and central nociceptive processing [10; 13; 26; 27; 29; 39]. However, compared with their role in nociception in the periphery, the role of supraspinal PPARs, and specifically within the PAG columns, is poorly understood.

The aim of the present study was to complete a comparative neurochemical and molecular analysis of the PPAR signalling system within the l, dl and vl columns of the PAG of WKY versus SD rats. Specifically, we measured the levels of endogenous PPAR ligands PEA and OEA, the expression of mRNA encoding for fatty acid amide hydrolase (FAAH), a catabolic enzyme for the hydrolysis of PEA and OEA, and the mRNA encoding for PPARα and PPARγ in WKY and SD rats that were exposed to the formalin test of tonic, persistent, inflammatory pain. To further elucidate the functional role of PPAR signalling in endogenous pain control in a genetic background of stress susceptibility, a pharmacological study was performed in WKY rats, targeting the specific PAG column where the levels of endogenous PPAR ligands and receptors were significantly different compared with SD counterparts.

2 Results

2.1 Strain differences and formalin-evoked changes in neurochemical and molecular components of the PPAR signalling system in the sub-columns of the PAG

2.1.1 lPAG

There were no differences in levels of PEA and OEA between saline-treated SD and WKY rats, however, formalin administration significantly reduced PEA and OEA levels in SD rats only (Fig 1A and B) [formalin, strain, and interaction effects for PEA ($F_{(1,10)} = 2.09$, $p = 0.1786$, $F_{(1,10)} = 9.54$, $p = 0.0194$ and $F_{(1,10)} = 4.26$, $p = 0.0659$) and OEA, ($F_{(1,10)} = 1.92$, $p = 0.1965$, $F_{(1,10)} = 8.18$, $p = 0.0170$, $F_{(1,10)} = 4.9$, $p = 0.0513$) respectively]. Post hoc analysis revealed a significant reduction in PEA and OEA levels in formalin-treated SD rats, compared with saline-treated SD rats ($p < 0.05$) and formalin-treated WKY rats ($p < 0.01$) (Fig1A and B). The expression of FAAH mRNA was significantly higher in SD rats compared with WKY counterparts [formalin strain and interaction effects for FAAH: $F_{(1,20)}=0.0063$, $F_{(1,20)}=25.82$, $p<0.0001$ and $F_{(1,20)}=0.19$, $p=0.67$] respectively. FAAH mRNA expression in saline- and formalin-treated WKY rats was significantly lower than in the corresponding SD groups ($p< 0.01$; Fig 1C). Moreover, levels of FAAH mRNA in formalin-treated SD rats were significantly lower than saline-treated counterparts ($p<0.05$; Fig 1C).

There were no strain differences ($F_{(1,20)} = 0.36$, $p = 0.5540$) or formalin-evoked alterations ($F_{(1,20)} = 0.03$, $p = 0.8552$) in levels of PPARα mRNA in SD or WKY rats (Fig 1D). Expression of PPARγ (Fig 1E) was significantly higher in both saline- and formalin-treated WKY rats, compared with SD counterparts ($F_{(1,17)} = 6.12$, $p = 0.0242$), and there was no further effect of formalin ($F_{(1,17)} = 0.01$, $p = 0.9082$). The differential expression of PPARγ in the lPAG of WKY versus SD rats was further investigated by western blotting methodology (Fig 1F i&ii). Western blot analysis revealed distinct molecular weight bands of ~52 and ~55kDa,

corresponding to the predicted molecular weights of PPARγ (1 and 2) isoforms. Analysis of densitometric data by 2-way ANOVA revealed significant formalin ($F_{(1,7)}$=12.31, p=0.0099), but not strain ($F_{(1,7)}$=3.66, p=0.0973), or strain x treatment ($F_{(1,7)}$=2.93, p=0.1306) effects on PPARγ protein expression. Post-hoc pairwise analysis revealed significantly (p<0.05) higher levels of PPARγ in saline-treated WKY rats compared with the corresponding SD group, Furthermore, formalin treatment significantly reduced PPARγ protein expression in WKY rats only, compared with saline-treated counterparts (Fig 1F- i & ii).

2.1.2 *dlPAG*

There were no statistically significant differences in PEA levels in the dlPAG between saline- or formalin-treated SD and WKY rats, as determined by 2-way ANOVA (formalin: $F_{(1,20)}$ = 0.24, p = 0.633; strain: $F_{(1,20)}$ = 3.63, p = 0.07130; formalin x strain: $F_{(1,20)}$ = 0.86, p = 0.3635; Fig 2A). OEA levels in the dlPAG were significantly different between the two strains ($F_{(1,20)}$ = 7.23, p = 0.0141), but were not affected by formalin administration ($F_{(1,20)}$ = 0.16, p = 0.6948) and there was no significant strain x formalin interaction ($F_{(1,20)}$ = 1.77, p = 0.1986). Post-hoc analysis revealed that saline-treated WKY rats exhibited significantly higher (p < 0.05) levels of OEA in the dlPAG compared with SD counterparts (Fig 2B). Analysis by 2-way ANOVA revealed a significant strain x formalin interaction ($F_{(1,19)}$=5.39, p<0.03), but no strain-related or formalin treatment effects *per se* on FAAH mRNA expression. FAAH mRNA expression was significantly (p< 0.05) higher only in formalin-treated SD rats, compared with the saline-treated group of the same strain (Fig 2C)

