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The hypothermic response to bacterial lipopolysaccharide critically depends on brain CB1, but not CB2 or TRPV1, receptors

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Non-technical summary Systemic inflammation and related disorders, including sepsis, are leading causes of death in hospitalized patients. In most severe cases, systemic inflammation is accompanied by a drop in body temperature (hypothermia). We know that inflammation-associated hypothermia is a brain-mediated response, but mechanisms of this response are unknown. We administered a bacterial product (endotoxin) to rats to cause systemic inflammation and hypothermia. We then used a variety of pharmacological tools to probe whether three different receptors are involved in this hypothermia. We have found that one of the receptors studied, the so-called cannabinoid-1 (CB1) receptor, is crucial for the development of hypothermia. This is the same receptor that is responsible for many effects of marihuana (cannabis). We further show that hypothermia associated with inflammation depends on CB1 receptors located inside the brain. These novel findings suggest that brain CB1 receptors should be studied as potential therapeutic targets in systemic inflammation and sepsis.

Abstract Hypothermia occurs in the most severe cases of systemic inflammation, but the mechanisms involved are poorly understood. This study evaluated whether the hypothermic response to bacterial lipopolysaccharide (LPS) is modulated by the endocannabinoid anandamide (AEA) and its receptors: cannabinoid-1 (CB1), cannabinoid-2 (CB2) and transient receptor potential vanilloid-1 (TRPV1). In rats exposed to an ambient temperature of 22°C, a moderate dose of LPS (25–100 μg kg^{-1} I.V.) induced a fall in body temperature with a nadir at ∼100 min postinjection. This response was not affected by desensitization of intra-abdominal TRPV1 receptors with resiniferatoxin (20 μg kg^{-1} I.P.), by systemic TRPV1 antagonism with capsazepine (40 mg kg^{-1} I.P.), or by systemic CB2 receptor antagonism with SR144528 (1.4 mg kg^{-1} I.P.). However, CB1 receptor antagonism by rimonabant (4.6 mg kg^{-1} I.P.) or SLV319 (15 mg kg^{-1} I.P.) blocked LPS hypothermia. The effect of rimonabant was further studied. Rimonabant blocked LPS hypothermia when administered I.C.V. at a dose (4.6 μg) that was too low to produce systemic effects. The blockade of LPS hypothermia by I.C.V. rimonabant was associated with suppression of the circulating level of tumour necrosis factor-α. In contrast to rimonabant, the I.C.V. administration of AEA (50 μg) enhanced LPS hypothermia. Importantly, I.C.V. AEA did not evoke hypothermia in rats not treated with LPS, thus indicating that AEA modulates LPS-activated pathways in the brain rather than thermoeffector pathways. In conclusion, the

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present study reveals a novel, critical role of brain CB1 receptors in LPS hypothermia. Brain CB1 receptors may constitute a new therapeutic target in systemic inflammation and sepsis.

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Abbreviations ACTH, adrenocorticotropic hormone; AEA, arachidonoyl *N*-ethanolamide (anandamide); CB1 and CB2, cannabinoid-1 and -2 receptors, respectively; CCK, cholecystokinin; HPA, hypothalamo–pituitary–adrenal; IL, interleukin; LPS, lipopolysaccharide; PG, prostaglandin; T_b, body temperature; TNF, tumour necrosis factor; TRPV1, transient receptor potential vanilloid-1.

Introduction

Thermoregulatory responses are hallmarks of sepsis-related syndromes. Although fever is the most common and better understood response, it is hypothermia that occurs in the most severe cases of sepsis and septic shock (Clemmer *et al.* 1992; Arons *et al.* 1999). In the laboratory, this fever-hypothermia dichotomy has been best characterized in a rat model of systemic inflammation induced by bacterial lipopolysaccharide (LPS); for a review, see Romanovsky *et al.* (2005). In rats exposed to an ambient temperature of 20–25°C, lower doses of LPS cause fever, whereas higher doses induce early (over the first 2 h) hypothermia that may or may not be followed by fever or by late hypothermia (Romanovsky *et al.* 1996*a*,*b*; Steiner *et al.* 2005). Both fever and hypothermia seem to be regulated thermoregulatory responses brought about by brain-driven changes in thermoeffector activity (Romanovsky *et al.* 1996*b*; Almeida *et al.* 2006*a*,*b*).

The neuroimmune mechanisms that operate the fever–hypothermia switch have been proposed to involve lipid-derived inflammatory mediators, including platelet-activating factor (Ephgrave *et al.* 1997; Ivanov *et al.* 2003*b*), prostaglandins (Engblom *et al.* 2002; Matsumura & Kobayashi, 2004; Romanovsky *et al.* 2005; Blatteis, 2006; Spencer *et al.* 2008), epoxyeicosatrienoic acids (Kozak *et al.* 2000), and leukotrienes (Paul *et al.* 1999). A new class of lipid-derived mediators was discovered as a result of the search for endogenous ligands of cannabinoid receptors (reviewed by Freund *et al.* 2003). This class consists of *N*-ethanolamides derived from fatty acids, which are commonly referred to as endocannabinoids. Anandamide (arachidonoyl *N*-ethanolamide, AEA) is the best known member of this class. It works as a full agonist at the cannabinoid-1 (CB1) receptor subtype (Vogel *et al.* 1993), as a partial agonist at cannabinoid-2 (CB2) receptor subtype (Gonsiorek *et al.* 2000), and as a full agonist at the transient receptor potential vanilloid-1 (TRPV1) receptor (Smart *et al.* 2000). The levels of AEA, both in the brain and in the periphery, are elevated during the systemic inflammatory response to LPS (Liu *et al.* 2003; Fernandez-Solari *et al.* 2006). Furthermore, the tissue and cell distribution of the AEA receptors is consistent with their possible participation in neuro-immune communication: CB1 receptors are expressed in leukocytes, microgliocytes and neurons (Mailleux & Vanderhaeghen, 1992; Bouaboula *et al.* 1993; Matsuda *et al.* 1993; Waksman *et al.* 1999); CB2 receptors are expressed in leukocytes and microgliocytes, but not in neurons (Munro *et al.* 1993; Galiegue *et al.* 1995; Walter *et al.* 2003); and TRPV1 receptors are expressed primarily in sensory neurons (Tominaga & Caterina, 2004; Dhaka *et al.* 2006) and, to a lesser extent, in other neural and non-neural cells (reviewed by Romanovsky *et al.* 2009). The roles of these receptors in LPS fever have been studied (Szekely & Szolcsanyi, 1979; Gourine *et al.* 2001; Dogan *et al.* 2004; Iida *et al.* 2005; Benamar *et al.* 2007; Fraga *et al.* 2009), but no study has investigated systematically whether any of these receptors are involved in LPS hypothermia. In the present study, we conducted a series of pharmacological experiments to determine whether LPS-induced hypothermia is affected by blockade of TRPV1, CB1, or CB2 receptors, and whether the receptors involved are located inside or outside of the brain. We then evaluated several potential mechanisms of the pharmacological effects found. Finally, we attempted to clarify the controversial issue of how AEA affects deep body temperature (T_b) under normal conditions (no systemic inflammation) and during systemic inflammation (LPS hypothermia).

Methods

Animals

The experiments were conducted in 294 rats. Two hundred and sixty-five male Long–Evans rats were purchased from Charles River Laboratories (Wilmington, MA, USA) and used in Experiments 1–5 (see below) conducted at St Joseph's Hospital and Medical Center (Phoenix, AZ, USA). Twenty-four male Long–Evans rats and five female Wistar rats were bred, raised, and studied (experiment 5) at the University of Pécs (Pécs, Hungary). The rats weighed 290–400 g at the time of experiments. At both centres, the rats were housed initially in groups; after surgery, they were

housed singly. They were housed in a thermally neutral environment (ambient temperature of 28°C) and under a 12:12 h light–dark cycle (lights on at 07.00 h). Standard rat chow and tap water were available *ad libitum*. At both centres, the rats were trained extensively to stay inside cylindrical confiners made of stainless steel wire. At St Joseph's Hospital, these confiners were also placed inside rat home cages where they enriched the cage space serving as artificial 'rat holes'. The rats spent some time inside the confiners voluntarily. Rodents are readily adaptable to confinement to an extent that habituated rodents respond to it with neither stress fever (Romanovsky *et al.* 1998*b*) nor other signs of stress (Abercrombie & Jacobs, 1987; Hashimoto *et al.* 1988; Melia *et al.* 1994; Stamp & Herbert, 1999). The same confiners were used later in the experiments. Each rat was used in an experiment once and killed with sodium pentobarbital (100 mg kg^{-1} I.V. or 400 mg kg^{-1} I.P.) immediately thereafter. All procedures at each centre were conducted under protocols approved by the Animal Care and Use Committee of the respective centre, St Joseph's Hospital or University of Pécs.

