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RESEARCH PAPER

Analgesic actions of *N*-arachidonoyl-serotonin, a fatty acid amide hydrolase inhibitor with antagonistic activity at vanilloid TRPV1 receptors

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Background and purpose: *N*-arachidonoyl-serotonin (AA-5-HT) is an inhibitor of fatty acid amide hydrolase (FAAH)-catalysed hydrolysis of the endocannabinoid/ endovanilloid compound, anandamide (AEA). We investigated if AA-5-HT antagonizes the transient receptor potential vanilloid-1 (TRPV1) channel and, as FAAH and TRPV1 are targets for analgesic compounds, if it exerts analgesia in rodent models of hyperalgesia.
Experimental approach: AA-5-HT was tested in vitro, on HEK-293 cells overexpressing the human or the rat recombinant TRPV1 receptor, and in vivo, in rats and mice treated with formalin and in rats with chronic constriction injury of the sciatic nerve. The levels of the endocannabinoids, AEA and 2-arachidonoylglycerol, in supraspinal (periaqueductal grey, rostral ventromedial medulla), spinal or peripheral (skin) tissues were measured.
Key results: AA-5-HT behaved as an antagonist at both rat and human TRPV1 receptors ($IC_{50} = 37$-40 nM against 100 nM capsaicin). It exerted strong analgesic activity in all pain models used here. This activity was partly due to FAAH inhibition, elevation of AEA tissue levels and indirect activation of cannabinoid CB_1 receptors, as it was reversed by AM251, a CB_1 antagonist. AA-5-HT also appeared to act either via activation/desensitization of TRPV1, following elevation of AEA, or as a direct TRPV1 antagonist, as suggested by the fact that its effects were either reversed by capsazepine and 5′-iodo-resiniferatoxin, two TRPV1 antagonists, or mimicked by these compounds administered alone.
Conclusions and implications: Possibly due to its dual activity as a FAAH inhibitor and TRPV1 antagonist, AA-5-HT was highly effective against both acute and chronic peripheral pain.
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Keywords: vanilloid; cannabinoid; FAAH; AEA; 2-arachidonoylglycerol; endocannabinoid; pain; neuropathic pain

Abbreviations: AA-5-HT, *N*-arachidonoyl-5-hydroxytryptamine; anandamide, *N*-arachidonoylethanolamine or AEA; 2-AG, 2-arachidonoylglycerol; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1*H*-indol-3-yl (4-methoxyphenyl)-methanone; capsaicin, (E)-*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide; CPZ, capsazepine or *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide; CCI, chronic constrictive injury; FAAH, fatty acid amide hydrolase; HEK-293, human embryonic kidney cells; I-RTX, 6,7-diepoxy-6,7-didehydroxy-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-(4-hydroxy-5-iodo-3-methoxybenzene acetate); NAGly, *N*-arachidonoylglycine; OL-135, 1-oxo-1[5-2-pyridyl)-2-yl]-7-phenylheptane; PEA, *N*-palmitoylethanolamine; TRPV1, transient receptor potential vanilloid type 1 channels; URB597, cyclohexyl carbamic acid-3′-carbamoyl-biphenyl-3-yl ester

Introduction

The endocannabinoid *N*-arachidonoylethanolamide (anandamide, AEA), and its naturally occurring cannabinoid receptor-inactive congeners or analogs, *N*-palmitoylethanolamide (PEA) and *N*-arachidonoylglycine (NAGly), exert potent analgesic and anti-inflammatory activities *in vivo* (see Bradshaw and Walker, 2005 for review). These com-

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pounds are all inactivated by enzymatic hydrolysis catalysed by amidases, the best characterized of which is the fatty acid amide hydrolase (FAAH) (Lichtman *et al*, 2004b). They act via various mechanisms: (1) activation of cannabinoid CB_1 and CB_2 receptors and activation and/or desensitization of transient receptor potential channels of vanilloid type 1 (TRPV1 receptors) (Di Marzo *et al*., 2002; Iversen and Chapman, 2002), in the case of AEA; (2) activation of peroxisome proliferation activating receptor-α, inhibition of FAAH expression (and subsequent enhancement of the levels of other analgesic/anti-inflammmatory mediators that are substrates for FAAH) or modulation of TRPV1 receptors, in the case of PEA (see Re *et al*., 2006, for review); and (3) inhibition of FAAH or activation of GPR18, in the case of NAGly (Bradshaw and Walker, 2005; Kohno *et al*., 2006). Because of these pharmacological effects of its substrates, pharmacological or genetical inhibition of FAAH was shown to exert analgesic and anti-inflammatory actions *in vivo* (Lichtman *et al*., 2004a, b; Holt *et al*., 2005; Jayamanne *et al*., 2006) through mechanisms that are not always mediated by cannabinoid receptors (Lichtman *et al*., 2004b).

The TRPV1 receptor is indeed another potential new target for the development of new analgesic and anti-inflammatory drugs, and has been described as a molecular transducer, expressed mostly in unmyelinated sensory fiber afferents of the C-type, of physical (high temperature and low pH) and chemical (plant toxins and endogenous fatty acid derivatives) nociceptive stimuli (Di Marzo *et al*., 2002). Agonists, through desensitization of TRPV1 and subsequent refractoriness of nociceptive neurons (Liu and Simon, 1996; McGaraugthy *et al*., 2003) or inhibition of voltage-activated Ca^{2+}-channels (Wu *et al*., 2006), and antagonists of TRPV1 receptors (Szallasi and Appendino, 2004) have been proposed as new therapies against inflammatory and chronic pain, two conditions that are often accompanied by sensitization and/or overexpression of these channels as well as by overproduction of their endogenous activators (Di Marzo *et al*., 2002). It has also been shown that 'dual' agonists of cannabinoid CB_1 and TRPV1 receptors cause analgesic effects that are stronger than those obtained by agonists of each single receptor alone (Brooks *et al*., 2002).

Based on this background, one possible strategy for the development of new analgesic drugs against inflammatory and chronic (e.g. neuropathic) pain is to concentrate in one molecule the capability of: (1) elevating the levels of the endogenous substrates of FAAH with analgesic and anti-inflammatory property (i.e. the fatty acid amides such as AEA, PEA and NAGly, but also the other major endocannabinoid 2-arachidonoylglycerol (2-AG), whose tissue concentrations under certain conditions are elevated by FAAH inhibitors (de Lago *et al*., 2005; Maione *et al*., 2006)); and (2) inactivating TRPV1 receptors, either through desensitization with agonists or through pharmacological antagonism. For these reasons the aims of the present study were: (1) to identify a 'dual' FAAH/TRPV1 blocker; (2) to examine its analgesic activity in rat models of acute or chronic peripheral pain; and (3) to assess the effect of the use of different administration routes (systemic or local), or different rodent species (mouse vs rat), on its analgesic activity in one of these models, the formalin test. This test was selected because it was used previously to assess the activity of FAAH inhibitors, such as 1-oxo-1[5-2-pyridyl)-2-yl]-7-phenylheptane (OL-135) (Lichtman *et al*., 2004a), and TRPV1 antagonists, such as capsazepine and 6,7-diepoxy-6,7-didehydroxy-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-(4-hydroxy-5-iodo-3-methoxybenzene acetate) (I-RTX) (Santos and Calixto, 1997; Kanai *et al*., 2006), in both rats and mice. On the other hand the effect against chronic pain was assessed in the model of loose ligatures of the sciatic nerve, which is mostly studied in rats (Bennett and Xie, 1988).

In a previous study (van der Stelt *et al*., 2005), we have reported that AEA produced upon activation of metabotropic receptors, acts as an intracellular mediator amplifying Ca^{2+} influx via TRPV1 receptors. In this study, we showed that FAAH inhibitors could potentiate acetylcholine- and ATP-induced and AEA- and TRPV1-mediated Ca^{2+}-influx in intact cells expressing TRPV1 receptors. However, we observed that the previously identified FAAH inhibitor *N*-arachidonoyl-5-hydroxytryptamine (AA-5-HT), a cannabinoid receptor-inactive, metabolically stable and mixed inhibitor of the enzyme (Bisogno *et al*., 1998; Holt *et al*., 2001; Fowler *et al*., 2003), previously found to elevate endocannabinoid levels following various types of administration protocols (de Lago *et al*., 2005; Suplita *et al*., 2005) (Table 1), could not enhance the TRPV1-mediated effect on Ca^{2+} influx of intracellular AEA, despite the fact that it did elevate endogenous AEA levels in TRPV1-expressing cells (M van der Stelt, L De Petrocellis and V Di Marzo, unpublished observations). This previous observation prompted us to speculate that AA-5-HT might, in some way, also counteract the activity of TRPV1 receptors, and, therefore, this compounds was assessed as a putative TRPV1 antagonist in the present study. We report that, indeed, AA-5-HT is a prototypic 'dual' FAAH/TRPV1 blocker with strong analgesic activity in all models used in this study.

Materials and methods

Animals

Male Wistar rats (250–300 g) and Swiss-Webster mice (40–45 g) (Harlan, Udine, Italy) were housed three per cage. Animals were housed under controlled illumination (12 h light/12 h dark cycle; light on 06.00 h) and standard environmental conditions (ambient temperature 20–22°C, humidity 55–60%) for at least 1 week before the commencement of experiments. Chow and tap water were available *ad libitum*. All surgery and experimental procedures were performed during the light cycle and were approved by the Animal Ethics Committee of The Second University of Naples. Animal care was in compliance with European regulations on the protection of laboratory animals (OJ of EC L358/1 18/12/86). All efforts were made to reduce both animal numbers and suffering during the experiments, in agreement with the Ethical Guidelines of the IASP.

