

RESEARCH PAPER

Lack of effect of chronic pre-treatment with the FAAH inhibitor URB597 on inflammatory pain behaviour: evidence for plastic changes in the endocannabinoid system

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BACKGROUND AND PURPOSE

Elevating levels of endocannabinoids with inhibitors of fatty acid amide hydrolase (FAAH) is a major focus of pain research, purported to be a safer approach devoid of cannabinoid receptor-mediated side effects. Here, we have determined the effects of sustained pharmacological inhibition of FAAH on inflammatory pain behaviour and if pharmacological inhibition of FAAH was as effective as genetic deletion of FAAH on pain behaviour.

EXPERIMENTAL APPROACH

Effects of pre-treatment with a single dose, versus 4 day repeated dosing with the selective FAAH inhibitor, URB597 (i.p. 0.3 mg·kg⁻¹), on carrageenan-induced inflammatory pain behaviour and spinal pro-inflammatory gene induction were determined in rats. Effects of pain induction and of the drug treatments on levels of arachidonoyl ethanolamide (AEA), palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA) in the spinal cord were determined.

KEY RESULTS

Single, but not repeated, URB597 treatment significantly attenuated the development of inflammatory hyperalgesia ($P < 0.001$, vs. vehicle-treated animals). Neither mode of URB597 treatment altered levels of AEA, PEA and OEA in the hind paw, or carrageenan-induced paw oedema. Single URB597 treatment produced larger increases in AEA, PEA and OEA in the spinal cord, compared with those after repeated administration. Single and repeated URB597 treatment decreased levels of immunoreactive *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) in the spinal cord and attenuated carrageenan-induced spinal pro-inflammatory gene induction.

CONCLUSION AND IMPLICATIONS

Changes in the endocannabinoid system may contribute to the loss of analgesic effects following repeated administration of low dose URB597 in this model of inflammatory pain.

Abbreviations

2-AG, 2-arachidonoylglycerol; AEA, arachidonoyl ethanolamide (anandamide); AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; FAAH, fatty acid amide hydrolase; GW6471, [(2S)-2-[[[(1Z)-1-methyl-3-oxo-3-[4-(trifluoromethyl) phenyl]-1-propenyl] amino]-3-[4-[2-(5-methyl-2-phenyl-4-oxazolyl) ethoxy] phenyl] propyl]-carbamic acid ethyl ester]; MAGL, monoacylglycerol lipase; NAE, N-acylethanolamine; mPGES-1, microsomal PGE synthase-1; NAPE-PLD, N-acylphosphatidylethanolamine phospholipase D; OEA, oleoyl ethanolamide; PEA, palmitoyl ethanolamide; TRPV1, transient receptor potential vanilloid type 1; URB597, cyclohexyl carbamic acid 3'-carbamoyle- biphenyl-3-yl ester; WY14643, chloro-6-(2, 3-xylylidino)-2-pyrimidinylthioacetic acid

Introduction

Cannabinoid-based medicines have analgesic activity, but their side effect profile, due to the global activation of cannabinoid CB₁ receptors, limits their utility (Pertwee, 2009; receptor nomenclature follows Alexander *et al.*, 2011). Novel strategies in this field have focused on the modulation of the endocannabinoid signalling system. The two major endocannabinoids are the N-acylethanolamine (NAE) anandamide (AEA) and the ester 2-arachidonoylglycerol (2-AG). AEA and other biologically active endogenous NAEs are synthesized by various enzymic pathways, of which the N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) pathway is the best characterized (see Wang and Ueda, 2009). These NAEs are predominantly degraded by fatty acid amide hydrolase (FAAH) (Cravatt *et al.*, 1996; see McKinney and Cravatt, 2005; Ahn *et al.*, 2008). Increased neuronal activity is associated with increased synthesis of the endocannabinoids and tissue-specific elevations in the endocannabinoids have been reported in various models of pain (see Sagar *et al.*, 2009).

Pharmacological inhibition of FAAH elevates levels of NAEs and attenuates inflammatory pain responses (Jayamanne *et al.*, 2006; Jhaveri *et al.*, 2008; Ahn *et al.*, 2009; Karbarz *et al.*, 2009; Pertwee, 2009) via the activation of CB₁, CB₂ (Jayamanne *et al.*, 2006; Naidu *et al.*, 2009) and PPAR α receptors (LoVerme *et al.*, 2006; Sagar *et al.*, 2008). We have shown that spinal inhibition of FAAH produces a CB₁ receptor-mediated attenuation of noxious-evoked responses of spinal neurones in a model of neuropathic pain (Jhaveri *et al.*, 2006) and joint pain (Sagar *et al.*, 2010). Studies using FAAH knockout mice have provided corroborative evidence that elevation of NAEs attenuates inflammatory pain responses (Cravatt *et al.*, 2004; Chang *et al.*, 2006; Naidu *et al.*, 2010). Significantly, there is no evidence for FAAH inhibition producing abuse potential (Justinova *et al.*, 2008; Adamczyk *et al.*, 2009). Given that inflammatory and chronic pain states are associated with tissue-selective elevations in endocannabinoids *per se* (see Sagar *et al.*, 2009), understanding the potential changes in the endocannabinoid system under these conditions, and the effect of prolonged pharmacological attenuation of FAAH activity on the endocannabinoid system under basal conditions and in a model of pain, is essential.

The aim of our study was to determine the effects of sustained pharmacological inhibition of FAAH on inflammatory pain behaviour and spinal nociceptive signalling pathways. To this end, we used a relatively short duration model of inflammatory pain (carrageenan model) to ensure that the

level of inhibition of FAAH activity was stable over the time course of the model. The carrageenan model of acute inflammatory pain produces a robust hind paw oedema and hyperalgesia, accompanied by the spinal induction of inflammation-associated enzymes, such as COX-2 and microsomal PGE synthase-1 (mPGES-1) (Hay and de Belleruche, 1997; Samad *et al.*, 2001; Guay *et al.*, 2004), and the elevation of spinal pro-inflammatory cytokines (Tao *et al.*, 2003; Song *et al.*, 2009) within 3 h. Systemic administration of URB597 produces an elevation in AEA for up to 6 h (Fegley *et al.*, 2005), ensuring stable elevation of NAEs over the time frame of the model. In this study we report that, despite acute and repeated treatment with the FAAH inhibitor URB597 producing a comparable inhibition of spinal cord FAAH activity and producing significant effects on carrageenan-induced spinal pro-inflammatory gene expression, only acute URB597 treatment attenuated behavioural hyperalgesia. Acute URB597 treatment produced greater elevations of spinal NAEs, compared with repeated treatment, and we demonstrate adaptive changes in elements of the endocannabinoid system in the spinal cord under these conditions. Our data suggest that there are differences in the effects of sustained pharmacological inhibition of FAAH, compared with those after genetic deletion of FAAH (FAAH knockout), on inflammatory pain behaviour, which may have important therapeutic implications.

Methods

All animal care and experimental procedures were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986) and the guidelines of the Committee for Research and Ethical Issues of IASP. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). Experiments were performed on 120 male Sprague-Dawley rats (225–275 g). Animals had free access to food and water and were group-housed during the experiments. To determine whether sustained inhibition of FAAH produced analgesia comparable to that after a single dose of URB597 in a model of acute inflammatory pain, some rats were given URB597 daily before the establishment of the pain model. Rats were treated once daily with URB597 (0.3 mg·kg⁻¹) or vehicle (i.p.) for 3 days before the day of the behavioural studies; these groups are described as repeated URB597 treatment or repeated vehicle treatment. On the fourth day, rats received a final dose of 0.3 mg·kg⁻¹ URB597 or vehicle (i.p.) 30 min before injection of λ -carrageenan

(Sigma, Gillingham, Dorset, UK; 2 mg in 100 μ L saline). Just before injection of carrageenan, the ipsilateral and contralateral paw volume was measured with a plethysmometer (average of three readings), and then carrageenan was injected into the plantar surface of the left hind paw under brief isoflurane (3%) inhalation anaesthesia (in a gas mixture: 34% O₂; 66% N₂O). The rationale for dosing with URB597 for 4 days was based on the earlier report of the effects of 4 day treatment with URB597 on levels of AEA in the brain and neurophysiological endpoints (Gobbi *et al.*, 2005). As intraplantar injection of carrageenan produces a dynamic inflammatory pain response, which varies over time, both in terms of modality and magnitude, pharmacological intervention to produce an acute versus sustained block of FAAH at a comparable stage of the inflammatory pain response was not achievable with treatments given after the carrageenan injection. Separate groups of rats received a single dose of URB597 (0.3 mg·kg⁻¹) or vehicle (i.p.) 30 min prior to intraplantar injection of carrageenan, described as acute URB597 treatment or acute vehicle treatment. Control groups received an intraplantar injection of physiological saline (100 μ L) in an identical manner to the injection of carrageenan.

