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RESEARCH PAPER

The fatty acid amide hydrolase (FAAH) inhibitor PF-3845 acts in the nervous system to reverse LPS-induced tactile allodynia in mice

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BACKGROUND AND PURPOSE

Inflammatory pain presents a problem of clinical relevance and often elicits allodynia, a condition in which non-noxious stimuli are perceived as painful. One potential target to treat inflammatory pain is the endogenous cannabinoid (endocannabinoid) system, which is comprised of CB_1 and CB_2 cannabinoid receptors and several endogenous ligands, including anandamide (AEA). Blockade of the catabolic enzyme fatty acid amide hydrolase (FAAH) elevates AEA levels and elicits antinociceptive effects, without the psychomimetic side effects associated with Δ^9-tetrahydrocannabinol (THC).

EXPERIMENTAL APPROACH

Allodynia was induced by intraplantar injection of LPS. Complementary genetic and pharmacological approaches were used to determine the strategy of blocking FAAH to reverse LPS-induced allodynia. Endocannabinoid levels were quantified using mass spectroscopy analyses.

KEY RESULTS

FAAH (–/–) mice or wild-type mice treated with FAAH inhibitors (URB597, OL-135 and PF-3845) displayed an anti-allodynic phenotype. Furthermore, i.p. PF-3845 increased AEA levels in the brain and spinal cord. Additionally, intraplantar PF-3845 produced a partial reduction in allodynia. However, the anti-allodynic phenotype was absent in mice expressing FAAH exclusively in the nervous system under a neural specific enolase promoter, implicating the involvement of neuronal fatty acid amides (FAAs). The anti-allodynic effects of FAAH-compromised mice required activation of both CB_1 and CB_2 receptors, but other potential targets of FAA substrates (i.e. μ-opioid, TRPV1 and PPARα receptors) had no apparent role.

CONCLUSIONS AND IMPLICATIONS

AEA is the primary FAAH substrate reducing LPS-induced tactile allodynia. Blockade of neuronal FAAH reverses allodynia through the activation of both cannabinoid receptors and represents a promising target to treat inflammatory pain.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids in Biology and Medicine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.165.issue-8. To view Part I of Cannabinoids in Biology and Medicine visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

Abbreviations

2-AG, 2-arachidonoylglycerol; AEA, arachidonoylethanolamide (anandamide); CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; i.pl., intraplantar; IRTX, 5′-iodoresiniferatoxin; THC, Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential channels of vanilloid type-1

Introduction

The endogenous cannabinoid (endocannabinoid) system consists of CB_1 and CB_2 receptors (Matsuda *et al.*, 1990; Gerard *et al.*, 1991) and endogenous signalling lipids that bind to and activate these receptors, such as anandamide (AEA) (Devane *et al.*, 1992). AEA levels are tightly regulated *in vivo* by the catabolic enzyme fatty acid amide hydrolase (FAAH) (Cravatt *et al.*, 1996). In contrast to Δ^9-tetrahydrocannabinoid (THC), the primary psychoactive constituent in *Cannabis sativa*, which globally activates cannabinoid receptors, endogenous cannabinoids are believed to be synthesized and released on demand and are regulated via enzymatic degradation. Accordingly, elevating endogenous levels of AEA in the CNS and periphery through genetic deletion or pharmacological inhibition of FAAH reduces nociceptive behaviour, but without the general cannabimimetic behavioural effects associated with THC (Cravatt *et al.*, 2001; Piomelli *et al.*, 2006). Importantly, blockade of FAAH leads to a hypo-algesic phenotype in several laboratory animal models of nociception (Lichtman *et al.*, 2004b). URB597, an irreversible FAAH inhibitor, as well as OL-135, a reversible FAAH inhibitor, reduces nociceptive responses in acute (Holt *et al.*, 2005; Naidu *et al.*, 2009, 2010) and chronic models of pain (Jayamanne *et al.*, 2006). More recently, a new FAAH inhibitor (PF-3845) was developed that is highly selective for FAAH and has a longer duration of action than previous FAAH inhibitors (Ahn *et al.*, 2009). The present study tested whether genetic deletion or pharmacological inhibition of the endocannabinoid catabolic enzyme FAAH, blocks tactile allodynia induced by the bacterial endotoxin LPS derived from the outer cell wall of gram (–) bacteria. LPS was injected into the plantar surface of the paw to elicit a mild innate inflammatory response, resulting in tactile allodynia.

The first goal of the present study was to modify the previously characterized LPS model of inflammation (Naidu *et al.*, 2010) to induce tactile allodynia without producing overt oedema of the paw or eliciting a general malaise (e.g. loss of body weight). To ensure validity of the LPS model, we tested the GABA analogue gabapentin, which possesses efficacy in treating various types of pain (Staahl *et al.*, 2009). In addition to this positive control, we examined the effects of global activation of cannabinoid receptors, using THC. In the second series of experiments, we examined whether genetic deletion of FAAH reduces LPS-induced allodynia. FAAH-NS mice, which express the enzyme under a neural specific enolase promoter (Cravatt *et al.*, 2004), were used to distinguish whether inhibiting FAAH expressed in the peripheral and/or nervous system(s) mediates the observed anti-allodynic effects. Thirdly, we examined whether pharmacological blockade of FAAH would reverse LPS-induced allodynia by comparing the anti-allodynic effects of the reversible, α-ketoheterocycle FAAH inhibitor OL-135 (Lichtman *et al.*, 2004a; Boger *et al.*, 2005), to two irreversible FAAH inhibitors, the carbamate URB597 (Piomelli *et al.*, 2006), and the piperidine urea PF-3845, which carbamylates FAAH's serine nucleophile (Ahn *et al.*, 2009). In addition, LC/MS/MS analysis was used to quantify endocannabinoid levels after systemic or local administration of PF-3845. Fourthly, we examined the receptor mechanism(s) of action underlying the observed anti-allodynic effects in FAAH-compromised mice. Several studies have implicated a role for CB_1 receptors in reducing hyperalgesia and CB_2 receptors in ameliorating oedema [see review (Anand *et al.*, 2009)]. Thus, we sought to determine whether these two cannabinoid receptors play a role in the anti-allodynic effects of FAAH (–/–) mice or wild-type mice treated with FAAH inhibitors. In addition, we examined various non-cannabinoid receptors, including the μ-opioid receptor shown to mediate the antihyperalgesic response in the rat spinal nerve ligation and mild thermal injury models (Chang *et al.*, 2006), the TRPV1 receptor reported to play a vital role in the antinociceptive effects of AEA in the thermal hyperalgesia model of inflammation (Horvath *et al.*, 2008), and the PPARα receptor which was shown to mediate the antihyperalgesic effects of URB597 in an acute model of inflammation (Jhaveri *et al.*, 2008).