Assays of intracellular $[Ca^{2+}]_i$ in HEK cells overexpressing human or rat TRPV1

Overexpression of human or rat TRPV1 cDNA into human embryonic kidney (HEK) 293 cells was carried out as

Table 1 Selectivity of the agents used in this study: summary of some of the available *in vitro* data

	FAAH (IC_{50})	*CB_1 (K_i)*	*CB_2 (K_i)*	*Human TRPV1 (IC_{50})*	*Other targets described to date*	In vivo *doses and administration routes shown to be selective for the corresponding target*	*References*
AA-5-HT	1–12 μM	>50 μM	>10 μM	No agonist activity up to 10 μM Antagonist activity (IC_{50} 36.8–39.9 nM, against capsaicin, 100 nM)	No activity on phospholipases A_2 No activity on serotonin receptors Inhibits soybean 15-lipoxygenase	5–10 mg kg^{-1}, i.p., in both mice and rats	(Bisogno *et al.*, 1998; Holt *et al.*, 2001; Fowler *et al.*, 2003; de Lago *et al.*, 2005; Suplita *et al.*, 2005; this study)
URB597	5–113 nM	>100 μM	>100 μM	No activity up to 10 μM	Inactive at acetylcholinesterase, butyryl cholinesterase and monoglyceride lipase. Inhibits some other lipases	0.3–3 mg kg^{-1}, i.p. in mice 1.2–4 nmol, intra PAG, in rats	(Kathuria *et al.*, 2003; Lichtmann *et al.*, 2004a; Maione *et al.*, 2006; authors' unpublished results)
OL-135	2.1 nM	>10 μM	>10 μM	No activity up to 10 μM	None	1–30 mg kg^{-1}, i.p. in mice	(Lichtmann *et al.*, 2004a; authors' unpublished results)
AM251	>50 μM	7.49 nM	2.29 μM	No activity up to 10 μM	None	0.3–3 mg kg^{-1}, i.p. in mice	(Lan *et al.*, 1999; authors' unpublished results)
Capsazepine	>50 μM	>10 μM	>10 μM	No agonist activity Antagonist activity (IC_{50} 40–100 nM, against capsaicin, 100 nM)	Inhibits the human hyperpolarization-activated cyclic nucleotide-gated 1 channel	up to 10 mg kg^{-1}, i.p. in mice and rats	(De Petrocellis *et al.*, 2000; Gill *et al.*, 2004; authors' unpublished results)

Abbreviations: AA-5-HT, *N*-arachidonoyl-5-hydroxytryptamine; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; FAAH, fatty acid amide hydrolase; i.p., intraperitoneally; OL-135, 1-oxo-1[5-2-pyridyl)-2-yl]-7-phenylheptane; s.c., subcutaneously; TRPV1, transient receptor potential vanilloid-1; URB597, cyclohexyl carbamic acid-3′-carbamoyl-biphenyl-3-yl ester.
The effects on FAAH, cannabinoid and TRPV1 receptors, as well as with other targets are reported. The doses and routes of administration previously shown to be sufficient for the selective interaction with the corresponding targets *in vivo* (i.e. FAAH inhibition and anandamide levels/action potentiation, for URB597 and OL135, and selective blockade of CB_1 and TRPV1 by AM251 and capsazepine, respectively) are also briefly summarized.

described previously (De Petrocellis *et al*., 2000). Cells were grown as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% foetal calf serum and 2 mM glutamine and maintained under 95%/5% O_2/CO_2 at 37°C. The effect of the substances on $[Ca^{2+}]_i$ was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+} (De Petrocellis *et al*., 2000). One day before experiments cells were transferred into six-well dishes coated with poly-L-lysine (Sigma-Aldrich, Milan, Italy) and grown in the culture medium mentioned above. On the day of the experiment the cells (50–60 000 per well) were loaded for 2 h at 25°C with 4 μM Fluo-3 methylester (Molecular Probes, Invitrogen, Milan, Italy) in dimethyl sulphoxide (DMSO) containing 0.04% Pluoronic. After the loading, cells were washed with Tyrode solution (pH 7.4), trypsinized, resuspended in Tyrode and transferred to the cuvette of the fluorescence detector (Perkin-Elmer LS50B, Monza, Italy) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25°C ($\lambda_{EX} = 488$ nm, $\lambda_{EM} = 540$ nm) before and after the addition of the test compounds at various concentrations. Response calibration was carried out by measuring the fluorescence intensity of intracellular fluo-3 with known extracellular $[Ca^{2+}]$ (Molecular Probes). The following equation was used to determine a ion dissociation constant (K_d) of 325 nM: $[Ca^{2+}]\text{free} = K_d [F-F_{min}]/[F_{max}-F]$, where F_{min} and F_{max} are the fluorescence intensities of fluo-3 without or with maximal $[Ca^{2+}]$, and F is the fluorescence intensity with an intermediate $[Ca^{2+}]$. Average F_{EM}/F_{EX} was 200 and this value was increased by $60 \pm 7\%$ in the presence of 4 μM ionomycin.

The efficacy of the agonists, (E)-*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide (capsaicin) and AEA, was determined by comparing it to the maximal effect on $[Ca^{2+}]_i$ observed with 4 μM ionomycin. Subsequently, the effects of all agonists are expressed as percent of the maximal observable effect, obtained with 4 μM ionomycin. Varying doses (1, 10, 100 and 1000 nM) of AA-5-HT or capsazepine were added 10 min before EC_{90} concentrations of capsaicin (100 nM) or AEA (1 μM). Data were expressed as the concentration exerting a half-maximal inhibition of agonist effect (IC_{50}), calculated by using GraphPad. The effect on $[Ca^{2+}]_i$ exerted by the agonist alone was taken as 100%. In some experiments with HEK-293 cells overexpressing human TRPV1, the effect of increasing concentrations of AA-5-HT were tested on the dose–response curve of capsaicin (where the effect of each dose of capsaicin was expressed as percent of the effect of 10^{-4} M capsaicin in the absence of the antagonist) to calculate Schild' plots. This was deemed necessary in order to gain further information as to whether the antagonism observed with this compound was competitive (Schild plot slope ~ 1) or non-competitive (Schild plot slope < 0.8). In some other experiments with HEK-293 cells overexpressing human TRPV1, AA-5-HT was tested on the effect of capsaicin (100 nM) at pH = 6.0. Also in this case, the effect on $[Ca^{2+}]_i$ exerted by the agonist alone was taken as 100%.

Treatments

Regarding the formalin test in the rat, a group of rats received a peripheral subcutaneous (s.c.) injection of vehicle (10% DMSO in 0.9%NaCl), AA-5-HT (1 mg per rat, 50 μl in the right paw) alone or in combination with AM251 (0.35 mg per rat) or capsazepine (CPZ, 50 μg per rat) 5 min before formalin (5%, 50 μl in the right paw). AM251 and capsazepine were co-injected with AA-5-HT in this set of experiments. Another group of rats received a peripheral s.c. injection of AA-5-HT (1 mg per rat, 50 μl in the right paw) 20 min before formalin.

In order to study the systemic effect of AA-5-HT in the formalin test in the rat, additional groups of rats received intraperitoneal (i.p.) injection of vehicle (10% DMSO in 0.9%NaCl), I-RTX (0.1 and 0.2 mg kg^{-1}), AA-5-HT (5 mg kg^{-1}) alone or in combination with AM251 (3 mg kg^{-1}), capsazepine (2.5 mg kg^{-1}) or I-RTX (0.1 and 0.2 mg kg^{-1}). All drugs were administered in a final volume of 1 ml kg^{-1}. AA-5-HT was given 15 min before the peripheral injection of formalin. When AA-5-HT was given in combination with AM251, capsazepine or I-RTX, these antagonists were injected 5 min before AA-5-HT. For formalin test experiments in the mouse, mice received i.p. injections of vehicle (10% DMSO in 0.9%NaCl), capsazepine (2.5 and 10 mg kg^{-1}, 50 μl), I-RTX (0.1 and 0.2 mg kg^{-1}, 50 μl), AA-5-HT (5 mg kg^{-1}, 50 μl) alone or in combination with AM251 (1 and 3 mg kg^{-1}, 50 μl), 6-iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1*H*-indol-3-yl (4-methoxyphenyl)methanone (AM630) (1 and 3 mg kg^{-1}, 50 μl), capsazepine (2.5 and 10 mg kg^{-1}, 50 μl) or I-RTX (0.1 and 0.2 mg kg^{-1}, 50 μl). AA-5-HT was given 15 min before the peripheral injection of formalin (1.25%, 25 μl). When AA-5-HT was given in combination with AM251, AM630 or capsazepine, these antagonists were injected 5 min before the AA-5-HT. Pretreatment times were chosen according to previous reports using the same or other FAAH inhibitors and CB_1/TRPV1 antagonists (Lichtman *et al*., 2004a; Suplita *et al*., 2005; Maione *et al*., 2006). For neuropathic rats, experimental treatments are described below in the section on chronic constriction injury of the sciatic nerve.

Test of anti-nociceptive activity in rats and mice treated with formalin

All behavioural tests were performed by experimenters blind to treatments. Formalin injection induces a biphasic, stereotypical nocifensive behaviour (Dubuisson and Dennis, 1977). In the current study, a concentration of 5% formalin was used in the rat because that concentration has initially been used in the rat, but concentrations of 0.2–5% are now generally used in mouse and rat (Sawynok and Liu, 2004). Nociceptive responses are divided into an early, short lasting first phase (0–7 min) caused by a primary afferent discharge produced by the stimulus, followed by a quiescent period and then a second, prolonged phase (15–60 min) of tonic pain. Mice or rats received formalin in the right dorsal surface of one side of the hindpaw. Each mouse or rat was randomly assigned to one of the experimental groups and placed in a Plexiglas cage and allowed to move freely for 15–20 min. A mirror was placed at a 45° angle under the cage to allow full view of the hindpaws. Nocifensive responses were recorded by numbering the cumulative time of lifting, favouring, licking, shaking and flinching of the injected

paw per unit of time (5 min) as described by Sufka *et al*. (1998). The total time of the nociceptive response measured every 5 min was expressed as the total time of the nociceptive responses in min (mean ± s.e.m.). Recording of nociceptive behaviour commenced immediately after the formalin injection and was continued for 60 min.

Chronic constriction injury of the sciatic nerve

Neuropathic pain was induced by chronic constrictive injury (CCI) of the sciatic nerve according to Bennett and Xie (1988). Briefly, rats were anaesthetized with sodium pentobarbital (60 mg kg^{-1} i.p.), the right sciatic nerve was exposed and four ligatures were loosely tied around the nerve just proximal to the trifurcation. Control rats underwent a sham surgery with exposure of the sciatic nerve without ligatures. Rats were divided into 26 groups of 10 rats per group: the first four groups consisted of rats with chronic constriction injury of the sciatic nerve 3 days after injury, treated every day with either vehicle (10% DMSO in 0.9% NaCl), AA-5-HT (5 mg kg^{-1}, s.c.), OL-135 (3 mg kg^{-1}, s.c.) or cyclohexyl carbamic acid-3′-carbamoyl-biphenyl-3-yl ester (URB597) (3 mg kg^{-1}, s.c.). The second set of 14 groups consisted of rats with chronic constriction injury of the sciatic nerve 7 days after injury, treated every day with either vehicle (10% DMSO in 0.9%NaCl), AA-5-HT (5 mg kg^{-1}, s.c.), capsazepine (2.5 and 10 mg kg^{-1}, s.c.), OL-135 or URB597 (same doses as above) or with AA-5-HT + AM251 (1 mg kg^{-1}, s.c.), AA-5-HT + AM630 (1 mg kg^{-1}, s.c.) or AA-5-HT + capsazepine (2.5 and 10 mg kg^{-1}, s.c.). A third set (four groups) consisted of sham operated rats 3 days after surgery, treated with either vehicle (10% DMSO in 0.9%NaCl), AA-5-HT, OL-135 or URB597 (same doses as above, every day). The final set (four groups) consisted of sham operated rats 7 days after surgery, treated with either vehicle, AA-5-HT, OL-135 or URB-597 (same doses as above, every day). All drugs were administered in a final volume of 1 ml kg^{-1}.