Assessment of hyperalgesia and hind paw volume

The following groups were used for the first study: repeated URB597 treatment plus hind paw carrageenan ($n = 9$); repeated vehicle treatment plus hind paw carrageenan ($n = 8$); acute URB597 plus hind paw carrageenan ($n = 10$), acute vehicle plus hind paw carrageenan ($n = 8$), repeated URB597 treatment plus hind paw saline ($n = 6$); repeated vehicle treatment plus hind paw saline ($n = 6$); acute URB597 plus hind paw saline ($n = 5$), acute vehicle plus hind paw saline ($n = 6$). Hyperalgesia was assessed as the difference in distribution of weight on the hind paws, which was measured using an incapacitance tester (Linton Instrumentation, Diss, Norfolk, UK) as previously described (Clayton *et al.*, 2002; Elmes *et al.*, 2005). The incapacitance tester consists of two sensitive strain gauge transducers, which measures the weight bearing on hind paws over a 3 s period. Differences in weight bearing between the inflamed and non-inflamed paw provide an index of basal hyperalgesia. This method of assessing inflammatory pain was employed in this study because there is mounting evidence that an over-reliance on measures of hypersensitivity based on evoked reflex responses, such as mechanical allodynia and hyperalgesia, and thermal hyperalgesia, may contribute to the lack of translational value of the outcomes of experimental pain studies to the development of clinically useful analgesics (Rice *et al.*, 2008; Mogil, 2009). Weight bearing measurements (means of 3 at each time point) were taken 30 min before the final injection of URB597 or vehicle, 20 min after injection of URB597 or vehicle and then at hourly intervals following intraplantar injection of carrageenan or vehicle.

The effects of URB597/vehicle on carrageenan-induced hyperalgesia were assessed for 3 h post-carrageenan injection. After the final measurement of weight bearing, the ipsilateral and contralateral paw volumes were measured with a plethysmometer (mean of three readings). Rats were then stunned and decapitated, and the ipsilateral and contralateral hind paw tissue, lumbar enlargement of the spinal cord and mid-

brain were rapidly dissected, frozen in liquid nitrogen and stored at -80°C . Samples were processed for the measurement of endocannabinoids and related NAEs. Experimenters were unaware of the treatments for the *ex vivo* studies.

Measurement of endocannabinoids and NAEs

A validated lipid extraction technique was employed, with some alterations (Richardson *et al.*, 2007). Briefly, tissue was finely minced and homogenized in acetonitrile (Fisher Scientific UK, Loughborough, UK) with 420 pmol of deuterated AEA (AEA-d₈) and 1 nmol of deuterated 2AG (2AG-d₈) internal standards (Cayman Europe, Tallinn, Estonia). The homogenized mixture was centrifuged (3200 $\times g$ for 15 min, repeated twice), the supernatant collected, the solvent was evaporated and the residue was reconstituted in 200 μ L acetonitrile (Fisher Scientific, Loughborough, UK). AEA, oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA) and 2AG were measured simultaneously by LC-MS/MS. Analytes were separated chromatographically using a Waters Symmetry C18 column (100 $\times$ 2.1 mm internal diameter, 3.5 μ m particle size; Hertfordshire, UK) with a mobile phase flowing at 0.3 mL·min⁻¹, using a gradient elution with mobile phases consisting of solvents A (water, 1 g·L⁻¹ ammonium acetate, 0.1% formic acid) and B (acetonitrile, 1 g·L⁻¹ ammonium acetate, 0.1% formic acid). Analytes were injected from a cooled autosampler maintained at 4°C. Analysis was carried out using an Agilent 1100LC system (Agilent Technologies, Böblingen, Germany) coupled to a triple quadrupole Quattro Ultima mass spectrometer (Waters Ltd, Manchester, UK) recording in electrospray positive mode. Compounds were identified using the mass to charge (m/z) ratios of precursor and product ions. The lower limit of quantification (in 0.1 g of tissue) of AEA, PEA and OEA was 2.5 pmol·g⁻¹ whilst that of 2AG was 50 pmol·g⁻¹.

Gene and protein expression

The requirement for larger amounts of tissue in order to quantify endocannabinoid levels meant that there was insufficient lumbar spinal cord tissue from this first study for the investigation of changes in gene and protein expression. Thus, the study was repeated, as described above, and following the measurement of behavioural hyperalgesia, lumbar spinal cord tissue was again collected 3 h after carrageenan. Treatment groups were: repeated URB597 treatment plus hind paw carrageenan ($n = 5$); repeated vehicle treatment plus hind paw carrageenan ($n = 6$); acute URB597 plus hind paw carrageenan ($n = 6$), acute vehicle plus hind paw carrageenan ($n = 5$), repeated URB597 treatment plus hind paw saline ($n = 5$); repeated vehicle treatment plus hind paw saline ($n = 5$); acute URB597 plus hind paw saline ($n = 5$), acute vehicle plus hind paw saline ($n = 5$). In a separate study, the contribution of the cannabinoid CB₁, versus PPAR α , receptor systems to the inhibitory effects of a single dose of URB597 was also studied. The ability of systemic pre-administration of the CB₁ receptor antagonist AM251 (5 mg·kg⁻¹, $n = 5$) versus the PPAR α antagonist GW6471 (10 mg·kg⁻¹, $n = 5$) to attenuate the effects of single systemic administration of URB597 ($n = 5$) on carrageenan-induced pain behaviour was compared with vehicle carrageenan treated rats ($n = 5$). AM251 and GW6471 (Tocris Bioscience, Abingdon, UK) were dissolved in 0.3% Tween 20/ saline. The doses of antagonists were based on

previous studies (Cuzzocrea *et al.*, 2008; Sticht *et al.*, 2011). Endocannabinoid levels were also measured in a subgroup of samples to ensure consistency between studies.

RNA extraction and cDNA synthesis

Approximately 50 mg of frozen tissue was homogenized in 2 mL of ice-cold Tri-reagent (Sigma-Aldrich, Gillingham, Dorset, UK) according to the manufacturer's instructions. Total RNA clean-up and on-column DNase digestion was performed using RNeasy purification columns (Qiagen, Crawley, West Sussex, UK). RNA concentration and purity were determined using a Nanodrop spectrophotometer. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using superscript III reverse transcriptase (Invitrogen, Paisely, UK) in a total volume of 20 µL for 1 h at 50°C, and the reaction was terminated at 70°C for 15 min. The final concentration of cDNA was 50 ng·µL⁻¹.

Taqman quantitative real-time PCR

The relative standard curve method based on Taqman quantitative real-time PCR (qRT-PCR) was used for quantifying gene expression. Samples were prepared in a total reaction volume of 25 µL [13 µL Taqman 2× reagent, 0.75 µL forward primer (10 µM), 0.75 µL reverse primer (10 µM), 0.5 µL Probe (10 µM), 5 µL water, and 5 µL cDNA]. qRT-PCR was performed using the StepOne Plus sequence detection system (Applied Biosciences, Warrington, UK). Gene expression was determined relative to β-actin.

Primers and probes for all genes (Table 1) were designed using Primer 3 software (Applied Biosystems, Warrington, UK) or obtained from published work and synthesized by MWG Biotech (Ebersberg, Germany).

Western blotting

Approximately 50 mg of tissue was homogenized in 1 mL of 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 Na₃VO₄, 10 mM NaF, 1 × Complete Protease inhibitor (Roche Applied Sciences, Burgess Hill, West Sussex, UK)]. The homogenate was placed on a rotating wheel for 45 min at 4°C followed by centrifugation at 15 000×g for 20 min. The supernatant layer was then separated from the pellet and assayed for

total protein concentration using a Pierce (Loughborough, UK) kit assay following the manufacturer's instructions.