Surgical preparation

Four to seven days before an experiment, a rat was implanted with an I.V. catheter or an I.C.V. guide cannula. The procedures were performed under anaesthesia with ketamine–xylazine–acepromazine (55.6, 5.5, and 1.1 mg kg^{-1} I.P.) and antibiotic protection (enrofloxacin, 1.1 mg kg^{-1} S.C.). Typically, no supplemental dose of the anaesthetic cocktail was required. During surgery, the rat was maintained on a board warmed to 37°C.

For I.V. catheterization, a small longitudinal incision was made on the left ventral surface of the neck. The left jugular vein was exposed, freed from its surrounding connective tissue, and ligated. A silicone catheter (ID 0.5 mm, OD 0.9 mm) filled with heparinized (10 U ml^{-1}) saline was passed into the superior vena cava through the jugular vein and secured in place with ligatures. The free end of the catheter was knotted, tunnelled under the skin to the nape, and exteriorized. The skin was sutured. The catheters were flushed daily with heparinized saline.

For I.C.V. cannulation, the rat was fixed to a stereotaxic apparatus (David Kopf, Tujunga, CA, USA). The skin was incised over the sagittal suture; the periosteum was excised; supporting microscrews were driven into the skull; and a steel guide cannula (Plastics One, Roanoke, VA, USA) was implanted. The tip of the cannula was placed 0.5 mm dorsal to the right lateral ventricle using the following stereotaxic coordinates: −0.5 mm from bregma, −1.5 mm from the midline, and 3.5 mm from the skull surface (Paxinos & Watson, 2004). The implanted cannula was attached to the supporting microscrews with acrylic cement.

Experimental set-up

On the day of the experiment, each rat was placed in a confiner to which it had been habituated (see Animals). For measurement of deep T_b, a copper–constantan thermocouple was inserted in the colon, 10 cm beyond the anal sphincter. The thermocouple was fixed to the base of the tail with adhesive tape and plugged into a data logger which conveyed the data to a personal computer. The rat was then transferred to an environmental chamber: a 3940 chamber from Forma Scientific (Marietta, OH, USA) was used in all experiments, except in the part of Experiment 5 conducted at the University of Pécs, for which a Plexiglas chamber was kept inside a temperature-controlled water bath. When present, the I.V. catheter was connected to a PE-50 extension. When the animal had an I.C.V. cannula, an injector needle (Plastics One) was fitted into the cannula and connected to a PE-50 extension; the needle protruded 1 mm beyond the guide cannula to reach the lateral ventricle. The I.V. or I.C.V. extension, filled with the drug of interest or its vehicle, was passed through a port in the chamber wall and connected to a syringe located outside of the chamber. This set-up permits drug administration without disturbing the rat and without causing a marked stress response that often presents a major limitation in thermoregulation experiments (Romanovsky *et al.* 1998*a*; Rudaya *et al.* 2005).

Experimental protocols

Experiment 1. The first experiment was performed to evaluate whether localized desensitization of TRPV1-bearing nerve afferents (or any other resiniferatoxin-sensitive cells) in the abdomen affects LPS-induced hypothermia. Abdominal visceral TRPV1 channels may play roles in thermoregulation (Steiner *et al.* 2007) and inflammation (Miranda *et al.* 2007). Although they are not involved in LPS fever (Dogan *et al.* 2004), they may be involved in LPS hypothermia. Under deep sedation with an acepromazine-enriched ketamine–xylazine–acepromazine cocktail (5.6, 0.6 and 1.2 mg kg^{-1} I.P.), a rat was injected I.P. with resiniferatoxin (20 μg kg^{-1}) or its vehicle. The sedation protocol was chosen based on our earlier study (Dogan *et al.* 2004) and resulted in a suppressed writhing response to resiniferatoxin. At the dose used, resiniferatoxin (a highly potent TRPV1 agonist) desensitizes TRPV1 receptors in abdominal viscera for at least 19 days, but does not desensitize TRPV1 receptors in other bodily compartments, as has been shown by several studies from our group using an extensive array of tests (Dogan *et al.* 2004; Steiner *et al.* 2007). On day 7 following administration of resiniferatoxin or its vehicle, each rat was implanted with an I.V. catheter. The experiment was conducted on day 11. Each rat was transferred to the

experimental set-up described above and exposed to an ambient temperature of 22°C. After 4 h of habituation to the experimental conditions, each rat was injected I.V. with a relatively high, hypothermia-inducing dose of LPS (100 μg kg^{-1}) or with saline. T_b was recorded from the beginning of the experiment to at least 240 min after the injection.

As in our previous study (Dogan *et al.* 2004), the extent of nerve afferent desensitization was assessed by the eye-wiping test (to confirm that desensitization did not reach systemic levels) and the cholecystokinin (CCK)-induced satiety test (to confirm abdominal desensitization). The eye-wiping test was conducted on day 9. It consisted of counting the number of eye-wiping movements for 30 s following corneal application of a chemical irritant (20 μl of 1% NH_4OH in saline). The two-measurement satiety test was conducted on day 15 (first measurement) and day 19 (second measurement), each time after 24 h of food deprivation. Each rat was injected I.P. with CCK (6 μg kg^{-1}) on one day and with saline on the other day; the order of injections was randomized. Standard chow was made available 5 min after the injections, and the mass of chow consumed over 30 min was determined. For each rat, the difference in food intake (CCK test minus saline test) was expressed as a percentage of the amount consumed in the saline test.

Experiment 2. In the second experiment we investigated whether LPS-induced hypothermia is altered in rats pretreated systemically with capsazepine, a reasonably selective (Romanovsky *et al.* 2009) and potent (Gavva *et al.* 2005) antagonist of the rat TRPV1 receptor in the chemical ligand and heat modes of activation. At an ambient temperature of 22°C, rats were pretreated I.P. with capsazepine (40 mg kg^{-1}) or its vehicle. Ninety minutes later they were injected I.V. with LPS (100 μg kg^{-1}) or saline. T_b was recorded from the beginning of the experiment to at least 240 min after LPS (or saline) administration.

To confirm the effectiveness of the capsazepine pretreatment, we tested whether it blocked capsaicin-induced hypothermia (Dogan *et al.* 2004). A separate group of rats were pretreated with capsazepine or its vehicle as described above and 90 min later injected I.P. with capsaicin (1 mg kg^{-1}). Their T_b was recorded.

Experiment 3. The third experiment was conducted to evaluate whether LPS-induced hypothermia is altered by systemic pretreatment with rimonabant (an established CB1 receptor antagonist and inverse agonist; Rinaldi-Carmona *et al.* 1994; Bouaboula *et al.* 1997) or SR144528 (a CB2 receptor antagonist and inverse agonist; Rinaldi-Carmona *et al.* 1998; Portier *et al.* 1999). At an ambient temperature of 22°C, each rat was pretreated I.P. with rimonabant at a dose of 4.6 mg kg^{-1} (10 μmol kg^{-1}), SR144528 at a dose of 1.4 mg kg^{-1} (3 μmol kg^{-1}), or their vehicle (1 ml kg^{-1}). The dose of rimonabant used has been shown to produce a maximum inhibition of the classic CB1-mediated *in vivo* effects, such as cannabinoid-induced analgesia, ring immobility, and barrel rotations (Rinaldi-Carmona *et al.* 1994). The dose of SR144528 used is known to exhibit a maximum competition for CB2 receptor binding sites in the spleen (Rinaldi-Carmona *et al.* 1998). Ninety minutes after the pretreatment with an antagonist or vehicle, rats were injected I.V. with LPS (100 μg kg^{-1}) or saline. The rats' T_b was recorded.