Thermal hyperalgesia was evaluated by the plantar test (Hargreaves *et al*., 1988). On the day of the experiment each animal was placed in a plastic cage (22 × 17 × 14 cm; length × width × height) with a glass floor. After a 30 min habituation period, the plantar surface of the hindpaw was exposed to a beam of radiant heat through the glass floor. The radiant heat source consisted of an infrared bulb (Osram halogen-bellaphot bulb; 8 V, 50 W). A photoelectric cell detected light reflected from the paw and turned off the lamp when paw movement interrupted the reflected light. Paw withdrawal latency was automatically displayed to the nearest 0.1 s; the cutoff time was 25 s in order to prevent tissue damage.

Paw withdrawal threshold to a mechanical stimulus, was measured by a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). Rats were allowed to move freely in one of the two compartments of the enclosure positioned on the metal mesh surface. Rats were adapted to the testing environment before any measurement was taken after that the mechanical stimulus was delivered to the plantar surface of the hindpaw of the rat from below the floor of the test chamber by an automated testing device. A steel rod (2 mm) was pushed against the hindpaw with ascending force (1–30 g in 10 s). When the rat withdrew its hindpaw, the mechanical stimulus was automatically withdrawn and the force recorded at the nearest 0.1 g. Nociceptive responses (the latencies to thermal stimulus and thresholds to mechanical stimulus) were measured in seconds and in grams, respectively, every 15 min for 3 h and averaged in order to establish the baseline for each, differently treated, group of rats. Each animal was used for one treatment only.

Endocannabinoid extraction and quantification

Extraction procedure. Rats were anaesthetized with a lethal dose of pentobarbital and decapitated. Brains were rapidly removed and immersed in ice-cold oxygenated sucrose artificial cerebrospinal fluid. A tissue block containing the rostral ventromedial medulla (RVM) was cut using a vibratome (Vibratome 1500, Warner Instruments, CT, USA). Coronal slices (1.4–1.6 mm thick) containing the RVM (interaural from –1.3 to –2.5 mm; Paxinos and Watson, 1986) were cut from the tissue block. The RVM was then isolated under optical microscope (M650, Wild Heerbrugg, Switzerland). Similarly, a tissue block containing the periaqueductal grey (PAG) matter was cut in order to obtain coronal slices (2 mm thick) containing the PAG area (interaural from 3 to 1 mm; Paxinos and Watson, 1986). The ventrolateral PAG area was then isolated under optical microscope (M650, Wild Heerbrugg, Switzerland). Finally, L4–L6 tissue of the spinal cord or ipsilateral paw skin were obtained using a optical microscope (M650, Wild Heerbrugg, Switzerland). Tissues were homogenized in 5 vol of chloroform/methanol/Tris-HCl 50 mM (2:1:1) containing 100 pmol of d_8-AEA, d_4-palmitoylethanolamide and d_8–2-AG. Deuterated standards were synthesized from d_8 arachidonic acid and ethanolamine or glycerol or palmitic acid and d_4-ethanolamine. Homogenates were centrifuged at 13 000 *g* for 16 min (4°C), the acqueous phase plus debris were collected and extracted again twice with 1 vol of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized samples were then stored frozen at −80°C under nitrogen atmosphere until analysed.

Analysis of endocannabinoid contents. Lyophilized extracts were resuspended in chloroform/methanol (99:1, v/v). The solutions were then purified by open bed chromatography on silica as described in Maione *et al*. (2006). Fractions eluted with chloroform/methanol (9:1, v v^{-1}) and containing AEA, pamitoylethanolamide and 2-AG, were collected and the excess solvent evaporated with a rotating evaporator, and aliquots analysed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (MS) carried out under conditions described previously (Maione *et al*., 2006) and allowing the separations of 2-AG, palmitoylethanolamide and AEA. MS detection was carried out in the selected ion-monitoring mode using *m/z* values of 356 and 348 (molecular ions + 1 for deuterated and undeuterated AEA), 304.0 and 300.0 (molecular ions + 1 for deuterated and undeuterated palmitoylethanolamide), and 384.35 and 379.35 (molecular ions + 1 for deuterated and undeuterated 2-AG). The area ratios between signals of

deuterated and undeuterated AEA varied linearly with varying amounts of undeuterated AEA and palmitoylethanolamide (30 fmol-100 pmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100 pmols-20 nmol interval. AEA and 2-AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas.

Statistics

Data from the formalin test (expressed as the total time of the nociceptive response in min and measured every 5 min, mean ± s.e.m.) were analysed by applying two-way analysis of variance (ANOVA) on each time point followed by the Bonferroni's post test. Statistical analysis of the data from chronic CCI nerve injury was performed by two-way ANOVA followed by Bonferroni's test on the complete dataset for each group of rats. Finally, the amounts of endocannabinoids (expressed as pmols or nmols per gram of wet tissue extracted) were compared by one-way ANOVA followed by Bonferroni's test. Differences were considered significant when $P<0.05$.

Drugs

AA-5-HT and AEA were synthesized in our laboratory as previously described (Bisogno *et al*., 1998). capsaicin, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251), *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide (capsazepine) and I-RTX were purchased from Tocris Cookson Ltd (Bristol, UK). URB597 was purchased from Alexis Biochemicals, USA. 1-Oxo-1[5-2-pyridyl)-2-yl]-7-phenylheptane (OL-135) was synthesized by Drs G Ortar and E Morera, University of Rome 'La Sapienza' and Istituto di Chimica Biomolecolare del CNR in Rome and kindly donated to us. Table 1 summarizes the rationale for selecting these pharmacological tools.

Results

AA-5-HT is a antagonist of TRPV1 receptors

The effects of AA-5-HT on FAAH *in vitro* (Bisogno *et al*., 1998; Fowler *et al*., 2003) and *in vivo* (Suplita *et al*., 2005; de Lago *et al*., 2005) have been already reported by many groups (see Table 1 for examples). Therefore, we report here only the newly discovered effects of this compound on TRPV1 receptors. When using the intracellular Ca^{2+} assay carried out in HEK-293 cells overexpressing the human recombinant TRPV1 receptor, AA-5-HT was inactive *per se* up to a 10 μM concentration but it antagonized the effect of both capsaicin (100 nM) and AEA (1 μM) ($IC_{50}=37\pm9$ and 105 ± 13 nM, respectively, means ± s.d., $N=3$, Figure 1a and b). The two agonists alone exerted similar and strong TRPV1-mediated effects on intracellular Ca^{2+} assay, at around 60% of the maximum observable effect with 4 μM ionomycin (65 ± 3% of maximal response, for capsaicin, and 56 ± 3% of maximal response, for AEA). Methylation of AA-5-HT on the aromatic hydroxyl group, as in VDM-13 (De Petrocellis *et al*., 2001), virtually abolished the antagonistic activity (not shown). As assessed by constructing different plots at different concentrations of capsaicin, where the effect of each dose of capsaicin is expressed as percent of the effect of the highest dose of capsaicin in the absence of the antagonist, AA-5-HT appeared to behave as a competitive antagonist from the nearly parallel sigmoid dose–response curves (Figure 1c), but not from the Schild' plot (slope = 0.73 ± 0.14, $r^2=0.96$, Figure 1d). AA-5-HT did not completely antagonize the effect of capsaicin on human TRPV1 at pH 6.0 (Figure 1e). Finally, AA-5-HT also antagonized the effect of capsaicin (100 nM) in HEK-293 cells overexpressing the rat recombinant TRPV1 receptor with $IC_{50}=40\pm6$ nM (Figure 1f, effect of capsaicin alone was 54 ± 3% of maximal response). Under the same assay conditions, the widely used TRPV1 antagonist, capsazepine antagonized the effect of 100 nM capsaicin with lower potency than AA-5-HT ($IC_{50}=74\pm9$ nM and 105 ± 10 nM for rat and human TRPV1, respectively, means ± s.d., $N=3$), whereas two more selective blockers, I-RTX and 6-iodonordihydrocapsaicin, were more potent ($IC_{50}=0.4\pm0.1$ and 10.0 ± 2.1 nM, against 100 nM capsaicin in HEK-293 cells overexpressing the human TRPV1, means ± s.d., $N=3$).

Systemic AA-5-HT causes anti-hyperalgesic effects in the formalin test in the mouse

S.c. injection of formalin in the mouse paw resulted in a typical biphasic nociceptive response. The first phase, lasting 5–10 min, occurred a few seconds after formalin injection and was characterized by a nociceptive response time of 3.48 ± 0.6 min as recorded 5 min post formalin. The second hyperalgesic phase started 25 min after formalin injection, reaching at this time point the maximal effect. Mice receiving peripheral s.c. injection of vehicle (0.9% NaCl) in the hindpaw, did not show pain behaviour (data not shown). Systemic AA-5-HT (5 mg kg^{-1}, i.p.) inhibited the first and, particularly, the second phase of the nocifensive behaviour induced by formalin, in a way blocked by AM251 (3 mg kg^{-1}, i.p.), but not AM630 (3 mg kg^{-1}, i.p) (Figure 2a). In this species, capsazepine (2.5 or 10 mg kg^{-1}, i.p.) caused a significant anti-hyperalgesic effect *per se* at both doses tested, and did not counteract the effect of AA-5-HT (Figure 2b and c). AM251 (Figure 2c) and AM630 (not shown) were, instead, inactive *per se* at the doses tested. Likewise, the anti-hyperalgesic effect of AA-5-HT (5 mg kg^{-1}, i.p.) was also not antagonized by I-RTX (0.1 and 0.2 mg kg^{-1}, i.p.), which again was able to counteract formalin-induced nocifensive behaviour *per se* (Figure 3a and b).