25–50 µg of protein was separated on a 10% SDS-PAGE. The protein was transferred onto a nitrocellulose membrane and incubated overnight at 4°C with either rabbit polyclonal primary antibody to FAAH, monoacyl glycerol lipase (MAGL) or NAPE-PLD (Cayman, Cambridge Biosciences, UK; 1:200 dilution) and mouse monoclonal primary antibody to β-actin (Sigma; 1:5000). Blots were washed in TBS/Tween (TBST) buffer and incubated with IRDye® conjugated goat polyclonal anti-rabbit or anti-mouse IgG (LI-COR® Biosciences, Abingdon Park, Oxford, UK; 1:10 000 dilution) as appropriate. Scanning and densitometric analysis of blots was performed using a LI-COR® ODYSSEY infrared imaging system.

Measurement of FAAH activity

Treatment groups were as described above. FAAH activity was assessed as reported in Jhaveri *et al.* (2008). Enzyme in total particulate preparations (prepared by two successive centrifugations at 30 000 × g for 30 min) was measured in the presence of 1, 5 and 20 µM *N*-arachidonoyl-[³H]-ethanolamine ([³H]-AEA, American Radiolabelled Chemicals, St Louis, MO, USA), using the equivalent of 0.5–1 mg original weight, incubated for 30 min at 37°C in 200 µL Tris EDTA, pH 7.4 buffer, halting the reaction with 400 µL activated charcoal (8% w/v) in 0.5 M HCl. [³H]-Ethanolamine in the supernatant layer following centrifugation (13 000 × g for 5 min) was quantified by liquid scintillation counting. Non-FAAH hydrolytic activity (defined by pre-incubation of preparations for 20 min in the presence of 1 µM URB597) was not different from background. Although some of the FAAH inhibitors, including URB597, have off-target effects, these are associated with the liver (Zhang *et al.*, 2007; Ahn *et al.*, 2009).

Data analysis

Data are expressed as mean ± SEM or median ± range. Two-way ANOVA, with a Bonferroni's *post hoc* test, was used to compare weight-bearing data between different treatment groups at different time points, as well as FAAH activity data. *K_m* and *V_{max}* values for FAAH activity in individual samples were calculated using non-linear regression fitting to a rectangular hyperbola using Prism (GraphPad, San Diego, CA).

Table 1

List of gene primer and probe sequences

Gene	Forward primer	Reverse primer	Probe
FAAH*	gcctcaaggaatgcttcagc	tgccctcattcaggctcaag	acaagggccacgactccacactgg
MAGL	tgccatctccatcctagcag	caaggatatgttgaggagga	atccggaatctgcatcgactttga
NAPE PLD	tcaagctcctctttggaacc	ctggaggaggacgtaaccaa	tatcccaaacgtgctcagatggct
COX-2**	ggcacaaatgatgttcgca	cctcgctctgatctgtcttga	tctttgccagacattcactcatcagttt
iNOS	cccagagtctctagacctcaacaaca	gccctcgaagggtgagttgaa	aagtcagccgcaccaccctcc
IL-1β ⁻	cctctcaagcagagcacag	gggtccatggtgaagtcaac	tgctccgaccattgctgttctcagg
mPGES-1 [#]	gcgaactgggccagaaca	ggcctacctgggcaaaatg	ccccggagcgaatgcgtgg
β-actin ^{##}	aggcatgtacgtagccatcca	tctccggagtccatcacaatg	tgctcctgtatgcctctggtctaccac

*Bortolato *et al.*, 2007, **Shafer and Slice, 2005, ⁻Rioja *et al.*, 2004, [#]Claveau *et al.*, 2003, ^{##}Erhuma *et al.*, 2007

Comparison of levels of endocannabinoids was carried out using a non-parametric Kruskal–Wallis test and Dunn's *post hoc* test. All protein and mRNA data were analysed using one-way ANOVA with a Bonferroni's selected pair *post hoc* test. The level of statistical significance levels was set at $P < 0.05$.

Materials

URB597, OEA, and PEA were from Cambridge Bioscience (Cambridge, UK); AEA from Tocris Bioscience, UK and activated charcoal from Sigma-Aldrich, UK.

Results

Acute, but not repeated, administration of URB597, attenuates inflammatory pain behaviour

Intraplantar injection of carrageenan resulted in a significant decrease in weight bearing on the ipsilateral hind paw, indicative of hyperalgesia (Figure 1) and an increase in paw volume (change in paw volume for acute vehicle carrageenan: 0.82 ± 0.05 mL; repeated vehicle carrageenan: 0.76 ± 0.13 mL). Acute treatment with the FAAH inhibitor URB597 ($0.3 \text{ mg}\cdot\text{kg}^{-1}$) significantly attenuated the carrageenan-induced hyperalgesia at 120 and 180 min post-carrageenan injection (Figure 1A), but did not alter carrageenan-induced increases in hind paw volume (change in paw volume for acute URB597 carrageenan: 0.63 ± 0.06 mL). The marked inhibitory effects of acute URB597 at 120 min were significantly attenuated by pre-administration of the selective PPAR α antagonist GW6471 and by the CB $_1$ cannabinoid receptor antagonist AM251 (Figure 1B). In contrast to the inhibitory effects of acute treatment, repeated administration of URB597 ($0.3 \text{ mg}\cdot\text{kg}^{-1}$, over 4 days) did not attenuate carrageenan-induced hyperalgesia at any time point (Figure 1C), nor carrageenan-induced increases in hind paw volume (change in paw volume for repeated URB597 carrageenan: 0.68 ± 0.13 mL).

Acute and repeated administration of URB597 attenuates FAAH activity

In order to determine whether the differences in the effects of URB597 on pain behaviour were due to a loss of its inhibitory action at FAAH, the effects of acute and repeated administration of URB597 on FAAH activity were determined in spinal cord homogenates. FAAH activity was comparable in rats which received intraplantar injection of saline ($5.4 \pm 0.8 \text{ pmol}\cdot\text{g}^{-1}\cdot\text{tissue}\cdot\text{min}^{-1}$) and carrageenan ($4.7 \pm 0.4 \text{ pmol}\cdot\text{g}^{-1}\cdot\text{tissue}\cdot\text{min}^{-1}$). Both acute and repeated treatment with URB597 reduced FAAH activity to the same extent (acute: $0.6 \text{ pmol}\cdot\text{g}^{-1}\cdot\text{tissue}\cdot\text{min}^{-1}$, $12 \pm 2\%$ of control, $n = 7$; repeated: $0.7 \text{ pmol}\cdot\text{g}^{-1}\cdot\text{tissue}\cdot\text{min}^{-1}$, $16 \pm 3\%$ of control, $n = 6$, $P = 0.31$ Mann–Whitney *t*-test). Thus, the two treatment strategies with URB597 produced comparable inhibition of FAAH activity in the spinal cord and therefore changes in the pharmacokinetics or activity of URB597 following repeated administration were unlikely to account for the differences in the effects of URB597 on pain behaviour.

Differential effects of acute and repeated administration of URB597 on levels of NAEs

To investigate further the basis for the reduced effectiveness of repeated administration of URB597 on pain behaviour, levels of the NAEs (AEA, PEA, OEA) and of 2-AG were measured in the ipsilateral spinal cord following acute and repeated treatment with URB597 in carrageenan-treated rats. Acute URB597 significantly elevated levels of AEA, PEA and OEA, but not 2-AG, in the ipsilateral spinal cord of carrageenan-treated rats compared with the control group (i.p. saline and intraplantar injection of carrageenan) (Figure 2). Repeated administration of URB597 did not significantly increase levels of AEA, PEA or OEA in the spinal cord of carrageenan-treated rats, compared with the control group (repeated i.p. saline and intraplantar injection of carrageenan), although there was a trend towards an increase (Figure 2). The magnitude of the elevations in NAEs in the ipsilateral spinal cord following repeated administration of URB597 was smaller than the effects of acute URB597 treatment (Table 2). It is noteworthy that when considering levels of AEA in the spinal cord following acute or repeated administration of vehicle in carrageenan-treated rats, there was a greater spread in the dataset from the repeated treatment group; however, these differences were not significant.