There was a significant effect of strain on levels of PPARα mRNA ($F_{(1,19)}$ = 4.69, p = 0.0432) which were significantly lower in saline-treated WKY rats, compared with saline-treated SD counterparts (p<0.01; Fig 2D). Formalin administration also had a significant effect on PPARα mRNA levels ($F_{(1,19)}$ = 6.81, p = 0.0172), with higher levels in WKY rats only, compared with

saline-treated counterparts (p<0.01; Fig 2E). There was no significant effect of strain on levels of PPARγ mRNA ($F_{(1,19)}$ = 0.19, p = 0.6674). However, formalin treatment was associated with a significant increase in PPARγ expression in the dlPAG ($F_{(1,19)}$ = 8.86, p = 0.0078) in both SD and WKY rats (p<0.01; Fig.1D).

2.1.3 *vlPAG*

In the vlPAG, levels of PEA were significantly higher in saline- and formalin-treated WKY rats than in their SD counterparts (Strain effect: $F_{(1,20)}$ = 16.87, p = 0.0005). Formalin administration *per se,* had no significant effect on PEA levels in SD or WKY rats ($F_{(1,20)}$ = 0.86, p = 0.3658) and there was no significant strain x formalin treatment effect ($F_{(1,20)}$ = 0.01, p=0.9363) (Fig 3A). There was a significant effect of strain on OEA levels ($F_{(1,20)}$ = 18.97, p = 0.0003), which were significantly higher only in saline-treated WKY rats, compared with SD counterparts (p<0.05; Figure 3B). There was no significant effect of formalin ($F_{(1,20)}$ = 2.25, p = 0.1491) or strain x formalin interaction ($F_{(1,20)}$ = 1.91, p = 0.1823) on levels of OEA in the vlPAG (Fig 3B). Analysis by 2-way ANOVA did not reveal any significant effects of strain, formalin treatment, or strain x formalin interaction on FAAH mRNA levels in the vlPAG (Fig 3C).

There was no significant effect of strain on levels of PPARα mRNA ($F_{(1,19)}$ = 0.94, p = 0.3438). Formalin administration had a significant effect on PPARα mRNA levels ($F_{(1,19)}$ = 12.12. p = 0.0025), with higher levels in SD rats only, compared with saline-treated counterparts (p<0.05; Fig 3D). There was no significant effect of strain on PPARγ expression ($F_{(1,18)}$ = 0.13, p = 0.7232). However, formalin administration was associated with a significant increase in levels of PPARγ mRNA in the vlPAG of both SD and WKY rats ($F_{(1,18)}$ = 10.71, p = 0.0042) (p<0.05; Fig 3E).

2.2 *Effects of pharmacological blockade of PPARγ in the lPAG on formalin-evoked nociceptive behaviour in SD and WKY rats*

Given the differential and dynamic effects of formalin treatment that we observed on levels of the endogenous PPAR ligands PEA and OEA within the lPAG, coupled with the differential expression of the PPARγ protein and mRNA in this PAG column between the two rat strains, we next investigated the effects of direct intra-lPAG administration of a selective PPARγ antagonist, GW9662, on formalin-evoked nociceptive behaviour in both SD and WKY rats. Analysis of composite pain scores in SD rats revealed a significant main effect of time ($F_{(5,70)}$=7.98, p<0.001) but not drug treatment ($F_{(1,70)}$=0.75, p=0.40), or strain x drug interaction ($F_{(5,70)}$= 0.19, p=0.96) (Figure 4A)

In contrast, analysis of composite pain scores in WKY rats by repeated measures ANOVA revealed a significant main effect of time ($F_{(11,132)}$=8.68, p<0.001), and a significant time x treatment interaction ($F_{(11,132)}$=1.94, p<0.05) on formalin-evoked nociceptive behaviour (Figure 4B). Further analysis by Student's unpaired t-test revealed a significant increase of both first (0-10 minutes) and second (21-50 minutes) phase formalin-evoked nociceptive behaviour in GW9662-treated rats, compared with vehicle-treated controls (p<0.05; Figure 4B).

Intra-lPAG administration of GW9662 did not alter pre-formalin locomotor activity (distance moved in arena) and general rat behaviours including the duration of rearing and grooming, compared with vehicle-treated controls (data not shown).