Methods

Subjects

Adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA), adult male and female FAAH (–/–) mice backcrossed for at least 13 generations onto a C57BL/6J background, and male and female FAAH (+/+) mice derived from the same line of FAAH (+/–) breeders used to derive FAAH (–/–) mice served as subjects. Additionally, male and female nervous system FAAH-restricted (FAAH-NS) (Cravatt *et al.*, 2004) mice backcrossed onto a C57BL/6J background for at least 13 generations were used. FAAH (+/–) littermates were used as controls, because they express wild-type levels of AEA and non-cannabinoid fatty acid amides (FAAs) (Cravatt *et al.*, 2001). Lastly, male and female CB_1 (–/–) and CB_2 (–/–) mice, along with respective matched CB_1 (+/+) and CB_2 (+/+) littermates were used to determine receptor mechanisms of action. All genetically modified mice were bred in the Center Transgenic Colony at Virginia Commonwealth University. The genotype of each genetically altered mouse was confirmed via rt-PCR. Subjects weighed between 20–30 g and were housed four to six per cage in a temperature-controlled (20–22°C) environment. Mice were randomly assigned to treatment conditions, although a block design was used to distribute transgenic and knockout mice by sex evenly across treat-

ments. Mice were kept on a 12 h light/dark cycle with food and water available *ad libitum*. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in concordance with the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). After testing was completed, mice were humanely killed by CO_2 asphyxiation, followed by cervical dislocation.

Drugs

URB597 (1–10 mg·kg^{-1} i.p.), gabapentin (3–30 mg·kg^{-1} i.p.), and MK886 [(1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid sodium salt] were purchased from Cayman Chemical (Ann Arbor, MI, USA). The 3 mg·kg^{-1} dose of MK886 used was shown to antagonize the PPARα receptor in a previous study (Kehrer *et al.*, 2001). This compound is also known to antagonize lipoxygenase, which will decrease leukotriene production, an action implicated in modulating inflammatory pain (Masferrer *et al.*, 2010). OL-135 (1–30 mg·kg^{-1} i.p.) (Boger *et al.*, 2005), and PF-3845 [1–10 mg·kg^{-1} i.p.; 0.1–10 μg intraplantar (i.pl.)] (Ahn *et al.*, 2009) were synthesized as described previously. THC, rimonabant (CB_1 receptor antagonist) and SR144528 (N-[(1S)-endo-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; CB_2 receptor antagonist) were obtained from the National Institute on Drug Abuse (Bethesda, MD, USA). 5′-Iodoresiniferatoxin (IRTX; TRPV1 receptor antagonist) was purchased from LC Laboratories (Woburn, MA, USA) and used at 0.5 mg·kg^{-1}, a concentration previously shown to antagonize TRPV1 receptors (Wahl *et al.*, 2001). The aforementioned drugs were dissolved in a vehicle consisting of ethanol, alkamuls-620, and 0.9% saline in a ratio of 1:1:18. Naltrexone HCl was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.9% saline. For all systemic injections, the i.p. route of administration was employed, using an injection volume of 10 μL·g^{-1} body weight. Gabapentin and OL-135 were administered 1 h before testing. THC, PF-3845 and URB597 were administered 2 h before testing. For the receptor antagonist experiments, rimonabant (3 mg·kg^{-1} i.p.), SR144528 (3 mg·kg^{-1} i.p.), MK886 (3 mg·kg^{-1} i.p.), IRTX (0.5 mg·kg^{-1} i.p.) or naltrexone (1 mg·kg^{-1} i.p.) was administered 10 min prior to the administration of the FAAH inhibitors/analgesic compounds. For experiments evaluating local effect of FAAH inhibition, PF-3845 (1, 3 or 10 μg) was administered via the i.pl. route of administration into either the LPS-treated paw or the saline-treated control paw 2 h prior to testing, using a total volume of 5 μL. The nomenclature of receptors and ligands follows BJP's *Guide to Receptors and Channels* (GRAC) (Alexander *et al.*, 2011).

Inflammatory pain model

Inflammatory pain was induced by injecting LPS from *Escherichia coli* 026:B6 Sigma (St. Louis, MO, USA) (Naidu *et al.*, 2010) in 20 μL of physiological saline into the plantar surface of one hind paw of each mouse (Kanaan *et al.*, 1996). Saline was administered into the opposite hind paw to assess whether the different genotypes or drug treatments affected withdrawal responses in non-inflamed paws. Thus, each mouse served as its own control, thereby reducing the total number of mice required.

Animals were tested for tactile allodynia 24 h post LPS administration using calibrated von Frey filaments, ranging from 0.16–6.0 g (Stoelting, Wood Dale, IL, USA), as described previously (Kinsey *et al.*, 2010). At 23 h after LPS administration, mice were placed into Plexiglas cylinders on an elevated wire mesh screen, allowing access to each hind paw by the filaments. Mice were allowed to acclimatize to the test apparatus for 60 min prior to testing. Paw withdrawal threshold was determined by using the 'up-down' method (Chaplan *et al.*, 1994). The plantar surface of each paw was stimulated five times each and a positive response was scored if each mouse clutched, lifted or fluttered the paw upon three of five stimulations. The experimenter was blinded with respect to genotype or drug treatment during von Frey testing. The thickness of the LPS-treated and saline-treated paws was measured both before and 24 h after LPS injection, using digital calipers (Traceable Calipers, Friendswood, TX, USA) and expressed to the nearest ±0.01 mm (Naidu *et al.*, 2010). The 24 h paw thickness values were measured immediately after allodynia assessment.

Extraction and quantification of endocannabinoids by LC/MS/MS

AEA and 2-AG levels were quantified in the whole brain, whole spinal cord, and paw tissue of male C57BL/6J mice treated with a systemic dose of PF-3845 (10 mg·kg^{-1}, i.p.) or local dose (1.0 μg, i.pl.) as described above. Two hours after drug or vehicle administration, the mice were decapitated and tissues were harvested. All tissue samples were snap frozen in liquid nitrogen and stored at –80°C until the endocannabinoids were extracted.

On the day of extraction, tissues were weighed and homogenized with 1.4 mL chloroform/methanol (2:1 v v^{-1} containing 0.0348 mg PMFS·mL^{-1}) after the addition of internal standards to each sample (2 pmol AEA-d8 and 1 nmol 2-AGd8) (Kinsey *et al.*, 2009). Homogenates were then mixed with 0.3 mL of 0.73% w/v NaCl, vortexed, and then centrifuged for 10 min at 3200× *g* (4°C). The aqueous phase plus debris were collected and extracted two more times with 0.8 mL chloroform. The organic phases from the three extractions were pooled, and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 mL chloroform and mixed with 1 mL ice-cold acetone. The mixtures were then centrifuged for 5 min at 1800× *g* and 4°C to precipitate the proteins. The upper layer of each sample was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 mL methanol and placed in autosampler vials for analysis. LC/MS/MS was used to quantify AEA and 2-AG. The mobile phase consisted of (10:90) water/methanol with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 4.6 × 15 cm, 3 μm (Supelco, PA, USA). The mass spectrometer was run in Electrospray Ionization, in positive mode. Ions were analysed in multiple-reaction monitoring mode, and the following transitions were monitored: (348 > 62) and (348 > 91) for AEA; (356 > 62) for AEAd8; (379 > 287) and (279 > 269) for 2-AG; and (387 > 96) for 2AG-d8. A calibration curve was constructed for each assay based on linear regression

using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.03 to 40 pmol for AEA and from 0.05 to 64 nmol for 2-AG.

Data analysis

The dependent measures included changes in paw oedema (24 h – baseline paw thickness values) and mechanical paw withdrawal thresholds 24 h after LPS. All data are reported as mean ± SEM and were analysed using one-way ANOVA or two-way ANOVA in the experiments evaluating the effects of PF-3845 in CB_1 (–/–) or CB_2 (–/–) mice versus the wild-type control mice. Dunnett's test was used for *post hoc* analysis in the dose–response experiments to compare the effects of each drug dose to those of vehicle. Tukey–Kramer *post hoc* analysis was used for all tests comparing different treatment groups, as well as genotype distinctions. Differences were considered significant at the $P < 0.05$ level.

