Neurobiology of Disease

Analgesic Effects of Fatty Acid Amide Hydrolase Inhibition in a Rat Model of Neuropathic Pain

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Cannabinoid-based medicines have therapeutic potential for the treatment of pain. Augmentation of levels of endocannabinoids with inhibitors of fatty acid amide hydrolase (FAAH) is analgesic in models of acute and inflammatory pain states. The aim of this study was to determine whether local inhibition of FAAH alters nociceptive responses of spinal neurons in the spinal nerve ligation model of neuropathic pain. Electrophysiological studies were performed 14–18 d after spinal nerve ligation or sham surgery, and the effects of the FAAH inhibitor cyclohexylcarbamic acid 3-carbamoyl biphenyl-3-yl ester (URB597) on mechanically evoked responses of spinal neurons and levels of endocannabinoids were determined.

Intraplantar URB597 (25 μg in 50 μl) significantly ($p < 0.01$) attenuated mechanically evoked responses of spinal neurons in sham-operated rats. Effects of URB597 were blocked by the cannabinoid 1 receptor (CB$_1$) antagonist AM251 [N-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide] (30 μg in 50 μl) and the opioid receptor antagonist naloxone. URB597 treatment increased levels of anandamide, 2-arachidonyl glycerol, and oleoyl ethanolamide in the ipsilateral hindpaw of sham-operated rats. Intraplantar URB597 (25 μg in 50 μl) did not, however, alter mechanically evoked responses of spinal neurons in spinal nerve ligated (SNL) rats or hindpaw levels of endocannabinoids. Intraplantar injection of a higher dose of URB597 (100 μg in 50 μl) significantly ($p < 0.05$) attenuated evoked responses of spinal neurons in SNL rats but did not alter hindpaw levels of endocannabinoids. Spinal administration of URB597 attenuated evoked responses of spinal neurons and elevated levels of endocannabinoids in sham-operated and SNL rats. These data suggest that peripheral FAAH activity may be altered or that alternative pathways of metabolism have greater importance in SNL rats.

Key words: electrophysiology; endocannabinoid; spinal nerve ligation; URB597; CB$_1$ receptor; opioid receptor

Introduction

Recent studies suggest that cannabis-based medicines have therapeutic potential for the treatment of neuropathic pain states (Hohmann, 2002). Activation of both the cannabinoid 1 receptor (CB$_1$) and CB$_2$ reduces nociceptive processing in animal models of neuropathic pain (Hohmann, 2002; Howlett et al., 2002; Elmes et al., 2004; Sagar et al., 2005). Furthermore, nociceptive processing is tonically modulated by the endocannabinoids. Both CB$_1$ receptor antisense oligonucleotides (Richardson et al., 1998; Dogrul et al., 2002) and CB$_1$ receptor antagonists facilitate nociceptive responses (Herzberg et al., 1997; Richardson et al., 1997, 1998; Calignano et al., 1998; Strangman et al., 1998; Chapman, 1999).

A novel experimental approach to treating pain is the augmentation of levels of endocannabinoids. Fatty acid amide hy-

drolase (FAAH) is the main enzyme responsible for the metabolism of several endogenous fatty acid amides, including anandamide (AEA), palmitoylethanolamide, and oleamide (Deutsch and Chin, 1993; Desarnaud et al., 1995; Maurelli et al., 1995; Ueda et al., 1995; Cravatt et al., 1996). FAAH inhibitors, such as cyclohexylcarbamic acid 3-carbamoyl biphenyl-3-yl ester (URB597) and 1-oxo-1[5-(2-pyridyl)-2-yl]-7-phenyl heptane (OL135), or deletion of the FAAH gene increases levels of anandamide *in vivo* and *in vitro* (Cravatt et al., 2001; Clement et al., 2003; Kathuria et al., 2003; Lichtman et al., 2004b; Fegley et al., 2005; Hohmann et al., 2005; Makara et al., 2005). FAAH-null mice are hypoalgesic in models of acute and inflammatory pain, effects that are blocked by a CB$_1$ receptor antagonist (Cravatt et al., 2001; Lichtman et al., 2004a). In contrast to studies of acute and inflammatory pain, it is less clear whether elevating levels of endocannabinoids modulates aberrant pain responses in models of neuropathic pain. Indeed, thermal hyperalgesia has been shown to develop in a similar manner in wild-type and FAAH-null mice in the chronic constriction injury model of neuropathic pain (Lichtman et al., 2004a). Furthermore, in the partial nerve ligation model of neuropathic pain, systemic administration of URB597 did not alter neuropathic pain behavior (Jayamanne et al., 2006).

The first aim of this study was to determine whether local

peripheral or spinal inhibition of FAAH modulates nociceptive responses in neuropathic rats compared with sham-operated rats and to determine the contribution of the CB_1 receptors to these effects. Endogenous opioids and peripheral opioid receptors have been implicated in cannabinoid receptor-mediated antinociception in models of acute pain (Ibrahim et al., 2005). The contribution of μ-opioid receptor mechanisms to the analgesic effects of endocannabinoids is unknown, and therefore, the ability of the μ-opioid receptor antagonist naloxone to attenuate the effects of URB597 has also been investigated. The second part of this study investigated the mechanism of action of URB597 on nociceptive responses by determining whether URB597 alters levels of endocannabinoids and related fatty acids in the hindpaw and spinal cord tissue of neuropathic and sham-operated rats.

Materials and Methods

All experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. Experiments were performed on 143 Sprague Dawley rats (240–300 g), which were group housed in a light-controlled room with *ad libitum* access to food and water. Rats were divided into 26 experimental groups, of which 11 were sham operated, and 15 were spinal nerve ligated (SNL). Electrophysiological experiments were performed in 17 groups, of which 7 were sham operated, and 10 were SNL. One neuron was recorded per rat. The remaining nine groups were used for endocannabinoid measurement.

Spinal nerve ligation. The spinal nerve ligation model of neuropathic pain was used in this study. Spinal nerves L5–L6 were ligated according to the procedures described by Kim and Chung (1992). Male Sprague Dawley rats (110–130 g) were anesthetized using isoflurane (induction, 3%; maintenance, 1–1.5%; in 33% O_2/67% N_2O) and placed in a prone position. A midline incision was made at the L3–S2 level, and the left paraspinal muscles at L4–S2 level were separated from spinal processes. Part of the L6 transverse process was removed with fine rongeurs, and the L4–L6 nerves were identified. The L5–L6 spinal nerves were isolated and tightly ligated distal to the dorsal root ganglia and proximal to the sciatic nerve formation with 6-0 silk. The wound was closed in two layers using absorbable sutures and wound clips, after complete hemostasis. A similar procedure was performed for the sham surgery, except spinal nerves were not ligated. After surgery, the sham-operated and SNL rats were group housed, and their posture and behavior were closely monitored for 48 h. From postoperative day 2 onwards, behavioral testing was performed to assess the development of mechanical allodynia up to day 11 after surgery. Rats were placed in Perspex cubicles with wire mesh grid floors and allowed to acclimatize before behavioral testing. Mechanical sensitivity of the ipsilateral and contralateral hindpaws was assessed by measuring the percentage of foot withdrawal in response to normally innocuous mechanical punctuate stimuli. Stimuli were delivered, from below, to the plantar surface of the foot using a 10 g von Frey hair stimulus. Each trial consisted of the application of a single von Frey hair 10 times. Trials were separated by 5 min. At 14–18 d after surgery, *in vivo* electrophysiological studies were performed, or tissue was collected for measurement of levels of endocannabinoids in hindpaw and spinal cord.