S.c. injection of AA-5-HT in the paw causes anti-hyperalgesic effects in the formalin test in rats

S.c. injection of formalin resulted in a typical biphasic nociceptive response in rats. The first phase, lasting 5–10 min, occurred a few seconds after formalin injection and was characterized by a nociceptive response time of 2.9 ± 0.6 min as recorded 5 min post formalin. The second hyperalgesic phase started 20–25 min after formalin injection reaching the maximal effect 40–45 min after the nociceptive stimulus. Rats receiving peripheral s.c. injection

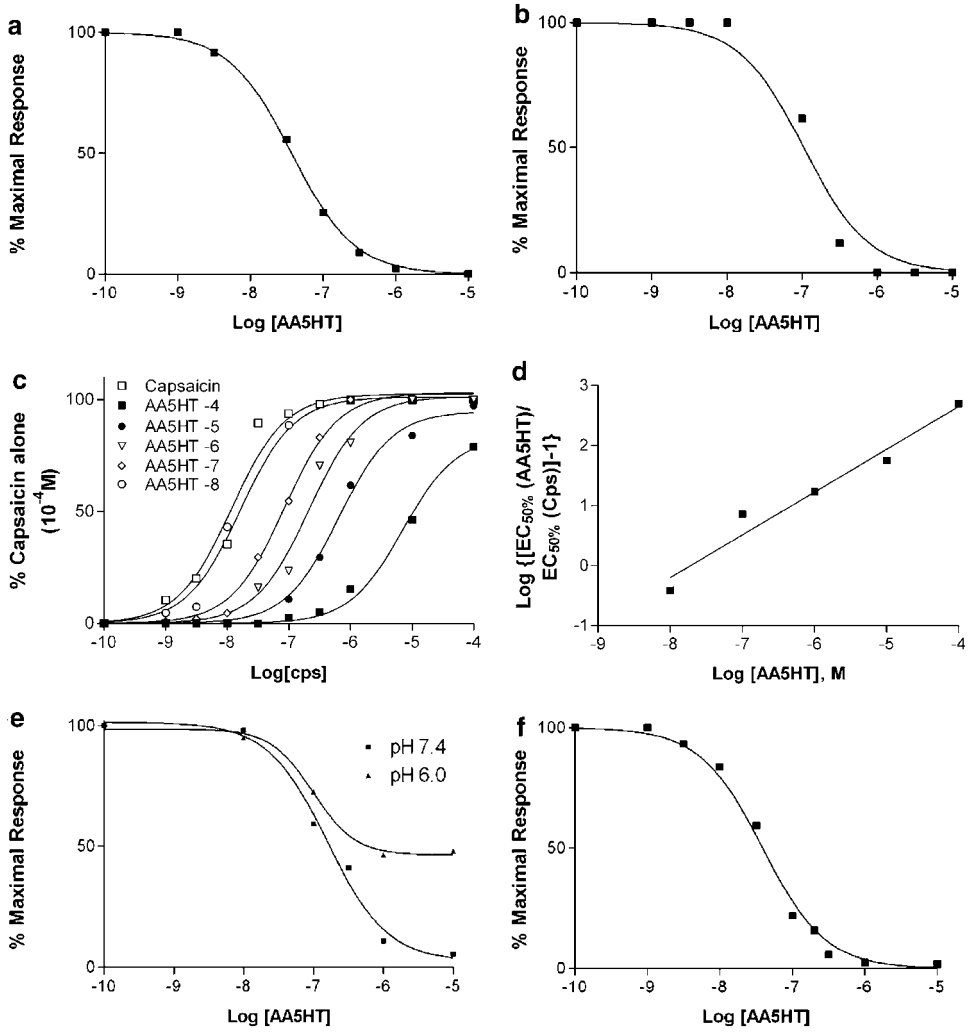

Figure 1 AA-5-HT is a potent antagonist of TRPV1 receptors. (**a**) Effect of increasing concentrations of AA-5-HT on the effect of capsaicin (100 nM, concentration eliciting ~90% of the maximal observable effect, determined with ionomycin (4 μM)) on intracellular calcium in HEK-293 cells overexpressing the human recombinant TRPV1. (**b**) Effect of increasing concentrations of AA-5-HT on the effect of anandamide (1 μM, concentration eliciting ~90% of the maximal observable effect, determined with ionomycin (4 μM)) on intracellular calcium in HEK-293 cells overexpressing the human recombinant TRPV1. (**c**) Effect of increasing concentrations of AA-5-HT on the dose–response curve of capsaicin on intracellular calcium in HEK-293 cells overexpressing the human recombinant TRPV1. Here we have expressed the effect of the agonist (in this case capsaicin) as percent of the effect of 10^{-4} M capsaicin in the absence of the antagonist, and not as percent of the maximum observable effect with ionomycin 4 μM. (**d**) Schild' plot calculated from the data in (**c**) and showing a slope of 0.73. (**e**) Effect of increasing concentrations of AA-5-HT on the effect of capsaicin (100 nM) on intracellular calcium in HEK-293 cells overexpressing the human recombinant TRPV1 at pH = 7.4 or 6.0. (**f**) Effect of increasing concentrations of AA-5-HT on the effect of capsaicin (100 nM, concentration eliciting ~90% of the maximal observable effect, determined with ionomycin (4 μM)) on intracellular calcium in HEK-293 cells overexpressing the rat recombinant TRPV1. All data are means of $n = 3$ separate experiments. The s.e. bars are not shown for clarity and were never higher than 10% of the means. In (**a**, **b**, **e** and **f**) the effect of AA-5-HT is expressed as percent of the effect on $[Ca^{2+}]_i$ exerted by the agonist alone. The net effect exerted by the agonists (100 nM capsaicin or 1 μM anandamide) are described in the Results section.

of vehicle (0.9% NaCl) in the hindpaw, did not show pain behaviour (data not shown). A 5 min pretreatment with s.c. AA-5-HT (1 mg per rat.) into the paw strongly reduced only the second phase of the nocifensive behaviour induced by formalin, in a way blocked by pretreatment with AM251 (0.35 mg per rat, s.c.) or capsazepine (50 μg per rat, s.c.) (Figure 4a). In this case, AM251, but not capsazepine, caused a slight hyperalgesic effect during both the first and second phase of the nocifensive behaviour (Figure 4b). In another set of experiments, AA-5-HT (1 mg per rat) was injected into the hindpaw 20 min before formalin. These experiments were carried out in order to assess whether the lack of effect of 5 min pretreatment with peripheral AA-5-HT (1 mg kg^{-1}) on the first phase of the formalin test was due to a pharmacokinetic problem. This possibility was ruled out by the observation that AA-5-HT (1 mg kg^{-1}), administered 20 min before formalin, again produced analgesia only during the second phase of the test (Figure 4c).

Systemic AA-5-HT causes anti-hyperalgesic effects in the formalin test in the rat

Systemic AA-5-HT (5 mg kg^{-1}, i.p.) also inhibited the second phase of the nocifensive behaviour induced by formalin in

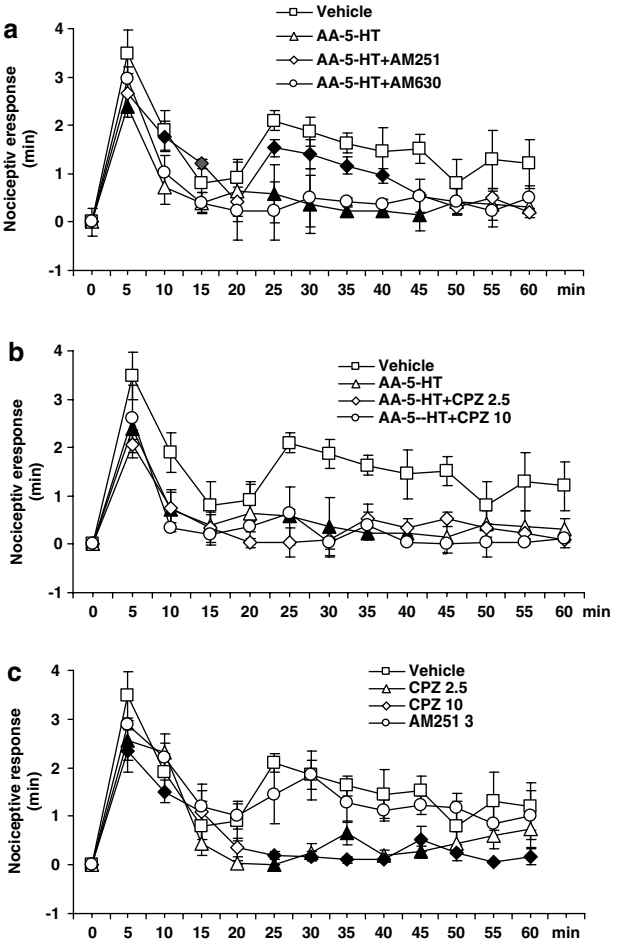

Figure 2 Effect of s.c. formalin (1.2%, 25 μl) injections into the hindpaw of mice on the time course of the nociceptive behaviour. Formalin was injected 15 min after the systemic administration of vehicle (10% DMSO in 0.9% NaCl, i.p.) (**a**–**c**), AA-5-HT (5 mg kg^{-1}, i.p.), alone or in combination with AM251 (3 mg kg^{-1}, i.p.), AM630 (3 mg kg^{-1}, i.p.) (**a**) or capsazepine (CPZ, 2.5 and 10 mg kg^{-1}, i.p.) (**b**). AM251 and CPZ were administered 5 min before AA-5-HT. The effects of AM251 (3 mg kg^{-1} i.p.) and capsazepine (CPZ, 2.5 and 10 mg kg^{-1}, i.p.) alone on the nociceptive behaviour induced by formalin are shown in (**c**). The data represent the total time of the nociceptive responses (mean$\pm$s.e.m.) of 10 mice per group, measured every 5 min. Significant differences from the corresponding controls are indicated by filled symbols. $P<0.05$ was considered statistically significant.

