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RESEARCH PAPER

A role for PPARα in the medial prefrontal cortex in formalin-evoked nociceptive responding in rats

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BACKGROUND AND PURPOSE

The nuclear hormone receptor, PPARα, and its endogenous ligands, are involved in pain modulation. PPARα is expressed in the medial prefrontal cortex (mPFC), a key brain region involved in both the cognitive-affective component of pain and in descending modulation of pain. However, the role of PPARα in the mPFC in pain responding has not been investigated. Here, we investigated the effects of pharmacological modulation of PPARα in the rat mPFC on formalin-evoked nociceptive behaviour and the impact of formalin-induced nociception on components of PPARα signalling in the mPFC.

EXPERIMENTAL APPROACH

The effects of intra-mPFC microinjection of a PPARα agonist (GW7647) or a PPARα antagonist (GW6471) on formalin-evoked nociceptive behaviour in rats were studied. Quantitative real-time PCR and LC-MS/MS were used to study the effects of intraplantar injection of formalin on PPARα mRNA expression and levels of endogenous ligands, respectively, in the mPFC.

KEY RESULTS

Intra-mPFC administration of GW6471, but not GW7647, resulted in delayed onset of the early second phase of formalin-evoked nociceptive behaviour. Furthermore, formalin-evoked nociceptive behaviour was associated with significant reductions in mPFC levels of endogenous PPARα ligands (*N*-palmitoylethanolamide and *N*-oleoylethanolamide) and a 70% reduction in PPARα mRNA but not protein expression.

CONCLUSIONS AND IMPLICATIONS

These data suggest that endogenous ligands may act at PPARα in the mPFC to play a facilitatory/permissive role in second phase formalin-evoked nociceptive behaviour in rats.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids 2013. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-6

Abbreviations

AEA, anandamide (N-arachidonoylethanolamide); CPS, composite pain score; FAM, fluorescein amidite; GAPDH, glyceraldehyde phosphate dehydrogenase; MGB, minor groove binder; mPFC, medial prefrontal cortex; OEA, *N-oleoylethanolamide;* PEA, *N*-palmitoylethanolamide

Introduction

PPARα is a member of the nuclear hormone receptor family of ligand-dependent transcription factors and is widely distributed within peripheral and CNS tissues (Braissant *et al.*, 1996). Endogenous ligands of the PPARα receptor include the bioactive fatty acid amides [*N*-palmitoylethanolamide (PEA), *N*-oleoylethanolamide (OEA)] (LoVerme *et al.*, 2006; Fu *et al.*, 2007) and the endocannabinoid anandamide (AEA) (Mackie and Stella, 2006; Sun *et al.*, 2007; Sun and Bennett, 2007). Early investigation and characterization of this receptor suggested a primary physiological role in lipid and lipoprotein metabolism and the regulation of inflammatory processes via the suppression of proinflammatory mediator gene transcription in pathophysiological states (Desvergne and Wahli, 1999; Delerive *et al.*, 2001; Chinetti *et al.*, 2003; Cuzzocrea *et al.*, 2008; Crisafulli and Cuzzocrea, 2009). However, subsequent studies in animal models of inflammatory and neuropathic pain (Calignano *et al.*, 2001; Benani *et al.*, 2004; LoVerme *et al.*, 2006; D'Agostino *et al.*, 2007; 2009) provided support for PPARα as an important modulator of pain processing and, as such, a potentially useful therapeutic target for the treatment of chronic pain states.

The importance of PPARα in pain modulation is underpinned by research demonstrating (i) the expression of the receptor at key sites within the peripheral and CNS involved in pain processing (Braissant *et al.*, 1996; Moreno *et al.*, 2004; Gofflot *et al.*, 2007; De Novellis *et al.*, 2012) and (ii) that endogenous ligands of the receptor, in particular the bioactive fatty acid amide, PEA, have antinociceptive effects which are mediated by PPARα expression (Calignano *et al.*, 2001; LoVerme *et al.*, 2006; Jhaveri *et al.*, 2008; Sagar *et al.*, 2008; De Novellis *Et Al.*, 2012). Indeed, pharmacological and gene knockout studies have shown that the antinociceptive effects of PEA are abolished either in the presence of selective PPARα antagonists or in PPARα$^{-/-}$ mice (LoVerme *et al.*, 2006; De Novellis *et al.*, 2012). Although the precise mechanisms involved are currently not well understood, the results of these studies suggest that PPARα activation is associated with rapid antinociceptive effects which do not appear consistent with transcription-dependent anti-inflammatory effects of PPARα activation, leading to suggestions of transcription-independent mechanisms (LoVerme *et al.*, 2006).

To date, the majority of studies of the role of PPARα in pain processing have focused on peripheral or spinal sites of action of endogenous ligands of the receptor. A relatively limited number of studies have investigated the role of supraspinal PPARα signalling in pain, despite credible immunohistochemical evidence for the presence of the receptor at key pain processing sites within the brain (Moreno *et al.*, 2004). Nonetheless, studies investigating the role of brain PPARα in pain processing have demonstrated that i.c.v. administration of PPARα agonists reduces nociceptive behaviour or oedema in animal models of inflammatory pain (Taylor *et al.*, 2005; D'Agostino *et al.*, 2007; 2009). More recently, pharmacological activation of PPARα within the midbrain periaqueductal grey (PAG), a key component of the descending pain pathway, has been shown to increase response latency of rats in the tail-flick test and to reduce activity of ON and OFF cells in the rostral ventromedial medulla (RVM) (De Novellis *et al.*, 2012). The descending pain pathway is subject to top-down modulation by virtue of its anatomical connections with other higher brain structures, in particular the medial prefrontal cortex (mPFC) (Jasmin *et al.*, 2004; Hadjipavlou *et al.*, 2006). The key role of the mPFC in supraspinal pain processing is supported by recent findings that nociceptive transmission within the CNS is associated with morphological and functional reorganization of cells in the mPFC, which may contribute to the development of chronic pain states (Metz *et al.*, 2009; Luongo *et al.*, 2013). Furthermore, key structures within the mPFC, in particular the anterior cingulate and prelimbic cortices are considered important for supraspinal affective and cognitive modulation of pain (Shyu and Vogt, 2009; Xie *et al.*, 2009; Giordano *et al.*, 2011). Significantly, immunohistochemical evidence has revealed a high expression of PPARα within the mPFC (Moreno *et al.*, 2004). To date, however, the role of mPFC PPARα in nociception has not been investigated.