Electrophysiology. Methods were similar to those described previously (Sokal and Chapman, 2001). Rats were anesthetized with isoflurane inhalation anesthetic (induction, 3%; surgery, 2%; maintenance, 1–1.5%; in 33% O_2/67% N_2O), and a tracheal cannula was inserted. Rats were then placed in a stereotaxic frame to maintain stability during recordings. A laminectomy was performed, lumbar vertebrae L1–L3 were located, and segments L4–L5 of the spinal cord were exposed using fine rongeurs. The spinal cord was held rigid by clamps rostral and caudal to the exposed section of spinal cord (L4/5), and a small well was formed with the surrounding muscle. Core body temperature was maintained at 36.5–37.5°C throughout the experiment by means of a heating blanket connected to a rectal temperature probe.

Extracellular single-unit recordings of deep (500–1000 μm) wide dynamic range (WDR) dorsal horn neurons were made with glass-coated tungsten microelectrodes. Electrodes were lowered vertically through the

cord with a SCAT-01 microdrive (Digitimer, Welwyn Garden City, UK), and depths of recorded neurons from the spinal cord surface were noted. Receptive fields of neurons covering one or two toes were identified using brush, pinch, and heat stimuli. Single-unit activity was amplified and filtered (Digitimer). Signals were digitized and analyzed using a Cambridge Electronic Design micro1401 interface and Spike 2 data acquisition software (Cambridge Electronic Design, Cambridge, UK). Responses of neurons to a train of 16 transcutaneous electrical stimuli (0.5 Hz; 2 ms pulse width) applied to the center of the receptive field were recorded. All neurons selected were WDR, exhibiting a short-latency Aβ-fiber-evoked response (0–20 ms after stimulus) and Aδ-fiber-evoked response (20–90 ms after stimulus). These neurons also exhibited longer-latency C-fiber-evoked responses (90–300 ms after stimulus) and postdischarge responses (300–800 ms after stimulus).

Responses of neurons to punctate mechanical stimulation of the peripheral receptive field of varying bending force (8, 10, 15, 26, and 60 g) were characterized. This range of von Frey hairs includes non-noxious and noxious stimuli, because the noxious stimulation-induced paw withdrawal threshold in awake animals is 15 g (Chaplan et al., 1994). Individual von Frey hairs were applied to the center of the receptive field for 10 s in ascending order. Mechanical stimulation of the receptive field was repeated every 10 min. Once stable control responses (<10% variation) were obtained, pharmacological studies were performed.

Drug treatment. In sham-operated rats, effects of intraplantar injections of URB597 (25 μg in 50 μl; $n = 6$; Alexis Biochemicals, Lausen, Switzerland) or vehicle (50 μl of 3% Tween 80 in physiological saline; $n = 4$) on mechanically evoked responses of WDR neurons were recorded for 60 min. Similarly, effects of intraplantar injection of URB597 (25 and 100 μg in 50 μl; $n = 6$) or vehicle ($n = 4$) were studied in SNL rats. The contributions of CB_1 versus μ-opioid receptors to the inhibitory effects of URB597 were investigated. In a separate groups of rats, the effects of intraplantar injection of the CB_1 antagonist N-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (AM251; 30 μg in 50 μl; $n = 6$; Tocris Bioscience, Bristol, UK) or the opioid antagonist naloxone (10 μg in 50 μl of physiological saline; $n = 6$; Sigma, Poole, UK), 30 min before intraplantar injection of URB597, on URB597-mediated inhibitions were investigated in sham-operated and SNL rats for an additional 60 min. To ascertain whether the effects of URB597 were attributable to a local site of action, the effects of a contralateral injection of URB597 (100 μg in 50 μl) on ipsilateral hindpaw stimulation-evoked neuronal responses were studied in a separate group of SNL rats ($n = 6$).

In separate groups of sham-operated ($n = 6$) and SNL ($n = 8$) rats, effects of spinal application of URB597 (10, 25, and 50 μg in 50 μl) directly onto the exposed spinal cord on mechanically evoked responses of WDR neurons were recorded for 50 min per dose. The ability of spinal administration of the CB_1 receptor antagonist AM251 (1 μg in 50 μl) 30 min before URB597 (50 μg in 50 μl) or naloxone 10 min before URB597 (50 μg in 50 μl) to attenuate the inhibitory effect of URB597 on mechanically evoked responses of WDR neurons was also studied in sham-operated ($n = 5$–6) and SNL rats ($n = 5$–6). The doses of AM251 (Johanek and Simone, 2004; Ibrahim et al., 2005) and naloxone (Dickenson and Sullivan, 1986; Johanek and Simone, 2004; Ibrahim et al., 2005) used were based on previously published literature.

Measurement of endocannabinoids in hindpaw skin and spinal cord. Separate groups of SNL and sham-operated rats were anesthetized as described above. URB597 (25 and 100 μg in 50 μl; $n = 6$) or vehicle (3% Tween 80 in saline; $n = 6$) was injected into the ipsilateral hindpaw of rats in a manner identical to that performed for the electrophysiological experiments. Twenty minutes later, the rats were killed, and the ipsilateral and contralateral hindpaw skin was dissected and stored at −80°C for measurement of endocannabinoids and related compounds. In separate groups of SNL and sham-operated rats, URB597 (50 μg in 50 μl; $n = 6$) or vehicle (3% Tween 80 in saline; $n = 4$) was applied directly to the spinal cord. Thirty minutes later, the rats were killed, the ipsilateral and contralateral spinal cord was dissected, and the tissue was stored at −80°C for 2 to 4 weeks before spectrometric analysis.

A lipid extraction method was used; in brief, tissue was homogenized in an ethyl acetate/hexane mixture with internal standards added in fixed

amounts to all samples [0.42 nmol of anandamide-d8, 1.5 nmol of 2-arachidonyl glycerol (2AG)-d8, and 0.2 nmol of heptadecanoyl ethanolamide (HEA)], followed by repeated centrifugation and supernatant collection stages. Anandamide-d8 was used as an internal standard for AEA, palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), and virodhamine; 2-arachidonyl glycerol-d8 was used as an internal standard for 2AG, 2-linoleoylglycerol (2LG), and noladin ether; and HEA was used as internal standard for arachidonyl glycine.

Solid-phase extraction was subsequently performed to purify samples. Simultaneous measurement of endocannabinoids and related compounds was then performed using liquid chromatography–tandem mass spectrometry. Analysis was performed on an Agilent (Agilent Technologies, Waldbronn, Germany) 1100 system coupled to a triple quadrupole Quattro Ultima MS (Waters, Manchester, UK) recording in electrospray-positive mode. Analytes were separated chromatographically on a HyPurity Advance C8 column and precolumn (internal diameter, 100 × 2.1 mm; particle size, 3 μm; Thermo Fisher Scientific, Rocurn, UK) with a mobile-phase flow rate of 0.3 ml/min. A gradient elution was used, with mobile phases consisting of A (water, 1 g/L ammonium acetate, and 0.1% formic acid) and B (acetonitrile, 1 g/L ammonium acetate, and 0.1% formic acid). Samples were injected from a cooled auto sampler maintained at 4°C. Multiple-reaction monitoring of individual compounds using specific precursor and product mass-to-charge (m/z) ratios allowed simultaneous measurement of AEA, 2AG, PEA, OEA, 2LG, virodhamine, noladin ether, and arachidonyl glycine. The peak area of each analyte was divided by the appropriate internal standard peak area, and this analyte/internal standard ratio was used to achieve quantitation by the internal standard method. Individual calibration lines were obtained during each analytical run by applying the method to a suitable range of concentrations of the nondeuterated forms of each analyte. Data is reported only for analytes above the limit of quantitation by this method [10 pmol/g except for 2AG (100 pmol/g)]. The method used here has been validated for the measurement of endocannabinoids and was described in detail previously (Richardson et al., 2006).