Since rimonabant blocked LPS hypothermia (see Results), we sought to test if SLV319, a structurally distinct CB1 receptor antagonist, was also capable of blocking LPS hypothermia. Because SLV319 is 3–4 times less potent than rimonabant (Lange *et al.* 2004), it was administered I.P. at a dose of 15 mg kg^{-1}. Following the systemic administration of this dose, SLV319 has been shown to occupy >80% of brain CB1 receptors (Need *et al.* 2006). At an ambient temperature of 22°C, rats received the I.P. pretreatment with SLV319 or its vehicle and 90 min later were injected I.V. with LPS or saline. It should be noted that we performed this test later than all other experiments of this study and had to use a newer lot of LPS (see Drugs). In separate experiments (data not shown), we determined a dose of the newer LPS that produced a hypothermic response comparable in magnitude to the response caused by the 100 μg kg^{-1} dose of the LPS lot used in all other experiments. This dose was found to be 25 μg kg^{-1}.

Experiment 4. In the fourth experiment we tested whether selective blockade of CB1 receptors in the brain affects LPS-induced hypothermia. Rimonabant was chosen for this experiment because it is the best studied of the two CB1 receptor antagonists employed in the present study. The experiment was conducted at an ambient temperature of 22°C. A low dose of rimonabant (4.6 μg) was administered into the lateral brain ventricle via a pre-implanted I.C.V. cannula. Control rats received an I.P. injection of rimonabant at the same low dose or an I.C.V. injection of the rimonabant vehicle. Ninety minutes after the pretreament (with rimonabant I.C.V., rimonabant I.P., or the vehicle I.C.V.), the rats received an I.V. injection of LPS (100 μg kg^{-1}) or saline. In a subset of the rats, T_b was monitored. In the other subset, blood samples were collected for determination of pro-inflammatory cytokines (viz. tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6), an anti-inflammatory cytokine (viz. IL-10), and hormones of the anti-inflammatory hypothalamo–pituitary–adrenal (HPA) axis (viz. adrenocorticotropic hormone (ACTH) and corticosterone). The blood samples were collected

50 min after the LPS (or saline) administration, a time that corresponds to the onset of the hypothermic response to LPS (Romanovsky *et al.* 1996*b*; Steiner *et al.* 2005). At the time of sample collection, a rat was anaesthetized I.V. with ketamine–xylazine–acepromazine (5.6, 0.6 and 0.1 mg kg^{-1}), its rib cage was opened, and blood was collected from the inferior vena cava. After the blood was collected, the animal was killed with sodium pentobarbital, as described in the Animals section above. Blood plasma was obtained by centrifugation (6000 *g*, 5 min, 4°C) of blood collected into EDTA-coated vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). Blood serum was obtained by allowing blood to clot at room temperature for 20 min and then centrifuging it (8000 *g*, 10 min, 4°C). Samples were stored at −80°C until assays.

The levels of TNF-α, IL-1β, IL-6 and IL-10 were determined in plasma samples by sandwich enzyme-linked immunosorbent assay (ELISA) using kits from R&D Systems (Minneapolis, MN, USA); the levels of ACTH were determined in plasma samples by sandwich ELISA using a kit from MD Biosciences (St Paul, MN, USA); and the levels of corticosterone were determined in serum samples by competitive ELISA using a kit from Assay Designs (Ann Arbor, MI, USA). Assay ranges (pg ml^{-1}) were: 13–800 for TNF-α; 31–2000 for IL-1β, IL-6, and IL-10; 5–500 for ACTH; and 32–20,000 for corticosterone. Samples were assayed undiluted (for IL-1β, IL-6, IL-10, and ACTH), diluted 1:10 (TNF-α), or diluted 1:100 (corticosterone). In each assay, all samples were run simultaneously, in duplicate.

Experiment 5. The fifth experiment was designed to evaluate whether and how AEA alters T_b under normal conditions and during LPS-induced hypothermia. Because blockade of CB1 receptors within the brain was sufficient to attenuate LPS hypothermia (see Results), AEA was administered I.C.V. in this experiment. The experiment was conducted at 22°C; T_b was recorded. The rats were injected I.V. with LPS (100 μg kg^{-1}) or saline and 50 min later treated I.C.V. with AEA (50 μg). Control rats were treated I.V. with AEA at the same low dose or I.C.V. with the AEA vehicle.

Because there is no agreement in the literature regarding the thermoregulatory effect of I.C.V. AEA (Crawley *et al.* 1993; Di Marzo *et al.* 2000; Fraga *et al.* 2009), and because the experiment described above did not reveal any thermoregulatory effect of I.C.V. AEA at 50 μg in the absence of systemic inflammation (see Results), additional experiments were conducted to test if the T_b of euthermic rats (not treated with LPS) could be changed by AEA administered I.C.V. in a wide dose range (0.07–520 μg) under various experimental conditions. Prostaglandin (PG) E_2 (200 ng), a potent fever-inducing agent (Sugimoto *et al.* 1999), was administered to test the patency of the I.C.V. cannula. These experiments were conducted at two centres (St Joseph's Hospital and the University of Pécs) in male Long–Evans rats from two different colonies and in female Wistar rats. The rats were exposed to an ambient temperature of 15, 22, 25, or 28°C. Some rats were cold-adapted (4°C for 21 days), while others were food-deprived (for 24 h) prior to the experiment. The experimental conditions were chosen based on the facts that the rat strain (Gordon, 1993; Ivanov *et al.* 2003*a*) and sex (Mouihate *et al.* 1998), as well as the ambient temperature during an experiment (Romanovsky *et al.* 1997; Ivanov *et al.* 2003*b*) and the prior thermal experience (e.g. the development of cold adaptation; see Petervari *et al.* (2003), can affect the direction and magnitude of a T_b response. The feeding status also influences thermoregulatory responses (Szekely, 1979; Steiner *et al.* 2009*b*; Krall *et al.* 2010), and at least some thermoregulatory consequences of the changes in the feeding status are TRPV1 dependent (Kanizsai *et al.* 2009). Furthermore, the strain (Arnold *et al.* 2001), sex (Farhang *et al.* 2009) and feeding status (Matias & Di Marzo, 2007) are determinants of the expression and responsiveness of CB receptors.

Drugs

E. coli 0111:B4 LPS was purchased from Sigma-Aldrich (St Louis, MO, USA). For all experiments except for the test with SLV319 (Experiment 3), we used lot 35H4086, the same lot that was used in our laboratory for years (Romanovsky *et al.* 1998*a*; Ivanov & Romanovsky, 2002). In the SLV319 test, we used LPS of a newer lot, 029K4022. The doses of the two LPS preparations used (100 μg kg^{-1} for 35H4086 and 25 μg kg^{-1} for 029K4022) were selected to cause a hypothermic response of a similar magnitude. LPS was suspended in saline (100 or 25 μg ml^{-1}) and bolus-injected I.V. at 1 ml kg^{-1}. CCK octapeptide sulfate (Tocris Cookson, Ellisville, MO, USA) was dissolved in saline (6 μg ml^{-1}) and bolus-injected I.V. at 1 ml kg^{-1}. Resiniferatoxin from *Euphorbia poisonii* (Sigma-Aldrich), capsaicin (Sigma-Aldrich), capsazepine (Tocris Cookson), SR144528 (Cayman Chemical, Ann Arbor, MI, USA), and SLV319 (Cayman Chemical) were dissolved in ethanol–Tween 80–saline (1:1:3 for capsazepine; 1:1:8 for the other drugs). The final concentrations of resiniferatoxin, capsaicin, capsazepine, SR144528 and SLV319 were 0.02, 1, 40, 1.4, and 15 mg ml^{-1}, respectively. These solutions were bolus-injected I.P. at 1 ml kg^{-1}. For I.P. administration, rimonabant (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in ethanol–Tween 80–saline (1:1:8) to a final concentration of 4.6 mg ml^{-1} (Experiment 3) or 0.046 mg ml^{-1} (Experiment 4), and the resulting solution was bolus-injected at 1 ml kg^{-1}.