3 Discussion

The results of the present study reveal higher levels of the endogenous PPAR ligands, PEA and OEA, as well as mRNA coding for PPARγ, but not PPARα, and a reduction in PPARγ protein, in the lPAG of formalin-treated WKY rats, compared with SD counterparts. In non-formalin (saline) treated rats, levels of PEA and OEA, but not PPARγ mRNA or protein, were comparable between the two rat strains. Furthermore, levels of mRNA coding for FAAH, a major hydrolysing enzyme for endogenous PPAR ligands, was significantly lower in saline- and formalin-treated WKY rats, compared with respective SD counterparts. Moreover, the functional relevance of higher levels of PEA, OEA, and PPARγ expression in the lPAG of WKY rats during inflammatory pain was further interrogated in a pharmacological study that demonstrated significantly enhanced first and second phase formalin-evoked nociceptive behaviour in WKY, but not SD, rats that had received intra-lPAG administration of the PPARγ antagonist GW9662. This result suggests that there may be an inhibitory role for endogenous PPARγ signalling in the lPAG on inflammatory pain-related behaviour in WKY rats. Furthermore, GW9662 did not alter pre-formalin locomotor activity or general rat behaviours such as rearing and grooming, indicating that it is unlikely that alterations in these parameters were confounding factors in the effects of GW9662 on formalin-evoked nociceptive behaviour. While there were differences in the levels of endogenous PPAR ligands, PEA and OEA, in the dlPAG and vlPAG of saline-treated SD versus WKY rats, the effects of formalin treatment on these analytes were indistinguishable between the two rat strains. Similarly, differences in the expression of mRNA coding for PPARα, PPARγ and FAAH were less pronounced in the dlPAG or vlPAG between the two rat strains. These findings suggest that alterations in the PPAR signalling components at the level of the dlPAG and vlPAG are unlikely to account for the differences in formalin-evoked nociceptive behaviour between the two rat strains. To our knowledge, this is the first study to characterise the neurochemical and molecular components

of the PPAR signalling system in a key neural substrate of the descending pain pathway, and to suggest a possible role for PPARγ signalling in the lPAG in a genetic background (WKY) prone to heightened stress, negative affect and hyperalgesia.

The PAG is a key component of the descending pain pathway [4; 23; 36]. Electrical stimulation of the different subunits of the PAG produces analgesia mediated by different receptor signalling systems. For example, stimulation of the vlPAG has been associated with opioid-mediated analgesia, whereas stimulation of the dlPAG or lPAG is thought to produce non-opioid mediated analgesia [8; 40]. Furthermore, the role of the PAG as a key substrate mediating anxiety- and panic-like behaviour has been demonstrated in both human and animal studies (for review see [18]). Thus, the PAG plays a pivotal role in mediating and modulating physiological responses to both stress and pain. As discussed in the Introduction, multiple lines of evidence from animal studies suggest that the PPAR signalling system plays a significant role in endogenous pain control. It is therefore feasible that dysfunction and/or alterations in this signalling system could result in impaired nociceptive processing. The findings from the present study suggest that behavioural responses to inflammatory pain in WKY rats is most likely influenced by enhanced endogenous PPAR signalling in the lPAG, possibly mediated by PPARγ, given the relatively higher expression in this PAG column compared with SD rats.

The higher levels of PEA and OEA in the lPAG of WKY rats were associated with a significantly lower expression of mRNA coding for FAAH, a major catabolic enzyme responsible for the hydrolysis of endogenous *N*-acylethanolamines including PEA and OEA [14]. Given that one of the functional consequences of increased levels of endogenous *N*-acylethanolamines is analgesia [9; 10; 20], it seems reasonable to suggest that the higher levels of PEA and OEA in the lPAG of formalin-treated WKY rats versus SD rats may represent adaptive or compensatory changes aimed at lowering the hyperalgesia exhibited by WKY rats,

with the antinociceptive effects of PEA and OEA mediated by PPARγ, the mRNA expression of which was higher in the lPAG of WKY rats. This hypothesis was supported by our observations of increased formalin-evoked-nociceptive behaviour following injection of a selective PPARγ antagonist, GW9662, into the lPAG of WKY rats. Thus, the endogenous activation of PPARγ in the lPAG may represent an important adaptive mechanism which functions to reduce nociceptive transmission in WKY rats in inflammatory pain states. Collectively, these findings suggest that a decrease in PPAR signalling components within the lPAG may contribute to the hyperalgesic phenotype of WKY rats. In this regard, the formalin-induced reduction in PPARγ protein in WKY rats as demonstrated by our western blotting data may represent a mechanism contributing to hyperalgesia in WKY rats. The finding of PPARγ-mediated endogenous analgesia at the level of the lPAG in WKY rats also raises the prospect of PPARγ signalling as a possible therapeutic target for the treatment of stress-related chronic pain disorders. Indeed, PPARγ agonists are already available clinically as therapeutic agents for treating disorders such diabetes mellitus [24]. The descending pain pathway is a major target for opioid drugs which comprise some of the most potent analgesics [25]. However, there are a myriad of side-effects associated with prolonged use of these analgesics [21; 33] and further research into novel receptor targets for improved treatment of chronic pain is required. In this regard, the emerging role of PPARs as mediators of both peripheral and central processing of pain, [11-13; 29; 39] offers an opportunity for the development of new therapies for chronic pain management

In conclusion, these data indicate differences in the PPAR signalling system in the lPAG between SD and WKY rats. The findings suggest that endogenous activation of the PPARγ isoform in this PAG column, facilitated by higher levels of PEA and OEA, may exert an

inhibitory effect on inflammatory pain processing in WKY rats, and may represent adaptive or compensatory mechanisms aimed at lowering the hyperalgesia exhibited by this strain.

