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N-palmitoylethanolamide in the anterior cingulate cortex attenuates inflammatory pain behaviour indirectly via a CB₁ receptor-mediated mechanism

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ABSTRACT

The neural substrates and mechanisms mediating the antinociceptive effects of the endogenous bioactive lipid, *N*-palmitoylethanolamide (PEA), require further investigation. We investigated the effects of exogenous PEA administration into the anterior cingulate cortex (ACC), an important brain region linked with cognitive and affective modulation of pain, on formalin-evoked nociceptive behaviour in rats. Potential involvement of peroxisome proliferator activated receptor isoforms (PPAR) α and γ or endocannabinoid-mediated entourage effects at cannabinoid$_1$ (CB$_1$) receptors or transient receptor potential subfamily V member 1 (TRPV1) in mediating the effects of PEA was also investigated. Intra-ACC administration of PEA significantly attenuated the first and early second phases of formalin-evoked nociceptive behaviour. This effect was attenuated by the CB$_1$ receptor antagonist AM251, but not by the PPARα antagonist GW6471, the PPARγ antagonist GW9662, or the TRPV1 antagonist 5'-iodo resiniferatoxin. All antagonists, administered alone, significantly reduced formalin-evoked nociceptive behaviour, suggesting facilitatory/permissive roles for these receptors in the ACC in inflammatory pain. Post-mortem tissue analysis revealed a strong trend for increased levels of the endocannabinoid anandamide in the ACC of rats that received intra-ACC PEA. Expression of *c-Fos,* a marker of neuronal activity, was significantly reduced in the basolateral nucleus of the amygdala, but not in the central nucleus of the amygdala, the rostral ventromedial medulla or the dorsal horn of the spinal cord. In conclusion, these data indicate that PEA in the ACC can reduce inflammatory pain-related behaviour, possibly via AEA-induced activation of CB$_1$ receptors and associated modulation of neuronal activity in the basolateral amygdala.

Keywords: Pain; *N*-palmitoylethanolamide; prefrontal cortex; anandamide; cannabinoid; PPAR alpha; PPAR gamma; TRPV1

List of Abbreviations

5-IRTX: 5-iodo resiniferatoxin

ACC: Anterior cingulate cortex

AEA: *N*-arachidonoylethanolamide (anandamide)

AM251: *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide

BLA: basolateral amygdala

DH: dorsal horn of the spinal cord

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)

GW9662: 2-Chloro-5-nitro-*N*-phenylbenzamide

OEA: *N*-oleoylethanolamide

PEA: *N*- palmitoylethanolamide

PPAR: peroxisome proliferator-activated receptor

RVM: rostral ventromedial medulla

TRPV1: transient receptor potential subfamily V member 1

1. INTRODUCTION

The antinociceptive effects of the endogenous fatty acid amide, *N*-palmitoylethanolamide (PEA), in the peripheral and central nervous systems, have been demonstrated in numerous studies employing animal models of inflammatory and neuropathic pain [6; 9-11; 20; 28]. These findings are also consistent with the anatomical expression of the primary endogenous target of PEA, specifically the peroxisome proliferator-activated receptor (PPAR)α, in key peripheral and central structures involved in pain processing [3; 11; 28; 36]. We previously reported that endogenous ligands of PPARα, including PEA, in the rat anterior cingulate cortex (ACC), are reduced during the expression of formalin-evoked nociceptive behaviour [36]. Given the key role of the ACC in pain processing and modulation [17; 25; 26; 46], and the widely documented antinociceptive properties of PEA [6; 9-11; 20; 28], herein we investigated the effects of intra-ACC administration of PEA on formalin-evoked nociceptive behaviour in rats.

The precise mechanisms underpinning the antinociceptive effects of PEA are not well understood. However, these effects occur rapidly, in a manner that is not consistent with transcription-dependent or genomic mechanisms, even if contingent upon PPARα expression [28]. The suggestion of a non-genomic mechanism of action is also consistent with an entourage hypothesis, which proposes that the pharmacological effects of PEA may be due to the indirect involvement of locally enhanced anandamide (AEA) signalling acting at endocannabinoid receptor targets [13; 27]. The biochemical evidence for the entourage hypothesis is based on the fact that both PEA and AEA are endogenous substrates of the enzyme, fatty acid amide hydrolase (FAAH) [8; 16; 43]. In this regard, increased PEA levels could competitively inhibit FAAH-mediated hydrolysis of AEA, resulting in enhanced AEA signalling at the cannabinoid$_1$ (CB$_1$) receptor [14], the non-selective cation channel, transient receptor potential subfamily V member 1, (TRPV)1 [13], or PPARγ [2]. In addition to PPARα

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[36], CB$_1$[45], TRPV1[44] and PPARγ [3] are all expressed within the ACC. However, their contribution to the effects of PEA on nociceptive processing within the ACC is unknown.

The primary aims of the present study were to investigate the effects of direct administration of PEA into the ACC on formalin-evoked nociceptive behaviour in rats, and the receptor mechanisms involved. We specifically investigated if there was direct involvement of PPARα in the effects of PEA, or indirect contributions of CB$_1$, PPARγ and TRPV1, mediated by entourage effects of PEA on AEA. In order to better understand the neural circuitry mediating the effects of PEA in the ACC, we also measured expression of mRNA coding for *c-Fos*, a marker of neuronal activity, in key brain regions associated with nociceptive processing downstream of the ACC, in particular the amygdala, rostral ventromedial medulla (RVM) and the dorsal horn of the spinal cord (DH).