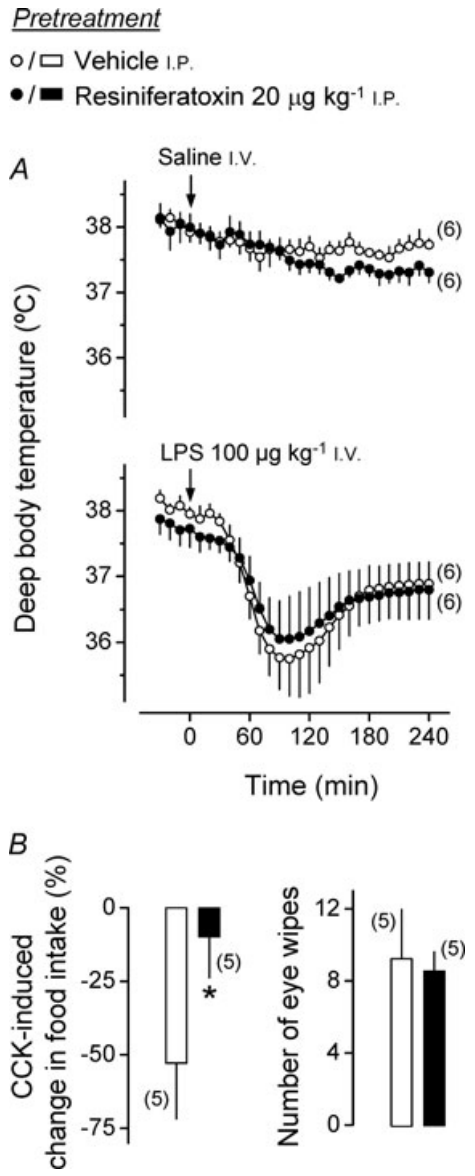

Figure 1. Intra-abdominal TRPV1 desensitization with resiniferatoxin does not affect LPS hypothermia
A, the T_b responses to I.V. LPS (dose indicated) or saline in rats pretreated I.P. with resiniferatoxin (dose indicated) or its vehicle and exposed to an ambient temperature of 22°C. *B*, the results of functional tests confirming the extent of resiniferatoxin-induced afferent nerve desensitization. The results of the CCK-induced satiety test (to assess abdominal desensitization) are shown as the relative difference in the amount of food consumed by food-deprived rats during a 30 min period after administration of CCK-8 sulfate (6 μg kg^{-1} I.P.). These results confirm that nerve fibres involved in CCK-mediated satiety were desensitized. The results of the eye-wiping test (to assess systemic desensitization) are shown as the number of eye wipes during a 30 s period after intraocular administration of an irritant. These results show that desensitization did not occur in remote extra-abdominal locations, such as the cornea. Here and in Fig. 2–7, the number of animals in each group is shown in parentheses. *$P < 0.05$ compared to vehicle pretreatment.

For I.C.V. administration, rimonabant was dissolved in ethanol–saline (1:1) to a concentration of 2.3 mg ml^{-1} and microinfused at a rate of 1 μl min^{-1} for 2 min. AEA (Tocris Cookson) was dissolved in ethanol–saline (1:1) to concentrations of 0.035–260 mg ml^{-1} and microinfused I.C.V. at 1 μl min^{-1} for 2 min or I.V. at 50 μl min^{-1} for 2 min. PGE_2 (Cayman) was administered I.C.V. by microinfusing a 100 μg ml^{-1} solution in ethanol–saline (1:1) at a rate of 1 μl min^{-1} for 2 min.

Statistical analyses

Statistical comparisons were made using Statistica Advanced 8.0 (StatSoft, Tulsa, OK, USA). Repeated measures ANOVA was employed to evaluate the effects of the pharmacological treatments on T_b, whereas factorial ANOVA was employed to evaluate the effects of treatments on the levels of TNF-α, IL-1β, IL-6, IL-10, ACTH and corticosterone. The ANOVA was followed by a *post hoc* analysis with Fisher's least significant difference test. Student's *t* test was employed to evaluate the changes in food intake and in the number of eye wipes in Experiment 1. The level of significance was set at $P < 0.05$. Data are reported as means ± SEM.

Results

Experiment 1: LPS hypothermia is not affected by desensitization of abdominal TRPV1 receptors

At an ambient temperature (22°C) known to be subneutral for rats in our experimental set-up (Romanovsky *et al.* 2002), the I.V. administration of a moderately high dose of LPS (100 μg kg^{-1}) to vehicle-pretreated rats caused a typical hypothermic response (a nadir at ∼100 min; $P < 0.02$), whereas the administration of saline caused no change in T_b (Fig. 1*A*). The hypothermic response to LPS also occurred in rats desensitized with resiniferatoxin (20 μg kg^{-1}), and it did not differ from the response of vehicle-pretreated rats.

Desensitization of intra-abdominal afferents was verified by testing rats for CCK-induced satiety, a response known to be mediated, at least partly, by abdominal vagal afferent fibres (Smith *et al.* 1985). CCK decreased the food intake of vehicle-pretreated rats by ∼50%, whereas it had a 5 times smaller effect in resiniferatoxin-desensitized rats ($P < 0.04$; Fig. 1*B*). Yet resiniferatoxin pretreatment did not impair the eye-wiping response to a chemical irritant (Fig. 1*B*), indicating that desensitization did not reach systemic levels.

Experiment 2: LPS hypothermia is not affected by systemic pharmacological blockade of TRPV1 receptors

Vehicle-pretreated controls responded to LPS with statistically significant ($P < 0.04$) hypothermia (Fig. 2*A*). Pretreatment with the TRPV1 receptor antagonist, capsazepine (40 mg kg^{-1} I.P.), affected neither the level of T_b in euthermic, saline-injected rats nor the hypothermic response to LPS. However, the same dose of capsazepine was effective ($P < 0.04$) in blocking the hypothermic response to capsaicin (1 mg kg^{-1} I.P.; Fig. 2*B*).

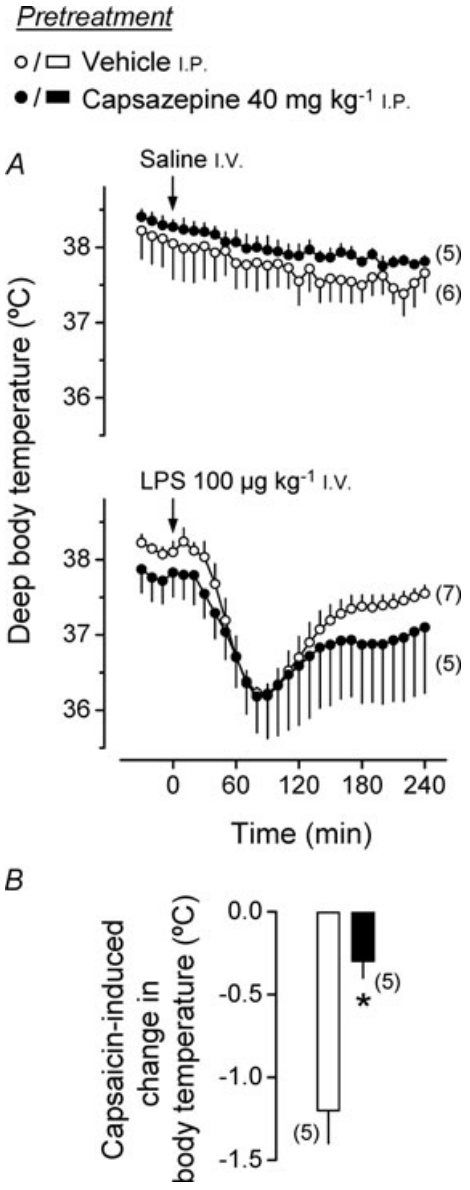

Figure 2. TRPV1 receptor antagonism with capsazepine does not affect LPS hypothermia
A, the T_b responses to I.V. LPS (dose indicated) or saline in rats pretreated I.P. with resiniferatoxin (dose indicated) or its vehicle. *B*, the results of a functional test confirming the effectiveness of the capsazepine pretreatment based on its ability to block the hypothermic response to the TRPV1 agonist, capsaicin (1 mg kg^{-1}, I.P.). Rats were exposed to an ambient temperature of 22°C during both experiments. The same capsazepine pretreatment that did not affect LPS hypothermia strongly attenuated the hypothermic response to capsaicin.