Results

Anti-allodynic effects of gabapentin and THC in the LPS model

Intraplantar LPS elicited profound allodynia in the LPS-treated paw, but not the saline-treated, control paw [$F(3,24) = 10.54$, $P < 0.001$; Figure 1A], and occurred at concentrations 10-fold less than those required to produce paw oedema [$F(3,24) = 11.18$, $P < 0.0001$; Figure 1B]. Whereas the high dose of LPS (25 μg) produced a significant increase in paw thickness, 2.5 μg LPS had no effect on oedema measurements, as compared with the saline-injected paw. In addition to the lack of oedema after i.pl. injection of 2.5 μg LPS, no weight loss occurred. Thus, this dose of LPS was used in all subsequent experiments.

The LPS injection significantly decreased mechanical paw withdrawal threshold, as compared with saline-treated paws, which remained constant throughout all of the studies. The GABA analogue gabapentin significantly reversed LPS-induced allodynia [$F(3,24) = 4.4$, $P < 0.05$; Figure 2A)]. Gabapentin did not affect paw withdrawal threshold in saline-injected control paws ($P = 0.64$; Figure S1A). Similarly, THC significantly reversed LPS-induced allodynia in the LPS-treated paw [$F(3, 28) = 5.71$, $P < 0.01$; Figure 2B], but did not modify the saline-treated paw threshold (Figure S1B).

FAAH (–/–) mice display an anti-allodynic phenotype

The objectives of this experiment were to determine whether FAAH (–/–) mice display a reduction in tactile allodynia, and to examine whether this phenotype would occur in mice that express the enzyme exclusively in the nervous system and not in non-neuronal tissue (Cravatt *et al.*, 2004). The subjects consisted of FAAH (–/–) mice, neural specific FAAH knock-in (FAAH-NS) mice and the FAAH (+/–) control group. FAAH (–/–) mice displayed significantly less allodynia to LPS treatment than either FAAH (+/–) control mice or FAAH-NS mice [$F(2,21) = 8.99$, $P < 0.01$; Figure 3A]. FAAH-NS mice showed wild-type responses to LPS-induced inflammatory pain, indicating that the FAAH anti-allodynic phenotype is mediated by neuronal FAAs. In addition, there were no genotype differences between groups in the saline-treated paw ($P = 0.96$, Figure S2A).

To determine the receptor mechanism of action by which FAAH inhibition caused anti-allodynic effects, FAAH (–/–) mice were pretreated with the CB_1 receptor antagonist, rimonabant or the CB_2 receptor antagonist, SR144528. Both rimonabant and SR144528 completely reversed the FAAH anti-allodynic phenotype ($P < 0.05$; Figure 3B). No significant effects were found in the saline-treated paw (Figure S2B).

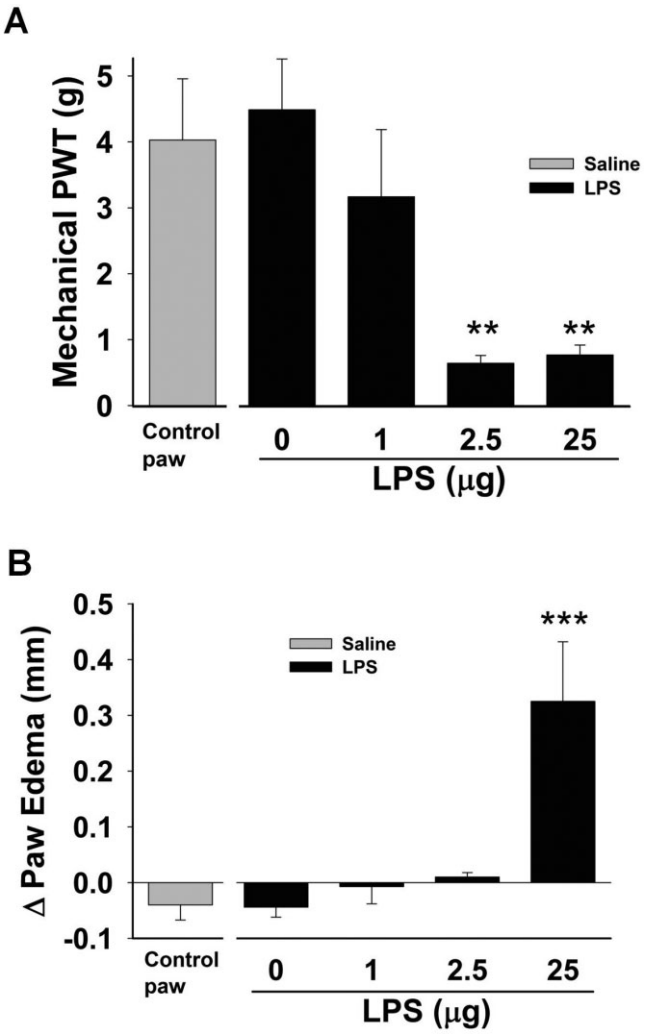

Figure 1

Intraplantar injection of LPS is more potent in eliciting tactile allodynia than in producing paw oedema. (A) Decreased mechanical paw withdrawal thresholds (PWTs) were reduced in the LPS-injected paw 24 h after injection. LPS injection, at doses of 2.5 μg or 25 μg per paw, increased sensitivity to tactile stimulation that was significantly different from PWT in the saline-injected paw of the same mice. (B) Paw oedema was significantly increased in hind paws injected with the high dose of LPS (i.e. 25 μg). Control paw represents the saline-injected paw of LPS treated mice. Values represent the mean (±SEM) mechanical PWT. $n = 6$–9 mice per group. $^{**}P < 0.01$; $^{***}P < 0.001$ versus saline-treated paw.