2. METHODS

2.1 Animals

Two cohorts of adult male Sprague-Dawley rats of n=44 (Experiment 1) and n=88 (Experiment 2), weighing 225-250 g, were obtained from Harlan (Bicester, UK). Animals were housed in groups of three to four per cage prior to surgery and singly housed post-surgery in plastic bottomed cages (45x20x20 cm) containing wood shavings as bedding. Animal housing rooms were maintained at a constant temperature (21 °C ± 0.5 °C) under standard lighting conditions (12:12 h light : dark, lights on from 07:00 to 19:00 h). Food and water were provided ad libitum. All *in vivo* procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland, Galway, and carried out 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 [24].

2.2 Intra-ACC guide cannulae implantation

Implantation of guide cannulae was carried out as previously described [36]. In brief, rats were implanted stereotaxically with stainless steel guide cannulae (5 mm length, Plastics One Inc, Roanoke, VA, USA), bilaterally 1 mm above the ACC (anteroposterior + 1.5 mm, mediolateral ± 1.3 mm relative to bregma at an angle of 12°, dorsoventral – 1.3 mm from dura) (Figure 1), under isoflurane anaesthesia (1-3 % in O2 ; 0.60 L/min). 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 each guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent carprofen (5mg/kg subcutaneously; Rimadyl, Pfizer, Kent, UK), was administered during surgery to manage postoperative pain. The antibiotic enrofloxacin (2.5 mg/kg subcutaneously; Baytril, Bayer Ltd., Dublin, Ireland) was administered on the day of surgery and for 3 consecutive days after surgery to prevent infection. Rats were allowed a period of 7 days to recover from surgery before formalin testing. Body weight and general health were monitored daily during the recovery period. The surgical procedures described here were also employed for off-target control experiments to determine the effects of intra-basolateral amygdala (BLA) administration of the antagonists used (anteroposterior + 2.8 mm, mediolateral ± 4.8 mm relative to bregma, dorsoventral – 7.5 mm from skull surface) using 12 mm guide cannulae (Plastics One Inc, Roanoke, VA, USA), stereotaxically implanted bilaterally, 1mm above the target region.

2.3 Chemicals and drug preparation

N-palmitoylethanolamide (PEA), the PPARα antagonist, GW6471, the CB$_1$ receptor antagonist AM251, the PPARγ antagonist, GW9662, and the TRPV1 antagonist, 5'-iodo resiniferatoxin (5'-IRTX) (Tocris Bioscience, Bristol, UK) were administered in 100 % dimethylsulfoxide (DMSO). Drug solutions were aliquoted and stored at -20 °C until the day of injection. Drug doses (Table 1) were chosen based on studies in the literature [11; 12; 20; 33; 36; 40] and a study in our laboratory demonstrating that the antagonists, administered alone at these doses, had no effect on formalin-evoked nociceptive behaviour when injected directly into the BLA (Supplementary Digital Content Figure S1). On the day of testing, stock drug aliquots were thawed, diluted to the required concentrations and kept at room temperature prior to injection. Rats receiving coadministration of PEA with an antagonist received a mixture of both drugs in a single injection. Formalin (2.5 %) was prepared from a 37 % stock solution (Sigma-Aldrich, Dublin, Ireland) diluted in 0.9 % sterile saline.

2.4 Intra-ACC microinjection

In Experiment 1, we investigated the role of PPARα, the primary receptor target for PEA, in mediating the effects of PEA administered directly into the ACC on formalin-evoked nociceptive behaviour. In Experiment 2, the potential involvement of endocannabinoid targets CB$_1$, PPARγ and TRPV1, per the entourage hypothesis, in mediating the effects of PEA on formalin-evoked nociceptive behaviour was studied in another cohort of rats. In both Experiments 1 and 2, drugs were microinjected into the ACC over 60 seconds (with injectors left in place for an additional 60 seconds to allow diffusion of drug away from the injection site) using an injector and Hamilton syringe attached to polyethylene tubing. Immediately thereafter, rats were placed in a Perspex chamber (30x30x40 cm, 30 lux) with blackened walls for the assessment of locomotor activity (distance moved, duration of rearing and grooming)

for 10 minutes. Rats were randomly assigned to each treatment group. In addition to this, the sequence and timings of drug treatment were randomized equitably across the experimental days to minimise their potential influence as a source of variability.

2.5 Formalin test

In both Experiment 1 and 2, rats received an intra-plantar injection of 50 µL formalin (2.5% in 0.9% sterile saline) into the right hind paw under brief isoflurane anaesthesia as described previously [36; 37; 40; 41], 10 minutes after intra-ACC drug administration (i.e. immediately after the 10 minute assessment of locomotor activity). All injections and animal handling were carried out by a male experimenter. Following formalin injection, rats were immediately returned to the perspex chamber for a period of 60 minutes. A video camera located beneath the chamber was used to record animal behaviour onto DVD for subsequent analysis. Post-formalin behaviours for each rat were recorded for 60 minutes and rated as described below. At the end of the 60-minute formalin trial, rats were killed by decapitation, brain and spinal cord tissues harvested and snap-frozen for post-mortem analysis of c-*Fos* mRNA in the amygdala (central and basolateral nuclei), RVM and DH (Experiment 1), or levels of endocannabinoids and *N*-acylethanolamines in the ACC (Experiment 2) of PEA-treated rats.

2.6 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 described by [47]. 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. Locomotor activity (distance moved, duration of rearing and grooming) during the 10 minute pre-formalin trial were also assessed with aid of Ethovision XT software.

2.7 Histological verification of intra-cerebral microinjection sites

Intra-cerebral microinjection sites were verified by bilateral injections of fast-green dye into the ACC post mortem. Representative cryosections of the ACC (30 µm thickness) were collected and mounted on gelatinised glass slides, stained with cresyl violet, and viewed under light microscope for precise verification of cannula / injector placement in the ACC. Only rats with the correct placement sites (~81% of total number of rats in both experiments) were included in final analysis of behavioural data (Figure 1). The majority of misplaced guide cannulae were located within the primary motor cortex area 2 (M2) sub-region which borders the ACC. Vehicle- (n=6) and PEA- (n=5) treated rats from Experiments 1 & 2 with microinjections in this M2 region were pooled together and analysed as out-of-ACC placement controls. The remainder of the ACC and other brain regions were cryosectioned and punched for measurement of c-*Fos* mRNA expression (Experiment 1) and endocannabinoid and N-acylethanolamine levels (Experiment 2) as described below.