Experiment 3: blockade of CB1 receptors, but not of CB2 receptors, abolishes LPS-induced hypothermia

Systemic pretreatment with the CB2 receptor antagonist, SR144528 (1.4 mg kg^{-1} I.P.), affected neither the T_b of saline-treated rats nor the hypothermic response to LPS (Fig. 3). The CB1 receptor antagonist, rimonabant (4.6 mg kg^{-1} I.P.), had no effect on the T_b of saline-treated rats, but it abolished the hypothermic response to LPS ($P < 0.004$). The effect of rimonabant was so

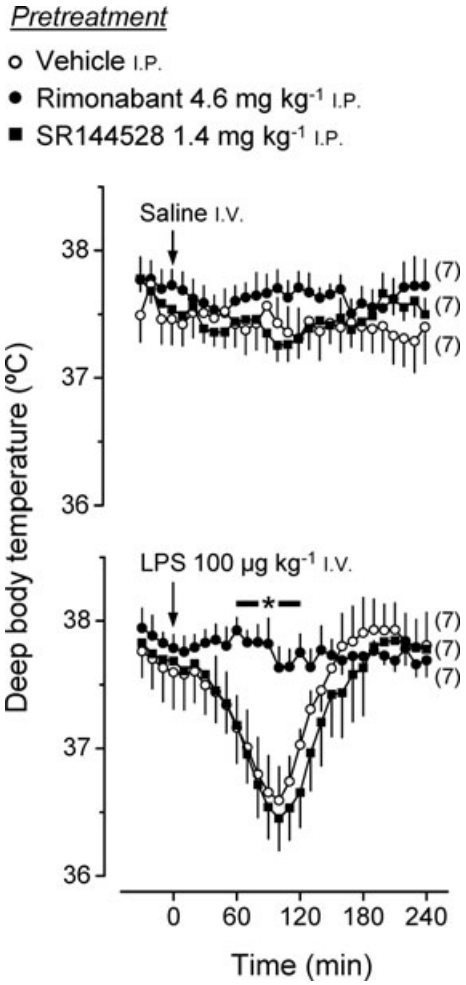

Figure 3. LPS hypothermia is blocked by a CB1 receptor antagonist (rimonabant), but unaffected by a CB2 receptor antagonist (SR144528)
Effects of I.P. pretreatment with rimonabant, SR144528, or their vehicle on the T_b responses to I.V. LPS or saline in rats exposed to an ambient temperature of 22°C.

pronounced that there was no statistical difference in T_b between rimonabant-pretreated rats injected with LPS and rimonabant-pretreated rats injected with saline.

The ability of CB1 receptor antagonism to block LPS hypothermia was confirmed using a distinct CB1 receptor antagonist, SLV319. Pretreatment with SLV319 (15 mg kg^{-1}) had no effect on the T_b of saline-treated rats, but it strongly attenuated the hypothermic response to LPS ($P < 0.03$; Fig. 4).

Experiment 4: LPS hypothermia is abolished by selective blockade of CB1 receptors in the brain

Figure 5*A* shows the effect of the I.V. administration of saline or LPS on T_b in rats pretreated I.C.V with either rimonabant at a low dose (4.6 μg) or with its vehicle. LPS lowered the T_b of rats pretreated with vehicle ($P < 0.01$), but not of rats pretreated with rimonabant. Comparison between LPS-induced effects in the rimonabant- and vehicle-pretreated groups revealed a

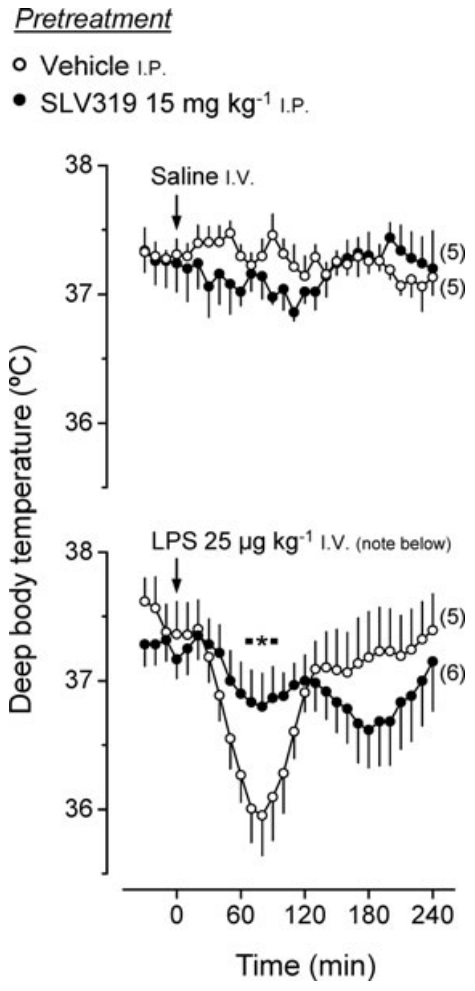

Figure 4. LPS hypothermia is blocked by SLV319, a CB1 receptor antagonist distinct from rimonabant
Effects of I.P. pretreatment with SLV319 or its vehicle on the T_b responses to I.V. LPS or saline in rats exposed to an ambient temperature of 22°C. Note that LPS of a different lot was used in this test, and that the dose of LPS was adjusted in order to produce a hypothermic response similar in magnitude to the responses observed in other experiments of this study (see Drugs for details).

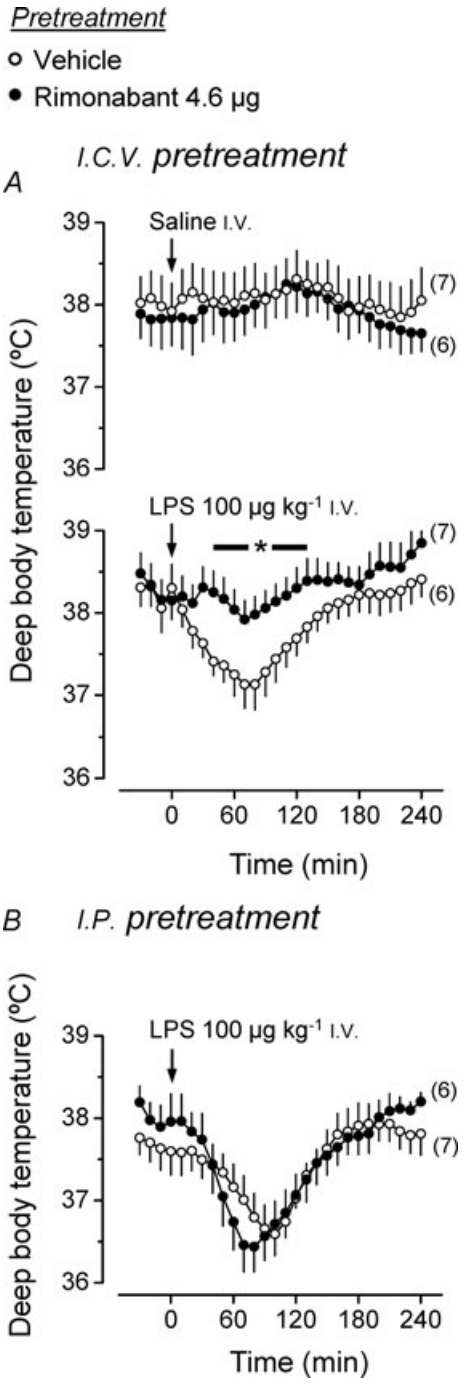

Figure 5. Intrabrain rimonabant at a systemically ineffective dose blocks LPS hypothermia
Effects of pretreatment with a low dose of rimonabant or its vehicle, either I.C.V. (panel *A*) or I.P. (panel *B*), on the T_b responses to I.V. LPS or saline.

significant difference ($P < 0.04$). However, when the same low dose of rimonabant was given I.P., it did not affect LPS hypothermia (Fig. 5*B*).