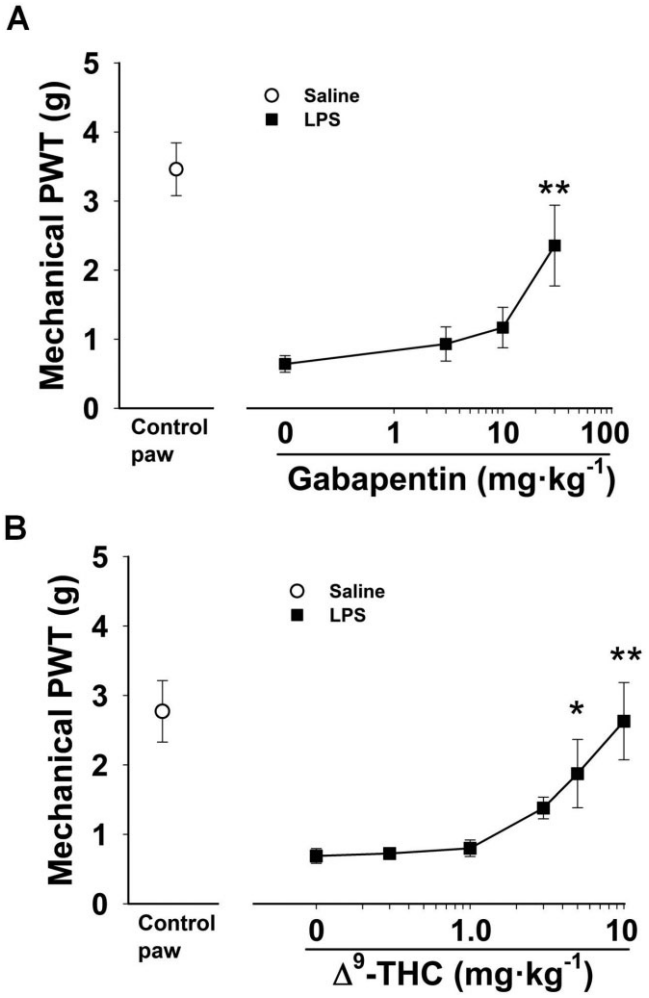

Figure 2

Systemic administration of gabapentin or THC reduces the tactile allodynia caused by i.pl. LPS (2.5 μg). (A) Gabapentin (30 mg·kg^{-1}) reversed tactile allodynia induced by LPS. (B) THC (5 or 10 mg·kg^{-1}) reversed LPS-induced allodynia in the treated paw. Control paw represents the saline-injected paw of LPS-treated mice. Values represent the mean (±SEM) mechanical paw withdrawal threshold (PWT). n = 6–10 mice per group. *P < 0.05; **P < 0.01 versus vehicle in the LPS-treated paw.

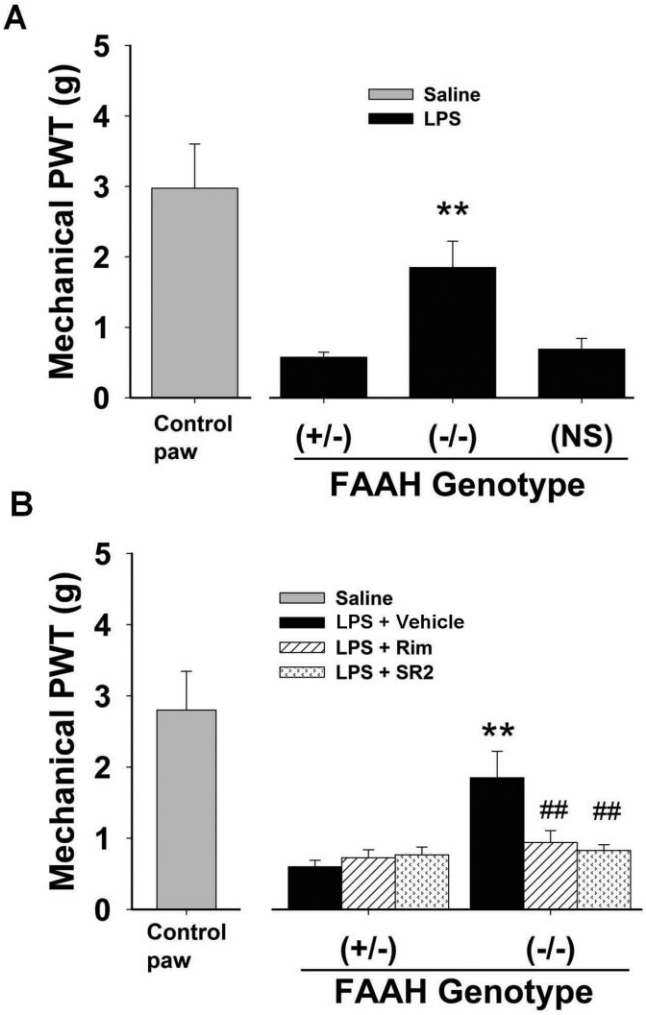

Figure 3

Deletion of FAAH within the nervous system reduces LPS-induced allodynia. (A) Control FAAH (+/–) mice displayed profound allodynic responses to i.pl. LPS (2.5 μg), whereas FAAH knockout mice (–/–) showed an anti-allodynic phenotype. FAAH-NS mice that express FAAH exclusively in neuronal tissue displayed a wild-type allodynic response to LPS. (B) The anti-allodynic phenotype in FAAH (–/–) mice was suppressed by the pretreatment of rimonabant (3 mg·kg^{-1}; Rim) and SR144528 (3 mg·kg^{-1}; SR2). Control paw represents the saline-injected paw of FAAH (+/–) LPS-treated mice. All values represent the mean (±SEM) mechanical paw withdrawal threshold (PWT). n = 6–10 mice per group. **P < 0.01 versus FAAH (+/–) mice or FAAH-NS mice (panel A); ##P < 0.01 versus LPS-treated FAAH (–/–) mice that received vehicle. Rim: rimonabant; SR2: SR144528.

Evaluation of endocannabinoid catabolic enzyme inhibitors on LPS-induced allodynia

As shown in Figure 4A, administration of each FAAH inhibitor, PF-3845 [F(3,24) = 6.07, P < 0.01], URB597 [F(3,30) = 5.95, P < 0.01] or OL-135 [F(4,41) = 3.82, P < 0.01], significantly reversed LPS-induced tactile allodynia, but did not modify paw withdrawal thresholds in the saline-injected paw (Figure S3A). As shown in Table 1, i.p. administration of PF-3845 (10 mg·kg^{-1}) significantly increased AEA levels, but not 2-AG levels, in the brain and spinal cord. In the light of the recent report by Clapper *et al.* (2010) showing that a peripherally restricted FAAH inhibitor reduces pain responses, we next evaluated whether i.pl. administration of PF-3845 reverses LPS-induced allodynia. Intraplantar administration of PF-3845 (1, 3 or 10 μg) partially reversed allodynia in the LPS administered paw P < 0.05 (Figure 4B), but did not affect withdrawal responses in the saline-treated paw (Figure S3B). Importantly, an i.pl. injection of PF-3845 (1 μg) to the saline-treated paw did not alter allodynia in the LPS-treated paw (Figure 4C), consistent with the notion that the drug effects were locally mediated. The observation that i.pl. administration of PF-3845 at 1 μg did not affect endocannabinoid levels in the brain or spinal cord (see Table 1) further supports the idea that these doses of PF-3845 were mediated through a local site of action. In contrast, systemic adminis-