2.8 Cryo-sectioning and punch microdissection

Frozen coronal brain sections (150 µm thickness) at the level of the ACC (AP +3.7 to -1.0 mm, based on rat brain atlas of [39], were cut on a cryostat (MICROM GMBH, Stuttgart, Germany). The ACC was then punch-dissected from sections using 2 mm cylindrical brain punchers (Harvard Apparatus, Holliston, MA, USA) as described previously [36; 40]. In Experiment 1, for the measurement of c-*Fos* mRNA, the central and basolateral nuclei of the amygdala

(Bregma, -1.8 —-3.3 mm, ML= ±4.8 mm, DV= 8.5 mm) and the RVM (encompassing the gigantocellular reticularis nucleus, raphe magnus nucleus, medial lemniscus, raphe pallidus nucleus, pyramidal tracts, reticular nucleus and the trigeminothalamic tract; Bregma, -9.16 — -11.6 mm, ML± 0.0, DV=8.3 mm), were punch-dissected and processed for quantitative real time polymerase chain reaction (qRT-PCR), together with the DH. Punched ACC tissues from rats in Experiment 2 were processed for quantification of PEA, AEA, OEA, and 2-AG levels by LC-MS/MS. The mean ± standard error of mean (SEM) weight per rat of the punch-dissected ACC tissue was 10 ± 2.0 mg.

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

Total RNA was extracted from post-mortem amygdala (central and basolateral nuclei) and RVM and DH of rats in Experiment 1 and processed for cDNA synthesis as previously described [36; 40]. Taqman gene expression assay for rat c-*Fos* (assay ID - Rn 02396759_m1, Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probe was used to quantify genes coding for c-*Fos* mRNA on a 'StepOne Plus' instrument (Applied Biosystems, Warrington, UK). VIC-labelled 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 $2^{-\Delta Ct}$, where ΔCt represents the magnitude of the difference between cycle threshold (Ct) values for c-*Fos* and GAPDH for each sample. The $2^{-\Delta Ct}$, value for each sample was then expressed as a percentage of the mean $2^{-\Delta Ct}$ of vehicle-treated rats.

2.10 Quantitation of PEA, AEA, OEA, and 2-AG in ACC tissue using liquid chromatography— tandem mass spectrometry (LC-MS/MS)

Measurement of PEA, AEA, OEA, and 2-AG levels in the ACC of rats from Experiment 2 was carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS) essentially as described previously [36; 37; 40]. Briefly, samples were homogenized in 400 μL 100% acetonitrile containing deuterated internal standards (0.014 nmol AEA-d8, 0.48 nmol 2-AG-d8, 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, AEA, 2-AG, PEA, and OEA eluted at the following retention times: 11.4 min 12.9 min 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 or pmol g−1 of tissue. The limit of quantification was 1.32 pmol/g, 12.1 pmol/g, 1.5 pmol/g, 1.41 pmol/g for AEA, 2-AG, PEA, and OEA, respectively.

2.11 Data analysis

SPSS statistical software was used to analyse all data. Pre-formalin locomotor activity data (distance moved) and general rat behaviours (duration of rearing and grooming) were analysed by one-way ANOVA. Formalin-evoked nociceptive behaviour (CPS) data were analysed by one-way repeated measures ANOVA with treatment and time considered as between-subject and within-subject factors, respectively. Fisher's LSD post-hoc tests for pairwise comparisons were performed where appropriate. Student's unpaired, two-tailed t-test was used to analyse PEA, AEA, OEA and 2-AG levels in the ACC and c-*Fos* mRNA levels in the CeA, BLA and

RVM. c-*Fos* mRNA in the DH was analysed by two-way ANOVA with treatment and side (relative to formalin injection) as factors, followed by Fisher's LSD post-hoc test for pairwise comparisons where appropriate. Data were considered significant when p<0.05. Results are expressed as group means ± SEM.

3. RESULTS

The effects of PEA on formalin-evoked nociceptive behaviour were studied in two series of experiments. In Experiment 1, we investigated the role of PPARα, the primary receptor target for PEA, in mediating the effects of PEA administered directly into the ACC on formalin-evoked nociceptive behaviour. In Experiment 2, the potential involvement of endocannabinoid targets CB_1, PPARγ and TRPV1, per the entourage hypothesis, in mediating the effects of PEA on formalin-evoked nociceptive behaviour was studied in another cohort of rats.

3.1 Effects of microinjection of drug into the ACC on locomotor activity, rearing or grooming behaviour

Locomotor activity, rearing and grooming behaviours were assessed in all rats immediately following intra-ACC administration of drug or vehicle and prior to intraplantar formalin injection. Locomotor activity was not altered by drug treatment in either Experiment 1 or 2 (Table 2), compared with vehicle-treated controls. Similarly, no effects of pre-formalin drug treatment were observed on rearing behaviour in (Table 2). Grooming behaviour was significantly (*p*<0.05) increased following treatment with the PPARγ antagonist GW9662 *per se*, or in combination with PEA, compared with vehicle-treated rats (Table 2, Experiment 2).