To investigate whether inhibition of LPS hypothermia by rimonabant might have been associated with stronger activation of the anti-inflammatory HPA axis or with an altered balance between pro- and anti-inflammatory cytokines, we measured the circulating levels of ACTH and corticosterone (HPA hormones), TNF-α, IL-1β and IL-6 (pro-inflammatory cytokines), and IL-10 (an anti-inflammatory cytokine). At the time corresponding to the onset of hypothermia (50 min), vehicle-pretreated rats responded to LPS with significant rises in the levels of ACTH ($P < 0.0007$) and corticosterone ($P < 0.05$), as well as in the levels of all cytokines measured ($P < 0.003$; Fig. 6). Pretreatment with rimonabant (4.6 μg I.C.V.) attenuated the LPS-induced rise in plasma ACTH ($P < 0.04$), but did not affect the rise in plasma corticosterone. The LPS-induced rise in plasma TNF-α was reduced by rimonabant ($P < 0.0002$), the rise in IL-1β tended to be attenuated ($P < 0.09$), and the rises in IL-6 and IL-10 were unaffected.

Experiment 5: central administration of AEA does not cause hypothermia, but enhances LPS-induced hypothermia

Figure 7 shows the effect of AEA at a low dose (50 μg) on the T_b of rats injected I.V. with LPS (100 μg kg^{-1}) or saline. The T_b of the saline-injected rats was not affected by the I.C.V. administration of AEA or its vehicle. However, LPS hypothermia was greater in the rats administered I.C.V. with AEA than in the rats administered with its vehicle ($P < 0.05$). When the same dose of AEA was given I.V., it did not affect LPS hypothermia.

The fact that I.C.V. AEA did not affect the T_b of saline-injected rats deserves attention, since there is controversy as to whether the action of AEA in the brain of euthermic rats causes hypothermia, a typical response to exogenous cannabinoid agonists (Crawley *et al.* 1993; Chaperon & Thiebot, 1999; Di Marzo *et al.* 2000; Fraga *et al.* 2009). We addressed this controversy by performing additional experiments, in which we studied thermoregulatory effects of I.C.V. AEA in rats not injected with LPS. AEA was administered over a wide dose range (0.07–520 μg) and under several experimental conditions. We studied two rat strains and sexes (male Long–Evans and female Wistar rats) and four ambient temperatures (15, 22, 25 or 28°C). In some experiments, we also used cold adaptation or food deprivation. Under no circumstance did the I.C.V. administration of AEA decrease (or cause any other change in) the T_b of the rats (Fig. 8). The patency of the I.C.V. cannulas and the overall ability of our methodology to detect a T_b response were verified by administering PGE_2 at a relatively low dose (200 ng) and registering a highly significant ($P < 0.0001$) T_b rise, as compared to the response of rats treated with the vehicle (0 μg AEA; Fig. 8).

Discussion

The present study was the first to investigate the potential involvement of TRPV1, CB1 and CB2 receptors in LPS-induced hypothermia. To assess the involvement of the TRPV1 receptor, we studied the effects of TRPV1 desensitization with resiniferatoxin and of the

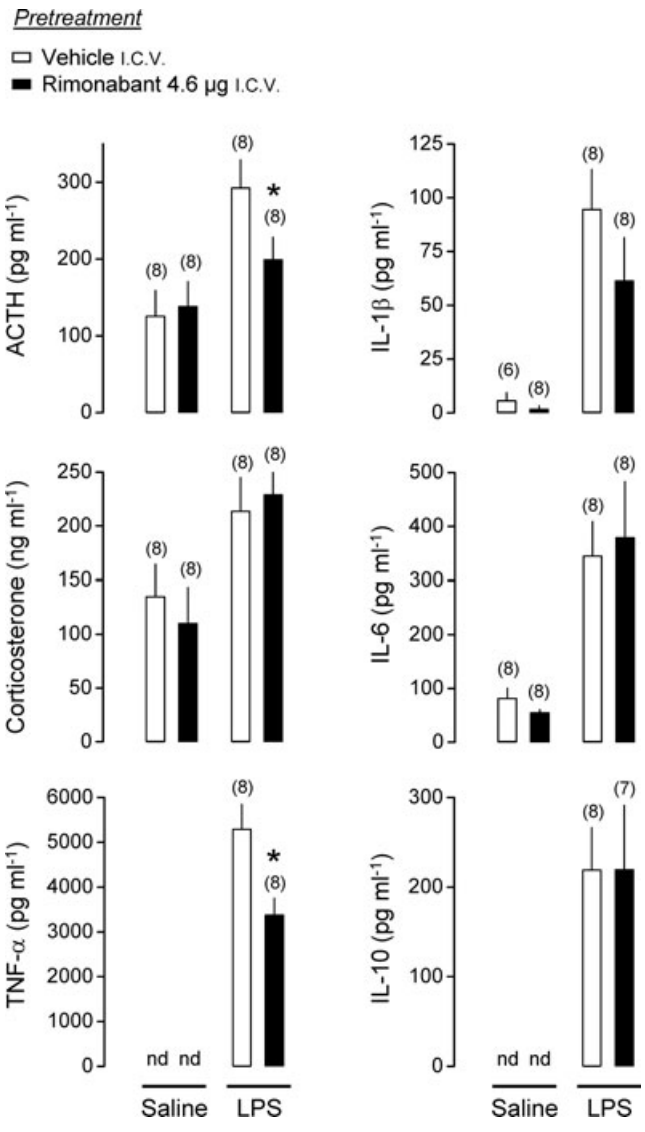

Figure 6. Intrabrain rimonabant attenuates the LPS-induced rises in circulating ACTH and TNF-α
Effects of LPS or saline on the circulating levels of ACTH, corticosterone, TNF-α, IL-1β, IL-6 and IL-10 of rats pretreated I.C.V. with a low dose of rimonabant (indicated) or its vehicle. Blood samples were collected 50 min after rats were administered I.V. with LPS (100 μg kg^{-1}) or saline at an ambient temperature of 22°C.

pharmacological antagonism with capsazepine on LPS hypothermia. Resiniferatoxin was our first-choice TRPV1 agonist for desensitization experiments because it is more potent and selective than capsaicin (Szallasi & Blumberg, 1989) and, most importantly, lacks the non-TRPV1-mediated effects of capsaicin, such as attenuation of the first febrile phase (Dogan *et al.* 2004; Nikami *et al.* 2008). Capsazepine was our first-choice TRPV1 antagonist because, at moderate doses, it does not exert a thermoregulatory effect of its own in rats (Garami *et al.* 2010), even though it blocks two modes of TRPV1 receptor activation, i.e. by ligands and heat, with moderate and high potency, respectively (McIntyre *et al.* 2001; Price *et al.* 2004; Gavva *et al.* 2005; Garami *et al.* 2010). Neither resiniferatoxin desensitization nor capsazepine antagonism altered LPS hypothermia, indicating that TRPV1 receptors are not involved in this response. To confirm this finding, we conducted a supplemental experiment employing AMG0347, a new-generation TRPV1 antagonist that blocks the proton, ligand and heat modes of activation of the rat TRPV1 channel with high potency (Steiner *et al.* 2007). Like many other TRPV1 antagonists, especially those that are potent blockers of the proton mode of activation (Garami *et al.* 2010), AMG0347 causes hyperthermia (Steiner *et al.* 2007; Garami *et al.* 2010). Indeed, in our supplemental experiment, AMG0347 elevated the basal T_b of rats (Suppl. Fig. 1). However, when these rats were injected with LPS, their deep T_b was decreased to the same level of vehicle-pretreated rats. Because neither TRPV1 desensitization nor pharmacological antagonism (by capsazepine or AMG0347) affects LPS hypothermia, we conclude that the hypothermic response to LPS does not depend on the activation of TRPV1 channels. A convincing way to further support this conclusion would be to study LPS hypothermia in *Trpv1* knockout mice. However, unlike rats, mice of several strains do not respond to a non-stressful I.V. injection of LPS with the early (over the first 2 h) hypothermic response (Rudaya *et al.* 2005). The non-responding strains also include *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ C57BL/6×129 mice from the Amgen colony at Charles River Laboratories (S. P. Wanner, N. R. Gavva & A. A. Romanovsky, unpublished observations).