Data analysis. Control mechanically evoked responses of WDR neurons were the average of three trains of stimulation before vehicle/drug injection. Properties (control evoked responses and depth) of WDR neurons before drug/vehicle administration were statistically compared using t test or one-way ANOVA as appropriate. Comparisons of the effects of vehicle versus URB597 on evoked activity of WDR neurons after injection into the receptive field were performed with one-way ANOVA. The time course of effects of drug treatments on evoked responses of WDR neurons of SNL and sham-operated rats were analyzed using repeated-measures ANOVA followed by Dunnett's *post hoc* test to identify the time point of maximal drug effect (data not shown). For comparison of the maximal effects of vehicle versus drug treatment on neuronal responses in SNL or sham-operated rats, data were normalized and expressed as a percentage of the predrug control value. This was performed to take into account any inherent variation in control evoked responses before drug treatment between individual rats. Given the size of groups compared, these data were analyzed with a nonparametric Mann–Whitney test, and $p < 0.05$ was considered significant. Data are expressed as mean ± SEM of percentage of control responses. Endocannabinoid levels were calculated as the ratio of the area of the endocannabinoid peak to its internal standard on the spectrogram and are expressed as moles per gram of wet weight of tissue. Spectrometric data were statistically compared using a Mann–Whitney nonparametric test and are presented as mean ± SEM.

Results

Development of mechanical allodynia in SNL rats

After spinal nerve ligation surgery, rats exhibited normal grooming behavior and weight gain similar to the sham-operated controls. The development of mechanical allodynia was assessed for up to 17 d after surgery. Before electrophysiological studies, rats exhibited significant mechanical allodynia. On day 11 after surgery, application of a 10 g mechanical stimulus to the ipsilateral hindpaw of SNL rats evoked a significant increase in paw withdrawal (58 ± 8% paw withdrawal; $p < 0.001$; mean ± SEM; $n = 28$) compared with sham-operated rats (1 ± 1% paw withdrawal; $n = 22$). Similarly, at day 17 after surgery, application of a 10 g stimulus to the ipsilateral hindpaw of SNL rats produced hindpaw withdrawal (78 ± 8% paw withdrawal; $n = 7$). In contrast, a 10 g stimulation of the contralateral hindpaw did not evoke a significant paw withdrawal in SNL or sham-operated rats (data not shown). Thus SNL rats developed marked mechanical allodynia after tight ligation of L5 and L6 nerves, which is in agreement with previous studies of this model (Kim and Chung, 1992; Chapman et al., 1998; Elmes et al., 2004; Jhaveri et al., 2005).

Characteristics of neuronal responses of SNL and sham-operated rats

In vivo single-unit extracellular recordings of dorsal horn neurons were performed between postoperative days 14 and 18 in SNL and sham-operated anesthetized rats. The mean depths of WDR neurons (500–1000 μm; corresponding to lamina V–VI) and control electrically evoked A- and C-fiber responses were similar for the population of neurons recorded from sham-operated and SNL rats (Table 1). Predrug control mechanically evoked responses of WDR neurons increased incrementally after the application of ascending weights of calibrated von Frey monofilaments (8–60 g) to the center of the receptive field on the hindpaw. The frequencies of mechanically evoked responses of WDR neurons in SNL rats were significantly lower than in sham-operated rats (Fig. 1), which is in keeping with a previous report (Chapman et al., 1998).

Effects of intraplantar injection of URB597 on responses of WDR neurons in SNL and sham-operated rats

Insertion of the needle and injection of URB597 or vehicle into the peripheral receptive field induced firing of WDR neurons, which often persisted after removal of the needle. The frequency and duration of firing of WDR neurons immediately after intraplantar injection of URB597 (25 μg in 50 μl per rat) into the peripheral receptive field was not significantly ($p > 0.05$) different from the effects of vehicle in both sham-operated and SNL rats (data not shown).

Intraplantar injection of vehicle produced minor increases in mechanically evoked responses of WDR neurons, compared with predrug control responses, in both sham-operated and SNL rats. Although these increases in response were not significant, they may reflect a small peripheral sensitization after intraplantar injection of vehicle. There were no significant differences between the effects of intraplantar injection of vehicle on mechanically evoked responses of WDR neurons in sham-operated and SNL rats, and therefore these data were pooled for statistical comparisons and clarity of presentation. Intraplantar injection of URB597 (25 μg in 50 μl) significantly reduced mechanically evoked responses of WDR neurons in sham-operated rats, with the exception of the 60-g-evoked response, compared with vehicle (Fig. 2). Maximal inhibitory effects of URB597 were observed at 20 min after injection. In contrast, intraplantar injection of URB597 (25 μg in 50 μl) had no significant effect on mechanically evoked responses of WDR neurons in SNL rats (Fig. 2). However, intraplantar injection of a higher dose of URB597 (100 μg in 50 μl) significantly inhibited the mechanically evoked responses of WDR neurons in SNL rats. Effects of the higher dose of URB597 in SNL rats were comparable with the effects of URB597 (25 μg in 50 μl) in sham-operated rats. Intraplantar injection of URB597 (100 μg in 50 μl) in the contralateral paw did not alter mechanically evoked responses of dorsal horn neurons in SNL

Table 1. Characteristics of dorsal horn neurons in the different treatment groups

	n	Depth (μM)	Evoked responses (Hz)			
			Aβ	Aδ	C	PD
Sham rats						
Intraplantar						
Vehicle	4	815 ± 51	203 ± 19	268 ± 56	622 ± 100	635 ± 188
URB (25 μg)	6	912 ± 28	178 ± 24	179 ± 38	418 ± 74	349 ± 99
AM251 plus URB (25 μg)	6	698 ± 64	143 ± 18	162 ± 34	514 ± 71	637 ± 106
Nal plus URB (25 μg)	6	692 ± 61	156 ± 16	177 ± 18	431 ± 58	428 ± 83
Spinal						
URB (10, 25, and 50 μg)	6	820 ± 40	163 ± 29	176 ± 29	585 ± 140	363 ± 120
AM251 plus URB (50 μg)	6	744 ± 70	113 ± 10	145 ± 40	522 ± 82	492 ± 130
Nal plus URB (50 μg)	5	730 ± 56	136 ± 9	149 ± 10	547 ± 46	519 ± 152
SNL rats						
Intraplantar						
Vehicle	4	760 ± 138	167 ± 14	167 ± 8	365 ± 68	245 ± 50
URB (25 μg)	6	723 ± 87	142 ± 21	151 ± 20	372 ± 57	333 ± 72
URB (100 μg)	6	755 ± 54	150 ± 12	219 ± 29	580 ± 73	722 ± 112
AM251 plus URB (100 μg)	6	712 ± 54	159 ± 9	184 ± 25	439 ± 68	434 ± 124
Nal plus URB (100 μg)	6	718 ± 64	153 ± 12	148 ± 13	327 ± 27	307 ± 75
URB (100 μg) contralateral paw	6	682 ± 48	147 ± 7	105 ± 16	274 ± 35	292 ± 36
Spinal						
Vehicle	5	610 ± 46	152 ± 11	177 ± 34	439 ± 119	385 ± 135
URB (10, 25, 50 μg)	8	666 ± 38	88 ± 8.4	132 ± 24	484 ± 77	554 ± 99
AM251 plus URB (50 μg)	5	854 ± 168	102 ± 14	147 ± 20	598 ± 99	647 ± 49
Nal plus URB (50 μg)	6	852 ± 73	102 ± 20	160 ± 13	417 ± 30	509 ± 84