3.2 Administration of PEA into the ACC attenuates first and early second phase formalin-evoked nociceptive behaviour

In Experiment 1 (Figure 2A), repeated measures ANOVA revealed significant main effects of treatment ($F_{(3,308)}$=3.2, $p<0.05$), time ($F_{(11,308)}$= 3.27, $p<0.001$) but not treatment X time interaction ($F_{(33,308)}$=1.23, $p>0.05$) on formalin-evoked nociceptive behaviour. Consistent with these findings, analysis of composite pain scores by repeated measures ANOVA in Experiment 2 (Figure 2B-D) also revealed significant treatment ($F_{(7,660)}$=3.52, $p<0.05$), time ($F_{(11,660)}$= 9.92, $p<0.05$) but not treatment X time interaction ($F_{(77,660)}$=0.80, $p>0.05$) effects. Compared with vehicle-treated controls, bilateral microinjection of PEA into the ACC in both experiments significantly ($p<0.05$) reduced formalin-evoked nociceptive behaviour during the first 25 minutes and from 30-35 minutes (in Experiment 2 only) of the formalin trial (Figure 2A-D).

The antagonists GW6471, AM251, GW9962 and 5'-IRTX were administered intra-ACC alone or co-administered with PEA to investigate the potential contribution of PPARα, CB_1, PPARγ and TRPV1, respectively, on the effect of PEA on formalin-evoked nociceptive behaviour. Co-administration with the CB_1 receptor antagonist/inverse agonist AM251 blocked PEA-induced reductions in formalin-evoked nociceptive behaviour, with this effect peaking and reaching statistical significance at 11-20 minutes post-formalin injection (Figure 2B). Furthermore, AM251 alone significantly ($p<0.05$) reduced formalin-evoked nociceptive behaviour when compared to vehicle-treated rats over this 11-20 minute period post-formalin (Figure 2B).

The PEA-induced reductions in formalin-evoked nociceptive behaviour were not blocked by co-administration with GW6471, GW9662 or 5'-IRTX, antagonists of PPARα, PPARγ and TRPV1 receptors, respectively (Figures 2A, 2C and 2D). These 3 antagonists did, however, significantly reduce ($p<0.05$) formalin-evoked nociceptive behaviour when administered

alone, compared with vehicle-treated counterparts (Figures 2A, 2C and 2D), with the antinociceptive effects of the PPARγ antagonist GW9662 being the most sustained over the duration of the formalin trial (Figure 2C). Analysis of data from out-of-ACC placement controls did not reveal any significant differences between vehicle and PEA-treated rats (Figure 2E-F).

In order to determine whether intra-ACC microinjection of the DMSO vehicle itself had any effect on formalin-evoked nociceptive behaviour, we collated data from previous studies carried out in our laboratory and performed a statistical analysis comparing composite pain scores of rats that received intra-ACC administration of 100% DMSO with those of naïve rats, for the first 30 minutes post-formalin, the period during which the majority of pharmacological effects were observed in the present study. Student's unpaired t-test revealed that there were no significant differences between the two groups of rats, suggesting strongly that intra-ACC administration of 100% DMSO has no effect on formalin-evoked nociceptive behaviour (Supplementary Digital Content Figure S2).

3.3 Increased levels of AEA in post-mortem ACC tissue from PEA-treated rats

There was a significant ($p<0.05$) increase in levels of PEA in the ACC of rats that had received bilateral microinjection of PEA, compared with vehicle-treated controls (Figure 3A). We also observed a robust increase in levels of AEA which just failed to reach statistical significance ($p=0.07$; Figure 3B). Levels of OEA (Figure 3C) and 2-AG (Figure 3D) were comparable between vehicle- and PEA-treated rats.

3.4 Administration of PEA into the ACC suppresses the induction of c-Fos mRNA following formalin administration in the basolateral amygdala but not in the CeA, RVM or dorsal horn of the spinal cord

Intra-ACC administration of PEA significantly (p<0.05) reduced levels of mRNA coding for *c-Fos* in the basolateral amygdala (BLA) (Figure 4), but had no effect on levels of *c-Fos* mRNA in the central nucleus of the amygdala (CeA), RVM or DH compared with vehicle-treated counterparts (Figure 4). For both the BLA and CeA, the ipsilateral and contralateral sides (relative to formalin injection) were initially analysed separately and the data subsequently pooled together following statistical analyses by Student's unpaired t-test which revealed no significant differences between the ispsilateral and contralateral sides in either the PEA- or vehicle treated rats.

4. DISCUSSION

The results of this study demonstrate that direct administration of the *N*-acylethanolamine PEA into the ACC rapidly reduces the first and early second phases of formalin-evoked nociceptive behaviour in rats, effects partially reversed by pharmacological blockade of CB_1 receptors. Antagonists at PPARα, PPARγ or TRPV1 in the ACC had no effect on the antinociceptive effects of PEA, but on their own reduced formalin-evoked nociceptive behaviour, suggesting key roles for these receptors in the ACC in facilitating inflammatory pain. Furthermore, the antinociceptive effects of PEA were associated with decreased *c-Fos* expression in the BLA, but not in the CeA, RVM or DH, suggesting involvement of brain circuitry involved in affective processing of pain rather than the descending pain pathway. Moreover, the lack of effect of PEA on pre-formalin locomotor activity or general rat behaviours including rearing or grooming, suggests that its effects on nociceptive behaviour were unlikely to be confounded

by overt effects on locomotor activity. In addition, the lack of effect of PEA injected "off-target" into the neighbouring M2 region on formalin-evoked nociceptive behaviour suggests that the effects of PEA were specifically mediated by the ACC.