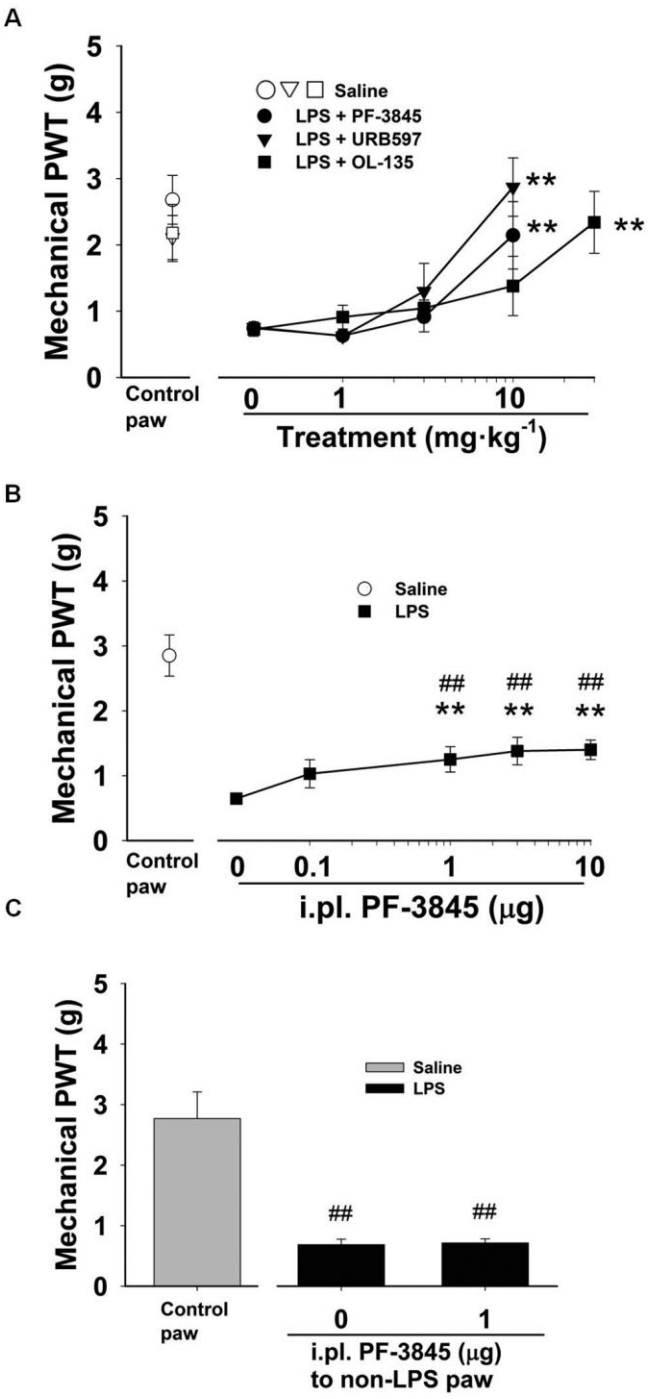

Figure 4

FAAH inhibitors reduce LPS-induced allodynia. (A) Three different FAAH inhibitors dose-dependently reversed tactile allodynia 24 h after i.pl. LPS administration. URB597 (10 mg·kg^{-1}), PF-3845 (10 mg·kg^{-1}) and OL-135 (30 mg·kg^{-1}), reversed the allodynic response produced by i.pl. injection of LPS (2.5 μg). Open symbols represent the control saline-injected paw of each respective treatment group. (B) Intraplantar administration of PF-3845 (1, 3, or 10 μg) partially reversed LPS-induced allodynia. (C) PF-3845 administered to the saline-treated, control paw did not reduce allodynic responses in the LPS-treated paw. Control paw represents the saline-injected paw of LPS only treated mice. Values represent the mean (±SEM) mechanical paw withdrawal threshold (PWT). n = 7–12 mice per group. **P < 0.01 versus vehicle treatment in the LPS-treated paw; ##P < 0.01 versus saline-treated paw.

tration of PF-3845 (10 mg·kg^{-1}) or i.pl. injection of a much higher dose of drug (i.e. 10 μg) caused significant increases in AEA, but not 2-AG levels in the brain and spinal cord. However, no differences in paw skin endocannabinoid levels after PF-3845 administration were detected at the highest concentration tested (see Table 1).

We next tested whether PF-3845 produces anti-allodynic effects in FAAH-NS mice. As shown in Figure 5, PF-3845 (10 mg·kg^{-1}) elicited significant anti-allodynic actions in FAAH (+/–) and FAAH (NS) mice, which significantly differed from mice treated with LPS only [F(2,52) = 4.35, P = 0.018]. In contrast, PF-3845 did not alter the anti-allodynic phenotype of FAAH (–/–) mice, suggesting that the anti-allodynic effects of this drug occurred because of its inhibition of FAAH in the peripheral NS and/or CNS(s). No effects of genotype or drug were found in the saline-treated paws (Figure S4).

In the final series of experiments, we evaluated the potential involvement of CB_1, CB_2, TRPV1, opioid and PPARα receptors in the anti-allodynic effects of PF-3845 (10 mg·kg^{-1}). First, we evaluated the effects of PF-3845 in CB_1 (–/–) and CB_2 (–/–) mice. PF-3845 increased the paw withdrawal threshold values in CB_1 (+/+) mice as well as in CB_2 (+/+) mice (two-way ANOVA P < 0.01 for each), but failed to increase paw withdrawal thresholds in either CB_1 (–/–) mice (Figure 6A) or CB_2 (–/–) mice (Figure 6B). As found previously, PF-3845 administration did not modify responses to the von Frey filaments in the saline-treated paw (Figure S5A and B).

Several other receptor systems may contribute to the anti-allodynic actions of PF-3845. For example, AEA acts as a full agonist of TRPV1 receptors (Smart *et al.*, 2000). In addition, FAAH regulates the degradation of non-cannabinoid FAAs, such as N-palmitoylethanolamine (PEA) and oleoylethanolamide (OEA), each of which possesses anti-inflammatory actions through the PPARα receptor. Also, naloxone has been shown to reduce the antinociceptive actions of the FAAH inhibitor OL-135 (Chang *et al.*, 2006). Thus, we explored whether these non-cannabinoid receptors also play a role in the anti-allodynic effects of PF-3845. Mice received the opioid receptor antagonist naltrexone (1 mg·kg^{-1}), the TRPV1 antagonist IRTX (0.5 mg·kg^{-1}), or the PPAR-α antagonist MK886 (3 mg·kg^{-1}) before administration of PF-3845 (10 mg·kg^{-1}). These antagonists did not modify the anti-allodynic effects of PF-3845 (Figure 6C) and had no effects on LPS-induced allodynia when given alone (Figure S6). As before, no effects of drug treatment were observed on the control paw (Figure S5C).

Discussion

Allodynia and hyperalgesia are common clinical features of many inflammatory diseases and disorders. Whereas hyperalgesia reflects an increased sensitivity to a noxious stimulus, allodynia is a painful response to a typically non-

Table 1

LC/MS/MS analysis of brain and spinal cord tissues after systemic (10 mg·kg^{-1}; i.p.) or local (0.1–10 μg; intraplantar) PF-3845 administration in mice

Administration route	Injection	Brain AEA (pm·g^{-1})	2-AG (nm·g^{-1})	Spinal cord AEA (pm·g^{-1})	2-AG (nm·g^{-1})	Paw tissue AEA (pm·g^{-1})	2-AG (nm·g^{-1})
I.p.	Vehicle	1.55 ± 0.11	9.04 ± 0.89	3.95 ± 0.18	9.35 ± 1.23	ND	ND
I.p.	PF-3845 (10 mg·kg^{-1})	16.67 ± 1.15**	9.80 ± 1.02	37.78 ± 2.15**	10.32 ± 2.22	ND	ND
Intraplantar	Vehicle	4.32 ± 0.84	6.82 ± 0.74	5.34 ± 0.60	10.34 ± 0.61	6.14 ± 0.50	2.47 ± 0.23
Intraplantar	PF-3845 (0.1 μg)	5.55 ± 0.62	7.42 ± 0.42	6.88 ± 1.14	12.41 ± 0.79	ND	ND
Intraplantar	PF-3845 (1.0 μg)	5.69 ± 0.81	7.00 ± 0.38	6.50 ± 0.54	11.84 ± 0.77	ND	ND
Intraplantar	PF-3845 (10.0 μg)	13.12 ± 0.40**	5.56 ± 0.58	28.42 ± 1.85**	7.34 ± 0.74	6.61 ± 0.41	2.48 ± 0.07

Systemic administration of PF-3845 significantly increased anandamide (AEA) levels in both brain and spinal cord $^{**}P < 0.001$. Local administration of PF-3845 (10 μg), but not 0.1 or 1.0 μg, increased AEA in the brain and spinal cord tissues. Values are calculated per wet tissue weight. $n = 6$ mice per group. Data represent the mean (±SEM) endocannabinoid content.
ND, not determined.