Depths from the surface of the spinal cord and electrically (Aβ, Aβ-fiber; Aδ, Aδ-fiber; C, C-fiber; PD, postdischarge) evoked responses of neurons were statistically compared using one-way ANOVA with their respective controls. Data are expressed as mean ± SEM. n, Number of neurons per group; URB, URB597; Nal, naloxone.
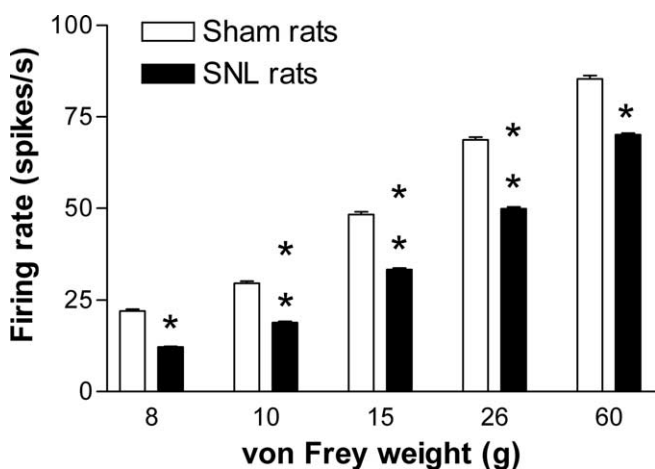

Figure 1. Control mechanically evoked responses of dorsal horn wide dynamic range neurons in sham-operated and SNL rats. Data were analyzed using a Mann–Whitney nonparametric test. *$p < 0.05$ and **$p < 0.01$ versus sham. Data are expressed as mean + SEM ($n = 39$–58 neurons per group).

rats ($p > 0.05$; Mann–Whitney test; $n = 6$) compared with the effects of injection of vehicle in ipsilateral paw (mean ± SEM; percentage of control evoked responses after contralateral URB597: 8 g, 108 ± 16; 10 g, 116 ± 22; 15 g, 102 ± 13; 26 g, 114 ± 16; 60 g, 99 ± 6 vs ipsilateral vehicle: 8 g, 155 ± 38; 10 g, 131 ± 12; 15 g, 109 ± 5; 26 g, 97 ± 4; 60 g, 109 ± 12).

Inhibitory effects of URB597 are attenuated by CB$_1$ receptor blockade
Intraplantar injection of the CB$_1$ receptor antagonist AM251 (30 μg in 50 μl) did not alter mechanically evoked responses of spinal neurons during the 30 min pretreatment period in sham-operated and SNL rats, except for 8-g-evoked responses in the former group. AM251 significantly attenuated the inhibitory effects of URB597 (25 μg in 50 μl) on mechanically evoked re-

sponses of WDR neurons in sham-operated rats (Fig. 2). Similarly, AM251 also attenuated the inhibitory effects of the higher dose of URB597 in SNL rats (Fig. 2). These data support the role of CB$_1$ receptors in mediating the inhibitory effects of URB597 in both sham-operated and SNL rats.

Differential contributions of μ-opioid receptors to the inhibitory effects of URB597 in sham-operated and SNL rats
Previous studies suggest that the inhibitory effects of peripherally administered cannabinoid agonists may be mediated, at least in part, by μ-opioid receptors (Ibrahim et al., 2005). To investigate whether this interplay with the opioid receptor system extends to the endocannabinoids, the ability of the μ-opioid receptor antagonist naloxone to attenuate the inhibitory effects of URB597 was also studied. Intraplantar injection of naloxone (10 μg in 50 μl) did not alter mechanically evoked responses of WDR neurons, compared with vehicle, during the 30 min pretreatment period in either sham-operated and SNL rats (Fig. 2). Pretreatment with naloxone did, however, attenuate the effects of URB597 on mechanically evoked responses of WDR neurons in sham-operated rats but not in SNL rats (Fig. 2).

Effects of spinal administration of URB597 on responses of WDR neurons in sham-operated and SNL rats
Spinal administration of vehicle (50 μl of 3% Tween 80 in saline) at 50 min intervals over a 150 min period did not alter 8- to 60-g-evoked WDR neuronal responses compared with prevehicle controls in SNL rats ($p > 0.05$; repeated-measures ANOVA; $n = 5$). All statistical comparisons were made with values from vehicle control data in SNL rats. Spinal administration of URB597 did not have significant effects on 8- and 10-g-evoked responses of WDR neurons in sham-operated and SNL rats, compared with vehicle controls (data not shown). URB597 (10, 25, and 50 μg in 50 μl) dose-relatedly inhibited 15-g-evoked responses of WDR neurons in both sham-operated [percentage control ± SEM, URB597 (10, 25, and 50 μg), 123 ± 33, 67 ± 32, and 36 ± 10,
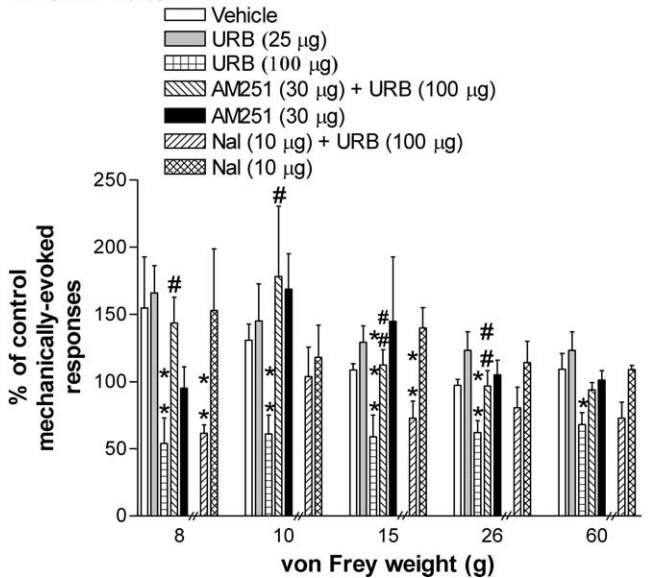
A. Sham rats
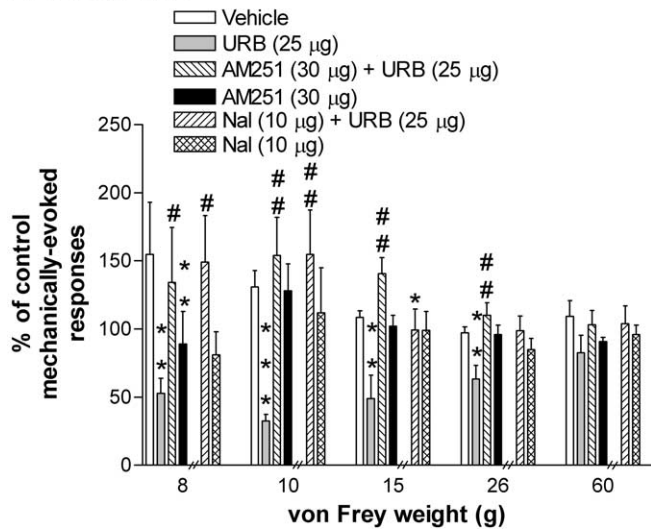
B. SNL rats

