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RESEARCH PAPER

Δ^9-Tetrahydrocannabinol (THC) and AM 404 protect against cerebral ischaemia in gerbils through a mechanism involving cannabinoid and opioid receptors

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Background and purpose: It has been suggested that the endocannabinoid system elicits neuroprotection against excitotoxic brain damage. In the present study the therapeutic potential of AM 404 on ischaemia-induced neuronal injury was investigated *in vivo* and compared with that of the classical cannabinoid receptor type 1 (CB_1) agonist, Δ^9-tetraydrocannabinol (THC), using a model of transient global cerebral ischaemia in the gerbil.
Experimental approach: The effects of AM 404 (0.015–2 mg kg^{-1}) and THC (0.05–2 mg kg^{-1}), given 5 min after ischaemia, were measured from 1 h to 7 days in terms of electroencephalographic (EEG) total spectral power, spontaneous motor activity, memory function, rectal temperature and hippocampal CA_1 neuronal count.
Key results: Over the dose range tested, AM 404 (2 mg kg^{-1}) and THC (1 mg kg^{-1}) completely reversed the ischaemia-induced behavioural, EEG and histological damage. Only THC (1 and 2 mg kg^{-1}) induced a decrease of body temperature. Pretreatment with the selective CB_1 receptor antagonist, AM 251 (1 mg kg^{-1}) and the opioid antagonist, naloxone (2 mg kg^{-1}) reversed the protective effect induced by both AM 404 and THC while the $TRPV_1$ vanilloid antagonist, capsazepine (0.01 mg kg^{-1}), was ineffective.
Conclusions and implications: Our findings demonstrate that AM 404 and THC reduce neuronal damage caused by bilateral carotid occlusion in gerbils and that this protection is mediated through an interaction with CB_1 and opioid receptors. Endocannabinoids might form the basis for the development of new neuroprotective drugs useful for the treatment of stroke and other neurodegenerative pathologies.
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Keywords: (Endo)cannabinoid; vanilloid; ischaemia; electroencephalogram; memory; motor activity; rectal temperature

Abbreviations: CB_1, cannabinoid receptor type 1; $TRPV_1$, transient receptor potential vanilloid type-1

Introduction

The therapeutic potential of the endocannabinoid system is yet to be fully determined, and the number of diseases that may be treated by interacting with this system will probably continue to grow (Sarne and Mechoulam, 2005; Bahr *et al*., 2006; Centonze *et al*., 2007). Until now the endocannabinoid system has been evaluated as a possible target for the treatment of several neurological disorders including Parkinson's disease (Lastres-Becker *et al*., 2005; Garcia-Arencibia *et al*., 2007; Kreitzer and Malenka, 2007), Huntington's disease (de Lago *et al*., 2006), neurodegeneration (Battista *et al*., 2006; Monory *et al*., 2006; Rotilio and Stella, 2006) and disorders associated with excessive excitatory neuronal activity, such as epilepsy. Endocannabinoids directly target hippocampal glutamatergic neurons to provide protection against acute epileptiform seizures in mice (Monory *et al*., 2006).

Special attention has been paid to the mechanism of endocannabinoid uptake through which the biological activity of endocannabinoids ceases (McFarland and Barker, 2004). These compounds, termed indirect agonists, act by potentiating the action of endogenous ligands, and hence they may be used in diseases where an increase in endocannabinoid transmission has been postulated to be of therapeutic value (Giuffrida *et al*., 2001; Pertwee, 2002). It may be possible to use these compounds to minimize the unwanted effects produced by the direct activation of