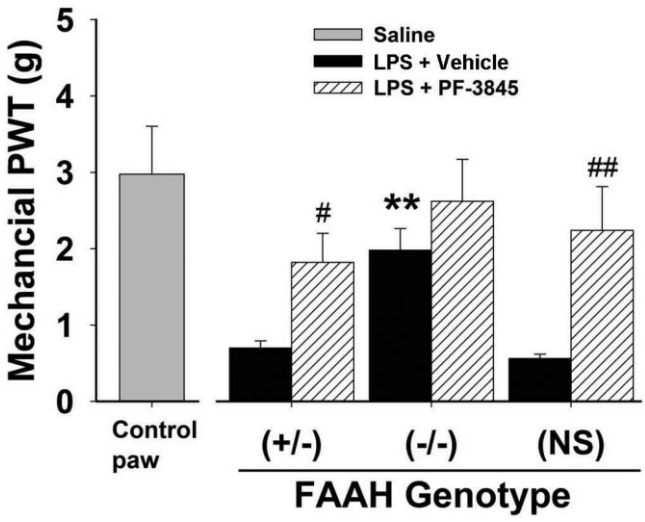

Figure 5

Blocking FAAH in the nervous system mediates the FAAH (–/–) anti-allodynic phenotypic in response to i.pl. LPS. FAAH (–/–) mice displayed an anti-allodynic phenotype that was not present in either the FAAH (+/–) mice or FAAH-NS (i.e. neural specific knock-in) mice. Pretreatment with PF-3845 (10 mg·kg^{-1}) restored the anti-allodynic phenotype in FAAH (+/–) mice and FAAH-NS mice. Control paw represents the saline-injected paw of FAAH (+/–) LPS only treated mice. All values represent the mean (±SEM) mechanical paw withdrawal threshold (PWT). $n = 8$–10 mice per group. $^{**}P < 0.01$ versus the vehicle-treated FAAH (+/–) or FAAH-NS mice; $\#P < 0.05$, $\#\#P < 0.01$ versus vehicle-treated mice for each respective genotype.

noxious stimulus. In a murine model of inflammatory pain, LPS injected into the hind paw leads to increased sensitivity to thermal nociceptive stimuli (Kanaan *et al.*, 1996; Naidu *et al.*, 2010). In the present study, we modified the LPS model by injecting a relatively low concentration of LPS (2.5 μg) into the plantar surface of a hind paw to elicit a profound tactile allodynic response at 24 h, without producing overt oedema or weight loss. A previous report from our laboratory showed maximum thermal hyperalgesic responses to 25 μg per paw LPS, although mice in that study also displayed severe oedema in the paw and significant weight loss, indicating systemic effects of LPS (Naidu *et al.*, 2010). Gabapentin, a commonly used anti-allodynic GABA analogue, as well as the phytocannabinoid THC, reversed LPS-induced allodynia. However, each of these drugs produces significant motor and cognitive side effects (Martin *et al.*, 1991; Backonja *et al.*, 1998). In contrast, elevating the endocannabinoid AEA, by blocking its catabolic enzyme FAAH, is well established to reduce nociceptive behaviour in a variety of animal models of pain, without eliciting the cannabimimetic side effects associated with THC. Therefore, we investigated whether genetic deletion or pharmacological inhibition of FAAH, the primary catabolic enzyme of AEA, would reduce LPS-induced allodynia. Both approaches reduced tactile allodynia via mechanism(s) that required both CB_1 and CB_2 receptors.

The FAAH (–/–) mice displayed a significant anti-allodynic phenotype. However, it is difficult to delineate whether the anti-allodynic responses of FAAH (–/–) mice resulted from increased levels of AEA and other FAAs at the time of testing or because the development of the LPS-induced allodynia was dampened. However, an acute injection of three different FAAH inhibitors reversed the peak allodynic effects of LPS. The reversible (OL-135) and irreversible (URB597, PF-3845) FAAH inhibitors attenuated LPS-induced allodynia. URB597 has antihyperalgesic effects in various models of inflammatory pain, such as carrageenan and complete Freund's adjuvant (CFA) models (Holt *et al.*, 2005; Jayamanne *et al.*, 2006). Similarly, the reversible FAAH inhibitor, OL-135 suppresses nociception in thermal pain models (i.e. tail immersion, hot-plate) and the formalin model of pain (Lichtman *et al.*, 2004a). Here, we show for the first time that OL-135 elicits anti-allodynic effects in an inflammatory pain model. Likewise, PF-3845, which has longer lasting effects than other FAAH inhibitors, also reduces allodynia in the rat CFA model (Ahn *et al.*, 2009).