4 Experimental procedures

4.1 Animals

Adult male SD and WKY rats in Experiment 1 (neurochemical and molecular characterisation studies, n = 27 rats in total per strain, 285-320g) and Experiment 2 (pharmacological study, n = 25 rats per strain, 225-250g) were obtained from Harlan, UK. Animals in Experiment 1 were singly housed on arrival and throughout the duration of the study. Animals in Experiment 2 were group-housed on arrival, followed by single housing post-surgery. Room temperature was maintained at 21 ± 20C under standard lighting conditions (12:12h light:dark, lights on from 07.00 to 19.00h). All experiments were carried out during the light phase between 08.00h and 17.00h. Food and water were available ad libitum. The experimental procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland Galway, and the work was carried out under license from the Department of Health and Children and the Health Products Regulatory Authority in the Republic of Ireland and in accordance with EU Directives 86/609 and 2010/63.

4.2 Experiment 1

4.2.1 Experimental design

Immediately after a 10-minute habituation exposure to the formalin test arena (30 x 30 x 40 cm, L x W x H; Perspex), rats from each strain (SD and WKY) received a single intra-plantar injection of 50 μL formalin (2.5% in 0.9% saline) or saline into the right hindpaw under brief

isoflurane anaesthesia, as described previously [7; 29; 35]. Rats were returned to the formalin test arena (Supplementary Video 1) and sacrificed by decapitation 30 minutes post-formalin injection, to coincide with the peak of the second phase of formalin-evoked nociceptive behaviour [1; 2]. Brains were removed and snap-frozen on dry ice, prior to storage at −80°C. The data on formalin-evoked nociceptive behaviour exhibited by the rats in Experiment 1 have been published previously in [35].

4.2.2 Cryo-sectioning and punch microdissection

Frozen coronal brain sections of 300μm thickness containing the PAG were cut on a cryostat (MICROM GMBH, Baden-Württemberg, Germany), and were punch-dissected as previously described [16; 31; 35], using cylindrical brain punchers (Harvard Apparatus, MA, USA, internal diameter 0.75mm for the different PAG columns, at the following rostro-caudal levels: dorsolateral (dl) PAG (Bregma, --6.3 — -7.8mm), lateral (l) PAG (Bregma, -6.3 — -7.8mm) and ventrolateral (vl) PAG (Bregma, -7.3 — -8.3mm, using the rat brain atlas [32]. Samples from all treatment groups were randomly split into two cohorts of n=6 per group for the purposes of measuring PEA and OEA levels and mRNA coding for PPARα , PPARγ and FAAH and n=3 for PPARγ protein measurement in the lPAG only.

4.2.3 Quantitation of PEA and OEA levels in the PAG

PEA and OEA levels were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously [15; 29; 31]. Briefly, samples were homogenized in 400 μL 100% acetonitrile containing deuterated internal standards (0.016 nmol PEA-d4 and 0.015 nmol OEA-d2). Lyophilized samples were re-suspended in 40 μL 65% acetonitrile and separated on a Zorbax® C18 column (150 × 0.5 mm internal diameter; Agilent Technologies, Cork, Ireland) by reversed-phase gradient elution initially with a mobile phase of 65%

acetonitrile and 0.1% formic acid, which was ramped linearly up to 100% acetonitrile and 0.1% formic acid over 10 min and held at this for a further to 20 min. Under these conditions, PEA and OEA eluted at the following retention times: 14.4 min and 15.0 min respectively. Analyte detection was carried out in electrospray-positive ionization and multiple reaction monitoring (MRM) mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Quantification of each analyte was performed by ratiometric analysis and expressed as nmol g−1 of tissue. The limit of quantification was 1.5 pmol g−1, 1.4 pmol g−1 for PEA and OEA respectively.

4.2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed according to methods described previously [29; 35]. Total RNA was extracted from post-mortem PAG, tissue using a Machery-Nagel extraction kit (Nucleospin RNA II, Technopath, Dublin, Ireland) according to manufacturer's instructions. RNA quality (1.8-2 260/280 ratio) and quantity were assessed using a Nanodrop spectrophotometer (ND-1000, Nanodrop, Labtech International, Uckfield, UK) and equalised to a concentration of 5ng/µL. 50ng 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 assay for rat PPARα (assay ID - Rn00566193_m1, Applied Biosystems, UK), rat PPARγ receptor (assay ID - Rn00440945_m1 Applied Biosystems, UK) or rat FAAH (assay ID - Rn00577086_m1 Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probe was used to quantify PPARα or PPARγ receptor mRNA on a StepOne plus instrument (Applied Biosystems, Warrington, UK). VIC-labelled glyceraldehydes phosphate dehydrogenase (GAPDH) (assay ID 4308313 Applied Biosystems, Warrington, UK) was used as the housekeeping gene and endogenous control. A no template control 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 expressed as a percentage of the mean value of the control (SD-saline) group.