Figure 2. *A*, *B*, Intraplantar injection of a low dose of URB597 (URB) reduced mechanically evoked responses of wide dynamic range neurons in sham-operated (*A*) but not SNL (*B*) rats. A higher dose of URB attenuated evoked responses in SNL rats. Inhibitory effects of URB were attenuated by AM251 (30 μg) in both sham-operated and SNL rats. Inhibitory effects of URB were also attenuated by naloxone (Nal; 10 μg) in sham-operated rats. All drugs were given in a volume of 50 μl. Data were statistically analyzed with a nonparametric Mann–Whitney test. Effects of vehicle in sham-operated and SNL rats did not differ significantly and were pooled ($n = 8$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus vehicle. #$p < 0.05$ and ##$p < 0.01$ versus URB (25 μg)–sham. #$p < 0.05$ and ##$p < 0.01$ versus URB (100 μg)–SNL. Data are expressed as mean + SEM ($n = 6$–8 neurons per group).

respectively] and SNL rats [percentage control ± SEM, URB597 (10, 25, and 50 μg), 77 ± 46, 87 ± 62, and 55 ± 40, respectively]. Effects of spinal URB597 on 26- and 60-g-evoked responses of WDR neurons are presented in Figure 3. The lowest dose of URB597 studied (10 μg in 50 μl) significantly ($p < 0.05$) inhibited 15- and 60-g-evoked responses of WDR neurons in SNL rats, compared with time-matched vehicle controls, but not in sham-operated rats. URB597 (25 μg in 50 μl) significantly ($p < 0.05$) inhibited 26- and 60-g-evoked responses of WDR neurons in SNL rats, compared with time-matched vehicle controls. URB597 (25 μg in 50 μl) only significantly ($p < 0.05$) inhibited
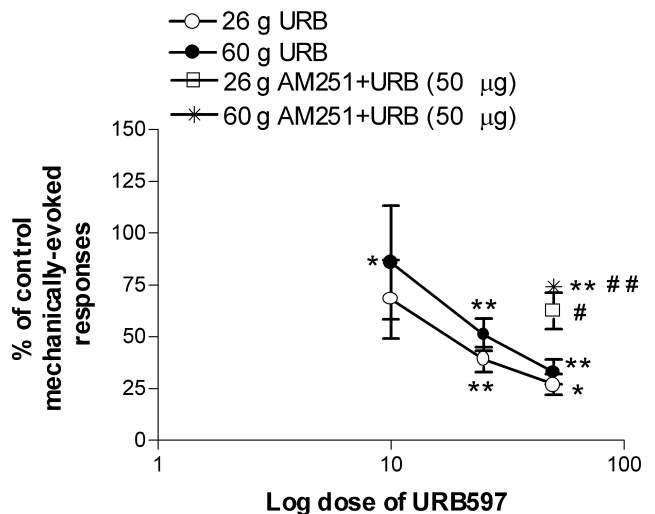
A. Sham rats
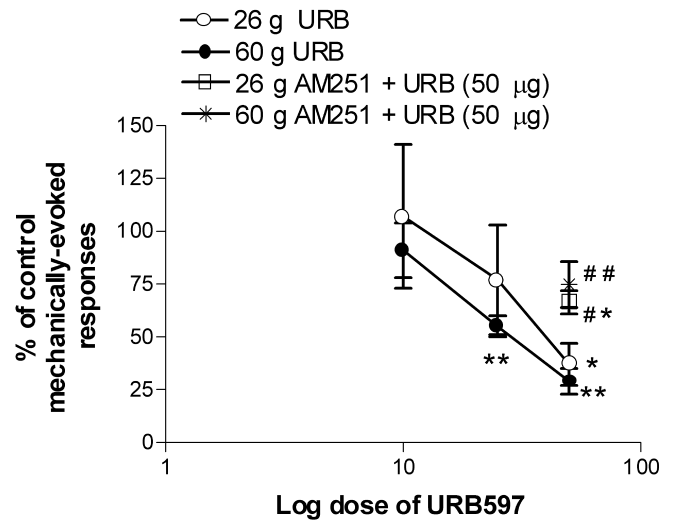
B. SNL rats

Figure 3. *A*, *B*, Spinal administration of URB597 (URB; 10, 25, and 50 μg) dose-relatedly reduced 26- and 60-g-evoked responses of WDR neurons in sham-operated (*A*) and SNL (*B*) rats. Effects of URB597 were blocked by spinal preadministration of AM251 (1 μg). All drugs were given in a volume of 50 μl. Data were statistically compared using a Mann–Whitney nonparametric test and are expressed as mean ± SEM ($n = 5$–8 neurons per group). *$p < 0.05$ and **$p < 0.01$ versus time-matched vehicle control. #$p < 0.05$ and ##$p < 0.01$ versus URB (50 μg).

60-g-evoked responses in sham-operated rats. The highest dose of URB597 studied significantly ($p < 0.05$) inhibited 15- to 60-g-evoked responses in both sham-operated and SNL rats, compared with time-matched vehicle controls.