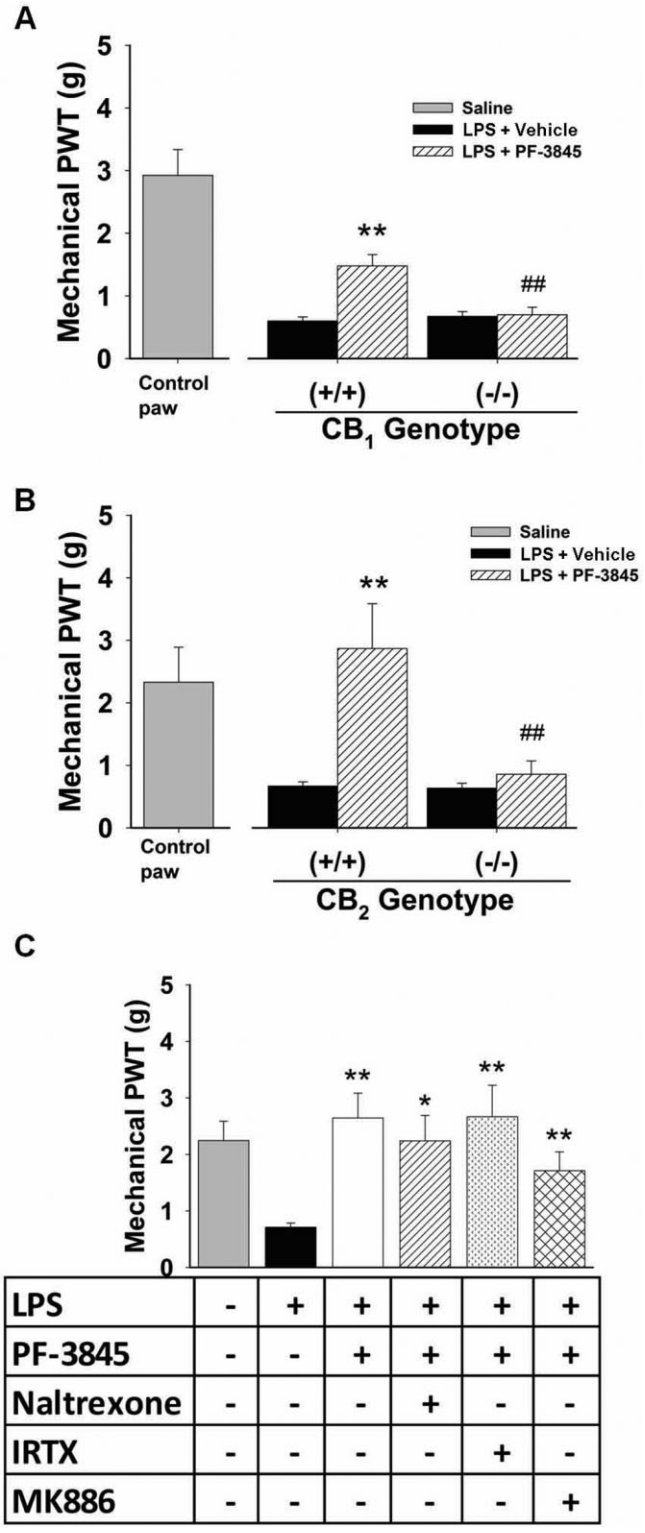

Figure 6

PF-3845 reduces LPS-induced allodynia through a cannabinoid receptor mechanism of action. (A) PF-3845 (10 mg·kg^{-1}) reduced LPS-induced allodynia in CB_1 (+/+) mice, but not in CB_1 (–/–) mice. Control paw represents the saline-injected paw of CB_1 (+/+) LPS only treated mice. (B) PF-3845 (10 mg·kg^{-1}) reduced LPS-induced allodynia in CB_2 (+/+) mice, but not in CB_2 (–/–) mice. Control paw represents the saline-injected paw of CB_2 (+/+) LPS only treated mice. (C) The anti-allodynic effects of PF-3845 (10 mg·kg^{-1}) in the LPS model were not blocked by the opioid receptor antagonist naltrexone (1 mg·kg^{-1}), the TRPV1 receptor antagonist IRTX (0.5 mg·kg^{-1}) or the PPARα antagonist MK886 (3 mg·kg^{-1}). Data shown in (C) represent two combined experiments contracted into a single figure. Control paw represents the saline-injected paw of LPS only treated mice. n = 6–10 mice per group. Values represent the mean (±SEM) mechanical paw withdrawal threshold (PWT). $^{**}P < 0.01$ versus vehicle-treated mice in the LPS-injected paw; $^{\#\#}P < 0.01$ versus PF-3845-treated CB_1 (+/+) or CB_2 (+/+) mice.

The results of the present study demonstrate that both CB_1 and CB_2 receptors play an essential role in mediating the anti-allodynic phenotype of FAAH-compromised mice in the LPS model. These data are congruent with other reports showing that both CB_1 and CB_2 receptors are required for the anti-allodynic effects of FAAH inhibition, such as the chronic constriction injury model (Russo *et al.*, 2007; Kinsey *et al.*, 2009, 2010) and the partial sciatic and spinal nerve injury models of pain (Jhaveri *et al.*, 2006; Desroches *et al.*, 2008). The results may be attributed to sequestration of the cannabinoid receptors. *In situ* hybridization revealed that CB_1 receptors are expressed in cells of the dorsal root ganglia inserted on nerve terminals in the periphery (Hohmann and Herkenham, 1999). Furthermore, the CB_2 receptor is expressed on activated mast cells (Facci *et al.*, 1995), which infiltrate peripheral nerve tissues during an innate inflammatory response, such as that initiated by LPS exposure. However, the two cannabinoid receptors play differential roles in mediating the antinociceptive actions of FAAH blockade in other pain models. Only the CB_1 receptor mediates the antinociceptive effects of FAAH (–/–) mice in the tail withdrawal and formalin test (Lichtman *et al.*, 2004b). In contrast to the data in the present study demonstrating FAAH inhibitors require both cannabinoid receptors to reduce tactile allodynia caused by LPS, we previously reported FAAH inhibition ameliorates thermal hyperalgesia through the activation of CB_1 receptors, only (Naidu *et al.*, 2010). The phenotypic anti-oedematous actions caused by FAAH deletion were mediated by the CB_2 receptor, not the CB_1 receptor, in carrageenan (Lichtman *et al.*, 2004b) and LPS (Naidu *et al.*, 2010) paw inflammatory assays.

FAAH-NS mice, which express FAAH only in nervous tissue (Cravatt *et al.*, 2004), displayed similar allodynic responses to i.pl. LPS as the FAAH (+/–) control mice. This finding, along with the observation that the highly selective FAAH inhibitor PF-3845 had anti-allodynic effects in FAAH-NS mice, supports the idea that the anti-allodynic effects induced by FAAH inhibition are mediated in the central and/or peripheral nervous system(s). In contrast, we recently reported that FAAH-NS mice show an anti-oedematous phenotype when given an i.pl. injection of 25 μg LPS (Naidu *et al.*, 2010). The results of these studies indicate that different pools of FAAs mediate the oedematous versus allodynic effects of LPS. Whereas elevating these lipid signalling molecules in non-neuronal tissue mediates the anti-oedematous effects, the anti-allodynic actions appear to be mediated through the nervous system. Of note, a recent study found that a peripherally restricted FAAH inhibitor

(URB937) reduced mechanical and thermal hyperalgesia in the carrageenan inflammatory pain model as well as the peripheral nerve injury pain model (Clapper *et al.*, 2010). Thus, targeting FAAH in the peripheral nervous system, as well as in the CNS (Suplita *et al.*, 2005), effectively blocks pain-related behaviour, possibly by differentially elevating distinct pools of FAAs.

In agreement with previous data (Ahn *et al.*, 2009), i.p. administration of PF-3845 increased AEA levels in the brain and spinal cord, without increasing 2-AG levels. Furthermore, we found local administration of PF-3845 into the LPS-injected paw produced a small, but significant, reduction in the tactile allodynic response to LPS at doses that did not alter levels of AEA in the brain or spinal cord, suggesting that the anti-allodynic effects of PF-3845 were not merely due to diffusion into the CNS. The lack of increased levels of AEA in paw skin by PF-3845 may be limited by challenges associated with extracting lipids from mouse skin or possibly low levels of FAAH on discrete sensory neurons relative to the large surface area of the paw. Regardless, the finding that PF-3845 administered into the contralateral paw did not reduce allodynia in the LPS-injected paw provides compelling indirect evidence supporting a local site of action. Interestingly, the highest dose of PF-3845 (10 μg) administered into the paw led to significant increases in brain and spinal cord AEA levels, although it did not produce a further increase in the magnitude of the anti-allodynic effects. According to the data presented in Table 1, this dose of drug administered i.pl. led to 3-fold and 5-fold increases in the levels of AEA in brain and spinal cord, respectively, while 10 $mg \cdot kg^{-1}$ of PF-3845 administered i.p., produced 10-fold increases in both the brain and spinal cord. These findings taken together indicate that PF-3845 administered into the paw is less efficacious in reversing LPS-induced allodynia than after i.p. administration.

The endotoxin model used in the present study differs from other models of inflammation, in that LPS is derived from the outer cell wall of gram negative bacteria and is commonly used to model an innate inflammatory response. Similar to other inflammatory pain models, such as injection of CFA (derived from a mycobacteria) or carrageenan (extracted from red seaweed), LPS mimics an inflammatory response to a non-self immunogen. Additionally, the LPS model offers a longer temporal dimension than the carrageenan or formalin injection model of inflammation, with peak effects at 24 h, thereby allowing for the detection of subtle anti-inflammatory effects.