4.3 Experiment 2

4.3.1 Chemicals and drug preparation

The PPARγ antagonist, GW9662 (Tocris Bioscience, Bristol, UK), was administered in 100% dimethylsulfoxide (DMSO) vehicle. Drug solutions were aliquoted and stored at -20°C until the day of injection. The dose of GW9662 (14.4nmols/0.2µL) was chosen based on studies in the literature and pilot studies in our laboratory demonstrating its efficacy in inflammatory pain models following intracerebral administration [26]. On the day of testing, the stock drug aliquot was thawed, diluted to the required concentration and kept at room temperature prior to injection. Formalin (2.5%) was prepared from a 37% stock solution (Sigma-Aldrich, Dublin, Ireland) diluted in 0.9% sterile saline.

4.3.2 Intra-lPAG guide cannulae implantation and post-surgical care

Under isofluorane anaesthesia (3% induction and 2% maintenance in 0.5L/min O2), stainless steel guide cannulae of 9mm length (Plastics One Inc, Roanoke, VA, USA) were implanted bilaterally 1mm above the lPAG (anteroposterior -6.8mm, mediolateral ± 1.9mm relative to bregma at an angle of 12°, dorsoventral – 5.3 mm from dura; (Supplementary Figure 1). Surgery and post-surgical care were performed according to methods that we have previously described [29; 31]. Rats were allowed a period of 7-8 days to recover from surgery before formalin testing.

4.3.3 Intra-lPAG microinjection

The selective GPR55 antagonist, GW9662, was microinjected into the lPAG using previously published protocols [29; 31; 35; 38]. Immediately after injection, rats were placed in a Perspex chamber (30x30x40 cm, 30 lux) with blackened walls and locomotor activity (distance moved, duration of rearing and grooming) was assessed for 10 minutes. Rats were randomly assigned to each treatment group and the sequence and timings of drug treatment were randomized equitably across the experimental days to minimise their potential influence as a source of variability.

4.3.4 Formalin test

Formalin was administered as described above for Experiment 1, 10 minutes after intra-lPAG microinjections. However, the duration of the formalin trial in Experiment 2 study was extended to 60 minutes to allow for a more comprehensive assessment of the effects of GW9662. A video camera located beneath the chamber was used to record animal behaviour onto DVR appliance for subsequent analysis. At the end of the 60-minute formalin trial, rats were killed by decapitation, brain tissues harvested and snap-frozen for post-mortem histological verification of microinjection sites.

4.3.5 Behavioural analysis

Behaviour was analysed using EthoVision XT software (Noldus Information Technology, Wageningen, The Netherlands), which allowed for continuous event recording over each 60-minute trial. A trained observer, blind to the experimental conditions, rated formalin-evoked nociceptive behaviour according to the weighted composite pain scoring technique [41]. According to this method, pain behaviours are categorized as time spent raising the right hindpaw above the floor without contact with any other surface (pain 1) and time spent holding,

licking, biting, shaking or flinching the paw (pain 2) to obtain a composite pain score (CPS). CPS was calculated as (pain 1 + 2[pain 2])/total duration of analysis period.

4.3.6 Histological verification of intra-cerebral microinjection sites

Intra-cerebral microinjection sites were verified according to previously published methods [29; 31; 35; 38]. Only rats with both microinjections correctly located within the lPAG (~ 72% of total number of rats used in study were included in final analysis of behavioural data (Figure 5).

4.3.7 Western blotting

Protein extraction from the lPAG and quantification were performed according to methods previously described [29; 35]. 42µg of lPAG protein from each sample was subjected to 9% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) electrophoresis at a constant voltage of 120 V for 2 hours. The separated protein samples were electro-blotted onto a nitrocellulose membrane (Nitrocellulose membrane, CAS# 9004-70-0, Bio-Rad, Dublin, Ireland) at 100V for 0.5hour. Membranes were blocked in 5% non-fat dry milk in 0.05% Tris-buffered saline / Tween 20 (TBST) solution for 1hr at room temperature and incubated with rabbit polyclonal antibody to PPAR-γ (1:200, cat# SC-7196, Santa Cruz Biotechnology) 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^{o}C. Membranes were subjected to 3 x 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 1:10000 in 1% milk/ 0.05% TBST for one hour. Three x 5 minute washing steps were then performed with washing solution and one final 5 minute wash in distilled H_2O. Blots were scanned on a LI-COR® Odyssey imager. The ratio of total PPARγ/β-actin staining

signal intensities was determined for each sample and expressed as a percentage of the of the mean ratio of the control (SD-saline) group.

4.4 Statistical analysis

The SPSS 17.0 statistical package was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene test, respectively. Neurochemical and molecular data (Experiment 1) were analysed by 2-way ANOVA with Fisher's LSD post hoc test for pairwise comparisons where appropriate. Pharmacological data (Experiment 2) were analysed by repeated measures ANOVA with time and treatment considered as within and between-subject factors respectively, followed by Student's unpaired t-test for pairwise comparisons. Data are expressed as group means ± standard error of the mean (± SEM) and were considered significant when $p<0.05$.

Author contributions

All authors have approved the final article. BNO, JCG, MKM WMO, BH, MR and DPF all contributed to study design and preparation of the manuscript. BNO, JCG, MKM, WMO and BH collected and analysed the data. MR and DPF supervised the work and contributed to data interpretation.