The contribution of spinal CB$_1$ receptors to the effects of spinal URB597 was investigated in sham-operated and SNL rats. Spinal preadministration of the CB$_1$ receptor antagonist AM251 (1 μg in 50 μl) alone had no significant effect on 10-, 15-, and 60-g-evoked responses of WDR neurons in sham-operated or SNL rats. AM251 did, however, significantly increase 8- and 26-g-evoked responses of WDR neurons in SNL rats compared with time-matched vehicle controls (8 g, $p = 0.02$; 26 g, $p = 0.02$; Mann–Whitney test; $n = 5$). Spinal preadministration of AM251 (1 μg in 50 μl) significantly attenuated the effects of URB597 (50 μg in 50 μl) on 15- to 60-g-evoked responses in sham-operated
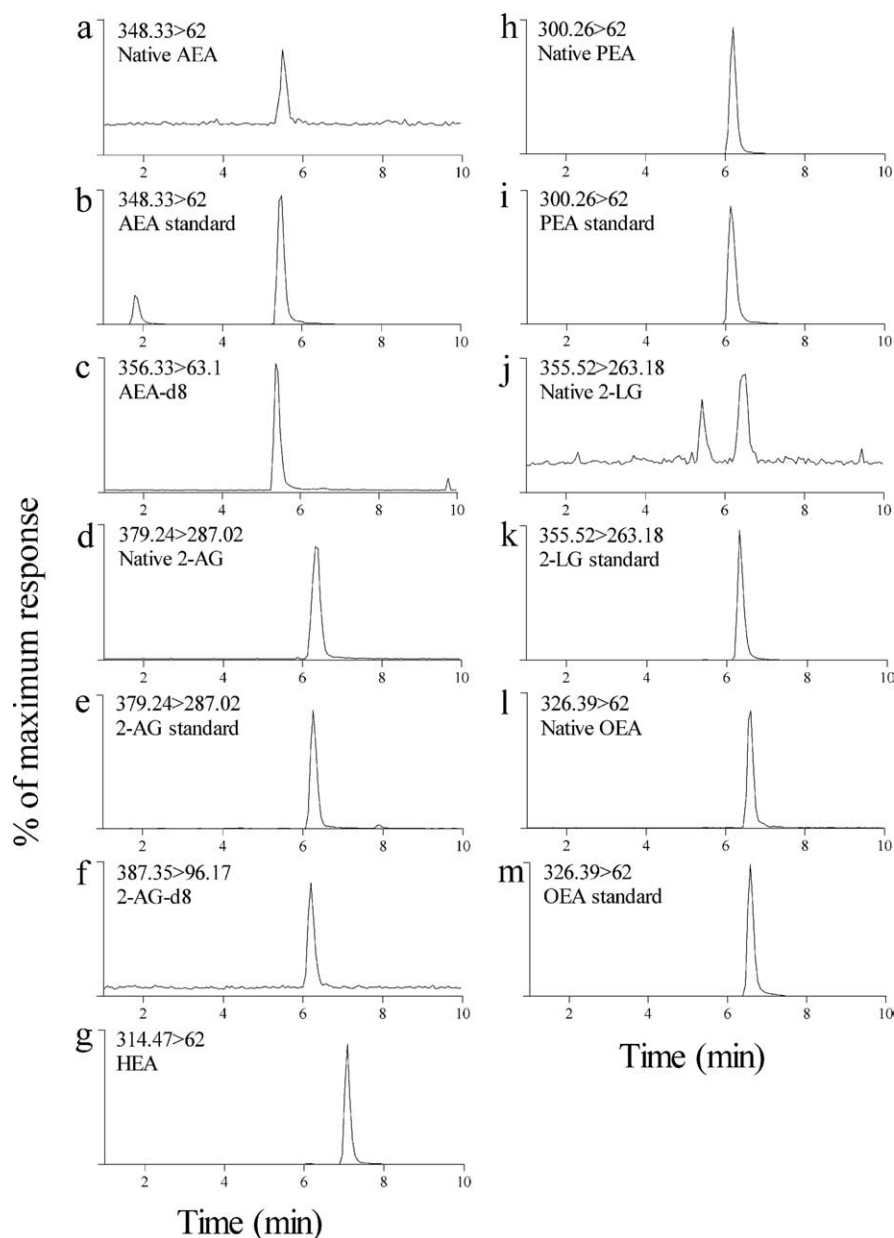
Figure 4. *a–m*, Representative chromatograms of native endocannabinoids and related fatty acids detected in rat paw tissue (*a*, *d*, *h*, *j*, *l*), along with the corresponding synthetic standards used to construct calibration curves (*b*, *e*, *i*, *k*, *m*) and the three internal standards, AEA-d8, 2AG-d8, and HEA (note differences in *m/z* values) used to quantify levels of these compounds (*c*, *f*, *g*). The additional peak in *b* corresponds with the virodhamine standard, which shares the precursor and product *m/z* values with AEA and is therefore solely differentiated by chromatographic separation.

15 g, 91 ± 11; 26 g, 97 ± 11; 60 g, 88 ± 14 vs URB597 alone: 15 g, 14 ± 5; 26 g, 27 ± 6; 60 g, 34 ± 6). Naloxone alone did not alter mechanically evoked responses of spinal neurons, compared with time-matched vehicle controls (data not shown).

Effects of intraplantar injection of URB597 on levels of endocannabinoids
In separate experiments, the effects of intraplantar injection of URB597 (25 μg in 50 μl), versus vehicle, on levels of endocannabinoids in the hindpaw were measured in sham-operated and SNL rats and compared with corresponding synthetic standards (Fig. 4). In sham-operated rats, intraplantar injection of vehicle had no significant effects on the levels of AEA, OEA, 2AG, or PEA in the ipsilateral hindpaw, compared with the contralateral paw (Fig. 5). Comparison of the effects of intraplantar injection of vehicle in SNL rats on levels of endocannabinoids and related fatty acids in the ipsilateral hindpaw revealed that injection of vehicle increased levels of AEA ($p < 0.05$) and OEA (not significant), compared with the contralateral paw of SNL rats (Fig. 5). Levels of AEA and OEA in the ipsilateral paw of vehicle-treated SNL rats were also higher than in the vehicle-treated hindpaw of sham-operated rats. In contrast, intraplantar injection of vehicle did not significantly alter levels of 2AG in the ipsilateral hindpaw of SNL or sham-operated rats. Effects of vehicle treatment on levels of PEA and 2LG were more complex and did not follow a similar profile.

Twenty minutes after intraplantar injection of URB597 (25 μg in 50 μl) in sham-operated rats, levels of AEA, 2AG, and OEA were significantly increased in the ipsilateral hindpaw, compared with the contralateral hindpaw (Fig. 5). Increases in the levels of 2AG and OEA in the URB597-treated ipsilateral hindpaw of sham-operated rats were statistically significant compared with the effects of vehicle (Fig. 5). Thus, on the basis of the changes in levels of endocannabinoids and related fatty acids after intraplantar injection of URB597, we can conclude that the lower dose of URB597 produces inhibitory effects on neuronal responses in parallel with increased levels of endocannabinoids and related fatty acids in sham-operated rats. In contrast to sham-operated rats, intraplantar injection of URB597 (25 μg in 50 μl) did not alter levels of AEA, 2AG, OEA, PEA, and 2LG in the ipsilateral hindpaw of SNL rats, compared with contralateral hindpaw. Because in the electrophysiological studies a higher dose of URB597 (100 μg in 50 μl) attenuated mechanically evoked responses of dorsal horn neurons in SNL rats, the ability of this dose of URB597 to modulate levels of endocannabinoids and related fatty acids in the hindpaw was also investigated. Intraplantar injection of a higher

rats and 26- to 60-g-evoked responses in SNL rats (Fig. 3; for clarity of presentation, 15 g data are not presented).

The role of spinal opioid receptors in mediating the effects of spinally administered URB597 (50 μg in 15 μl) was also investigated. Preadministration of spinal naloxone (15 μg in 50 μl) attenuated the inhibitory effects of URB597 in both sham-operated and SNL rats to a similar extent. Naloxone significantly (mean ± SEM; percentage of control: 15 g, 85 ± 15; 26 g, 79 ± 11; 60 g, 92 ± 9; $p < 0.05$; Mann–Whitney test; $n = 5$–6) blocked the effects of URB597 (15 g, 36 ± 10; 26 g, 37 ± 10; 60 g, 29 ± 6) on mechanically evoked responses of dorsal horn neurons in sham-operated rats. Naloxone also significantly ($p < 0.05$; Mann–Whitney test; $n = 6$) blocked the effects of URB597 in SNL rats (mean ± SEM; percentage of control, naloxone plus URB597:

dose of URB597 (100 μg in 50 μl) in SNL rats decreased levels of AEA, 2AG, OEA, and PEA in the ipsilateral hindpaw, compared with the contralateral hindpaw; significance was only reached for OEA and PEA (Fig. 5). Both the low and high dose of URB597 decreased levels of AEA and OEA in the ipsilateral hindpaw compared with vehicle treatment in SNL rats. It is important to note, however, that levels of AEA and OEA were significantly higher in the ipsilateral hindpaw of vehicle-treated SNL rats compared with vehicle-treated sham-operated rats. These data indicate that there is a greater sensitivity of sham-operated rats to the effects of URB597 and that in SNL rats, endocannabinoids and related fatty acids are not elevated by URB597, suggesting that they are metabolized by alternative pathways.