The finding that PF-3845 was ineffective in CB_1 (–/–) and CB_2 (–/–) mice indicates that cannabinoid receptors play a necessary role in mediating the anti-allodynic actions caused by FAAH inhibition. CB_2 receptors are highly expressed on immune cells and may play an essential role in modulating the release of inflammatory cytokines/chemokines involved in pain sensitization (Klein *et al.*, 2003; Roche *et al.*, 2006). Therefore, changes in the inflammatory pain response may be attributed to the activation of the CB_2 receptors, which alternatively decrease the release of cytokines and the resulting allodynia. Although the CB_2 receptor is expressed at low levels in the nervous system and much higher levels on mast cells (Facci *et al.*, 1995), previous evidence suggests that it is up-regulated in the dorsal horn in neuropathic pain models (Beltramo *et al.*, 2006; Hsieh *et al.*, 2011), hence changes in CB_2 expression during a disease state may be responsible for the strong role of CB_2 in modulating LPS-induced allodynia. Furthermore, a recent report by Hsieh *et al.* (2011) also illustrated that in the CFA model of inflammation, CB_2 receptor mRNA, but not CB_1 receptor mRNA, is up-regulated in the dorsal root ganglia and paw tissue, but not in the spinal cord. Conversely, the CB_1 receptor is expressed at much higher levels in the nervous system than in other tissues (e.g. immune cells) and has a more prominent role in modulating neurotransmitter signalling than the CB_2 receptor. However, due to the limitations of the test (e.g. floor effect) it is difficult to determine whether genetic deletion of CB_2, for example, would exacerbate LPS-induced allodynia. Notably, local administration of low doses of PF-3845 partially reversed allodynia, suggesting FAAH in the peripheral nervous system modulates inflammatory pain in the LPS-induced allodynia model.

A growing body of research suggests non-cannabinoid receptors and FAAs play a role in the anti-inflammatory actions of FAAH inhibitors (Tognetto *et al.*, 2001; Chang *et al.*, 2006; Sun *et al.*, 2006). For example, although AEA is an agonist at the cannabinoid receptor, it also activates the TRPV1 receptor; albeit its affinity at TRPV1 is lower than its affinity at cannabinoid receptors (Zygmunt *et al.*, 1999; Howlett *et al.*, 2002). In the LPS-induced inflammatory pain model, the TRPV1 antagonist, IRTX failed to block the anti-allodynic effects of PF-3845, indicating that the TRPV1 receptor is not responsible for the anti-allodynic effects of PF-3845. In addition to increasing systemic AEA, FAAH inhibition also increases levels of non-cannabinoid FAAs, such as OEA, oleamide and PEA (Clement *et al.*, 2003). In particular, the PPARα receptor has been implicated in anti-inflammatory effects of OEA and PEA (Jhaveri *et al.*, 2008). In the present study, the PPARα antagonist MK886 did not block the anti-allodynic effects of FAAH inhibition, indicating PPARα does not play a necessary role in the anti-allodynic effects of PF-3845. Lastly, it was reported that naloxone reversed analgesia induced by the FAAH inhibitor, OL-135 in the mild thermal injury and spinal nerve injury rat pain models (Chang *et al.*, 2006). Here, we found no evidence for involvement of the opioid receptor in the anti-allodynic effects of PF-3845. Additionally, these data support previous work from our laboratory negating the role of opioid receptors in FAAH inhibition in mouse peripheral nerve injury (Kinsey *et al.*, 2009) and visceral pain models (Naidu *et al.*, 2009).

In conclusion, the results of the present study indicate genetic deletion or pharmacological inhibition of FAAH reduces LPS-induced pain responses. In particular, we demonstrated that three distinct FAAH inhibitors reverse LPS-induced tactile allodynia. Furthermore, these data reveal that inhibiting FAAH expressed in the nervous system is primarily responsible for the anti-allodynic response to LPS via a mechanism requiring both cannabinoid receptors. While administration of the FAAH inhibitor PF-3845 into the LPS-treated paw reduced allodynia without increasing brain or spinal cord AEA levels, the efficacy of this route of administration appears rather low compared with i.p. administration of this drug. Previous research has shown that FAAH inhibitors lack abuse potential, dependence potential and cannabimimetic side effects. Taken together, these data indicate

inhibition of FAAH in the peripheral nervous system and CNS provides a promising strategy to treat inflammatory pain.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Systemic administration of neither gabapentin (A) nor THC (B) affected paw withdrawal thresholds (PWTs) in the control saline-injected paw. Values represent the mean (±SEM) mechanical PWT. Data from the LPS-treated paws are depicted in Figure 2 of the manuscript. n = 6–10 mice per group.

Figure S2 (A) FAAH (–/–), FAAH-NS and FAAH (+/–) mice showed identical paw withdrawal thresholds (PWTs) in the control saline-injected paw. (B) FAAH (–/–) and FAAH (+/–) mice displayed similar withdrawal thresholds in the control saline-injected paw and neither rimonabant (3 mg·kg^{-1}; Rim) nor SR144528 (3 mg·kg^{-1}; SR2) affected control responses. All values represent the mean (±SEM) mechanical PWT. Data from the LPS-treated paws are depicted in Figure 3 of the manuscript. n = 6–10 mice per group. Rim: rimonabant, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl; SR2: SR144528.

Figure S3 (A) Intraperitoneal administration of three different FAAH inhibitors did not affect paw withdrawal thresholds (PWTs) in the control saline-injected paw. (B) Intraplantar administration of PF-3845 into the LPS-treated paw did not affect withdrawal thresholds in the contralateral saline-treated paw. (C) PF-3845 administered to the saline-treated, control paw did not affect withdrawal responses. Values represent the mean (±SEM) mechanical PWT. Data from the LPS-treated paws are depicted in Figure 4 of the manuscript. n = 7–12 mice per group.

Figure S4 PF-3845 (10 mg·kg^{-1}; i.p.) did not affect paw withdrawal thresholds (PWTs) in the control saline-injected paw. All values represent the mean (±SEM) mechanical PWT. Data from the LPS-treated paws are depicted in Figure 5 of the manuscript. n = 8–10 mice per group.

Figure S5 (A) CB_1 (+/+) and (–/–) mice show similar paw withdrawal thresholds (PWTs) in the control saline-injected paw and PF-3845 (10 mg·kg^{-1}; i.p.) had no effects in either genotype. (B) CB_2 (+/+) and (–/–) mice show similar PWTs in the control saline-injected paw and PF-3845 (10 mg·kg^{-1}; i.p.) had no effects in either genotype. (C) None of the drug treatments from Figure 6 affected PWTs in the control saline-injected paw. Values represent the mean (±SEM) mechanical PWT. Data from the LPS-treated paws are depicted in Figure 6 of the manuscript. n = 6–10 mice/group.

Figure S6 None of the antagonists administered in the absence of an FAAH inhibitor affected tactile paw withdrawal thresholds (PWTs) in either allodynic LPS-injected paws or control saline-injected paws. Values represent the mean (±SEM) mechanical PWTs in LPS- and saline-treated paws. Rimonabant (Rim; 3 mg·kg^{-1}), SR144528 (SR2; 3 mg·kg^{-1}), naltrexone (Nal; 1 mg·kg^{-1}), MK886 (MK; 3 mg·kg^{-1}), and IRTX (0.5 mg·kg^{-1}). n = 6–14 mice·group^{-1}. Rim: rimonabant; SR2: SR144528.

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