The rapid antinociceptive effects of PEA in the present study are consistent with previous findings in animal models of inflammatory and neuropathic pain [28]. These rapid effects more likely reflect transcription-independent mechanisms, possibly mediated by AEA signalling at CB_1 receptors in the ACC given the effects of selective CB_1 receptor blockade on the antinociceptive effects of PEA. Our observation of a strong trend for increased levels of AEA in post mortem PEA-treated ACC tissue supports previous *in vitro* and *in vivo* reports of a role for PEA as an enhancer of AEA signalling [6; 13; 15; 21], and therefore a possible indirect mechanism underlying the rapid antinociceptive effects of PEA. The potential implication of increased AEA is that multiple receptor signalling pathways are likely to be activated concurrently in the ACC, given the promiscuous nature of AEA as an endogenous partial or full agonist acting at CB_1 receptors, TRPV1 and PPARs [2; 4; 5]. Evidence for both antinociceptive [23; 28; 32; 33; 37; 38; 40] and pronociceptive [12; 36] roles of these targets of AEA further complicate the issue. However, the results of the present study suggest that the early inhibitory effects of PEA on formalin-evoked nociceptive behaviour are primarily and preferentially mediated via AEA activation of CB_1 receptors in the ACC, since the CB_1 receptor antagonist AM251, but not the PPAR or TRPV1 antagonists, was able to reverse the effects of PEA on formalin-evoked nociceptive behaviour. One possible reason that might account, at least in part, for the preferential activation of the CB_1 receptor over PPARs or TRPV1 is access by AEA to the ligand-binding domain of the receptor. The extracellular ligand binding sites of the CB_1 receptor [1] may provide ready access for its activation by AEA, as opposed to the intracellular binding sites associated with the TRPV1 and PPARs [22; 35]. The relative expression of CB_1, TRPV1, PPARα and PPARγ in the ACC could also underlie the

preferential activation of the CB_1 over TRPV1, PPARα and PPARγ. Whilst the literature is replete with evidence of CB_1, TRPV1, PPARα and PPARγ expression in the ACC, to our knowledge, a direct comparison of their expression densities in the ACC has not been reported or studied. However, unpublished mRNA data based on cycle threshold (Ct) values from our laboratory reveal a relative expression order of CB_1>PPARα>TRPV1>PPARγ in the ACC. Thus, in addition to a readily accessible receptor activation binding site, the density of CB_1 relative to TRPV1, PPARα and PPARγ in the ACC may also contribute to the preferential activation of the CB_1 receptor in this region.

Evidence from previous studies suggests a possible co-localisation of CB_1 receptor [45] and [44] expression on pyramidal (glutamatergic) neurones in forebrain regions including the ACC. If AEA were to preferentially activate the CB_1 receptor over TRPV1 on glutamatergic neurones, then the expected outcome would be a reduction of glutamate-mediated neuronal hyperexcitability in the ACC. Moreover, the suggestion of a modulatory influence of PEA on glutamatergic neurones in the ACC is also consistent with more recent findings by Guida and colleagues, demonstrating a similar effect of systemically administered PEA on glutamatergic synapses in the medial prefrontal cortex in a mouse model of neuropathic pain [20]. However, CB_1 receptors are also prominently expressed on GABAergic interneurons in the prefrontal cortex [30]. In this instance, activation of CB_1 receptors on inhibitory GABAergic interneurones would result in the disinhibition of GABAergic synapses resulting in neuronal excitation within the ACC. Thus, the effects of PEA on formalin-evoked nociceptive behaviour are likely to reflect the net balance between the simultaneous AEA-mediated activation of CB_1 receptors on glutamatergic (inhibitory effects) and GABAergic (disinhibitory effects) neurones in the ACC. The antinociceptive effects of PEA were however not sustained over the entire duration of the formalin trial. This might reflect the dose-limiting effects of the drug or possibly

changes in pharmacokinetics and pharmacodynamics of the drug *in vivo* as a result of metabolism by endogenous hydrolases including FAAH [8].

Administration of the CB_1 receptor antagonist AM251 alone was associated with significant reductions in nociceptive behaviour at discrete time points of the formalin trial. These antinociceptive effects of AM251 are not particularly surprising given that similar antinociceptive effects of CB_1 receptor blockade have been reported in previous studies employing intracerebral [41] or systemic [7] administration of CB_1 receptor antagonists in animal models of inflammatory and neuropathic pain. The findings from the present study reveal a pronociceptive endogenous cannabinoid signalling tone in the ACC in inflammatory pain states which is blocked by AM251 administration. This mechanism may be physiologically relevant in the sense that it allows the animal to engage in escape or avoidance behaviour, consistent with the role of the ACC in supraspinal pain processing (For review see [17]). In the presence of PEA however, the AEA levels increase, likely through FAAH substrate competition, thus amplifying CB_1 receptor-mediated signalling with a consequent net antinociceptive effect as the outcome.

The results of the present study also demonstrated that pharmacological blockade of PPARα, PPARγ or TRPV1 in the ACC, independently or in the presence of PEA, reduced formalin-evoked-nociceptive behaviour in rats. These findings suggest that endogenous activation of both PPARs and TRPV1 in the ACC facilitate nociceptive responding during inflammatory pain states. To our knowledge, the present study is the first demonstration of a facilitatory role for PPARγ and TRPV1 in the ACC in inflammatory pain behaviour. We have previously reported a similar facilitatory role for PPARα in the ACC in formalin-evoked nociceptive behaviour [36] and our data for TRPV1 blockade support those of de Novellis and co-workers in a rat model of neuropathic pain [12]. Thus, while PPARα, PPARγ or TRPV1 do not appear to mediate the rapid antinociceptive effects of PEA in the ACC, endogenous tone at PPARα,

PPARγ or TRPV1 does play a key facilitatory/permissive role in the expression of formalin-evoked nociceptive behaviour. However differences in pharmacokinetic properties may account for the different durations of the antinociceptive effects of each antagonist following intra-ACC administration. None of the antagonists administered, had any effect on pre-formalin locomotor activity or rearing behaviour; although the PPARγ antagonist GW9662 increased grooming behaviour, the significance of which remains to be determined.