We hypothesized that alterations in PPARα signalling within the mPFC may be associated with altered nociceptive processing within the CNS. Thus, the specific aims of the study were to investigate (i) the effects of pharmacological stimulation or blockade of PPARα in the mPFC on formalin-evoked nociceptive behaviour and (ii) the effects of intraplantar injection of formalin on PPARα expression and levels of the endogenous ligands, PEA and OEA, in the rat mPFC. Given that AEA is an endogenous ligand at both PPARα and cannabinoid$_1$ (CB_1) receptor, we also measured levels of AEA and CB_1 receptor expression in the mPFC of formalin-treated rats. The results suggest that nociceptive processing is associated with alterations in PPARα, but not CB_1 receptor, signalling in the mPFC and that PPARα in the mPFC may play a facilitatory/permissive role in second phase formalin-evoked nociceptive behaviour in rats.

Methods

Animals

Three cohorts of adult male Sprague-Dawley rats weighing 225–250 g were obtained from Harlan (Bicester, UK). Animals in experiment 1 were housed in groups of three prior to surgery and housed singly post-surgery. Animals in experiment 2 were housed singly on arrival and throughout the experiment. All animals were housed in plastic bottomed cages (45 × 20 × 20 cm) containing wood shavings as bedding. Animal housing rooms were maintained at a constant temperature (21°C ± 1°C) under standard lighting conditions (12:12 h light: dark, lights on from 0700 to 1900 h). Food and water were provided *ad libitum*. Subjects were randomly assigned to experimental groups and the sequence of testing was randomized throughout the experiment in order to minimize any confounding effects associated with the order of testing. All *in vivo* experiments were carried out following approval from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Department of Health and Children in the Republic of Ireland and in accordance with EU Directive 86/609 and ARRIVE guidelines (Kilkenny *et al.*, 2010).

Experiment 1

Intra-mPFC guide cannulae implantation

Implantation of guide cannulae was carried out as previously described (Ford *et al.*, 2008; Roche *et al.*, 2009; Olango *et al.*, 2011; Rea *et al.*, 2013). In brief, one cohort of rats were implanted stereotaxically with stainless steel guide cannulae (5 mm length, Plastics One Inc., Roanoke, VA, USA) bilaterally above the mPFC (anteroposterior + 1.5 mm, mediolateral ± 1.3 mm relative to bregma at an angle of 12°, dorsoventral –1.3 mm from dura) under isoflurane anaesthesia (1–3% in O_2; 0.60 L min^{-1}). Rats were deemed to be sufficiently anaesthetised if there was no withdrawal reflex in response to tail / toe pinch, as well as the absence of a corneal reflex. A second cohort of rats used as off-target controls were implanted stereotaxically with stainless steel guide cannulae just above the corpus callosum and outside the borders of the mPFC (anteroposterior + 1.5 mm, mediolateral ± 1.6 mm relative to bregma at an angle of 12°, dorsoventral –1.3 mm from dura). The cannulae were permanently fixed to the skull using stainless steel screws and carboxylate cement. A stainless steel stylet (Plastics One Inc.) was inserted into the guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent carprofen (5 mg kg^{-1} s.c.; Rimadyl, Pfizer, Kent, UK), was administered during surgery to manage post-operative pain. The antibiotic enrofloxacin (2.5 mg kg^{-1} s.c.; Baytril, Bayer Ltd, Dublin, Ireland) was administered during surgery and for 3 days post-surgery to prevent infection. Rats were allowed 5–8 days to recover from surgery before formalin testing. During this period, the rats were handled and their body weight and general health monitored daily.

Chemicals and drug preparation

The PPARα agonist, GW7647 (2-[[4-[2-[[(cyclohexylamino) carbonyl](4-yclohexylbutyl) amino]ethyl]phenyl]thio]-2-methylpropanoic acid), and the PPARα antagonist, GW6471(N-(2S)-2-(((1Z)-1-methyl-3-oxo-3-(4-(trifluoromethyl) phenyl) prop-1-enyl)amino)-3-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy)phenyl) propyl) propanamide) (Tocris Bioscience, Bristol, UK) were prepared in 100% DMSO. Drug solutions were aliquoted and stored at –20°C until the day of injection. The dose of GW7647 (10 µg 0.5 $µL^{-1}$) was chosen on the basis of similar studies in the literature demonstrating efficacy of the drug in inflammatory pain models following intracerebral administration (D'Agostino *et al.*, 2007, 2009) and being the highest possible dose based on solubility in DMSO. Two lower doses of GW7647 (0.1 and 1.0 µg 0.5 $µL^{-1}$) were also tested and were found to be without effect on formalin-evoked nociceptive behaviour. For GW6471, the highest possible dose based on solubility in DMSO was also employed (i.e. 10 µg 0.5 $µL^{-1}$). On the day of testing, stock drug aliquots were thawed, diluted to the required concentrations and kept at room temperature before injection. A solution of 2.5% formalin (Sigma-Aldrich, Dublin, Ireland) was prepared from a 37% stock solution diluted with 0.9% sterile saline.