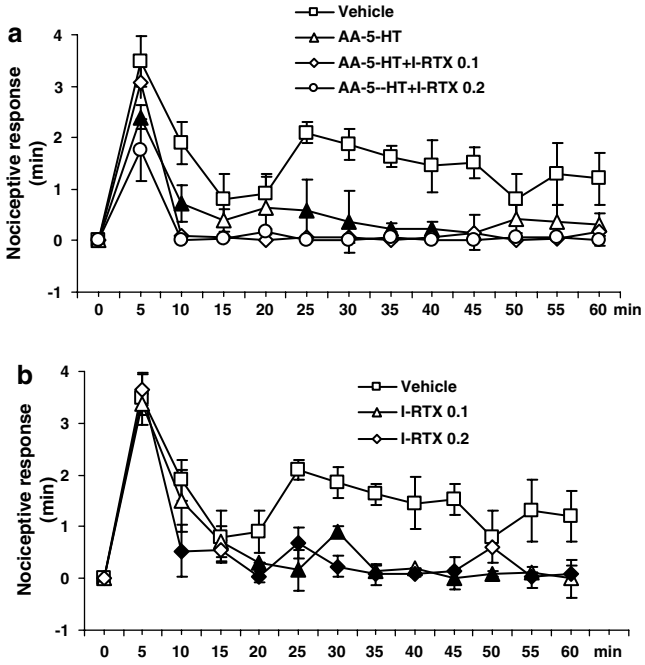

Figure 3 Effect of s.c. formalin (1.2%, 25 μl) injections into the hindpaw of mice on the time course of the nociceptive behaviour. Formalin was injected 15 min after the systemic administration of vehicle (10% DMSO in 0.9% NaCl, i.p.) (**a** and **b**), AA-5-HT (5 mg kg^{-1}, i.p.), alone or in combination with 5′-iodoresiniferatoxin (I-RTX, 0.1 and 0.2 mg kg^{-1}, i.p.) (**a**) or 5′-iodoresiniferatoxin alone (I-RTX, 0.1 and 0.2 mg kg^{-1}, i.p.) (**b**). I-RTX was administered 5 min before AA-5-HT. The data represent the total time of the nociceptive responses (mean$\pm$s.e.m.) of 10 mice per group, measured every 5 min. Significant differences from the corresponding controls are indicated by filled symbols. $P<0.05$ was considered statistically significant.

the rat, an effect prevented by AM251 (3 mg kg^{-1}, i.p.) or capsazepine (2.5 mg kg^{-1} i.p.) (Figure 5a). Capsazepine was inactive *per se* at the dose tested, whereas AM251 exerted a very slight hyperalgesic effect in the 1st phase and at 30 and 45 min (Figure 5b). Moreover, the anti-hyperalgesic effect of AA-5-HT (5 mg kg^{-1}) was also blocked by the more selective TRPV1 antagonist, I-RTX (0.1 and 0.2 mg kg^{-1}, i.p.), which was again inactive *per se* on formalin-induced nocifensive behaviour (Figure 6a and b).

Systemic AA-5-HT inhibits thermal hyperalgesia and mechanical allodynia in rats with CCI

Chronic administration with AA-5-HT (5 mg kg^{-1}, s.c.) strongly and significantly inhibited mechanical allodynia and thermal hyperalgesia in rats with CCI of the sciatic nerve (Figure 7a and b). Interestingly, AA-5-HT was as efficacious as URB597 (3 mg kg^{-1}, s.c.) or OL-135 (3 mg kg^{-1}, s.c.) 7 days after surgery, but unlike these two more potent FAAH inhibitors, did not affect thermal hyperalgesia after 3 days (Figure 7c and d). None of the three inhibitors affected mechanical allodynia when this was assessed 3 days after surgery. The effect of AA-5-HT on mechanical allodynia at 7 days was reversed by AM251 (1 mg kg^{-1}, s.c.), but not by AM630 (1 mg kg^{-1}, s.c.), a selective antagonist of cannabinoid CB_2 receptors. The two antagonists were inactive *per se* at the doses used (Figure 8a, not shown for AM630). Capsazepine dose dependently inhibited the effect of AA-5-HT on mechanical allodynia but was inactive *per se*. The effect of AA-5-HT on thermal hyperalgesia at 7 days was counteracted by AM251 (1 mg kg^{-1}, s.c.), but not by AM630 (1 mg kg^{-1}, s.c.). Also in this model the two antagonists were inactive *per se* at the doses used (Figure 8b, not shown for AM630). Capsazepine inhibited thermal hyperalgesia and did not antagonize the effect of AA-5-HT at both doses tested (Figure 8b).

AA-5-HT exerts a variety of effects on endocannabinoid and PEA levels

We have previously observed that endocannabinoid levels are elevated not only in the spinal cord but also in the PAG

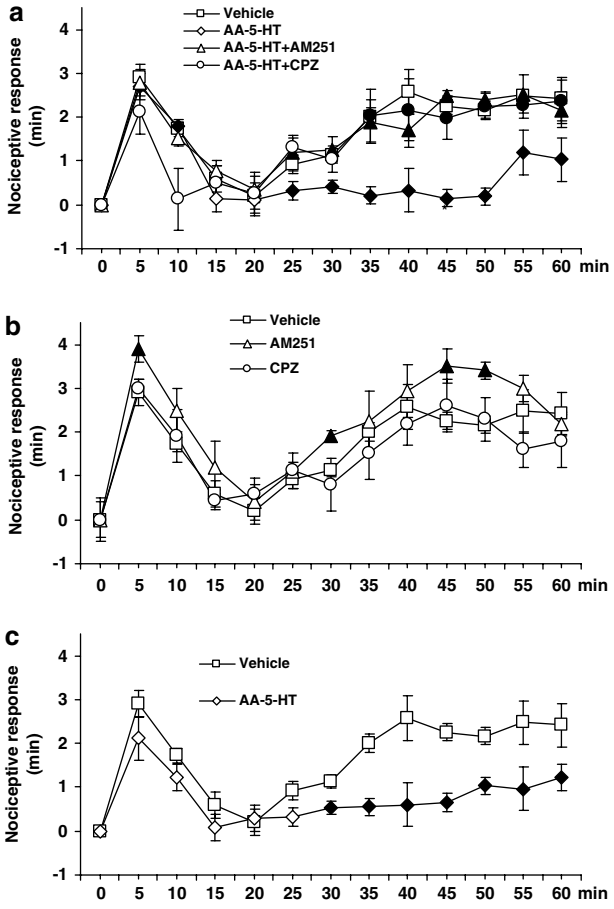

Figure 4 Effect of s.c. formalin (5%, 50 μl) injections into the hindpaw of rat on the time course of the nociceptive behaviour. Formalin was injected 5 min after vehicle (10% DMSO in 0.9% NaCl, s.c. intra paw), AA-5-HT (1 mg per rat, s.c. intra paw) alone or in combination with AM251 (0.35 mg per rat, s.c. intra paw) or capsazepine (CPZ, 50 μg per rat, s.c. intra paw) into the dorsal surface of the hindpaw (**a**). AM251 and CPZ were co-injected with AA-5-HT. (**b**) The effect or lack thereof of AM251 (0.35 mg per rat, s.c. intra paw) or capsazepine (CPZ, 50 μg per rat, s.c. intra paw) on the time course of the nociceptive behaviour induced by formalin. In (**c**) AA-5-HT (1 mg rat^{-1}, s.c.) was administered into the hindpaw 20 min before formalin. The data represent the total time of the nociceptive responses (mean$\pm$s.e.m.) of 10 rats per group, measured every 5 min. Significant differences from the corresponding controls are indicated by filled symbols. $P<0.05$ was considered statistically significant.

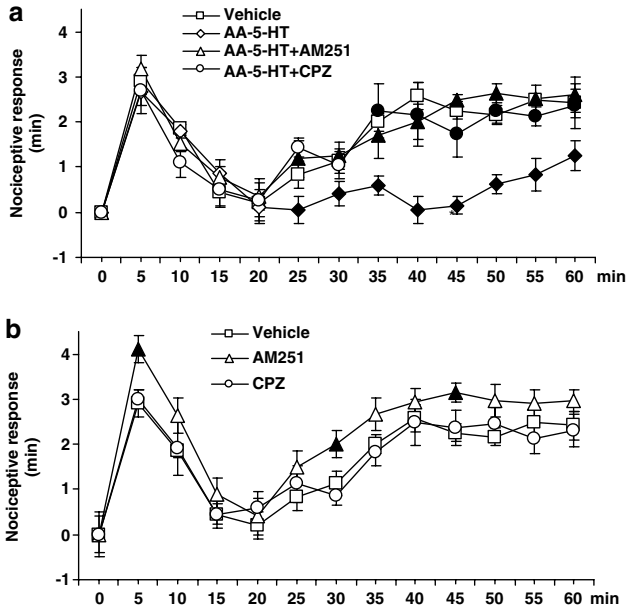

Figure 5 Effect of s.c. formalin (5%, 50 μl) injections into the hindpaw of rats on the time course of the nociceptive behaviour. Formalin was injected 15 min after the systemic administration of vehicle (10% DMSO in 0.9% NaCl, i.p.) (**a** and **b**), AA-5-HT (5 mg kg^{-1}, i.p.), alone or in combination with AM251 (3 mg kg^{-1}, i.p.) or capsazepine (CPZ, 2.5 mg kg^{-1}, i.p.). AM251 and CPZ were administered 5 min before AA-5-HT. The effect, or lack thereof, of AM251 (3 mg kg^{-1} i.p.) and capsazepine (CPZ, 2.5 mg kg^{-1}, i.p.) alone on the nociceptive behaviour induced by formalin are shown in (**b**). The data represent the total time of the nociceptive responses (mean$\pm$s.e.m.) of 10 rats per group, measured every 5 min. Significant differences from the corresponding controls are indicated by filled symbols. $P<0.05$ was considered statistically significant.

and RVM of rats with CCI of the sciatic nerve (Petrosino *et al.*, 2006). For this reason, we investigated in this model, the effects of AA-5-HT on endocannabinoid levels in these three tissues. However, when administered systemically and chronically, AA-5-HT (5 mg kg^{-1}, s.c.) did not elevate, or even decreased, endocannabinoid levels in the PAG and in the spinal cord (Table 2) and it caused a significant increase of AEA, but not of 2-AG, levels only in the RVM, and of PEA in the spinal cord.

As previously reported (Beaulieu *et al.*, 2000), formalin did not elevate endocannabinoid or PEA paw skin levels. Instead, it occasionally caused an apparent decrease of endocannabinoid and PEA tissue concentrations, which was very probably due to the fact that formalin-injected paws exhibited a significantly higher wet weight than vehicle-injected paws (23.3$\pm$2.5 g vs 14.8$\pm$2.1 g, $P<0.02$, $N=8$, in mice; 162.8$\pm$14.1 g vs 49.9$\pm$4.5 g, $P<0.01$, $N=8$, in rats). A single administration of AA-5-HT (5 mg kg^{-1}, i.p.), caused a strong elevation of endocannabinoid levels in the skin of the paw of rats treated with formalin, but not in vehicle-treated mice (Table 3).