The ACC has strong anatomical connections with sub-nuclei of the amygdaloid complex [31]. Thus, it is feasible that modulation of neuronal activity in the ACC by PEA could in turn directly influence neuronal activity in the amygdala and downstream components of the descending pain pathway. This view is supported by our findings of reduced expression of the marker of neuronal activity, c-*Fos,* in the BLA, a key anatomical substrate for modulation of the affective component of pain [19; 29; 48], following the injection of PEA into the ACC. Interestingly, these effects of PEA were limited to the BLA and did not appear to extend to the adjacent central nucleus of the amygdala (CeA) or the RVM and spinal cord DH, components of the descending pain pathway. BLA neurones are activated by chemical somatic noxious stimulation, such as that evoked by intra-plantar injection of formalin, via the spino-thalamo-cortico-amygdaloid pathway [34; 42]. Taken together with other work implicating the ACC and amygdala in modulating the affective components of pain [18], these findings suggest that the modulation of the ACC-BLA neuronal circuitry may underlie the pharmacological effects of PEA. In this regard, the reduction in formalin-evoked nociceptive behaviours may represent a behavioural consequence of direct modulation of ACC-BLA circuitry by PEA.

In conclusion, our data suggest that the entourage effect of PEA on AEA-CB$_1$ receptor signalling in the ACC reduces inflammatory pain-related behaviour, possibly via modulation of neuronal activity in ACC-BLA circuitry and the affective component of pain rather than via recruitment of the descending inhibitory pain pathway.

Acknowledgements

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Table 1. Drugs, doses and group sample sizes.

Table 2. Pre-formalin locomotor activity, rearing and grooming duration in rats, *$p<0.05$ vs vehicle. Data are Mean $\pm$ SEM

Figure 1. Schematic depicting microinjection sites in **A)** Experiment 1 and **B)** Experiment 2 in the rat ACC. Images were taken from the rat brain atlas of Paxinos and Watson (2006) AP=+1.6mm.

Figure 2. The effects of intra ACC-administration of PEA and/or (A) PPARα antagonist GW6471, (B) CB$_1$ receptor antagonist AM251, (C) PPARγ antagonist GW9662, (D) TRPV1 antagonist 5'- IRTX on formalin-evoked nociceptive behaviour in rats. Data in panels B-D were part of an 8-group experiment (Experiment 2), which were analysed together but are presented separately for each antagonist for clarity of presentation. Data are means $\pm$ SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, veh vs PEA, #$p<0.05$, ##$p<0.01$, ###$p<0.001$ veh vs GW6471, AM251, GW9662 or 5-IRTX , ^$p<0.05$, PEA vs PEA+AM251, 2-way repeated measures ANOVA with Fisher's LSD post–hoc test. n = 7-10 rats per group. (E) and (F): Results of out-of-ACC Vehicle vs PEA control microinjections into the primary motor cortex area 2 (M2). Data are means $\pm$ SEM. n=6 rats for vehicle- and n=5 rats for PEA-treated groups.

Figure 3. LC-MS/MS analysis of PEA, AEA, 2-AG and OEA levels in the ACC. Levels of PEA were significantly higher in rats that received intra-ACC administration of PEA and there was a strong trend for an increase in levels of AEA, but not 2-AG or OEA in the ACC of PEA-treated rats. Data are means ± SEM. ** $p<0.01$ veh vs PEA, Student's unpaired t-test. n = 7-8 rats per group. PEA, N-palmitoylethanolamide, OEA, N-Oleoylethanolamide, 2-AG, 2-arachidonoylethanolamide, AEA-anandamide

Figure 4. Effects of intra-ACC administration of PEA on levels of *c-Fos* mRNA in the amygdala (BLA and CeA), RVM and DH. Intra-ACC administration of PEA significantly reduced levels of *c-Fos* mRNA in the BLA, but not in the CeA, RVM or DH. Data are means ± SEM. * $p<0.05$ veh vs PEA, Student's unpaired t-test was used to analyse BLA, CeA and RVM data and 2-WAY ANOVA with Fisher's LSD post hoc used for the DH. n = 5-7 rats per group. BLA: basolateral nucleus of amygdala, CeA: central nucleus of the amygdala, RVM: rostral ventromedial medulla, DH: dorsal horn of the spinal cord.
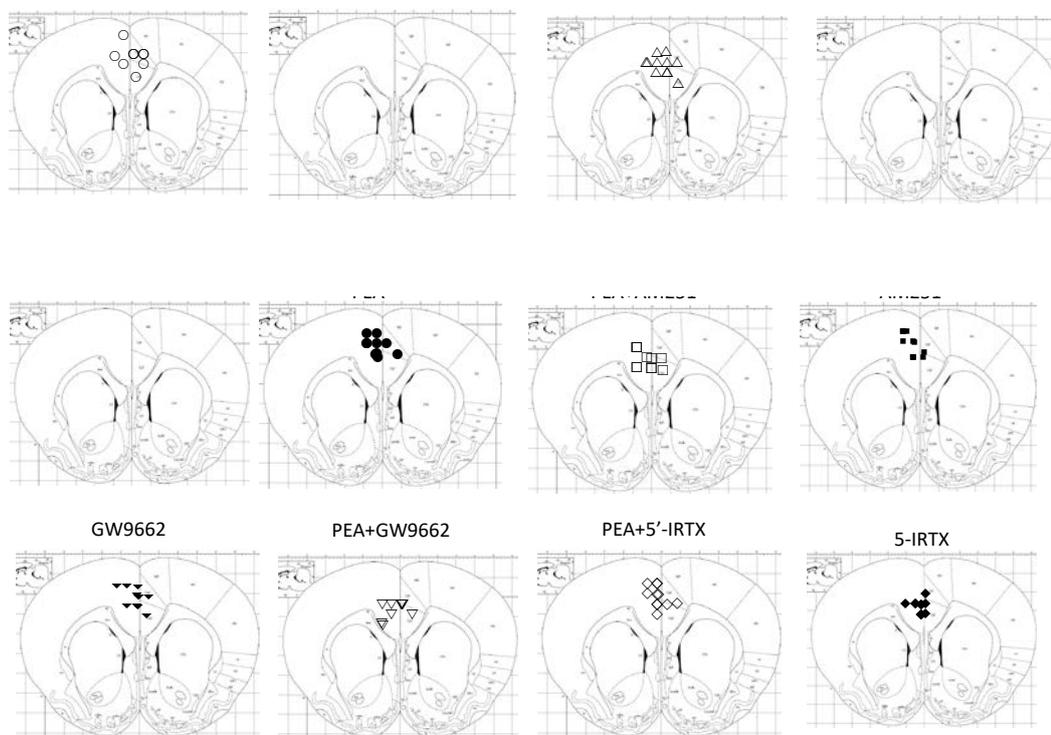
Figure 1