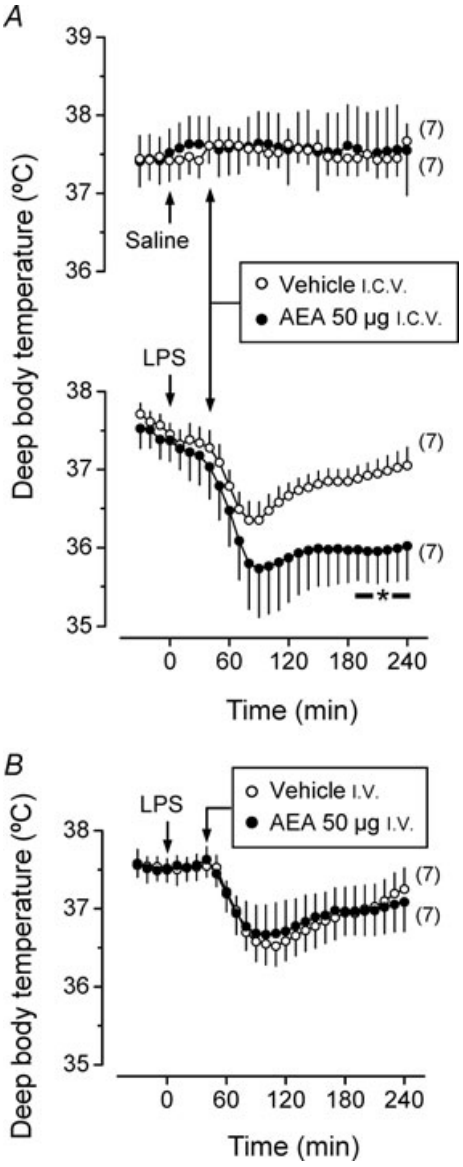

Figure 7. Intrabrain AEA enhances LPS hypothermia
Effects of a low dose of AEA given I.C.V. (panel *A*) or I.V. (panel *B*) on the T_b responses to I.V. LPS (100 μg kg^{-1}) or saline. The ambient temperature was 22°C.

Our present experiments also show that the hypothermic response to LPS is not affected by CB2 receptor antagonism with SR144528. In line with this finding are reports that CB2 receptor antagonists have no impact on other brain-mediated components of the systemic inflammatory response, e.g. hyperalgesia (Naidu *et al.* 2010) and fever (Benamar *et al.* 2007; Fraga *et al.* 2009). Even though CB2 receptors have been shown to play anti-inflammatory roles in some immune cells (Sacerdote *et al.* 2000; Germain *et al.* 2002; Eisenstein *et al.* 2007), such roles are likely to be limited to localized inflammatory processes (Oka *et al.* 2005; Naidu *et al.* 2010).

The most striking finding of the present study was that blockade of CB1 receptors by rimonabant or SLV319 prevented the hypothermic response to LPS. The effect was so strong that the T_b response of rats treated with LPS looked like that of rats treated with saline. Having worked with LPS hypothermia for years (Romanovsky *et al.* 1996*b*, 1997, 1998*a*; Steiner *et al.* 2004, 2005, 2009*a*; Rudaya *et al.* 2005; Almeida *et al.* 2006*a*,*b*; Krall *et al.* 2010), we have never seen such a complete pharmacological blockade of the robust, highly reproducible hypothermic response of rats to LPS. Rimonabant prevented LPS

hypothermia not only when administered systemically at a relatively high dose, but also when administered I.C.V. at a dose that was too low to produce systemic effects. These findings indicate that CB1 receptors in the brain play an essential role in the development of LPS-induced hypothermia. Such a role is unlikely to reflect a direct thermoregulatory (hypothermic) effect because, in our experiments, I.C.V. administration of AEA over a wide dose range and under a variety of experimental conditions failed to produce any hypothermic response in normal (no systemic inflammation) rats. This observation may seem paradoxical, as hypothermia is considered a classic effect of exogenous cannabinoid agonists (Fitton & Pertwee, 1982; Ovadia *et al.* 1995; Rawls *et al.* 2002). However, in agreement with our study, I.C.V. AEA failed to cause hypothermia in the studies by Lichyman *et al.* (1996) and Fraga *et al.* (2009). In fact, Fraga *et al.* (2009) have shown that AEA causes fever rather than hypothermia. Only in a study by Porter *et al.* (2002) did I.C.V. AEA produce a hypothermic effect, possibly by leaking into the systemic circulation and causing hypothermia via an action on peripheral targets. That peripheral administration of AEA causes hypothermia in rats and mice has been shown repeatedly (Crawley *et al.* 1993; Di Marzo *et al.* 2000; Fegley *et al.* 2004; Garami *et al.* 2011), including in our present study (Suppl. Fig. 2). It has also been recognized that many effects of exogenous cannabinoid agonists (such as the hypothermic effect) do not correspond to intrinsic effects of endogenous AEA (Chaperon & Thiebot, 1999). From this point of view, inhibitors of the AEA-hydrolysing enzyme, fatty acid amide hydrolase, are now being used as a powerful tool to assess the roles of endogenous AEA. It has been shown that inhibition of fatty acid amide hydrolase with URB597 does not result in hypothermia, even when it markedly elevates the brain level of AEA (Fegley *et al.* 2005).

The present study sheds some light on the physiological and molecular mechanisms of the hypothermic effect of