Effects of spinal administration of URB597 on levels of endocannabinoids

The effects of spinal administration of URB597 (50 μg in 50 μl) versus vehicle on levels of endocannabinoids and related fatty acids was investigated in SNL and sham-operated rats. URB597 significantly ($p < 0.05$) increased levels of AEA and 2AG in the ipsilateral spinal cord of SNL rats, compared with vehicle treatment in SNL rats (Fig. 6). In contrast, URB597 did not alter levels of AEA in the ipsilateral or contralateral spinal cord of sham-operated rats compared with vehicle treatment (Fig. 5). URB597 did, however, significantly ($p < 0.05$) increase levels of 2AG in the ipsilateral and contralateral spinal cord of sham-operated rats compared with vehicle treatment (Fig. 6). URB597 significantly ($p < 0.05$) increased the levels of PEA in the ipsilateral spinal cord, compared with vehicle treatment in SNL rats, but had no significant effect on levels of PEA in sham-operated rats (data not shown). Spinal levels of OEA and 2LG were not altered by spinal administration of URB597 in SNL or sham-operated rats (data not shown).

Discussion

Intraplantar injection of URB597 elevated levels of AEA, OEA, and 2AG in the ipsilateral hindpaw and significantly reduced mechanically evoked responses of dorsal horn neurons in sham-operated rats. Inhibitory effects were attenuated by AM251, suggesting that elevated levels of AEA and 2AG act via CB_1 receptors to produce their functional effects. In contrast, in neuropathic rats, the same intraplantar dose of URB597 had no effect, although a higher dose attenuated responses of spinal neurons in neuropathic rats, without increasing levels of endocannabinoids in the ipsilateral hindpaw.
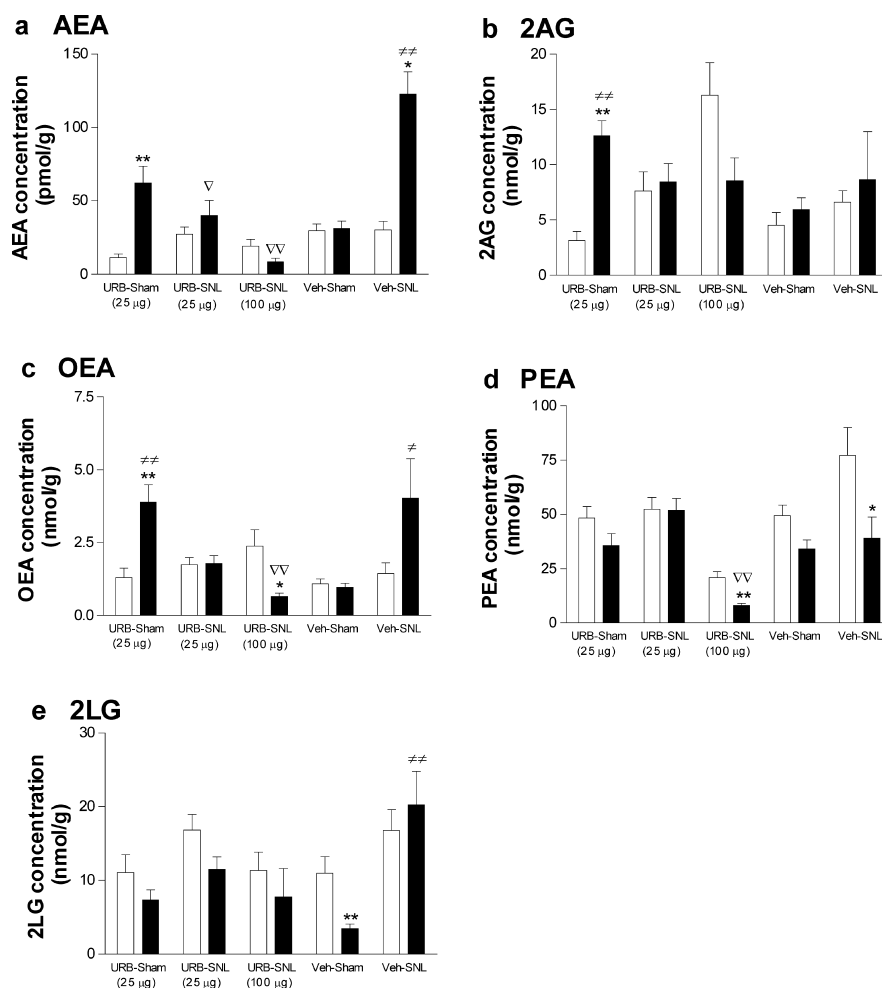
Figure 5. *a–e*, Effects of intraplantar injection of URB597 (URB; 25 or 100 μg in 50 μl) on the levels of endocannabinoids (*a*, *c*) and related fatty acids (*b*, *d*, *e*) in the ipsilateral (filled bars) and contralateral (open bars) hindpaw of sham-operated and SNL rats. Data were analyzed using a Mann–Whitney nonparametric test and are expressed as mean + SEM picomoles per gram (*a*) or nanomoles per gram (*b–e*) ($n = 6$ rats per group). $*p < 0.05$ and $**p < 0.01$ versus contralateral paw; $\neq p < 0.05$ and $\neq\neq p < 0.01$ versus ipsilateral vehicle (Veh)–sham; $\triangledown p < 0.05$ and $\triangledown\triangledown p < 0.01$ versus ipsilateral vehicle–SNL.
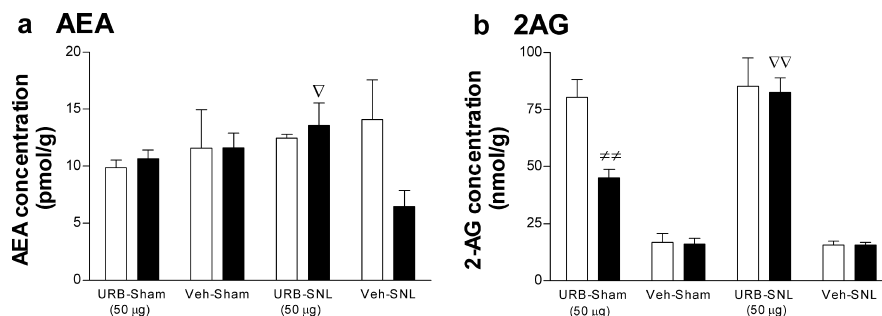
Figure 6. Effects of spinal administration of URB597 (URB; 50 μg in 50 μl) on the levels of endocannabinoids in the ipsilateral (filled bars) and contralateral (open bars) spinal cord of sham-operated and SNL rats. *a*, *b*, Data were analyzed using a Mann–Whitney nonparametric test and are expressed as mean + SEM picomoles per gram (*a*) or nanomoles per gram (*b*) ($n = 4 – 6$ rats per group). $\neq\neq p < 0.01$ versus ipsilateral vehicle (Veh)–sham; $\triangledown p < 0.05$ and $\triangledown\triangledown p < 0.01$ versus ipsilateral vehicle–SNL.

Metabolism of endocannabinoids and related fatty acid amides plays a major role in limiting their biological effects. Although FAAH is the predominant enzyme responsible for the metabolism of AEA, OEA, and PEA in the brain (Fegley et al., 2005), the contribution of different metabolic pathways (Mulder and Cravatt, 2006) in peripheral tissue or under conditions of

pathology is unclear. Increased FAAH activity or changes in pH (Paylor et al., 2006) in the hindpaw of neuropathic rats could account for the reduced potency of URB597. Alternative routes of endocannabinoid metabolism, such as cyclooxygenase-2 (COX-2) (Kozak et al., 2004), may also be engaged in peripheral tissue in neuropathic pain states and contribute to endocannabinoid metabolism in the presence of FAAH inhibition. Indeed, increased COX-2 metabolism of AEA has been reported in FAAH-null mice (Weber et al., 2004). The inhibitory effects of the higher dose of URB597 in neuropathic rats were sensitive to a CB_1 receptor antagonist but were not associated with elevated levels of AEA or 2AG, indicating that, in the presence of FAAH inhibition, endocannabinoids are metabolized by alternative pathways to metabolites that modulate nociceptive processing or nonselective effects of this dose of URB597.