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Figure Legends

Figure 1 Levels of (A) PEA and (B) OEA and the expression of mRNA coding for (C) FAAH (D) PPARα and (E) PPARγ and (F-i&ii) PPARγ protein in the lPAG following intra-plantar injection of saline or formalin in SD and WKY rats. +p<0.05, ++p<0.01 vs. SD-Formalin, *p <0.05 vs. corresponding saline-injected counterparts, ^p< 0.05 vs SD-Saline. Data are expressed as mean + SEM, n=6 per group for neurochemical and mRNA data and n=3 per group for western blotting data. SD, Sprague-Dawley; WKY, Wistar-Kyoto; PEA, *N*-palmitoylethanolamide; OEA, *N*-oleoylethanolamide; PPAR, peroxisome proliferator-activated receptor.

Figure 2 Levels of (A) PEA and (B) OEA and the expression of mRNA coding for (C) FAAH (D) PPARα and (E) PPARγ in the dlPAG following intra-plantar injection of saline or formalin in SD and WKY rats., *p <0.05, **p<0.01, ***p<0.001 vs. corresponding saline-injected counterparts, ^p<0.05, ^^p< 0.01 vs SD-Saline. Data are expressed as mean + SEM, n=6 per treatment group. SD, Sprague-Dawley; WKY, Wistar-Kyoto; PEA, *N*-palmitoylethanolamide; OEA, *N*-oleoylethanolamide; PPAR, peroxisome proliferator-activated receptor.

Figure 3 Levels of (A) PEA and (B) OEA and the expression of mRNA coding for (C) FAAH (D) PPARα and (E) PPARγ in the vlPAG following intra-plantar injection of saline or formalin in SD and WKY rats. +p<0.05 vs. SD-Formalin, *p<0.05 vs. corresponding saline-injected counterparts, ^^p< 0.01 vs SD-Saline. Data are expressed as mean + SEM, n=6 per treatment group. SD, Sprague-Dawley; WKY, Wistar-Kyoto; PEA, N-palmitoylethanolamide; OEA, N-oleoylethanolamide; PPAR, peroxisome proliferator-activated receptor.

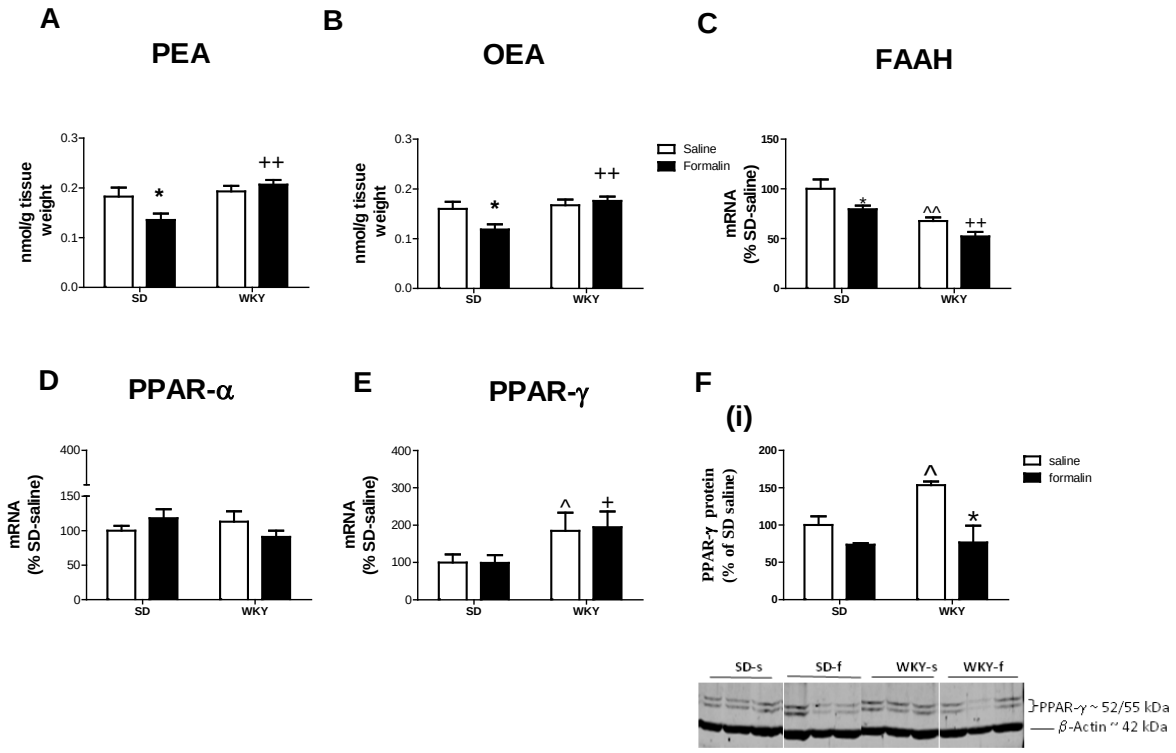
Figure 4 Effects of pharmacological blockade of PPARγ in the lPAG on formalin-evoked nociceptive behaviour in (A) SD and (B) WKY rats. *p <0.05 for GW9662 vs. Vehicle at corresponding time point. Repeated measures ANOVA with Student's unpaired t-test for pairwise comparisons. Data are mean ± SEM, n=7-9 SD rats and 6-8 for WKY rats per treatment group. WKY;Wistar-Kyoto, GW9662; selective PPARγ antagonist.