To evaluate further the potential site of action of URB597, the effects of systemically administered URB597 on levels of AEA, PEA and OEA were determined in the hind paw and the midbrain. Neither acute, nor repeated, treatment with systemic URB597 altered levels of the NAEs in the carrageenan-inflamed hind paw (Table 3). At the level of the midbrain, both acute and repeated administration of URB597 significantly elevated levels of AEA and PEA in carrageenan-treated rats (Table 3). Consistent with the observations in the spinal cord, the elevation of the NAEs in the midbrain was greater following acute than after repeated administration of URB597.

Given that the inhibitions of FAAH activity were comparable after acute and repeated administration of URB597, the differences in the effects of acute versus repeated administration of URB597 on the levels of the NAEs suggests that sustained pharmacological blockade of FAAH may have modulated the synthesis of these mediators. Thus, we investigated whether inhibition of FAAH influenced the expression and protein levels of NAPE-PLD, the major synthetic enzyme for long chain NAEs. Both acute and repeated administration of URB597 were associated with marked decreases in the expression of NAPE-PLD protein in the ipsilateral spinal cord of carrageenan and saline-treated rats (Figure 3A, B). URB597 did not alter FAAH protein in the the ipsilateral spinal cord of carrageenan and saline-treated rats (Figure 3A). Neither NAPE-PLD, nor FAAH, mRNA expression was altered by either acute or repeated administration of URB597 (Figure 4). In keeping with the established divergence of catabolic pathways for the NAEs and 2-AG, mRNA (acute vehicle + carrageenan: 1.12 ± 0.12 ; acute URB + carrageenan: 1.10 ± 0.17 , relative to β -actin) and protein (acute vehicle + carrageenan: 1.45 ± 0.16 ; acute URB + carrageenan: 1.59 ± 0.27 , relative to β -actin) levels of the major 2-AG catabolic enzyme MAGL were comparable in the spinal cord. Similarly, repeated administration of URB597 did not alter levels of MAGL mRNA or protein in the spinal cord of saline or carrageenan-treated rats (data not shown).

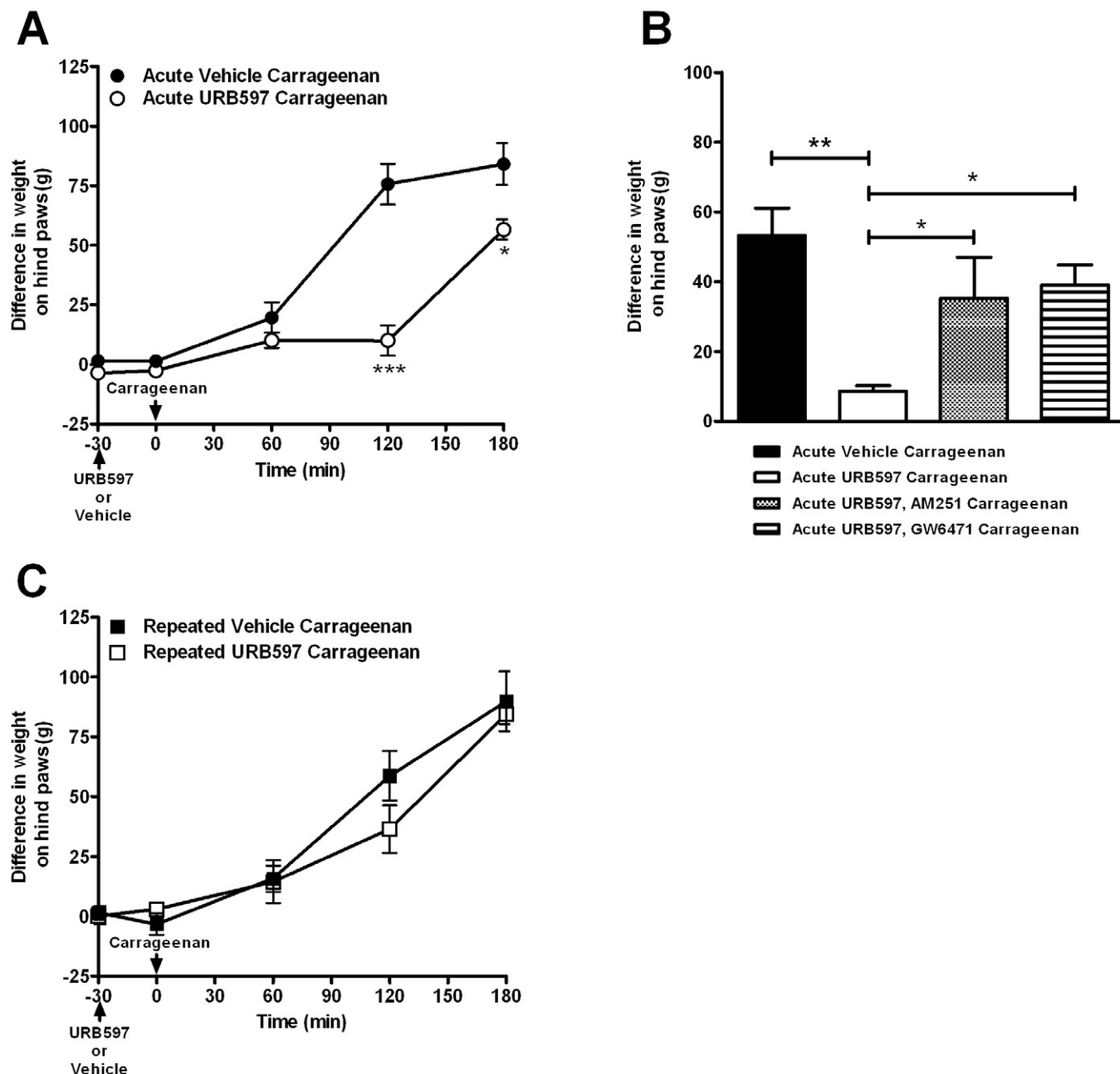


Figure 1

Comparison of the effects of acute and repeated administration of the FAAH inhibitor URB597 ($0.3 \text{ mg}\cdot\text{kg}^{-1}$) on carrageenan-induced pain behaviour. (A) Acute URB597 attenuated carrageenan-induced changes in weight bearing. Data were analysed using two-way ANOVA followed by Bonferroni's *post hoc* test, $*P < 0.05$, $***P < 0.001$. (B) Inhibitory effects of acute URB597 at 120 min after carrageenan injection were significantly blocked by co-administration of the PPAR α antagonist GW6471 ($10 \text{ mg}\cdot\text{kg}^{-1}$) with URB597 and by co-administration of the CB $_1$ receptor antagonist AM251 ($5 \text{ mg}\cdot\text{kg}^{-1}$). Data were analysed using one-way ANOVA with Bonferroni's *post hoc* test, $*P < 0.05$, $**P < 0.01$. (C) Repeated administration of URB597 did not alter carrageenan-induced changes in weight bearing. Data shown are differences in weight bearing between contralateral and ipsilateral hind paw and are expressed as mean $\pm$ SEM.