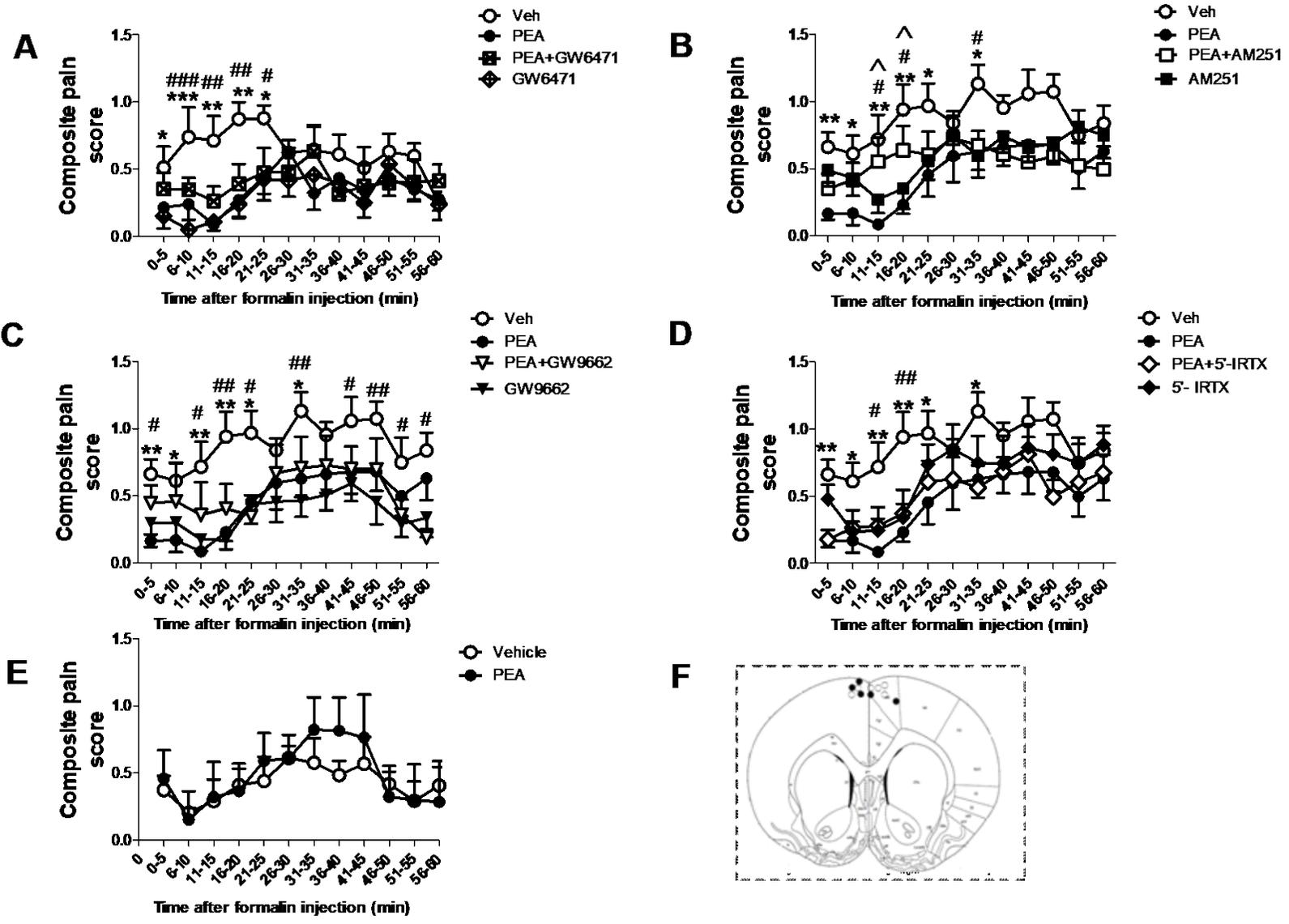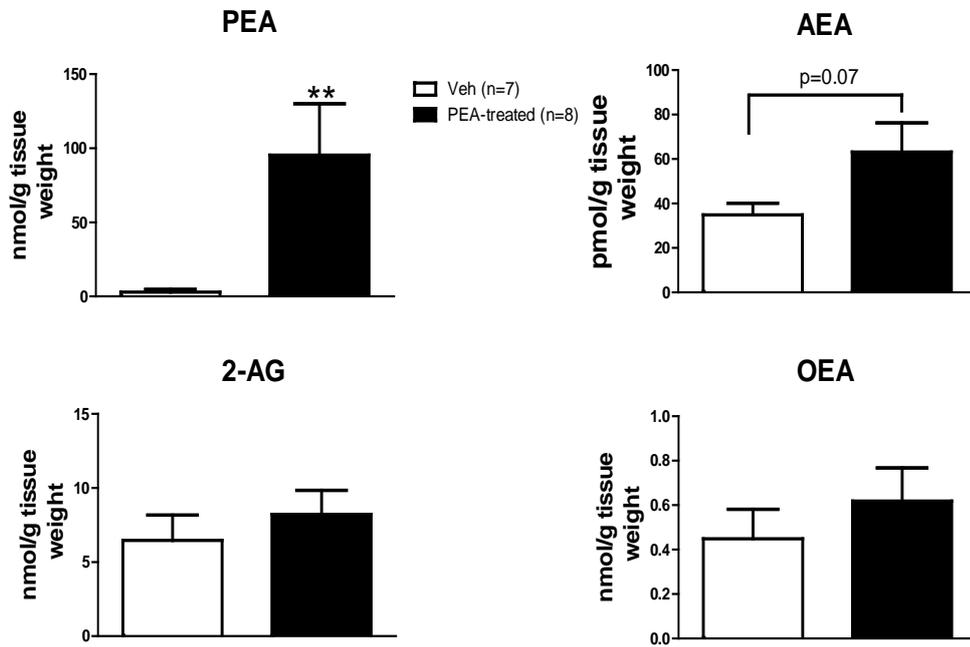GW9662   PEA+GW9662   PEA+5'-IRTX   5-IRTX