Figure 5 Schematic depicting microinjection sites in SD and WKY rats receiving intra-lPAG administration of GW9662 (closed circles) or vehicle (open circles), n=7-9 rats per group for SD and n=6-8 rats per group for WKY. Images were taken from the rat brain atlas of Paxinos and Watson (2006) AP = -6.72 to -7.04mm as depicted in left panel (top to bottom) with abscissa and ordinate axes.

Supplementary Video 1 Representative 30-minute video recordings of formalin-evoked nociceptive behaviour in (A) SD and (B) WKY rats.

Supplementary Figure 2 Representative image of rat in stereotaxic frame under isoflurane anaesthesia.

Fig 1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SD
WKY
B
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Saline
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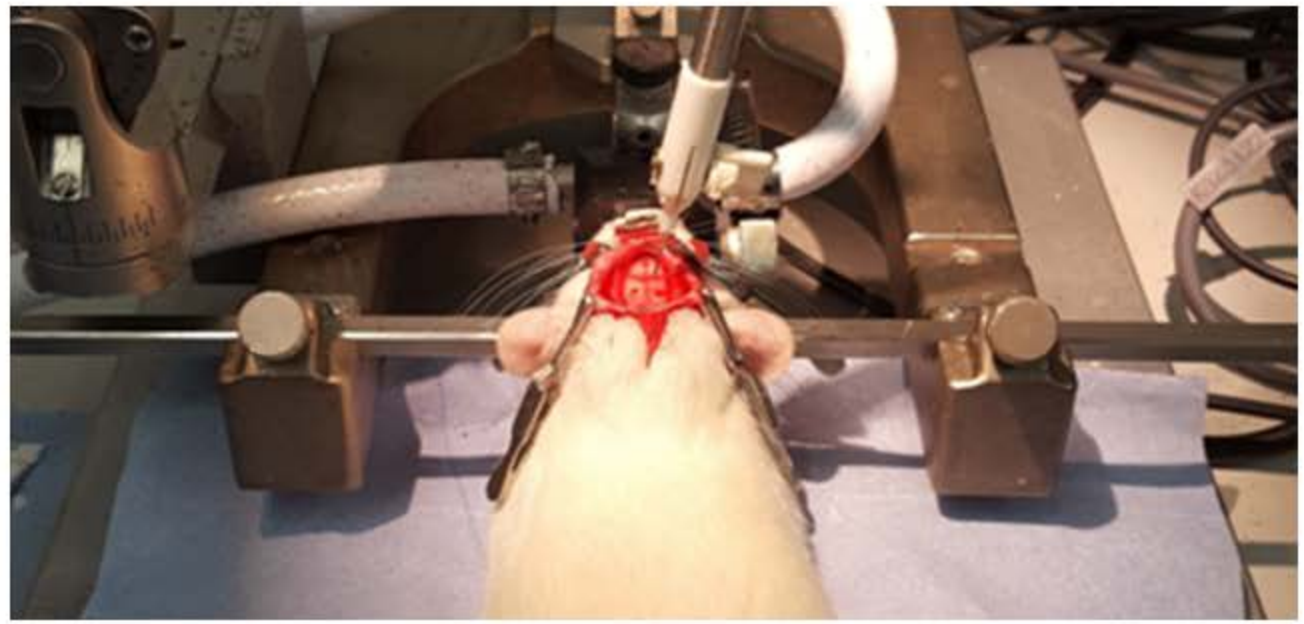
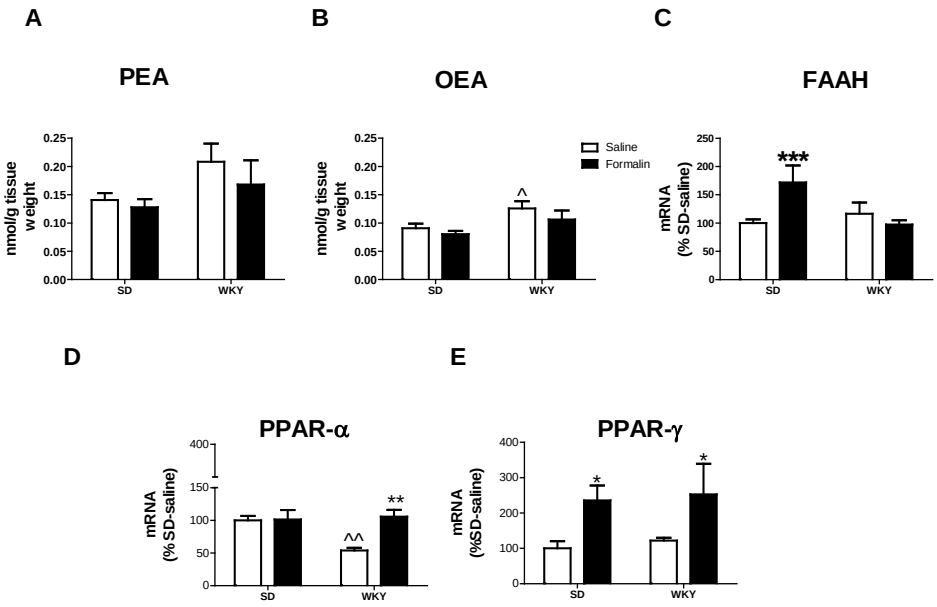
PPAR-γ ~ 52/55 kDa
β-Actin ~ 42 kDa