Effects of acute and repeated administration of URB597 on pro-inflammatory spinal responses

As the spinal cord plays a critical role in the processing of noxious sensory inputs and our biochemical data demon-

strate a spinal site of action of acute and repeated administration of URB597, the effects of URB597 on the spinal pro-inflammatory responses associated with inflammatory pain behaviour were determined. As described previously, carrageenan-induced hyperalgesia was associated with an

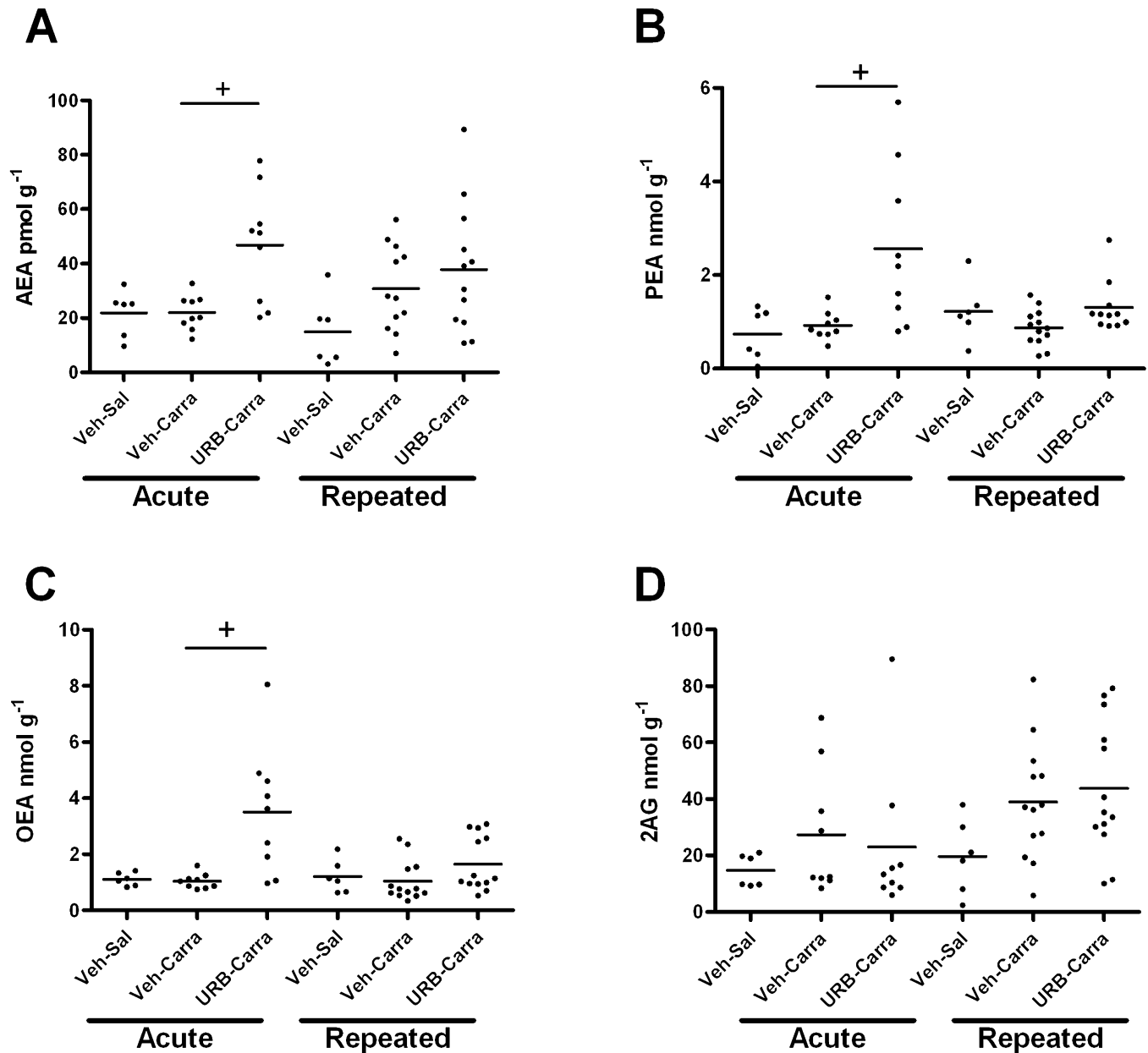


Figure 2

Effects of acute and repeated administration of URB597 or vehicle on intraplantar injection of carrageenan (carra)-, or saline (sal)-, induced changes in levels of (A) AEA, (B) PEA, (C) OEA and (D) 2-AG in the ipsilateral spinal cord 3 h following intraplantar injection (see Figure 1 legend for further details of drug treatments). Data are expressed as median and range ($n = 9$ – 13 rats per group) and were analysed using Kruskal–Wallis and Dunn's *post hoc* test: $^+P < 0.05$, compared with vehicle carrageenan.

increased expression of mRNA encoding COX-2 and mPGES-1 in the ipsilateral spinal cord, compared to rats receiving intraplantar injection of saline (Figure 5). Carrageenan-induced hyperalgesia was also associated with an increased expression of iNOS and IL1 β mRNA in the ipsilateral spinal cord (Figure 5). Acute URB597 treatment significantly decreased the carrageenan-induced increases in spinal COX-2 ($60 \pm 14\%$ decrease), mPGES-1 ($65 \pm 15\%$ decrease), IL-1 β ($57 \pm 5\%$ decrease) and iNOS ($67 \pm 9\%$ decrease) expression, compared with vehicle (Figure 5). Alongside the

effects of acute URB597 on FAAH activity and endocannabinoid levels in the spinal cord, these data suggest that a spinal site of action contributes to the effects of acute systemically administered URB597 on behavioural hyperalgesia and that inhibition of FAAH attenuates pro-inflammatory spinal mechanisms. Repeated URB597 treatment had a smaller effect on carrageenan-induced COX-2 and mPGES-1 expression compared with the effect of acute URB597 (Figure 5). Although there were some small differences in the absolute levels of spinal COX-2, induced by carrageenan, following

Table 2

Comparison of the effects of acute and repeated administration of URB597 (0.3 mg·kg⁻¹) on levels of AEA, PEA, OEA and 2-AG in the ipsilateral spinal cord of carrageenan-treated rats

		Relative levels of endocannabinoids and related NAEs (% control)			
		AEA	PEA	OEA	2-AG
Spinal Cord	Acute URB597	213 ± 31*	278 ± 62	337 ± 72*	84 ± 32
	Repeated URB597	123 ± 22	149 ± 17	158 ± 26	112 ± 17

Data are expressed as a percentage of the mean level in the ipsilateral spinal cord of carrageenan-treated rats receiving either acute or repeated administration of vehicle ($n = 9$ – 13 rats per group). Statistical comparisons between the effects of acute and repeated administration of URB597 was carried out using a Mann–Whitney non-parametric test (* $P < 0.05$)

Table 3

Levels of NAEs in the hind paw (A) and midbrain (B) of rats receiving an acute or repeated treatment with URB597 (0.3 mg·kg⁻¹) or vehicle prior to intraplantar injection of carrageenan

A: Hind paw			
Treatment	AEA (pmol g ⁻¹) median (range)	OEA (nmol g ⁻¹) median (range)	PEA (nmol g ⁻¹) median (range)
Acute vehicle + carrageenan	5.22 (4.34–6.89)	0.12 (0.04–0.19)	0.12 (0.05–0.34)
Acute URB597 + carrageenan	6.47 (4.26–11.15)	0.09 (0.07–0.17)	0.14 (0.06–0.30)
Repeated vehicle + carrageenan	8.50 (1.45–12.28)	0.15 (0.06–0.28)	0.18 (0.14–0.24)
Repeated URB597 + carrageenan	4.14 (1.38–14.14)	0.15 (0.07–0.19)	0.13 (0.08–0.19)

B: Midbrain			
Treatment	AEA pmol g ⁻¹ median (range)	OEA nmol g ⁻¹ median (range)	PEA nmol g ⁻¹ median (range)
Acute vehicle + carrageenan	16.41 (14.0–34.98)	0.85 (0.72–1.34)	1.34 (0.83–1.58)
Acute URB597 + carrageenan	29.2 (18.6–44.78)*	2.97 (0.98–3.36)*	3.21 (1.30–3.89)*
Repeated vehicle + carrageenan	11.69 (8.84–22.69)	0.70 (0.32–0.75)	0.73 (0.38–1.01)
Repeated URB597 + carrageenan	23.27 (15.57–51.58)*	1.83 (0.51–2.67)	2.28 (0.67–3.71)*

Data are expressed as median and range, $n = 9$ – 13 rats per group. Comparisons between the effects of acute vehicle and acute URB597 or repeated vehicle and repeated URB597 were performed using a Mann–Whitney non-parametric test, * $P < 0.05$, significantly different from vehicle + carrageenan.

acute or repeated vehicle treatment, these were not significant and carrageenan-induced levels of mPGES-1 were comparable for both vehicle treatment groups. In contrast to COX-2, carrageenan-induced spinal IL1 β and iNOS expression were significantly attenuated by repeated administration of URB597 (Figure 5). Indeed, both acute and repeated administration of URB597 abolished any effect of carrageenan on IL1 β and iNOS expression in the spinal cord.