Intra-mPFC microinjection and formalin test

Rats received bilateral microinjections of GW7647 (10 µg) or GW6471 (10 µg) or vehicle (100% DMSO) in an injection volume of 0.5 µL over 60 s using an injector and Hamilton syringe attached to polyethylene tubing, 10 min before intraplantar formalin injection (n = 7 or 8 rats per group). Immediately following the intra-mPFC injections, rats were placed in a perspex chamber (30 × 30 × 40 cm, 30 lux) with blackened walls for a 10-min habituation, after which time they received an intraplantar injection of 50 µL formalin (2.5% in 0.9% sterile saline) or 0.9% saline into the right hindpaw under brief isoflurane anaesthesia as described previously (Finn *et al.*, 2003; Roche *et al.*, 2007). Following formalin injection, rats were immediately returned to the perspex chamber for a period of 90 min. A video camera located beneath the chamber was used to record animal behaviour onto DVD for subsequent analysis. Formalin-induced oedema was assessed by measuring the change in the diameter of the right hindpaw measured immediately before formalin administration and at the end of the experiment using Vernier callipers. Pre- and post-formalin behaviours for each rat were recorded for 10 and 90 min, respectively and rated as described later. At the end of the 90-min formalin trial, rats were transcardially perfused as described below.

Transcardial perfusion

Rats were deeply anaesthetized (5% isoflurane in O_2) and transcardially perfused with heparin-treated saline solution (5000 IU L^{-1}) until blood cleared (2 min) followed by 4% paraformaldehyde fixative (in 0.1 M phosphate buffer), pH 7.4, at an initial flow rate of 50 mL min^{-1} for 2 min, followed by a flow rate of 25 mL min^{-1} for 6 min (Watso-Marlow Bredal Pumps, 323E/D Pump; Watson-Marlow Pumps Limited, Cornwall, UK). A total of 0.5 µL of 1% fast green dye was then slowly injected via each cannula to mark the mPFC injection site position. Rats were decapitated and brain tissues excised. All tissues were stored in 4% paraformaldehyde fixative overnight and then transferred into 25% sucrose solution with 0.1% sodium azide for cryoprotection before sectioning on a cryostat.

Histological verification of intracerebral microinjection sites

The sites of intracerebral microinjection were determined before data analysis. Brain sections with fast green dye mark were collected (30 µm thickness) using a cryostat, mounted on gelatine-treated glass slides and counterstained with cresyl violet to locate the precise position of microinjection sites under light microscopy.

Behavioural analysis

Behaviour was analysed using EthoVision XT software (Noldus Information Technology, Wageningen, The Netherlands), which allowed for continuous event recording over each 90-min trial. A trained observer, blind to the experimental conditions, rated formalin-evoked nociceptive behaviour according to the weighted composite pain scoring technique described by Watson *et al.* (1997). 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. In the 10-min

pre-formalin trial, total distance moved was tracked automatically by Ethovision XT and the duration of rearing and grooming and number of faecal pellets excreted were scored manually.

Experiment 2

Experimental procedure

Rats (n = 5 or 6 per group) were placed in a perspex chamber (30 × 30 × 40 cm, 30 lux) with blackened walls for a 10-min habituation after which time they received an intraplantar 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 (Finn *et al.*, 2003; Roche *et al.*, 2007). Rats were returned to their home cage for a further 3 min, at which point they were returned to the same perspex chamber for a period of 30 min. Rats were killed by decapitation after the 30-min trial, brains removed rapidly, snap-frozen on dry ice and stored at –80°C until subsequent molecular and neurochemical analyses of components of the PPARα signalling system in the mPFC. The 30-min post-formalin time point was chosen based on the results of experiment 1 where the effects of pharmacological modulation of PPARα were evident within the first 30 min of the formalin trial.

Cryosectioning and punch microdissection

Frozen coronal brain sections (300 µm thickness) at the level of the mPFC [AP + 3.7 to –1.0 mm, based on rat brain atlas of Paxinos and Watson (2006)], were cut on a cryostat (MICROM GMBH, Stuttgart, Germany). The mPFC was then punch-dissected from sections using 2 mm cylindrical brain punchers (Harvard Apparatus, Holliston, MA, USA) as described previously (Ford *et al.*, 2008; Olango *et al.*, 2011). Punched mPFC tissues were weighed and processed for measurement of PPARα and CB_1 mRNA expression by quantitative real-time PCR (qRT-PCR), PPARα protein expression by western blotting or quantification of PEA, OEA and AEA levels by LC-MS/MS. The average weight per rat of the punch-dissected mPFC tissue was 10 ± 2.0 mg.

qRT-PCR

Total RNA was extracted from post-mortem mPFC tissue using a Machery-Nagel extraction kit (Nucleospin RNA II, Technopath, Dublin, Ireland) according to manufacturer's instructions and as described previously (Kerr *et al.*, 2012; 2013). RNA quality (1.8-2.0 as determined by λ260/λ280 ratio) and quantity were assessed using a Nanodrop spectrophotometer (ND-1000, Nanodrop, Labtech International, Uckfield, UK) and equalized to a concentration of 5 ng μL^{-1}. A total of 50 ng 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, Warrington, UK) or rat CB_1 receptor (assay ID Rn00562880_m1, Applied Biosystems) containing forward and reverse primers and a FAM-labelled MGB Taqman probe was used to quantify PPARα or CB_1 receptor mRNA on a '*StepOne plus* instrument (Applied Biosystems). VIC-labelled *GAPDH* (assay ID 4308313 Applied Biosystems) was used as the house keeping 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 $2^{-\Delta Ct}$, 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 saline-treated control group.