Discussion

AA-5-HT is a potent antagonist at human and rat recombinant TRPV1 receptors

We have reported here for the first time that AA-5-HT, a previously described and widely used inhibitor of FAAH-catalysed endocannabinoid inactivation and, hence, an 'indirect' activator of cannabinoid receptors (Bisogno *et al.*, 1998; Holt *et al.*, 2001; Fowler *et al.*, 2003; Suplita *et al.*, 2005; de Lago *et al.*, 2005), also antagonizes human and rat recombinant vanilloid TRPV1 receptors with potency slightly higher than that of the widely used TRPV1 antagonist, capsazepine (Figure 1a and f). The assay used in this study, the TRPV1-mediated increase of intracellular Ca^{2+} caused by capsaicin or AEA in HEK-293 cells over-

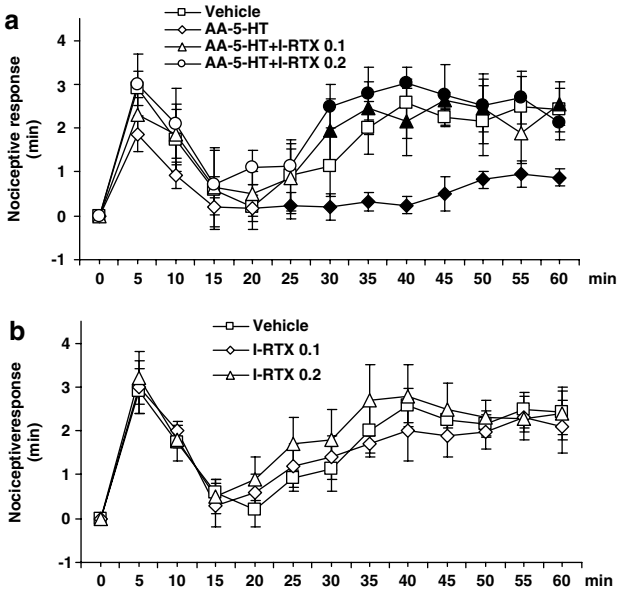

Figure 6 Effect of s.c. formalin (5%, 50 μl) injections into the hindpaw of rats on the time course of the nociceptive behaviour. Formalin was injected 15 min after the systemic administration of vehicle (10% DMSO in 0.9% NaCl, i.p.) (**a** and **b**), AA-5-HT (5 mg kg^{-1}, i.p.), alone or in combination with I-RTX (0.1 and 0.2 mg kg^{-1}, i.p.) (**a**) or I-RTX (0.1 and 0.2 mg kg^{-1}, i.p.) (**b**). I-RTX was administered 5 min before AA-5-HT. The data represent the total time of the nociceptive responses (mean $\pm$ s.e.m.) of 10 rats per group, measured every 5 min. Significant differences from the corresponding controls are indicated by filled symbols. $P<0.05$ was considered statistically significant.

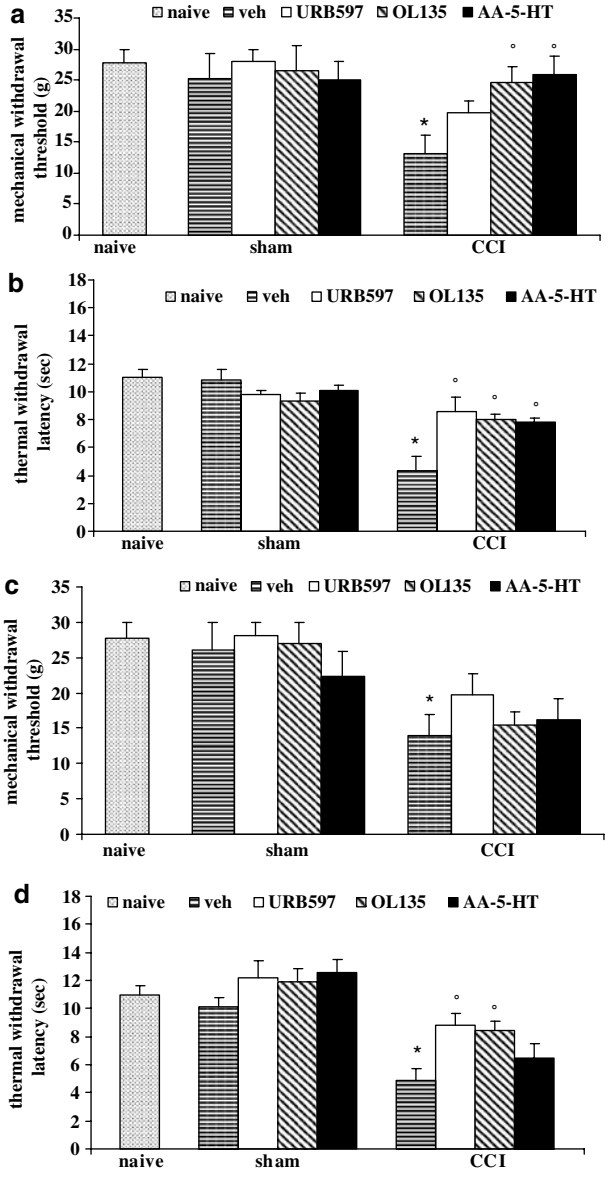

Figure 7 Effects of 7-day repeated treatment with vehicle (veh, 10% DMSO in 0.9% NaCl, s.c.), URB597 (3 mg kg^{-1} s.c.), OL-135 (3 mg kg^{-1} s.c.) or AA-5-HT (5 mg kg^{-1} s.c.) on mechanical withdrawal threshold (**a**) and thermal withdrawal latency (**b**) in sham and CCI rats. (**c** and **d**) The effects in sham and CCI rats of 3-day repeated treatment with vehicle (veh, 10% DMSO in 0.9% NaCl, s.c.), URB597 (3 mg kg^{-1} s.c.), OL135 (3 mg kg^{-1} s.c.) or AA-5-HT (5 mg kg^{-1} s.c.) on mechanical withdrawal threshold and thermal withdrawal latency, respectively. Each point represents the mean $\pm$ s.e.m. of 10 animals per group. (*) Indicates significant differences vs sham/veh, (°) significant differences vs CCI/veh. *P*-values <0.05 were considered statistically significant.

expressing either the rat or the human recombinant TRPV1 receptor, has been widely used in previous studies to assess the effect of agonists and antagonists (De Petrocellis *et al.*, 2000, 2001). AA-5-HT was slightly more efficacious against capsaicin than against the endovanilloid AEA (Figure 1a and b). Although not very abundant, FAAH is present in HEK-293 cells (De Petrocellis *et al.*, 2001), where it does control AEA levels, which, however, in the absence of stimuli, never reach concentrations higher than 10^{-7} M in these cells (van der Stelt *et al.*, 2005). Therefore, considering that AA-5-HT was tested against a 1 μM concentration of AEA, it is unlikely that the antagonism observed here with this compound against TRPV1-mediated elevation of intracellular Ca^{2+} by AEA was significantly reduced by its indirect elevation of AEA intracellular levels. As capsaicin was more efficacious than AEA at both rat and human TRPV1 receptors, it is even less likely that AA-5-HT-induced elevation of AEA levels in HEK-293 cells reduced to a significant extent the capsaicin-induced activation of TRPV1. Importantly, AA-5-HT appeared to be less efficacious at antagonizing the TRPV1-mediated effects of capsaicin on intracellular Ca^{2+} at pH 6 (Figure 1e), an experiment that we have performed because low pH is known to gate TRPV1 channels by interacting with a site different than that responsible for capsaicin or AEA binding (Jordt and Julius, 2002). This finding might suggest that AA-5-HT becomes less efficacious at antagonizing TRPV1 receptors during chronic inflammatory conditions, which lead to a decrease of the tissue pH. Moreover, as some FAAH inhibitors, including AA-5-HT, become less efficacious at inhibiting AEA hydrolysis at pH between 5.4 and 6 (Holt *et al.*, 2001; Paylor *et al.*, 2006), thus also becoming less likely to elevate AEA levels, this finding also argues against a possible indirect interference of the FAAH-inhibitory activity of AA-5-HT on its antagonism of capsaicin activity at TRPV1. It is possible, instead, that AA-5-HT, in the presence of

low pH, interacts with both FAAH and TRPV1 in a less efficacious way. In fact, the aromatic hydroxyl group of AA-5-HT, which becomes less nucleophilic at low pH, is likely to be important for the interaction with both FAAH and TRPV1 as VDM-13 (De Petrocellis *et al*., 2000), which is identical to AA-5-HT except for fact that the aromatic hydroxyl group is methylated, is unable to inhibit FAAH or to antagonize capsaicin (100 nM) effect at human TRPV1 at concentrations up to 5 μM (De Petrocellis *et al*., 2000 and data not shown).

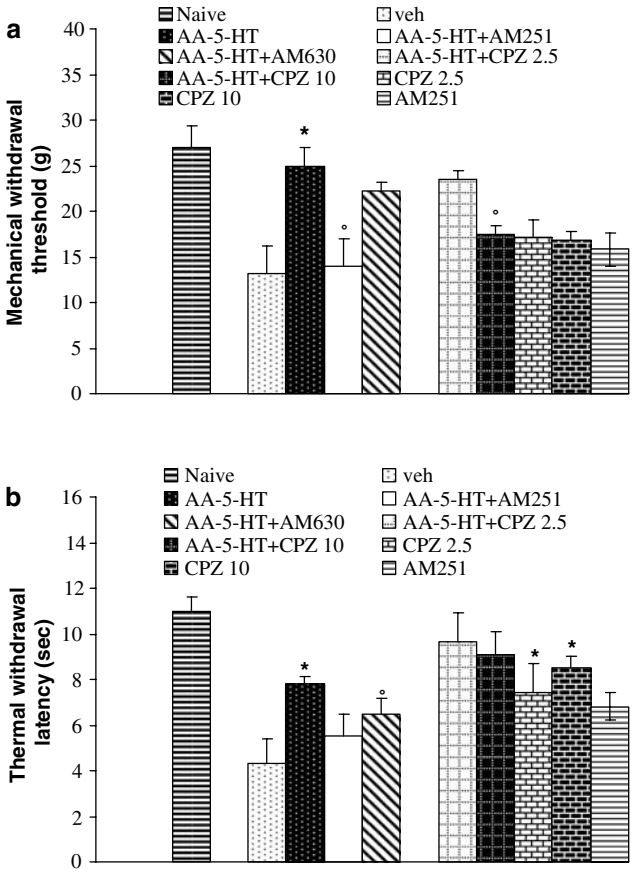

Figure 8 Effects of 7-day treatment with vehicle (veh, 10% DMSO in 0.9% NaCl, s.c.), capsazepine (CPZ, 2.5 and 10 mg kg^{-1}, s.c.), AM251 (1 mg kg^{-1}, s.c.), AA-5-HT (5 mg kg^{-1} s.c.) alone or in combination with AM251 (1 mg kg^{-1}, s.c.), AM630 (1 mg kg^{-1}, s.c.) or capsazepine (CPZ, 2.5 and 10 mg kg^{-1}, s.c.) on mechanical withdrawal threshold (**a**) and on thermal withdrawal latency (**b**) in CCI rats. Each point represents the mean ± s.e.m. of 10 animals per group. (*) Indicates significant differences vs veh, (°) significant differences vs AA-5-HT (5 mg kg^{-1}, s.c.). *P*-values <0.05 were considered statistically significant.