Figure 2

Figure 3

Figure 4
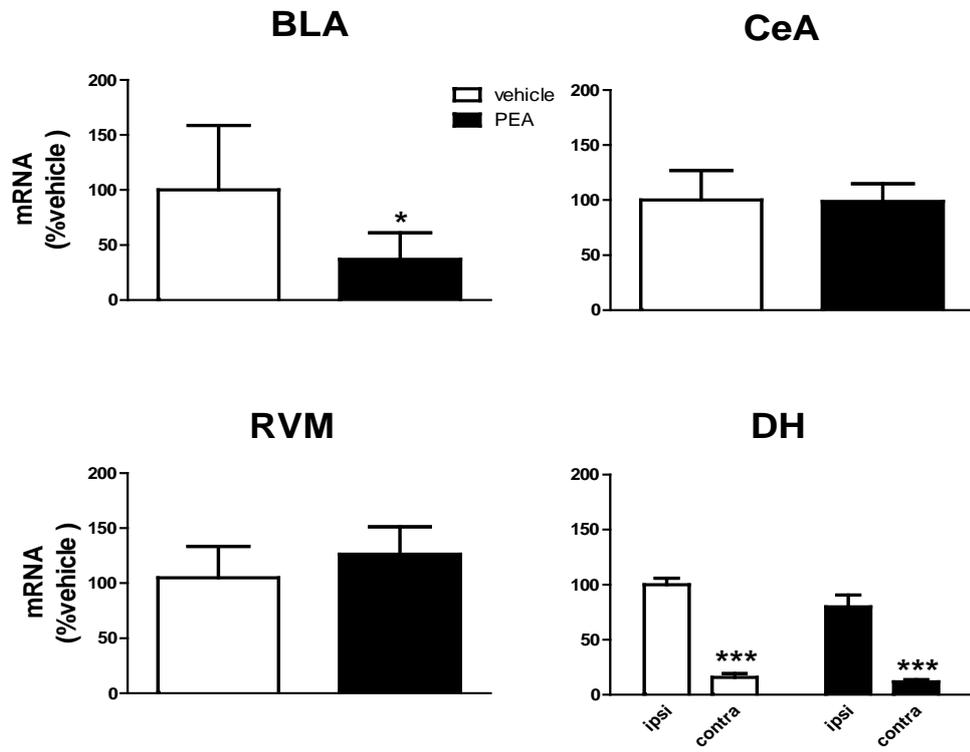
Table 1. Drugs, doses and group sample sizes

Drug	Dose/0.5 µL	n per group
Experiment 1		
Vehicle (100% DMSO)	-	7
PEA *(PPAR agonist)*	6 nmol	8
PEA+GW6471	(6 nmol+3 nmol)	8
GW6471 *(PPARα antagonist)*	3 nmol	8
Experiment 2		
Vehicle (100% DMSO)	-	7
PEA (PPAR agonist)	6 nmol	8
PEA+AM251	(6 nmol+1.25 nmol)	10
AM251 (*CB$_1$ antagonist*)	1.25 nmol	9
PEA+GW9662	(6 nmol+36 nmol)	7
GW9662 (*PPARγ antagonist*)	36 nmol	9
PEA+5'-IRTX	(6 nmol+1 nmol)	9
5'-IRTX (*TRPV$_1$ antagonist*)	1 nmol	9

Table 2. Pre-formalin locomotor activity, rearing and grooming duration in rats, *p<0.05 vs. vehicle. Data are Mean ± SEM

Treatment	Distance (cm)	Rearing duration (s)	Grooming duration (s)
Experiment 1			
Vehicle (n=7)	1227 ± 75.8	69.5 ± 10.3	49.1 ± 9.9
PEA (n=8)	932.3 ± 118.7	30.2 ± 7.6	46.3 ± 8.9
GW6471 (n=8)	1107 ± 148.0	49.0 ± 12.2	69.3 ± 15.1
PEA + GW6471 (n=8)	1129 ± 135.5	45.4 ± 10.2	50.4 ± 14.6
Experiment 2			
Vehicle (n=7)	678.5 ± 76.6	138.6 ± 47.1	50.7 ± 10.3
PEA (n=8)	665.9 ± 83.6	105.1 ± 31.4	88.7 ± 16.9
AM251 (n=9)	627.4 ± 51.2	62.3 ± 21.9	43.6 ± 16.2
GW9662 (n=9)	483.8 ± 50.3	98.8 ± 30.1	122.4 ± 23.8*
IRTX (n=9)	558.8 ± 55.5	58.1 ± 11.9	77.6 ± 18.9
PEA + AM251 (n=10)	617 ± 64.5	82.4 ± 23.1	82.8 ± 23.9
PEA + GW9662(n=7)	610 ± 52.5	54.6 ± 19.5	132.5 ± 16.7*
PEA + IRTX (n=9)	515.8 ± 64.5	89.6 ± 35.4	57.4 ± 22.0