Western blotting

Western blotting was performed according to methods described by Okine *et al.* (2012) with minor modifications. Briefly, frozen punches of PFC weighing approximately 10 mg were lysed by brief (3 s) sonication in RIPA lysis buffer [150 mM NaCl, 25 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 1 mM Na_3VO_4, 10 mM NaF containing 1% protease inhibitor cocktail (Sigma-Aldrich)] at a ratio of 1:10 (w v^{-1}) in a 1.5 mL microcentrifuge tube and homogenate placed on a shaker for 45 min at 4°C with gentle agitation to allow for complete dissociation of nucleoprotein complexes and centrifuged at 16168 *g* (Eppendorf Centrifuge 5415R, Stevenage, UK) for 20 min at 4°C. The supernatant was collected and protein content determined by Bradford assay (Bradford, 1976). A total of 36 µg of protein sample in 4X loading buffer [25% v v^{-1} 1 M Tris HCl pH 6.8, 5 % w v^{-1} SDS, 20% v v^{-1} glycerol, 2.5% bromophenol blue (0.2% w v^{-1} in 100% ethanol), 20% v v^{-1} of 2-mercaptoethanol and made up to total volume of 20 mL in distilled H_2O] was boiled at 100°C for 5 min, briefly centrifuged and subjected to 9% SDS-PAGE electrophoresis at a constant voltage of 120 mV for 2 h. 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 h. Protein transfer efficiency was verified by Ponceau dye (0.1% 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 h at room temperature and incubated with goat polyclonal antibody to PPARα (1:200, cat# sc1985, Santa Cruz Biotechnology, Heidelberg, Germany) and mouse monoclonal antibody to β-Actin (1:10 000, A5441 Sigma-Aldrich) diluted in 5% milk/0.05% TBST overnight at 4°C. Membranes were subjected to 3 × 10 min washes in 0.05% TBST and incubated with secondary antibody solution containing IRDye® conjugated donkey anti-goat (λ800) and donkey anti-mouse (λ700) (LI-COR® Biosciences, Abingdon Park, Oxford, UK) diluted 1:10 000 in 1% milk/ 0.05% TBST for 1 h. Three washes of 5 min duration were performed, followed by one final 5 min wash in distilled H_2O. Blots were scanned on a LI-COR Odyssey imager. Infrared band intensities for PPARα protein expression (~52 kDa) 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 PPARα intensity to β-actin intensity was then calculated for each sample, and then expressed as a % of mean saline-treated group values.

Quantification of PEA, OEA and AEA in mPFC tissue using LC-MS/MS

Measurement of PEA, OEA and AEA levels in the mPFC using LC-MS/MS was essentially as described previously (Ford *et al.*,

2008; Olango *et al.*, 2011; Kerr *et al.*, 2012; 2013; Rea *et al.*, 2013). Briefly, samples were homogenized in 400 μL 100% acetonitrile containing deuterated internal standards (0.016 nmol PEA-d4, 0.015 nmol OEA-d2 and 0.014 nmol AEA-d8). Lyophilized samples were resuspended in 40 μL 65% acetonitrile and separated on a Zorbax® C18 column (150 × 0.5 mm internal diameter; Agilent Technologies Ltd, 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, OEA and AEA eluted at the following retention times: 14.4, 15.0 and 11.4 min respectively. Analyte detection was carried out in electrospray-positive ionization and multiple reaction monitoring mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd). Quantification of each analyte was performed by ratiometric analysis and expressed as nmol or pmol g^{-1} of tissue. The limit of quantification was 1.5, 1.4 and 1.32 pmol g^{-1} for PEA, OEA and AEA respectively.

Data analysis

SPSS statistical software (IBM House, Dublin, Ireland) was used to analyse all data. Pre-formalin behavioural data were analysed by one-way ANOVA followed by Fisher's least significant difference (LSD) *post hoc* test if normally distributed (distance moved and duration of grooming) or by Kruskal–Wallis test followed by Mann–Whitney *U*-tests if not normally distributed (rearing duration and defecation). Formalin-evoked nociceptive behaviour (CPS) data were analysed by repeated measures ANOVA with treatment as the between-subjects factor and time as the within-subjects factor. Pairwise group comparisons at discrete time points were made using Fisher's LSD *post hoc* test where appropriate. Student's unpaired, two-tailed *t*-test was used to analyse PEA, OEA and AEA levels and PPARα mRNA and protein or CB_1 mRNA expression in the mPFC. Data were considered significant when $P < 0.05$. Results are expressed as group means ± SEM.

Results

Experiment 1

Histological verification of intracerebral microinjection sites. Ninety per cent of the injectors were placed within the borders of the mPFC (Figure 1, defined in this experiment as Cg1, the cingulate cortex area 1; Cg2, cingulate cortex area 2 and PrL, prelimbic cortex) with the remaining 10% positioned in the secondary motor cortex or the genu of the corpus callosum on one or both sides. Only the results of the experiments in which both injections were correctly positioned in the mPFC were included for analyses. The final *n* numbers per group were Vehicle, $n = 8$; GW7647, $n = 7$; GW6471, $n = 7$. For off-target experiments, all of the cannulae were successfully placed outside the borders of the mPFC, with 75% of those specifically placed within the genu of the corpus callosum, results of which are presented in this manuscript. The final *n* numbers per group for the off-target experiments were $n = 6$ per treatment group.