Regarding the mechanism of action of AA-5-HT at TRPV1, our data are insufficient to draw a definitive conclusion as to whether the compound behaves as a competitive or non-competitive antagonist. Although the dose–response curves of capsaicin in the presence of increasing doses of AA-5-HT appeared to be mostly parallel (Figure 1c), as with competitive antagonists, the corresponding Schild' plot yielded a slope of 0.73 (Figure 1d), which is different from the value of 1 expected for competitive antagonists. It is possible that the compound behaves as a mixed antagonist, a behaviour previously observed also for the FAAH-inhibitory properties of AA-5-HT (Bisogno *et al*., 1998). The high lipophilic nature of AA-5-HT might have masked a competitive antagonism, particularly at the highest concentration used for this compound (10^{-4} M, Figure 1c).

AA-5-HT administration affects tissue endocannabinoid levels and causes analgesic actions in vivo

As discussed in the Introduction, its 'dual' inhibitory activity at FAAH and TRPV1 should endow AA-5-HT with efficacious analgesic properties. Therefore, this compound was tested here *in vivo* with the aim of investigating this possibility, and was indeed found to exert anti-hyperalgesic effects. As its potency *in vitro* against FAAH was lower than that as a TRPV1 antagonist, AA-5-HT was expected to antagonize TRPV1 receptors *in vivo* at systemic doses not higher than those previously shown to inhibit rat FAAH and to elevate brain endocannabinoid levels (Table 1). For this reason we used the 5 mg kg^{-1} dose, administered i.p. or s.c., to assess analgesic activity in the formalin test and in neuropathic rats, respectively, whereas for local treatments in the former test we selected a lower dose (1 mg rat^{-1}, s.c. in the right hindpaw). We found that acute i.p. administration of 5 mg kg^{-1} AA-5-HT elevated AEA and 2-AG levels in hindpaw skin in both mice and rats treated with formalin (Table 3), while causing a significant decrease of the second phase of the nocifensive response caused in both species by this chemical agent (Figures 2–6). On the other hand, AA-5-HT, given at the same dose s.c. and chronically to CCI rats, elevated AEA levels only in the RVM, and reduced them in the PAG and spinal cord (Table 2), while still causing

Table 2 Levels of the two endocannabinoids, AEA and 2-AG, and of PEA, in the spinal cord, PAG and RVM of neuropathic rats injected for 7 days with vehicle or AA-5-HT, 5 mg kg^{-1}, s.c.

	Spinal cord			*PAG*			*RVM*		
	AEA (pmol g^{-1})	*PEA (pmol g^{-1})*	*2-AG (nmol g^{-1})*	*AEA (pmol g^{-1})*	*PEA (pmol g^{-1})*	*2-AG (nmol g^{-1})*	*AEA (pmol g^{-1})*	*PEA (pmol g^{-1})*	*2-AG (nmol g^{-1})*
CCI + vehicle	87 ± 17	270 ± 22	1.2 ± 0.1	181 ± 5	900 ± 70	4.3 ± 0.1	139 ± 15	470 ± 25	3.3 ± 0.8
CCI + AA-5-HT	25 ± 4**	388 ± 48*	1.4 ± 0.1	86 ± 9***	670 ± 30*	3.5 ± 0.2	213 ± 33*	530 ± 35	3.0 ± 0.1

Abbreviations: AA-5-HT, *N*-arachidonoyl-5-hydroxytryptamine; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CCI, chronic constrictive injury; PAG, periaqueductal grey; PEA, *N*-palmitoylethanolamine; RVM, rostral ventromedial medulla.
Data are means ± s.e. of $n=4$ determinations in 4 rats per group. Means were compared by ANOVA followed by the Bonferroni's test.
*$P<0.05$; **$P<0.01$; ***$P<0.005$.

Table 3 Levels of the two endocannabinoids, AEA and 2-AG, and of PEA, in the ipsilateral paw skin from mice or rats treated with either vehicle or AA-5-HT, 5 mg kg^{-1}, i.p., and whose paws were treated with formalin or saline, respectively

	AEA (pmol g^{-1})	*PEA (nmol g^{-1})*	*2-AG (nmol g^{-1})*
Mice			
Vehicle	176±35	1.3±0.2	4.1±0.6
AA-5-HT	171±79	1.0±0.1	4.4±0.6
Vehicle + formalin	71±24*	0.6±0.1*	1.5±0.01**
AA-5-HT + formalin	150±27$^{\#}$	1.1±0.2§	2.3±0.1$^{\#\#\#}$
Rats			
Vehicle	143±14	0.75±0.10	6.1±0.08
AA-5-HT	143±21	0.59±0.04	5.8±0.13
Vehicle + formalin	107±18	0.61±0.06	2.4±0.07**
AA-5-HT + formalin	279±88$^{\#}$	1.28±0.45	3.8±0.9$^{\#}$

Abbreviations: AA-5-HT, *N*-arachidonoyl-5-hydroxytryptamine; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; PEA, *N*-palmitoylethanolamine.
Data are means±s.e. of $n=4$ determinations in 4 animals per group. Means were compared by ANOVA followed by the Bonferroni's test.
*$P<0.05$; **$P<0.01$, vs vehicle. $^{\#}P<0.05$; $^{\#\#\#}P<0.005$ vs vehicle + formalin. $^{\S}P=0.1$ vs vehicle + formalin.

inhibition of both mechanical allodynia and thermal hyperalgesia (Figures 7 and 8). Upregulation of tissue endocannabinoid levels as a possible adaptive response to pain was found to occur following CCI of the sciatic nerve (Petrosino *et al*., 2006), but not after formalin treatment (present results and Beaulieu *et al*., 2000). Therefore, reductions of tissue endocannabinoid levels following AA-5-HT administration to neuropathic rats might be the consequence of pain reduction by this compound (except in the RVM where, perhaps, the activity of certain neurons might be opposite to that of some PAG neurons), whereas in formalin-treated rodents the elevation of skin endocannabinoid levels by AA-5-HT might be instead the cause of its analgesic effect. Indeed, based on the results presented here and discussed below, we speculate that AA-5-HT, depending on the route of administration or animal species used, produces analgesia not only by elevation of endocannabinoid levels and indirect activation of CB_1, or indirect activation/desensitization of TRPV1, but also by direct antagonism of the latter receptor (Table 4).

Analgesic effects of AA-5-HT in formalin-treated mice: 'indirect' agonism at cannabinoid CB_1 receptors and TRPV1 antagonism?

Systemic administration of FAAH inhibitors like OL-135 was previously shown to inhibit the second phase of the nocifensive response to formalin in mice (Lichtman *et al*., 2004b). For this reason we began here by testing AA-5-HT in this assay, and we did not judge it necessary to test again OL-135. On the first phase of the nocifensive behaviour, AA-5-HT was very weak, although it produced a statistically significant effect, whereas it totally blocked the second phase of nociception. From our data, we can suggest that AA-5-HT causes analgesia by elevating AEA and 2-AG levels (Table 3), and by leading to indirect activation of CB_1, but not CB_2, receptors, as indicated by the blockade of its effect by AM251, a CB_1 antagonist, but not AM630, a CB_2 antagonist (Figure 2). These effects are similar to the ones observed previously with pharmacological (i.e. with OL-135) or genetic (i.e. in FAAH knockout mice) inhibition of FAAH, which inhibit the second phase of the formalin response in a way antagonized by CB_1, but not CB_2, antagonists (Lichtman *et al*., 2004a, b). In this assay, analgesic compounds are likely to act mostly at the level of the spinal cord or sensory afferents, where both CB_1 and TRPV1 receptors are also expressed. However, spinal and peripheral TRPV1 receptors are known to mediate pain, rather than counteracting it, as indicated by the analgesic actions against formalin-induced nociception found previously in mice with TRPV1 antagonists (Santos and Calixto, 1997). Indeed, we found here that two chemically distinct TRPV1 antagonists, the widely used capsazepine and the more selective and less species-specific I-RTX, not only behaved like AA-5-HT by reducing the second phase of the nocifensive behaviour induced by formalin, but they also did not influence, at both doses tested, the analgesic effect of AA-5-HT (Figures 2 and 3). In view of the present finding that AA-5-HT is a TRPV1 antagonist, it is tempting to suggest that also this compound causes analgesia in formalin-treated mice in part by antagonizing TRPV1 receptors. Further studies, for example by comparing the analgesic efficacy of AA-5-HT in wild-type and TRPV1-null mice, are needed in order to conclusively demonstrate this hypothesis.

Analgesic effects of AA-5-HT in formalin-treated rats: 'indirect' agonism at cannabinoid CB_1 receptors and desensitization of TRPV1-expressing nociceptors?