Discussion

The major finding of the present study is that repeated administration of URB597 did not attenuate inflammatory pain behaviour, in contrast to the inhibitory effects of a single dose of the FAAH inhibitor, which were significantly blocked

by the PPAR α -selective antagonist GW6471 and by the CB₁ receptor-selective antagonist AM251. Both acute and repeated administration of URB597 inhibited FAAH activity in the spinal cord to a similar extent. Despite this, levels of AEA, PEA and OEA in the spinal cord were elevated to a greater extent by URB597 administered as a single dose, compared with repeated administration. Our data suggest that sustained pharmacological inhibition of FAAH results in an adaptation in the synthesis of the endocannabinoids, and related NAEs, or their catabolism by alternative pathways, which are not dependent upon FAAH (Kozak *et al.*, 2002), in the spinal cord and are not evident following FAAH knock-out. The demonstration that sustained pharmacological inhibition of FAAH does not mimic the robust inhibitory effects of FAAH knockout (see below) on inflammatory pain behaviour (both experimental approaches inhibit FAAH before the

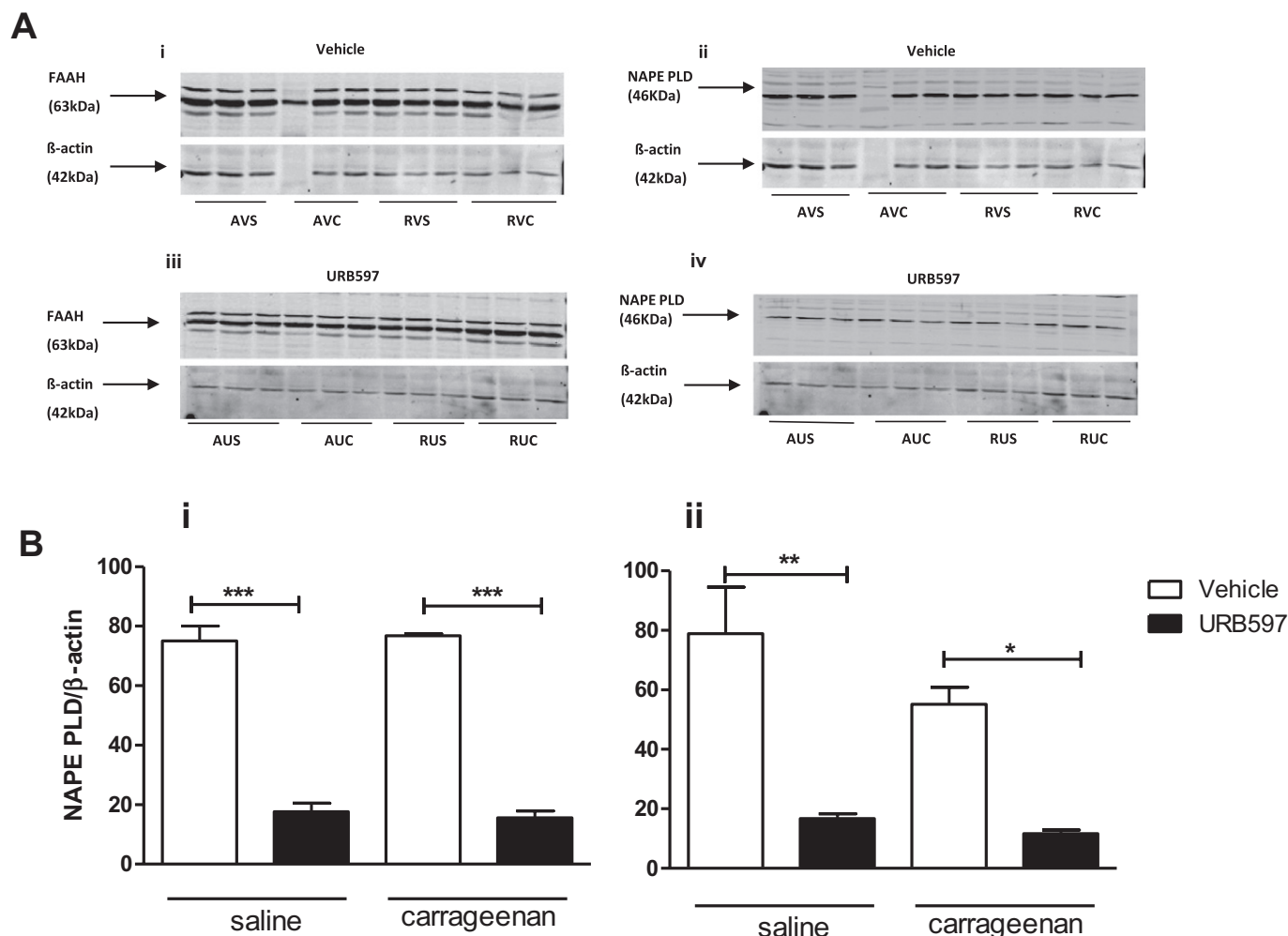


Figure 3

Effects of URB597 on FAAH and NAPE PLD protein expression in the ipsilateral spinal cord (see Figure 1 legend for further details of drug treatments). A (i, iii): Neither acute, nor repeated, URB597 altered FAAH protein levels. A (ii, iv), B (i, ii): Acute and repeated administration of URB597 reduced NAPE-PLD protein levels by approximately two-thirds, compared with the corresponding vehicle treated groups. AVS (Acute vehicle + saline), AUS (Acute URB597 + saline), AVC (Acute Vehicle + carrageenan), AUC (Acute URB597 + carrageenan), RVS (Repeated vehicle + saline), RUS (Repeated URB597 + saline), RVC (Repeated Vehicle + carrageenan), RUC (Repeated URB597 + carrageenan), $n = 3$, per each treatment group, performed in duplicate. Statistical analysis with one-way ANOVA with a Bonferroni's post test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

establishment of the pain state), indicates the importance of multiple experimental approaches in the identification of potential analgesic targets.

Comparison of the effects of acute versus repeated treatment with URB597 in the model of inflammatory pain revealed that peripheral hind paw levels of NAEs were unaltered by either treatment, suggesting that this is not a major site of action of URB597 following systemic administration. Furthermore, neither acute nor sustained inhibition of FAAH with URB597 altered carrageenan-induced increases in hind paw volume. These data suggest that the ability of acute URB597 treatment to attenuate the carrageenan-induced hyperalgesia is via mechanisms distinct from an anti-inflammatory action. Our data are consistent with the observation that higher doses of systemic URB597 (1 and 3 mg·kg⁻¹) are required to attenuate carrageenan-induced increases in paw volume, whereas 0.3 mg·kg⁻¹ URB597

attenuates carrageenan-induced thermal hyperalgesia without influencing paw volume (Costa *et al.*, 2010). This dose of URB597 has, however, been shown to attenuate carrageenan-induced increases in paw volume in the anaesthetized mouse (Holt *et al.*, 2005). Previously, we have shown that local hind paw injection of URB597 can elevate levels of NAEs and attenuate pain behaviour but does not alter paw volume in this model (Jhaveri *et al.*, 2008).