Effects of intra-mPFC administration of GW6471 and GW7647 on behaviour prior to formalin injection. The effects of GW6471 and GW7647 on total distance moved, duration of rearing and grooming and number of faecal pellets excreted during the 10 min pre-formalin trial were determined. No significant differences between the treatment groups were observed for any of these behaviours (Table 1).

Effects of intra-mPFC administration of GW7647 and GW6471 on formalin-evoked nociceptive behaviour. The effects of intra-mPFC injection of the selective PPARα agonist GW7647 and the selective PPARα antagonist GW6471 on formalin-evoked nociceptive behaviour in rats were determined. Intraplantar injection of formalin was associated with biphasic nociceptive behaviour characterized by robust licking, flinching, biting and elevation of the right hindpaw. The early phase (5–10 min) and late phase (20–90 min) marked two distinct periods of this formalin-evoked nociceptive behaviour (expressed as CPS) in vehicle-treated rats (Figure 2A). Repeated measures ANOVA revealed a significant effect of time ($F_{(17, 323)} = 6.065$, $P < 0.05$) but not treatment ($F_{(2,19)}$, 0.101, $p > 0.05$) or time × treatment interaction ($F_{(34,323)} = 1.317$, $P > 0.05$) over the 90-min trial period. *Post hoc* analysis by Fisher's LSD indicated that intra-mPFC administration of the selective PPARα antagonist GW6471 was associated with a significant ($P < 0.05$) delay in the onset of the second phase of formalin-evoked nociceptive behaviour from 15 to 25 min, compared with vehicle-treated controls (Figure 2A). In contrast, off-target injection of the selective PPARα receptor antagonist GW6471 into the corpus callosum had no significant effect on formalin-evoked nociceptive behaviour (Figure 2B). Intra-mPFC administration of GW6471 or GW7647 had no significant effect on formalin-induced right hindpaw oedema as determined by measurements of change in paw diameter post-formalin injection (Vehicle: 1.9 ± 0.247 mm, GW6471: 1.8 ± 0.155 mm, GW7647: 1.77 ± 0.196 mm).

Experiment 2

Effects of intraplantar injection of formalin on levels of endogenous PPARα ligands and PPARα mRNA and protein or CB_1 mRNA expression in the rat mPFC. The effects of formalin treatment on components of the PPARα signalling system in the rat mPFC were determined. LC-MS/MS revealed significant reductions in tissue levels of the endogenous ligands, PEA and OEA ($P < 0.05$), in rats injected with formalin, compared with saline-treated rats (Figure 3). Furthermore, intraplantar formalin injection was associated with a significant reduction ($P < 0.05$) in PPARα mRNA but not protein expression in the mPFC compared with saline-treated counterparts (Figure 4). Formalin injection had no significant effects on levels of AEA (Figure 3) or CB_1 mRNA in the mPFC (Figure 4), compared with saline-treated controls.

Discussion

The results of the present study demonstrate that pharmacological blockade of PPARα in the mPFC significantly delays the onset of the second phase of formalin-evoked nociceptive behaviour in rats. These effects were not observed following