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cannabinoid receptor type 1 (CB_1) receptors by classical cannabinoids, through the control of endocannabinoid levels in a concentration range that avoids psychoactive side-effects (Felder and Glass, 1998). One of these compounds, *N*-(4- hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (AM 404), an inhibitor of anandamide cellular reuptake and inactivation (Khanolkar *et al*., 1996; Beltramo *et al*., 1997), has been shown to provide some relief of symptoms associated with neurological disorders. AM404 produced a marked recovery of 6-hydroxydopamine-induced dopamine depletion and tyrosine hydroxylase deficit in the lesioned side of rats submitted to unilateral lesions of nigrostriatal dopaminergic neurons, induced by local application of 6-hydroxydopamine (Pertwee, 2005). Amelioration of akinesia and sensorimotor orientation and reduction of amphetamine-induced turning behaviour in 6-hydroxydopamine-lesioned rats has also been observed after an injection of AM 404 (Fernandez-Espejo *et al*., 2004). AM 404 significantly decreased restraint-induced serum corticosterone release in mice, suggesting a role of the endocannabinoid system in the signs of environmental stress (Patel *et al*., 2004; Patel and Hillard, 2006). AM 404 has also been shown to inhibit the anandamide transporter in rat brain neurons and slices (Beltramo *et al*., 1997; Beltramo and Piomelli, 2000) and human astrocytoma cells (Piomelli *et al*., 1999) *in vitro*. When given intravenously, AM 404 induced vasodilatation (Calignano *et al*., 1997) in guinea-pigs and enhanced and prolonged anandamide-induced analgesia (Beltramo *et al*., 1997) in mice, in *vivo*. However, exogenously administered AM404 did not elicit catalepsy or analgesia in rats (Beltramo *et al*., 2000), but was found to potentiate the effects of anandamide (Rodriguez de Fonseca *et al*., 2005). In Wistar rats, treatment with AM 404, at a dose of $10\,mg\,kg^{-1}$, was found to have a hypokinetic effect which was reversed by the CB_1 receptor antagonist, rimonabant (Giuffrida *et al*., 2000). In addition, AM 404 ($5\,mg\,kg^{-1}$) has been found to act as a psychodysleptic drug, altering prepulse inhibition in mice through a cannabinoid CB_1 receptor (Fernandez-Espejo and Galan-Rodriguez, 2004) and elicit antidepressant-like effects in the forced swimming test in rats (Hill and Gorzalka, 2005).

There is some evidence from experiments with mice that increasing anandamide or 2-arachidonoyl glycerol content may lead to neuroprotection. After an excitotoxic insult in rat hippocampal slices, an infusion of the fatty acid amide hydrolase inhibitor palmitylsulphonyl fluoride (AM374) and AM404, in combination, protected against cytoskeletal damage and synaptic decline (Karanian *et al*., 2005, 2007). AM404 *per se* elicited cytoskeletal and synaptic protection *in vivo* when coinjected with excitotoxin into the dorsal hippocampus. The potentiated endocannabinoid responses also prevented alterations in behaviour and memory impairment that are characteristics of excitotoxic damage. These protective effects were blocked by a selective CB_1 antagonist. The endocannabinoid 2-arachidonoyl glycerol is released in mouse brain after closed head injury, and treatment with exogenous 2-arachidonoyl glycerol exerts neuroprotection via the central CB_1 receptor (Panikashvili *et al*., 2006).

AM404, structurally similar to transient receptor potential vanilloid type-1 ($TRPV_1$) vanilloid receptor agonists, anandamide and capsaicin, may also activate vanilloid $TRPV_1$ receptors (Zygmunt *et al*., 2000) and, then, exhibit direct effects by itself (Gonzalez *et al*., 1999; Beltramo and Piomelli, 1999). Capsaicin has been shown to effect neuroprotection through the activation of $TRPV_1$ vanilloid receptors in a model of transient cerebral ischaemia in gerbils (Pegorini *et al*., 2005, 2006).