In formalin-treated rats, both local (intra paw, s.c.) and systemic (i.p.) treatment with AA-5-HT inhibited the second phase of the nocifensive behaviour (Figures 4 and 5). In this species, the effect of AA-5-HT was never statistically significant in the first phase of the nocifensive behaviour, even following more prolonged pretreatment with the drug. Also in rats, CB_1 receptors participate in the analgesic effect of AA-5-HT, as shown by the blockade of this effect observed with AM251, which in this species also produced slight hyperalgesia *per se*, and by the elevation of AEA and 2-AG levels (Table 3). However, unlike mice, in rats capsazepine did not exert any analgesic effect *per se*, and instead it always blocked AA-5-HT effect on formalin-induced nociception. One possible way to explain these data comes from the previous observation that AEA can also activate and readily

Table 4 Summary of the analgesic effects observed with AA-5-HT in the present study

Model	*Test*	*Administration route*	*Effect of AA-5-HT*	*Effect of antagonists per se*	*Effect of antagonists on AA-5-HT*	*Possible mechanism*
Formalin-treated rat	Nocifensive behaviour	i.p.	Reversal of the second phase of nociception	Slight anti-hyperalgesic effect with AM251. No effect of capsazepine or I-RTX	Blocked by both AM251 and capsazepine Blocked by I-RTX	Inhibition (via CB_1 stimulation) and desensitization/ refractoriness (via TRPV1 stimulation) of TRPV1-expressing sensory pro-nociceptive neurons where TRPV1 is not directly involved in nociception
Formalin-treated rat	Nocifensive behaviour	Intra paw, s.c.	Reversal of the second phase of nociception	Slight anti-hyperalgesic effect with AM251. No effect of capsazepine	Blocked by both AM251 and capsazepine	Inhibition (via CB_1 stimulation) and desensitization/ refractoriness (via TRPV1 stimulation) of TRPV1-expressing sensory pro-nociceptive neurons where TRPV1 is not directly involved in nociception
Formalin-treated mouse	Nocifensive behaviour	i.p.	Reversal of the second phase of nociception	No effect of AM251 or AM630. Anti-hyperalgesic effect by capsazepine and I-RTX	Blocked by AM251 but not capsazepine nor I-RTX	Inhibition, via CB_1 stimulation and TRPV1 antagonism, of TRPV1-expressing sensory pro-nociceptive neurons where TRPV1 is directly involved in nociception
CCI rat	Thermal hyperalgesia	s.c.	Anti-hyperalgesic effect	No effect of AM251 or AM630. Anti-hyperalgesic effect by capsazepine	Blocked by AM251 but not capsazepine	Inhibition, via CB_1 stimulation and TRPV1 antagonism, of TRPV1-expressing sensory pro-nociceptive neurons mediating thermal pain transmission where TRPV1 is directly involved in nociception
CCI rat	Mechanical allodynia	s.c.	Anti-allodynic effect	No effect of AM251, AM630 or capsazepine	Blocked by both AM251 and capsazepine	Inhibition (via CB_1 stimulation) and desensitization/ refractoriness (via TRPV1 stimulation) of TRPV1-expressing, sensory mechanical pain transducing neurons where TRPV1 is not directly involved in pain

Abbreviations: AA-5-HT, *N*-arachidonoyl-5-hydroxytryptamine; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1*H*-indol-3-yl (4-methoxyphenyl)methanone; CCI, chronic constrictive injury; i.p., intraperitoneal; I-RTX, 6,7-diepoxy-6,7-didehydroxy-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-(4-hydroxy-5-iodo-3-methoxybenzene acetate); s.c., subcutaneous.
Doses and timing of treatments are described in the Materials and methods section and in the legends to Figures 2–8.

desensitize TRPV1 receptors, or participate in capsaicin-induced TRPV1 desensitization (Baamonde *et al*., 2005; Lizanecz *et al*., 2006). This is a process that not only eventually prevents TRPV1-mediated nociception, but also causes refractoriness of TRPV1-expressing nociceptive sensory neurons to other painful stimuli (Karai *et al*., 2004). TRPV1 stimulation also causes analgesia via inhibition of voltage-activated Ca^{2+} channels (Wu *et al*., 2006). Therefore, it is possible that in formalin-treated rats, the elevated AEA levels caused by administration of AA-5-HT help in activating/desensitizing those TRPV1 channels that do not participate directly in nociception but are expressed in sensory neurons involved in the second phase of the formalin response, neurons that consequently are made refractory to pain induced by formalin. The possibility that capsazepine was inactive *per se* against hyperalgesia because it might not be as efficacious in mice or rats, as previously suggested (Walker *et al*., 2003), is unlikely because of four reasons: (1) As shown above, capsazepine was indeed found here to be efficacious *per se* in formalin-treated mice (Figure 2b); (2) the compound exhibited exactly the same behaviour in rats when administered either s.c. or i.p. (Figures 4 and 5) and did antagonize the analgesic effect of s.c. or i.p. AA-5-HT, thus arguing against a pharmacokinetic effect; (3) the other TRPV1 antagonist, I-RTX, previously shown to be efficacious in rats (Almasi *et al*., 2003), also did not inhibit formalin-induced hyperalgesia in rats in the present study, although again it was capable of blocking the analgesic effects of AA-5-HT, thus behaving exactly like capsazepine (Figure 6); and (4) capsazepine was previously shown to inhibit the formalin-induced response in rats when administered intrathecally (Kanai *et al*., 2006). These observations taken together suggest that there is in rats also a population of TRPV1-expressing neurons, possibly expressed in the spinal cord (Kanai *et al*., 2006), where TRPV1 is directly involved in formalin-induced nociception, and which is targeted by TRPV1 antagonists by using intrathecal, but not s.c. or i.p., administration. In view of the fact that even when the same administration route (i.p.) was used in both species, capsazepine and I-RTX behaved in rats in a way opposite to that observed above for mice, we can only conclude that the mechanism of action of these two antagonists, and hence of AA-5-HT, must be strongly dependent on the animal species under investigation.

Analgesic effects of AA-5-HT in neuropathic rats: antagonism of TRPV1-receptors involved in thermal hyperalgesia, desensitization of TRPV1-expressing nociceptors involved in mechanical allodynia and 'indirect' agonism at cannabinoid CB_1 receptors?

The three possible mechanisms hypothesized above to explain the effects of AA-5-HT against formalin-induced pain in mice and rats might also apply to the analgesic effects of this compound observed here in neuropathic rats following its chronic systemic administration (Table 4). In this case, the possible occurrence of more than simple inhibition of FAAH was suggested by two findings: (1) the lack of effect of AA-5-HT on endocannabinoid levels in the spinal cord following CCI of the sciatic nerve, a condition that we have shown elsewhere to be accompanied *per se* by strong elevation of both AEA and 2-AG levels in this area (Petrosino *et al*., 2006) (Table 2); (2) the similar or even higher efficacy, 7 days from CCI, of AA-5-HT as compared to two much more potent FAAH inhibitors, OL-135 and URB597 (Figures 7 and 8) (Table 1), respectively. These two FAAH inhibitors have been tested here because either they had never been tested before in this assay, as in the case of OL-135 (Lichtman *et al*., 2004b), or had been tested only on mechanical allodynia and only following acute administration, and found to be inactive, as in the case of URB597 (Jayamanne *et al*., 2006). We found here that:

(1) when thermal hyperalgesia was monitored, capsazepine inhibited nociception and did not modify the effect of AA-5-HT. This might suggest that, as hypothesized above for formalin-treated mice, the two compounds act in a similar way, that is, by directly antagonizing TRPV1-mediated thermal hyperalgesia, and only 7 days from surgery, when TRPV1 upregulation in the spinal cord is more pronounced (Kanai *et al*., 2005);
(2) when mechanical allodynia was measured, capsazepine was inactive *per se* but inhibited AA-5-HT analgesia. This suggested that in the sensory fibres mediating tactile allodynia: (a) TRPV1 is not directly involved in pain transmission, (b) AA-5-HT, by locally elevating AEA levels, activates/desensitizes TRPV1 and causes inhibition or refractoriness of TRPV1-expressing nociceptive neurons involved in pain transmission (Karai *et al*., 2004; Wu *et al*., 2006), as suggested above for AA-5-HT analgesic actions in formalin-treated rats. This might explain not only capsazepine blockade of the anti-allodynic effect of AA-5-HT, but also why this effect of this as well as two other FAAH inhibitors developed only after 7 days from surgery. In agreement with these hypotheses are the observations that TRPV1-expressing sensory neurons are involved in both thermal and mechanical pain, although TRPV1 directly participates mostly in thermal hyperalgesia (Caterina *et al*., 2000; Kanai *et al*., 2005). However, using the same species but different hyperalgesic stimuli, that is, rats treated with complete Freund's adjuvant or capsaicin and rats with osteoarthritic pain, or using again rats with CCI of the sciatic nerve but in which compounds are administered intrathecally, other authors have shown that TRPV1 antagonists do reduce mechanical allodynia (Kanai *et al*., 2005; Cui *et al*., 2006). This suggests that also in this case different populations of TRPV1-expressing neurons in different tissues participate, via either TRPV1-mediated or non-TRPV1-mediated mechanisms, to mechanical nociception.
(3) With both mechanical allodynia and thermal hyperalgesia, blockade of CB_1, but not CB_2, receptors also reduced the anti-hyperalgesic action of AA-5-HT. Considering that this compound is inactive at CB_1 receptors (Table 1), this finding suggests that, although we could detect this effect only in one out of three tissues analysed, this FAAH inhibitor, like the FAAH-selective compounds OL-135 and URB597, does elevate AEA levels in a way to not only desensitize TRPV1 channels, but also to activate CB_1 receptors indirectly, and hence exert analgesia.

Conclusions

We have shown here that AA-5-HT is the first 'dual' FAAH inhibitor and TRPV1 antagonist ever reported. Whereas the activity of this compound in inhibiting FAAH and in elevating tissue endocannabinoid levels was already known, we have found here that AA-5-HT can effectively antagonize the effects of both exogenous (capsaicin) and endogenous (AEA) agonists at TRPV1 receptors. The capability of this compound to target simultaneously two distinct targets involved in different ways in the transmission and control of nociception might open the possibility to treat chronic and inflammatory pain more efficaciously than with other analgesics. In fact, opiates and tricyclic antidepressants can be less potent than AA-5-HT in the tests used here ($IC_{50} > 5\,mg\,kg^{-1}$, i.p. or s.c., Idanpaan-Heikkila and Guilbaud, 1999; Otsuka *et al*., 2001; Joshi *et al*., 2006), and their effects might undergo tolerance upon chronic administration (Gonzalez *et al*., 2000). Indeed, we presented here examples of how AA-5-HT produces anti-nociceptive effects against two types of pro-algesic stimuli. Although we have provided here some evidence that the analgesic activity of this compound is based on its capability not only to elevate endocannabinoid levels (thus affecting both cannabinoid and TRPV1 receptors), but also to antagonize TRPV1 receptors directly (Table 4), the full understanding of the mechanisms through which AA-5-HT causes analgesia awaits further investigation. Future studies will have to be aimed at addressing this question as well as enhancing further, by chemical modification, the potency of AA-5-HT at both FAAH and TRPV1, and examining more in detail the advantages deriving from its use as an anti-hyperalgesic drug as compared to 'pure' FAAH inhibitors.

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Conflict of interests

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