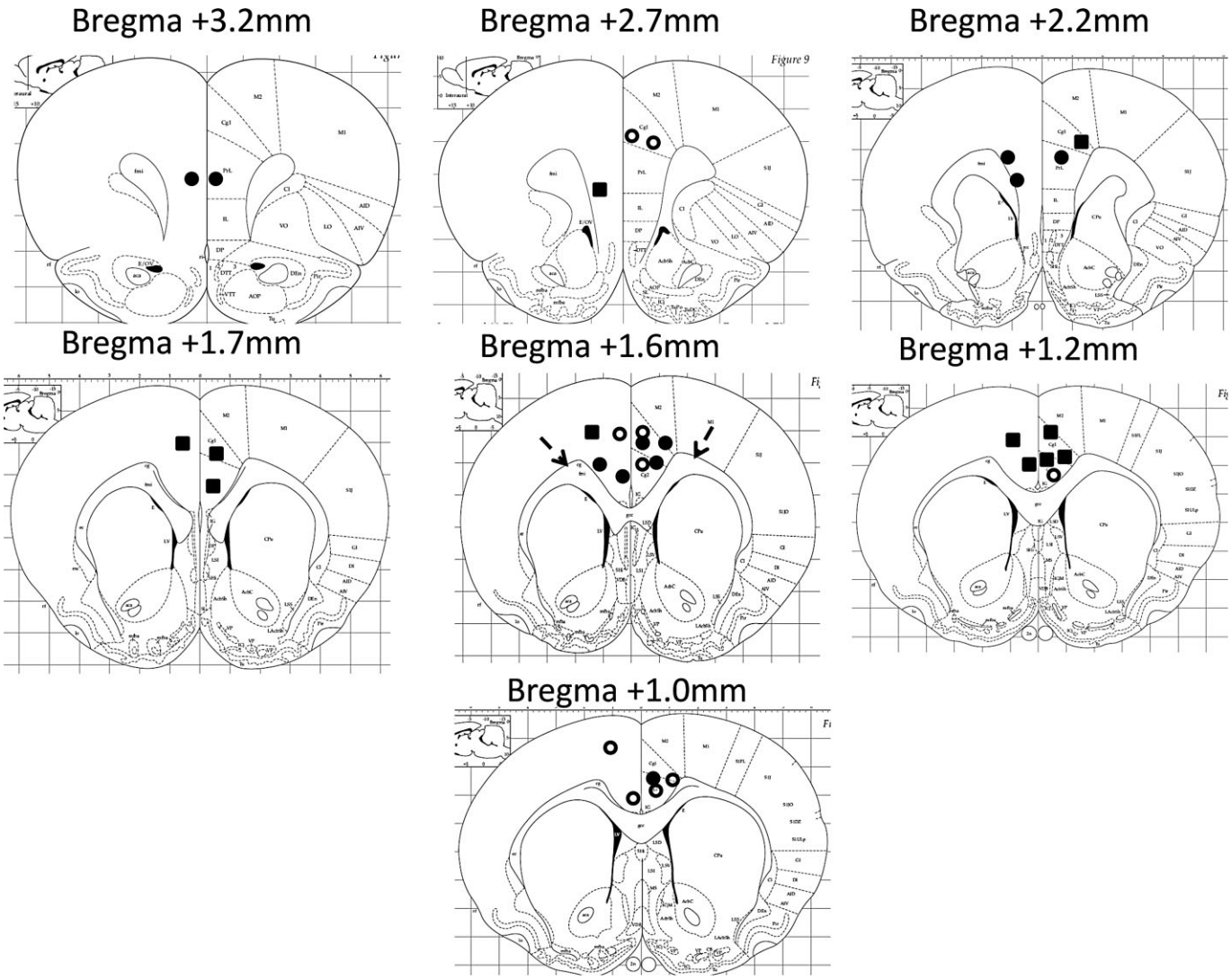

Figure 1

Schematic depicting of the sites of the bilateral injector placement in the rat medial prefrontal cortex (mPFC). Rats received microinjections of either vehicle (open circles) or GW6471 (closed circles) or GW7647 (closed squares) bilaterally into the mPFC. Bilateral off-target injection sites are indicated by black arrows. Images were taken from the rat brain atlas of Paxinos and Watson (2006).

Table 1

Summary of preformalin distance moved, rearing, grooming and defecation

Treatment	Distance(cm)	Rearing duration (s)	Grooming duration (s)	Defecation
Vehicle	128.0 ± 20.2	34.2 ± 7.1	64.1 ± 18.08	1.7 ± 0.8
GW6471	94.9 ± 8.9	35.6 ± 15.8	40.7 ± 8.6	1.8 ± 0.8
GW7647	122.9 ± 13.4	37.1 ± 14.3	46.3 ± 11.24	2.5 ± 1.2

No significant differences observed between groups. Data expressed as mean ± SEM, $n = 8$ rats per group for vehicle and $n = 7$ rats per group for GW6471 or GW7647.

administration of the antagonist into the corpus callosum which has significant PPARα expression (Moreno *et al.*, 2004). In contrast, pharmacological activation of PPARα in the mPFC had no significant effect on formalin-evoked nociceptive behaviour. Intraplantar injection of formalin resulted in a significant reduction in levels of the endogenous PPARα ligands, PEA and OEA, and a concomitant reduction in the expression of the gene coding for PPARα, but no change in

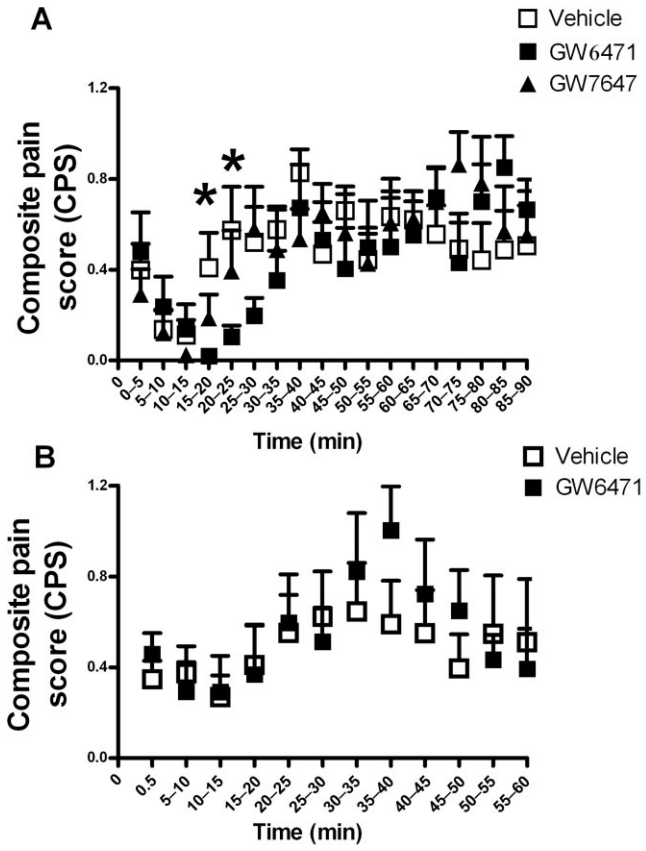

Figure 2

The effects of pharmacological modulation of PPARα signalling in the mPFC on formalin-evoked nociceptive behaviour in rats. (A) Time-course of formalin-evoked nociceptive behaviour in rats treated with intra-mPFC vehicle, GW7647 (10 μg) or GW6471 (10 μg). (B) Off-target injection of GW6471 into the corpus callosum did not alter formalin-evoked nociceptive behaviour compared with vehicle-treated rats. Data are means ± SEM. *$P < 0.05$ for GW6471 versus vehicle, two-way repeated measures ANOVA with Fisher's LSD *post hoc* test. (A) Vehicle $n = 8$, GW6471 $n = 7$, GW7647 $n = 7$ rats per group; (B) $n = 6$ rats per group.