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Saline
Formalin
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C
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D
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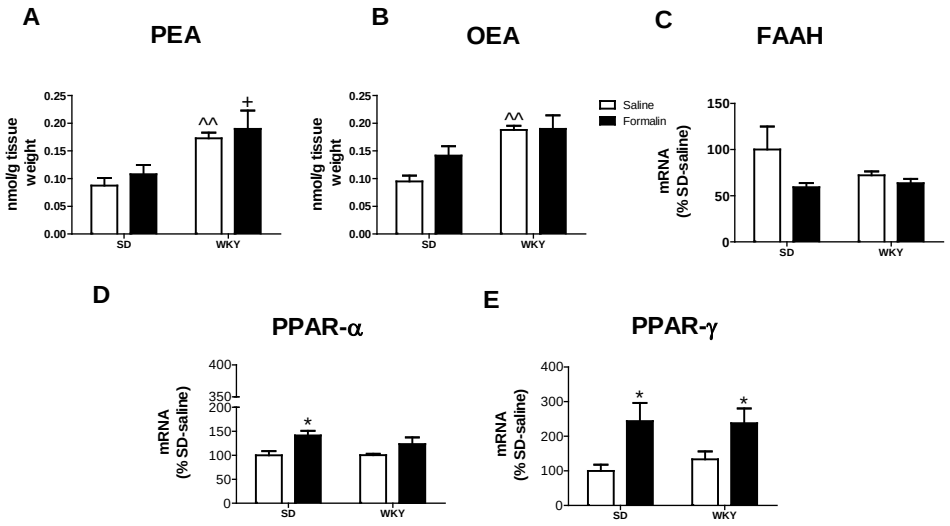
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mRNA (% SD-saline)
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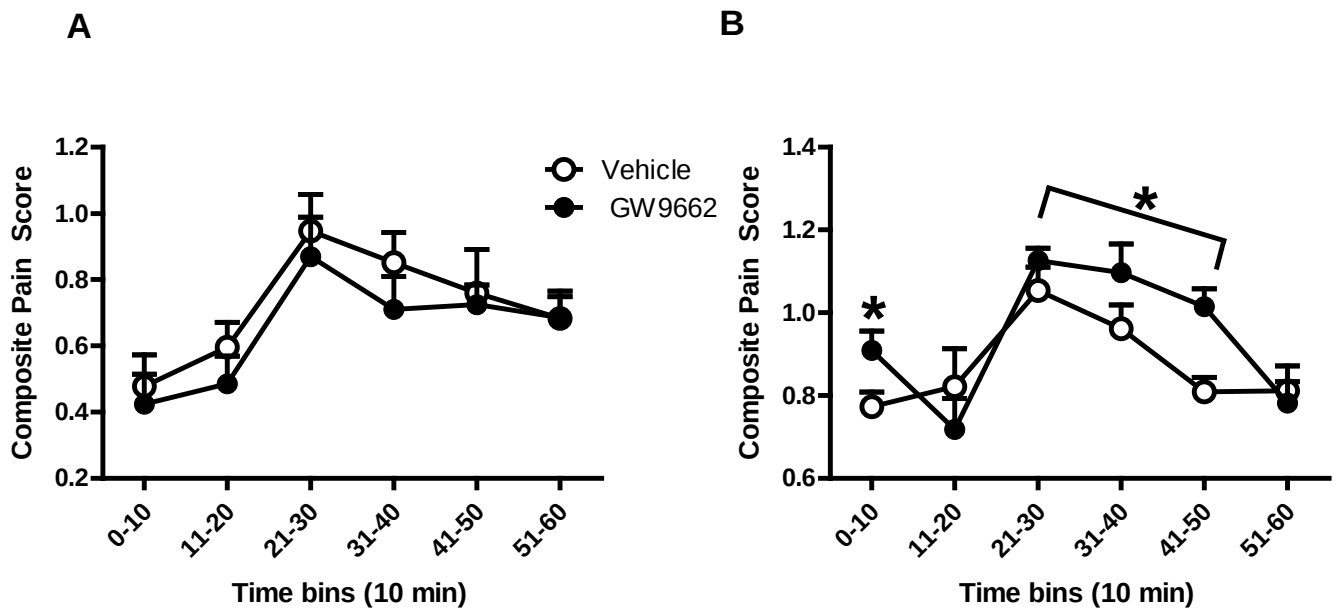
Fig 4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B
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0.6
0.4
0.2
Vehicle
GW9662
0-10
11-20
21-30
31-40
41-50
51-60
Time bins (10 min)
1.4
*
*