Acute administration of URB597 significantly elevated levels of NAEs in both the spinal cord and midbrain, which is consistent with the original report that this dose of URB597 produces a maximal inhibition of basal AEA hydrolysis in the brain from 15 min to 6 h after administration and elevates brain levels of AEA and PEA from 1–6 h (Fegley *et al.*, 2005). In contrast to the effects of acute URB597, levels of NAEs in the spinal cord were not altered following repeated administration of URB597 but were elevated in the midbrain. The

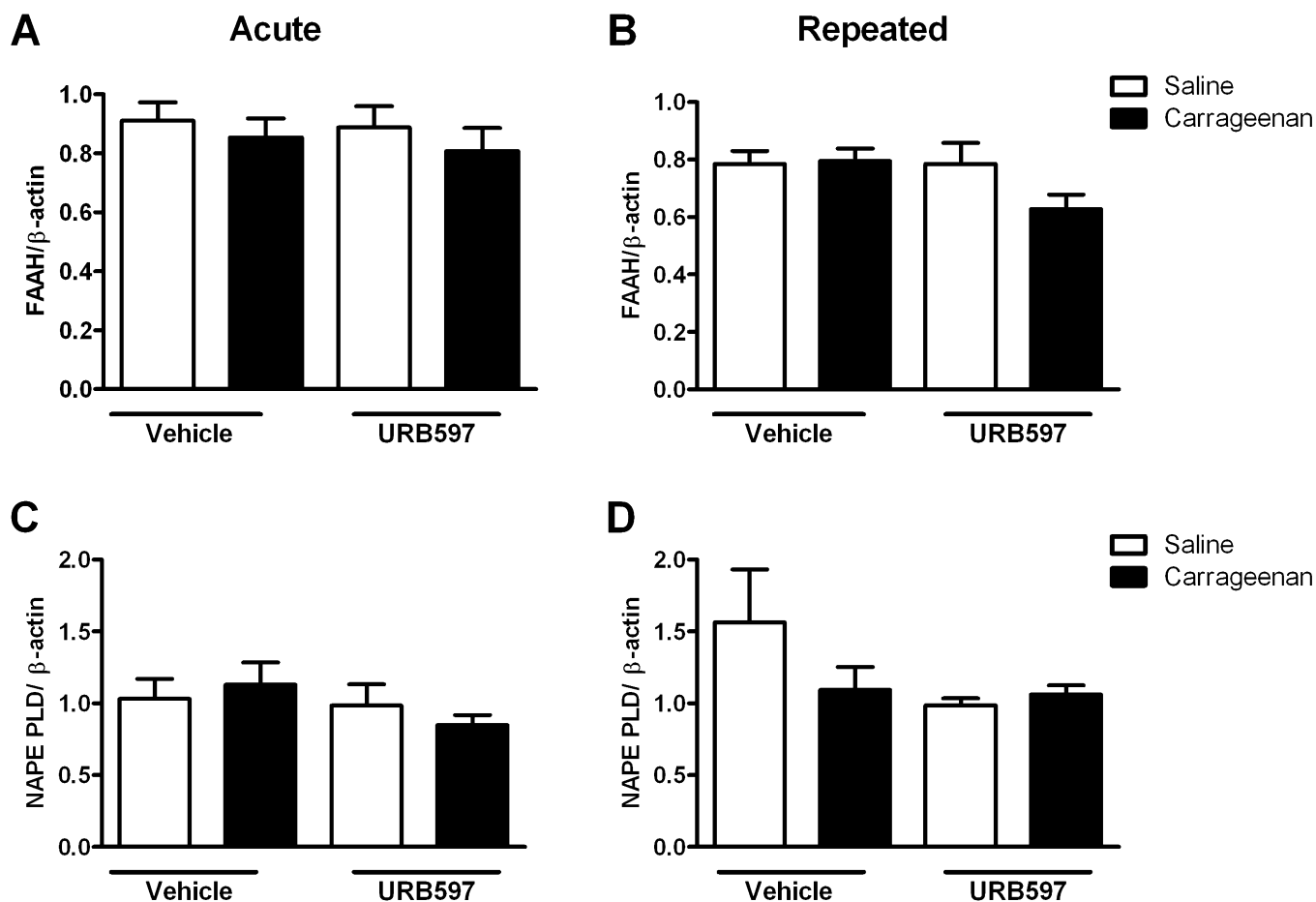


Figure 4

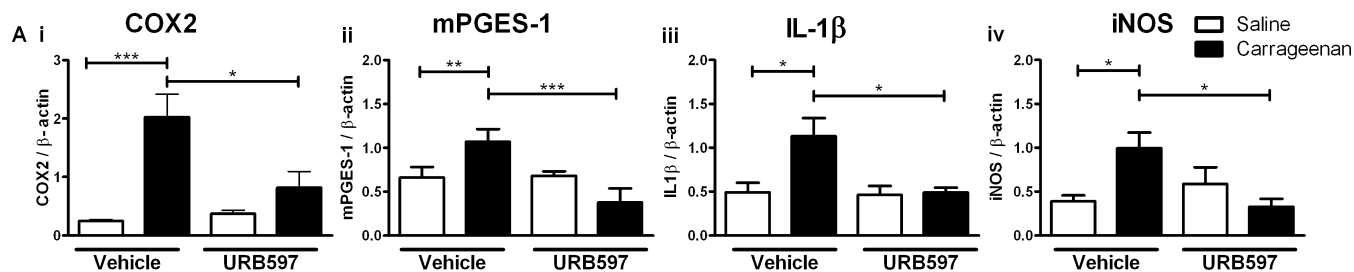
Effects of acute and repeated administration of URB597 on FAAH and NAPE-PLD mRNA expression in the ipsilateral spinal cord. (A and B) FAAH mRNA levels, relative to β -actin, in the ipsilateral spinal cord of saline and carrageenan-treated rats were not altered by acute or repeated administration of URB597. (C and D) NAPE PLD mRNA levels, relative to β -actin, in the ipsilateral spinal cord of saline and carrageenan-treated rats were not altered by acute or repeated administration of URB597 ($n = 5$ per treatment group).

demonstration of tissue-specific differences in the effects of URB597 is consistent with earlier reports (Gobbi *et al.*, 2005; Bortolato *et al.*, 2007; Long *et al.*, 2011). For example, 5 weeks of daily administration of URB597 increased levels of AEA in some brain regions; however, despite hippocampal FAAH activity being significantly reduced, levels of AEA were unaltered (Bortolato *et al.*, 2007). In our study, acute and repeated treatment with URB597 produced a comparable inhibition of hydrolytic activity; thus, it is unlikely that the lack of effect of repeated URB597 treatment on pain behaviour and levels of NAEs in the spinal cord reflects an inadequate blockade of enzyme activity at this level. This notion is further supported by the ability of repeated URB597 treatment to elevate levels of NAEs in the midbrain. The differences in the behavioural effects of sustained pharmacological inhibition of FAAH, compared to FAAH knockout, on inflammatory pain responses (Lichtman *et al.*, 2004; Naidu *et al.*, 2010), suggest that the endocannabinoid system may influence the development of pain circuits that are subject to considerable neonatal and postnatal influence (Fitzgerald, 2005), as well as

possible effects on other neuronal systems which affect inflammatory pain pathways.

There are marked differences between the effects of pharmacological intervention with URB597 in models of inflammatory and neuropathic pain, which are likely to reflect differences in the mechanisms underlying these different pain states, or differences in the behavioural tests employed (changes in weight bearing versus stimulus-evoked nociceptive responses, and the modality of stimulus). Indeed, the original report of the effects of pharmacological inhibition of FAAH in inflammatory and neuropathic rats indicated a lack of effect of URB597 on mechanical allodynia in neuropathic rats but a pronounced inhibitory effect on thermal hyperalgesia in an inflammatory pain model (Jayamanne *et al.*, 2006). Repeated (4 day) oral administration of a far higher dose of URB597 in mice did, however, attenuate established neuropathic pain behaviour via a CB_1 receptor-dependent mechanism (Russo *et al.*, 2007). The more recent description of the dose-response effects of URB597 on neuropathic pain behaviour in mice demonstrates that the lower doses of

Acute



Repeated

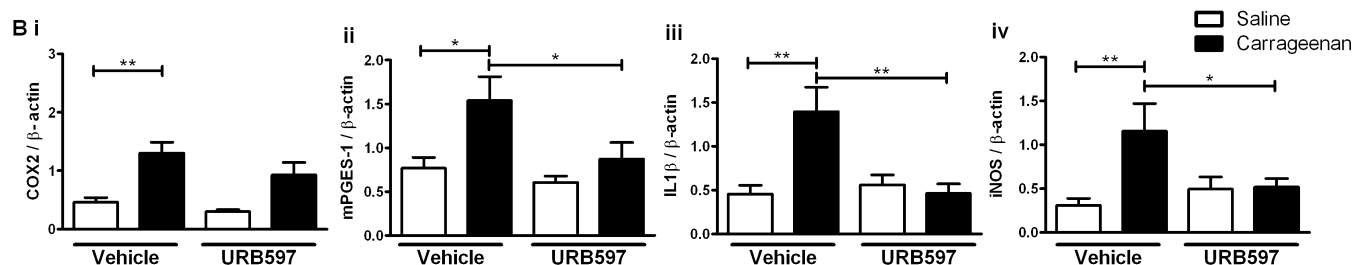


Figure 5

Effects of acute and repeated administration of URB597 on carrageenan induced pro-inflammatory gene expression in the ipsilateral spinal cord. Intraplantar injection of carrageenan produced a robust elevation in COX-2, mPGES-1, IL1 β and iNOS mRNA in the ipsilateral spinal cord. Acute administration of URB597 significantly reduced carrageenan-induced pro-inflammatory gene expression. By contrast, repeated administration of URB597 did not significantly attenuate carrageenan-induced COX-2 expression in the spinal cord and had less effect on mPGES-1 expression compared with acute URB597. Repeated administration of URB597 attenuated carrageenan-induced IL1 β and iNOS expression. Statistical analysis with one-way ANOVA with Bonferroni's *post hoc* test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ ($n = 4-6$ per treatment group).