The peripheral effects of URB597 in sham-operated rats were mirrored by inhibitory effects of spinal URB597 in sham-operated rats, corroborating previous reports that FAAH inhibitors are antinociceptive in models of acute and inflammatory pain (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004a,b; Holt et al., 2005; Jayamanne et al., 2006). Intrathecal URB597 increased levels of 2AG, but not AEA or PEA, in the ipsilateral spinal cord of sham-operated rats. Spinal administration of URB597 attenuated evoked responses of neurons in neuropathic rats and significantly increased levels of AEA, PEA, and 2AG in the ipsilateral spinal cord. Previously, it has been shown that a single dose of systemic URB597 does not alter neuropathic pain behavior (Jayamanne et al., 2006), whereas the FAAH inhibitor OL135 attenuated allodynia in neuropathic rats (Chang et al., 2006). Our data suggest that there may be tissue-specific changes in the sensitivity to URB597 in neuropathic rats, which may arise as a result of changes in FAAH activity, metabolic pathways, and tissue pH (Paylor et al., 2006).

Inhibitory effects of peripheral and spinal URB597 in SNL and sham-operated rats were blocked by AM251, demonstrating the contribution of CB_1 receptors. CB_1 receptor-mediated antinociception has been widely described in neuropathic rats (Herzberg et al., 1997; Mao et al., 2000; Bridges et al., 2001; Fox et al., 2001; Monhemius et al., 2001; Helyes et al., 2003; Lim et al., 2003; Scott et al., 2004; Pascual et al., 2005; Sagar et al., 2005). Our data are consistent with reports that CB_1 receptors mediate the inhibitory effects of FAAH inhibitors (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004a,b; Wilson et al., 2004; Hohmann et al., 2005; Jayamanne et al., 2006). URB597 has been shown to produce a rapid and sustained (>6 h) inhibition of FAAH activity in the brain and to increase levels of AEA and other related fatty acids (Kathuria et al., 2003; Fegley et al., 2005; Hohmann et al., 2005; Makara et al., 2005). URB597 does not bind to CB_1 or CB_2 receptors (Kathuria et al., 2003), suggesting that the effects of URB597 on evoked responses of spinal neurons arise as a result of CB_1 receptor activation by the increased levels of AEA and 2AG in the ipsilateral hindpaw and spinal cord reported herein.

Inhibitory effects of peripherally and spinally administered URB597 in sham-operated rats were attenuated by the opioid antagonist naloxone, supporting evidence for interactions between the endocannabinoid and opioid receptor systems (Maldonado and Valverde, 2003; Ibrahim et al., 2005). In SNL rats, the inhibitory effects of the higher dose of intraplantar URB597 were not altered by naloxone. Inhibitory effects of spinal URB597, however, were significantly attenuated by naloxone in SNL rats. It has been reported recently that inhibitory effects of OL135 are also blocked by naloxone in SNL rats (Chang et al., 2006). Thus, it appears that opioid receptors contribute to cannabinoid-

mediated analgesia in neuropathic pain states in a tissue-specific manner.

A major benefit of our analytical approach is the simultaneous measurement of endocannabinoids and related fatty acids. In sham-operated rats, inhibitory effects of intraplantar URB597 on nociceptive responses occurred in parallel with increased hindpaw levels of AEA, 2AG, and OEA. In contrast, levels of PEA and 2LG were not altered by intraplantar administration of URB597. FAAH is the main enzyme responsible for the metabolism of PEA in the brain. Previously, levels of PEA were shown to be increased in the duodenum of FAAH knock-out mice, but URB597 did not alter PEA levels in wild-type or FAAH knock-out mice, suggesting that additional pathways metabolize PEA in the periphery (Fegley et al., 2005). Indeed, an acid amidase enzyme is reported to be also responsible for the metabolism of PEA in peripheral tissue and macrophages (Ueda et al., 1999, 2001; Tsuboi et al., 2004; Sun et al., 2005). PEA is the preferred substrate for N-acylethanolamine-hydrolyzing acid amidase, which is insensitive to URB597 (Sun et al., 2005). The lack of effect of URB597 on levels of PEA in hindpaw suggests that N-acylethanolamine-hydrolyzing acid amidase may play a role in the metabolism of PEA in the hindpaw, as has been described for other peripheral tissue.

Another important observation of the present study was that levels of 2AG in the hindpaw and spinal cord were elevated by URB597. 2AG has been shown to be metabolized by monoacylglycerol (MAG) lipase (Goparaju et al., 1999; Dinh et al., 2002; Saario et al., 2004) and FAAH in cell preparations (Cravatt et al., 1996; Bisogno et al., 1997; Goparaju et al., 1998, 1999; Ueda et al., 1998, 2002; Lang et al., 1999; Fowler et al., 2001). Our data using local administration of URB597 *in vivo* are in agreement with *in vivo* studies demonstrating that intraperiaqueductal gray injection of URB597 increases levels of AEA and 2AG (Maione et al., 2006) and that intraperitoneal injection of the FAAH inhibitor N-arachidonyl-serotonin increases levels of 2AG in the brain (de Lago et al., 2005). In contrast, systemic administration of URB597 does not increase levels of 2AG (Kathuria et al., 2003; Fegley et al., 2005), and levels of 2AG are not altered in FAAH knock-out mice (Fegley et al., 2005). Because URB597 is reported to have no inhibitory effects on MAG lipase (Kathuria et al., 2003; Lichtman et al., 2004b; Hohmann et al., 2005; Makara et al., 2005), the present results suggest that 2AG may be metabolized by FAAH *in vivo*. Alternatively, the increases in 2AG after administration of URB597 may arise because of indirect circuit effects of FAAH inhibition on the production and metabolism of 2AG.

An important consideration when using FAAH inhibitors is that some endocannabinoids, such as AEA, are also agonists (Zygmunt et al., 1999; Smart and Jerman, 2000; Smart et al., 2000) at pronociceptive transient receptor potential vanilloid subfamily member 1 (TRPV1) receptors, which are upregulated in primary afferent fibers in neuropathic rats (Macdonald et al., 2001; Fukuoka et al., 2002; Rashid et al., 2003a,b). In the present study, levels of AEA and OEA, but not 2AG, were increased after injection of vehicle in the hindpaw of neuropathic but not sham-operated rats. The increased tissue content of endocannabinoid may reflect either increased synthesis or reduced metabolism in the neuropathic rats, but, given the attenuated response to URB597 in the neuropathic rats, the latter seems unlikely. The mechanism responsible for increased levels of AEA and OEA in response to vehicle injection in neuropathic rats is unknown, although one possibility is an upregulation of TRPV1 receptor, which has been suggested to act as an integrator and amplifier of

calcium-mobilizing signals via intracellular AEA synthesis (van der Stelt et al., 2005).

In conclusion, peripheral and spinal administration of URB597 increased levels of AEA, 2AG, and OEA in the hindpaw and reduced nociceptive processing in sham-operated rats in a CB$_1$ receptor-dependent manner. The requirement for a higher intraplantar dose of URB597 in neuropathic rats to attenuate nociceptive responses suggests that the contribution of FAAH to endocannabinoid metabolism is altered in neuropathy. These changes do not appear to be global, because spinal inhibition of FAAH was equally effective in sham-operated and neuropathic rats and was associated with increased levels of AEA, PEA, and 2AG.

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