PPARα protein expression, in the mPFC. The formalin-induced reductions in levels of PEA and OEA demonstrate for the first time that inflammatory pain state is associated with a reduction in endogenous PPARα signalling at the level of the mPFC and that pharmacological blockade of PPARα signalling within the mPFC can delay the onset of second phase formalin-evoked nociceptive behaviour.

Our data on the effects of pharmacological modulation of PPARα suggest that blockade of the receptor at the level of the mPFC attenuates formalin-evoked pain responding, while stimulation of this receptor in the mPFC has no effect. Previous studies have demonstrated that activation of supraspinal PPARα results in reduced pain responding (D'Agostino *et al.*, 2009; De Novellis *et al.*, 2012). However, these studies employed different routes of administration and different models to that used in the present study. For example, D'Agostino *et al.* (2009) investigated the effects of i.c.v. administration of the endogenous PPARα agonist PEA on mechanical hyperalgesia in the mouse carrageenan model of inflammatory pain. The study by De Novellis *et al.* was based on local intraventrolateral periaqueductal grey administration of PEA in rats which resulted in increased latency of nociceptive response in the tail-flick test and a reduction of ON/OFF neuronal cell firing in the RVM. However, the role of PPARα in the mPFC in pain responding has not been studied and there are no reasons to believe that the effects of PPARα modulation that result from i.c.v. or intra-periaqueductal grey PAG administration of agonists would be the same as those resulting from intra-mPFC administration. Indeed, one might expect the effects of intracerebral administration of PPARα modulators to be region-dependent given (i) the differential distribution and expression of PPARα throughout the brain (Braissant *et al.*, 1996; Moreno *et al.*, 2004), (ii) differences in the neuronal subtypes and circuitry between the mPFC and other brain regions (Ryan *et al.*, 2013) and (iii) evidence for differential roles of the mPFC versus the PAG and other brain regions in pain responding and its regulation (Zaki *et al.*, 2007; Etkin *et al.*, 2010; Duncan *et al.*, 2013). Indeed, it is interesting to note that PPARα in the PAG (De Novellis *Et Al.*, 2012) and mPFC (present study) may have opposing roles in the

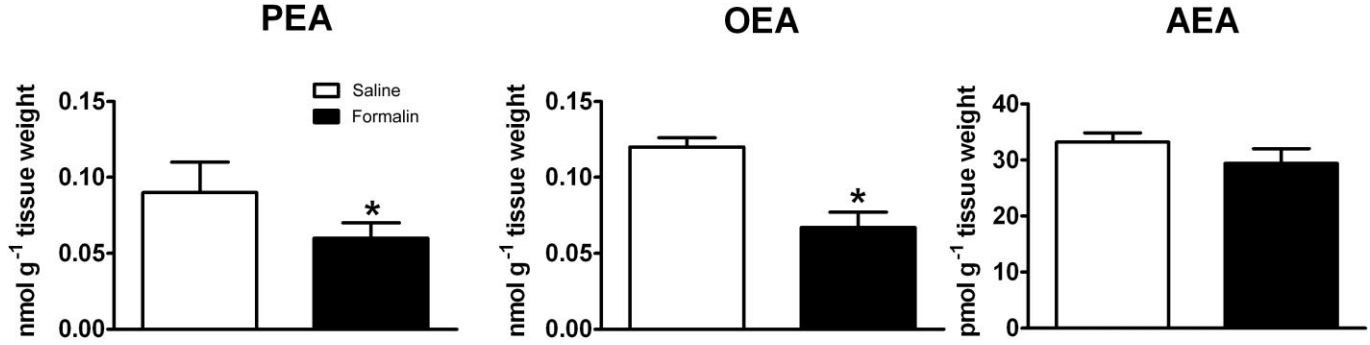

Figure 3

Intraplantar injection of formalin results in a significant reduction in tissue levels of the endogenous PPARα ligands, *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA), but not endocannabinoid anandamide (AEA), in the medial prefrontal cortex, compared with saline-treated controls. Data are means ± SEM. *$P < 0.05$ versus saline-treated controls, Student's unpaired *t*-test. $n = 6$ for all groups except AEA in saline-treated rats where $n = 4$.