URB597, which inhibit inflammatory pain behaviour in rats (present study, Jayamanne *et al.*, 2006) and mice (Costa *et al.*, 2010), do not alter mechanical allodynia in neuropathic mice and that far higher doses are required to inhibit these responses with both oral and i.p. routes of administration (Kinsey *et al.*, 2009). These data suggest that differences in the mechanisms underlying acute inflammatory pain responses from those underlying nerve damage-induced pain, and the effect these pain states have on the endocannabinoid system *per se* (Sagar *et al.*, 2009), may account for the differences in efficacy of URB597 in these two different pain states.

In the present study, the inhibitory effects of a single dose of URB597 were significantly blocked by a PPAR α -selective antagonist, which is consistent with our earlier work (Jhaveri *et al.*, 2008; Sagar *et al.*, 2008), as well as a CB $_1$ receptor-selective antagonist. These data are consistent with FAAH metabolizing a number of NAEs which have multiple targets, including PPAR α , CB $_1$ receptors and CB $_2$ receptors, which modulate inflammatory pain responses. Although not investigated in this study, CB $_2$ receptors may also contribute to the inhibitory effects of URB597 seen in this model of inflammatory pain (Jayamanne *et al.*, 2006). Previous studies have shown that PEA, a ligand for the nuclear receptor PPAR α , has rapid anti-inflammatory and analgesic effects (LoVerme *et al.*, 2005; 2006). PEA has also been reported to have antinociceptive effects in a neuropathic pain model, apparently via activation of CB $_1$, PPAR γ and TRPV1 receptors (Costa *et al.*, 2008). Thus, the reduced effectiveness of repeated administration of URB597 to elevate spinal levels of PEA may indeed

contribute to the lack of behavioural analgesia seen with this treatment. It is feasible that secondary changes in receptor levels, for example CB $_1$ or PPAR α down-regulation, as a result of a sustained elevation in NAEs contributes to the lack of effect of repeated administration of URB597 on pain behaviour. Equally, effects of URB597 on levels of endovanilloids and the activation state of TRPV1 may also affect our data (Maione *et al.*, 2006). There is, however, no evidence for these, or other, changes in receptor function occurring in FAAH knockout mice, which have elevated levels of NAEs for a prolonged period of time.

On the basis that there were differential effects of URB597 treatment on pain behaviour and levels of NAEs in the spinal cord, but not in the attenuation of hydrolytic activity or FAAH expression, we investigated whether this treatment might influence the synthesis of the NAEs by the best characterized NAE synthetic enzyme, NAPE-PLD (Wang and Ueda, 2009). Single and repeated administration of URB597 reduced NAPE-PLD protein, but not mRNA, expression in the spinal cord, suggesting that changes in protein levels arose as a result of translational or post-translational mechanism(s). The functional effect of acute and repeated administration of URB597 on NAPE-PLD and NAE levels should be considered in the context of NAE turnover. A single dose of URB597 increases NAE levels for at least 6 h after treatment (Fegley *et al.*, 2005); thus, decreased NAPE-PLD protein levels must take six or more hours to affect NAE levels. On this basis, it is feasible that it is only with the repeated dosing strategy over a longer period of time that the reduced NAPE-PLD protein

levels affect the rate of NAE synthesis and have a physiological effect. Indeed, if the rate of NAE synthesis is lower in the repeated treatment group, then the effects of inhibition of FAAH in terms of increasing levels of NAEs would be less pronounced. It is important to bear in mind that the contribution of the NAPE-PLD pathway, relative to other synthetic routes of synthesis (Ueda *et al.*, 2010), to the generation of NAEs has not been widely studied. Genetic deletion of NAPE-PLD decreases basal brain levels of saturated long chain NAEs (including PEA), but not unsaturated NAEs (including AEA; Leung *et al.*, 2006). Thus, if NAPE-PLD contributes in a similar manner to stimulated levels of NAEs, the reported decrease in protein levels of NAPE-PLD would be predicted to have a greater influence on the levels of PEA than on those of AEA.

Previously, we have reported an increased expression of spinal cord NAPE-PLD protein, but not mRNA, in a chronic model of osteoarthritic pain (Sagar *et al.*, 2010). However, in this shorter term, carrageenan-induced, model of inflammatory pain neither NAPE-PLD mRNA nor protein levels in the spinal cord were altered. Inflammatory signalling decreased NAPE-PLD mRNA and protein levels locally at the site of injury (Marquez *et al.*, 2009). LPS, which, like carrageenan, stimulates Toll-like receptor 4, has been proposed to cause an increased histone deacetylase activity, leading to decreased NAPE-PLD mRNA and protein levels in RAW264.7 macrophages (Zhu *et al.*, 2011). The demonstration that NAPE-PLD expression is regulated in a tissue and stimulus-specific manner, and that this pathway is influenced by FAAH inhibitors, supports the further investigation of the transcriptional and post-transcriptional control of NAPE-PLD.

The spinal activation of pro-inflammatory signalling pathways, in particular the induction of COX-2 and PGE₂ production plays a critical role in inflammatory pain states (Samad *et al.*, 2001). Thus, we investigated whether the differential effects of acute versus repeated URB597 treatment on pain behaviour reflected differences in the ability of URB597 to modulate pro-inflammatory signalling at this level. Despite the lack of effect of repeated URB597 treatment on pain behaviour, both acute and repeated URB597 treatment attenuated carrageenan-induced pro-inflammatory signalling at the level of the spinal cord, in particular iNOS and IL1 β induction. These data suggest that iNOS and/or IL1 β have a supporting, but not essential, role in these models of inflammatory pain which is consistent with the report that genetic disruption of iNOS (Tao *et al.*, 2003) or IL1 β (Fantuzzi and Dinarello, 1996) did not alter inflammatory responses to carrageenan, or LPS respectively. The effects of acute and repeated administration of URB597 on carrageenan-induced expression of COX-2 and mPGES-1 were, however, less clear-cut. Acute URB597 produced a robust inhibition of carrageenan-induced COX-2 and mPGES-1 gene induction. Repeated administration of URB597 had less robust inhibitory effects on carrageenan-induced COX-2 and mPGES-1 gene induction, but there was a tendency for these to be inhibited. Small differences in the absolute levels of carrageenan-induced COX-2 induction in the control groups (acute saline vs. repeated saline), which probably reflected the biological variation associated with these types of complex *in vivo* studies, prevented further conclusions being drawn.

In conclusion, we have demonstrated that repeated administration of low-dose URB597 did not produce a behav-

ioural analgesia, in contrast to the robust inhibitory effect of acute URB597 treatment. These behavioural effects were consistent with the differential effect of acute and repeated URB597 treatment on levels of NAEs in the spinal cord, but not the midbrain, but did not reflect differences in their ability to attenuate FAAH activity. Our data support the accumulating evidence that there are tissue-specific differences in the synthetic and catabolic pathways that regulate levels of NAEs, which may have further significance under pathological conditions, such as the spinal processing of inflammatory pain.

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Conflicts of interest

There are no conflicts of interest to be declared.

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