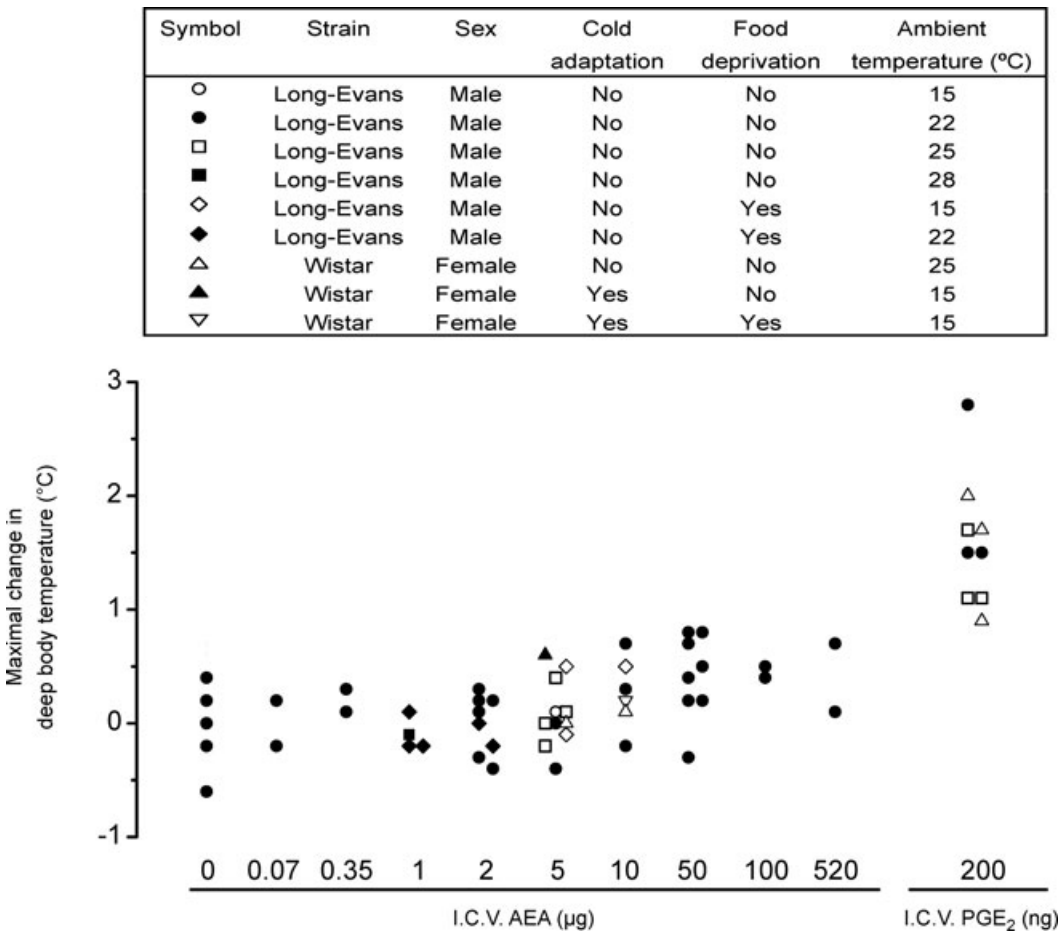

Figure 8. Intrabrain AEA does not cause hypothermia
The figure shows the effects of I.C.V. AEA on the T_b of individual rats under various experimental conditions (indicated). The body temperature responses to I.C.V. PGE_2 are shown to confirm the correct placement and patency of the I.C.V. cannulas. Doses of AEA and PGE_2 are indicated. Each data point denotes the maximal change in T_b during 0–120 min after the I.C.V. injection.

peripheral AEA. In supplemental experiments (Suppl. Fig. 2), we have found that the hypothermic response to I.V. AEA is associated with tail-skin vasodilatation, even though these experiments were conducted at a subneutral ambient temperature, which tends to counteract cutaneous vasodilatatory responses. In the same experiment, we could not block the hypothermic response to I.V. AEA by a combined treatment with CB1 and CB2 receptor antagonists (Suppl. Fig. 2). Cumulatively, these findings suggest that peripheral AEA causes hypothermia, at least in part, by triggering cutaneous vasodilatation via an action on non-CB1, non-CB2 receptors, perhaps on TRPV1 channels. Recently, we have shown the proposed TRPV1 mediation decisively by establishing that *Trpv1* knockout mice do not respond with hypothermia to peripheral AEA, whereas control mice develop marked hypothermia (Garami *et al.* 2011). Furthermore, the vasodilatatory effect of AEA has been shown to be mediated by TRPV1 channels (Zygmunt *et al.* 1999). Not all literature data can be readily reconciled with the present results. Experiments with inhibitors of the degradation of 2-arachidonyl glycerol suggest that this endogenous cannabinoid can decrease T_b via a CB1-mediated mechanism (Burston *et al.* 2008; Long *et al.* 2009). However, it is unclear whether the proposed mechanism involves central or peripheral CB1 receptors, and it is puzzling that the hypothermic response attributed to CB1 receptors still occurred (even though it was attenuated) in mice deficient of the *Cnr1* (CB1 receptor) gene (Burston *et al.* 2008). Clearly, further studies are needed.

While I.C.V. AEA did not cause hypothermia in the present study, it markedly enhanced the hypothermic response to LPS. This finding, together with our observation that a CB1 receptor antagonist (rimonabant) blocks LPS hypothermia, suggests that endocannabinoids act on brain CB1 receptors not within thermoregulatory circuits, but rather on some LPS-activated signalling pathway that ultimately interferes with thermoregulatory circuitry. Consistent with this idea, CB1 receptors have been shown to modulate LPS-induced changes in the hypothalamic concentration of noradrenaline (Villanueva *et al.* 2009), which, in turn, can drive thermoregulatory responses (Feleder *et al.* 2007*a*,*b*). Moreover, it is important to note that the blockade of LPS hypothermia by I.C.V. rimonabant in the present study was associated with an attenuated rise in the circulating level of TNF-α, a mediator of LPS hypothermia (Kozak *et al.* 1995; Leon *et al.* 1998; Tollner *et al.* 2000). Recent studies have similarly shown that the LPS-induced rise in circulating TNF-α is strongly attenuated by rimonabant, not only when this CB1 antagonist is administered systemically (Croci *et al.* 2003), but also when it is administered I.C.V. at systemically ineffective doses (Villanueva *et al.* 2009; De Laurentiis *et al.* 2010). It is plausible, therefore, that CB1 receptors in the brain are 'permissive' for the LPS-induced rise in circulating TNF-α. Such a permissive role of brain CB1 receptors may explain why inhibitors of degradation or reuptake of endocannabinoids enhance the TNF-α response to LPS (Roche *et al.* 2008), even though a peripheral action of endocannabinoids (on leukocytes) suppresses TNF-α production (Berdyshev *et al.* 1997; Cencioni *et al.* 2010).

Mechanisms by which blockade of brain CB1 receptors suppress the systemic production of TNF-α in response to LPS are unknown, but they are unlikely to involve activation of the anti-inflammatory HPA axis, because rimonabant enhanced neither the LPS-induced rise in plasma ACTH nor the LPS-induced rise in plasma corticosterone in the present study. In fact, the rise in ACTH was attenuated by rimonabant. It is of interest, however, that the vagus nerve (and possibly sympathetic nerves) can convey descending anti-inflammatory signals that suppress the production of TNF-α (Rosas-Ballina *et al.* 2008). Indeed, surgical vagotomy exaggerates LPS-induced hypothermia (Romanovsky *et al.* 1997), as well as other systemic effects of LPS, including TNF-α production (Borovikova *et al.* 2000), while electric stimulation of the peripheral end of the transected vagus attenuates these responses (Borovikova *et al.* 2000). Furthermore, many areas of the brain (including the hypothalamus) express CB1 receptors (Herkenham *et al.* 1991; Matsuda *et al.* 1993; Hirasawa *et al.* 2004) and project to the dorsal motor nucleus of the vagus (Saper *et al.* 1976; Chiba & Murata, 1985). Importantly, Derbenev *et al.* (2004) have provided functional evidence for a connection between CB1 receptors in the brain and vagal output by showing that pharmacological activation of CB1 receptors suppresses excitatory and inhibitory inputs to the motor nucleus of the vagus.

In conclusion, the main novel finding of the present study is that CB1 receptors in the brain are essential for the development of LPS-induced hypothermia, a prominent manifestation of severe systemic inflammation. We also show that brain CB1 receptors are permissive for the rise in the systemic level of TNF-α, an early event thought to contribute to morbidity and mortality in systemic inflammation and sepsis. Hence, brain CB1 receptors should be evaluated as potential therapeutic targets in sepsis and related conditions. The CB1-mediated mechanisms of LPS hypothermia remain speculative, but are likely to involve modulation of TNF-α production in the periphery by a descending pathway, perhaps involving the vagus nerve. In addition, the present study shows that central AEA exaggerates LPS hypothermia, but does not cause hypothermia under normal (without systemic inflammation) conditions. Only when injected peripherally does AEA cause hypothermia under normal conditions, possibly by triggering cutaneous vasodilatation via a TRPV1-mediated action. The present study

also provides evidence against an involvement of CB2 and TRPV1 receptors in LPS hypothermia.

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A.A.S., A.Y.M., M.D.D., M.C.A., M.S. and A.A.R. designed the study, with the help of S.P., E.P., M.B,. S.P.W. and N.R.G.; A.A.S., A.Y.M., M.D.D., S.P., E.P., M.B., S.P.W., J.E., D.L.O. and M.C.A. conducted the experiments; A.A.S., A.Y.M., M.D.D., S.P., E.P., M.B., J.E., M.C.A., M.S. and A.A.R. analysed the data; and A.A.S. and A.A.R. wrote the manuscript, with the help of A.Y.M., M.D.D., S.P., M.C.A. and M.S.

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