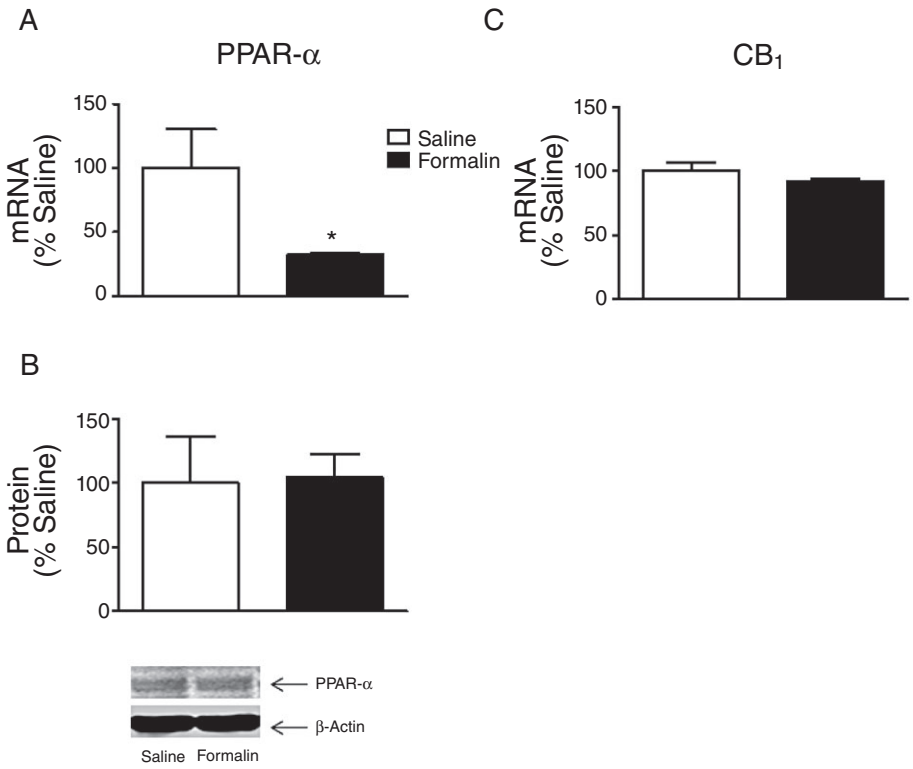

Figure 4

Intraplantar injection of formalin results in a significant reduction in (A) PPARα mRNA but not (B) PPARα protein or (C) CB_1 mRNA expression in the medial prefrontal cortex compared with saline-treated controls. Data are mean percentage of saline-treated controls ± SEM. *$P < 0.05$ versus saline-treated rats, Student's unpaired *t*-test, $n = 5$ (saline) or 6 (formalin) rats per group for mRNA data and $n = 6$ rats per group for Western blot data.

regulation of nociceptive behaviour, in a manner akin to that reported for transient receptor potential cation channel subfamily V member 1 (TRPV1) in the PAG (Starowicz *et al.*, 2007) versus the mPFC (Giordano *et al.*, 2011). Moreover, OEA and PEA are capable of activating both PPARα and TRPV1.

One possible interpretation of our data is that PPARα activation in the mPFC mediates, permits or facilitates, but does not modulate, formalin-evoked nociceptive behaviour. Such an interpretation is compatible with our findings that PPARα activation in this brain region was without effect (lack of modulatory role) while PPARα blockade delayed the onset of second phase formalin-evoked nociceptive behaviour (suggesting that endogenous ligands may act normally at PPARα in the mPFC to mediate/facilitate nociception). In this context, the formalin-evoked reductions in tissue levels of the endogenous PPARα ligands PEA and OEA in the mPFC may represent a protective response whereby the organism responds to the noxious inflammatory insult of formalin injection with a reduction in PPARα tone within the mPFC, which, in turn, serves to reduce/delay nociceptive responding. The second phase of formalin-evoked nociceptive behaviour is largely driven by peripheral and central inflammatory processes leading to central sensitization. Thus, the delayed onset of this second phase following administration of the antagonist suggests a permissive or facilitatory role for PPARα in the mPFC in these processes. The formalin-induced reduction in PPARα mRNA levels was not reflected at the level of PPARα protein expression measured at the same time point (30 min post-formalin). It is possible, however, that the formalin-induced reduction in PPARα mRNA has functional consequences for the animal at later time points and may be reflected at the level of protein expression at later time points also.

The inflammatory pain-related reductions in PEA and OEA signalling observed in the present study are also consistent with findings reported in the carrageenan model of inflammatory pain. Intraplantar injection of the proinflammatory substance λ-carrageenan was associated with reductions in levels of endogenous PPARα ligands in the rat hindpaw (Jhaveri *et al.*, 2008) and reduced PPARα receptor expression in the mouse spinal cord (D'Agostino *et al.*, 2009). These reductions were, however, associated with enhanced nociceptive responding which was reversed by restoration of PPARα tone via administration of PPARα agonists or inhibition of the hydrolytic enzyme, fatty acid amide hydrolase, which is primarily responsible for the degradation of most bioactive fatty acid amides including PEA, OEA and AEA. These findings, when considered together, reveal dynamic alterations of the PPARα signalling system in response to inflammatory pain stimuli, and suggest that the functional consequences of these inflammatory pain-induced reductions in PPARα signalling tone may be tissue-dependent. Thus, a decrease in peripheral and spinal PPARα signalling

tone may favour nociceptive responding whereas a decrease at the level of the mPFC may serve to counter nociceptive input and transmission within the CNS. The significant involvement of PPARα signalling in the mPFC is further supported by the lack of formalin-induced changes in the levels of AEA or CB_1 mRNA expression in this brain region in the present study. Moreover, neither the antagonist nor the agonist had any significant effects on distance moved, rearing, grooming or defecation during the 10-min pre-formalin trial, suggesting that drug effects on locomotor/general activity were not confounding factors in this study.

Formalin-evoked paw oedema was not significantly affected by intra-mPFC administration of either the PPARα agonist or the antagonist. Previous studies have shown that i.c.v. injection of the PPARα agonists PEA and perflouroacetic acid reduce carageenan-induced paw oedema in rats (Taylor *et al.*, 2005; D'Agostino *et al.*, 2007), suggesting that PPARα in the brain may have a role in controlling peripheral inflammation although the precise mechanisms or supraspinal sites of action remain unknown. The results of the present study suggest that such effects are unlikely to be mediated at the level of the mPFC.

In conclusion, the results presented here indicate that reduced PPARα signalling in the mPFC accompanies formalin-evoked nociceptive behaviour and that pharmacological blockade of PPARα in the mPFC delays the onset of the second phase of the formalin test. Taken together, these data suggest that PPARα in the mPFC may play a permissive or facilitatory role in formalin-evoked nociceptive responding. These data provide a foundation for future studies aimed at elucidating the precise neurochemical and molecular mechanisms underpinning pain regulation by PPARα in the mPFC and other supraspinal regions and for studies aimed at investigating a potential role for mPFC PPARα in the cognitive-affective dimension of pain.

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Conflict of interest

None.

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