The aim of the present work was to assess the therapeutic potential of AM 404 *in vivo*, and compare its effects with those of the classical CB_1 receptor agonist, Δ^9-tetraydrocannabinol (THC), on ischaemia-induced neuronal injury using a model of transient global cerebral ischaemia in the gerbil. In principle, we aimed to inject the drugs 5 min after ischaemia, as previously performed for other cannabinoid and vanilloid agonists (Braida *et al*., 2001, 2003; Pegorini *et al*., 2005, 2006) in order to compare their potential neuroprotective effects with those of previously tested drugs. To quantify the ischaemic damage, from 1 h to 7 days after reperfusion, we measured different parameters known to be influenced by global cerebral ischaemia: electroencephalogram (EEG) spectral power, spontaneous motor activity, memory function and hippocampal CA_1 neuronal count. We measured motor activity on day 1, since ischaemia-induced hyperlocomotion is maximal 24 h after occlusion (Araki *et al*., 1986), memory function on day 3 according to Sala *et al*. (1997). Since the decrease in electroencephalogram has been related to pronounced damage of neurons on day 7 (Suzuki *et al*., 1983; Hunter *et al*., 1995; Peruche *et al*., 1995), EEG spectral power and neuronal counts were measured at that time. In addition, receptor antagonists were used to investigate a potential role for CB_1, opioid and $TRPV_1$ receptors. Collectively, our data demonstrate that AM 404 and THC protect against neuronal ischaemia-induced injury through a mechanism involving cannabinoid and opioid receptors but not vanilloid receptors.

Methods

Animals

Male Mongolian gerbils (*Meriones unguiculatus*) (Charles River, Calco, Como, Italy) weighing 60–80 g were housed singly in standard laboratory conditions: air-conditioned room ($22 \pm 2\,^{\circ}C$), 12-h light/12-h dark light cycle, free access to food and water. The gerbils were allowed to acclimatize themselves to the environment for 1 week before surgical implantation of cortical electrodes. After the EEG electrodes had been implanted, the gerbils were divided into different groups on the basis of the treatment assigned. Each animal received all tests. All procedures were approved under the Italian Governmental decree no. 32/2004.

Surgical procedure

Gerbils were anaesthetized with an i.p. injection of chloral hydrate ($450\,mg\,kg^{-1}$) dissolved in saline and given in a volume of $9\,ml\,kg^{-1}$. Four electrodes (Bilaney, Dusseldorf, Germany) were implanted for EEG recordings, as described previously (Sala *et al*., 1997), on the right and left of the parieto-occipital cortex according to brain atlas coordinates

(anterior +2, posterior −3, lateral 2, ventral 1.6 from bregma) (Loskota *et al*., 1974). Another electrode was inserted into the nasal bone as a reference location. The five electrodes were connected to a pedestal (Bilaney) and fixed with acrylic cement. The animals were allowed a week to recover from surgery before the experiments were started.

EEG recording

Freely moving, awake gerbils were acclimatized in a sound-proofed Faraday chamber, then their electroencephalogram was recorded for 1 h a day, for 3 days, to determine the basal total and relative spectral power. The signals were recorded and processed for fast Fourier transform spectral analysis by means of PC software (PowerLab, AD Instruments Pty Ltd, Castle Hill, NSW, Australia). Electroen Cephalograph (EEG) recordings were also made during and 7 days after ischaemia. Each 1-h spectral power was calculated as the mean of six 1-min recordings taken at 10-min intervals.

Cerebral ischaemia

After basal EEG recordings, each gerbil was again lightly anaesthetized with 2,2,2- tribromoethanol (200 mg kg^{-1}, 10 ml kg^{-1}). Throughout surgery, body temperature was kept at 37 °C with a heating lamp and 10-min period of ischaemia was induced by occlusion of the bilateral common carotid arteries, as described previously (Braida *et al*., 2000). The ischaemia was verified qualitatively on paper by the complete absence of the electroencephalogram. A group of animals (sham-operated) underwent the same surgical procedure except that the carotid arteries were not clamped.

Locomotor activity

Spontaneous motor activity was evaluated as described previously (Braida *et al*., 2000) in an activity cage (43 cm long × 43 cm wide × 32 cm high: Ugo Basile, Varese, Italy), placed in a sound-proofed room. The cage was fitted with two parallel horizontal infrared beams 2 cm off the floor. Cumulative horizontal movements were counted every 5 min for 30 min, 1 day after ischaemia.

Passive avoidance task

On the third day after ischaemia, each gerbil was examined in the passive avoidance task. The apparatus (Ugo Basile) consisted of a box divided by a guillotine door into two compartments of the same size (22 cm long × 22 cm wide × 21 cm high) in which the floor was a stainless rod grid. One compartment was lit with a 10 W electric light bulb, while the other was dark. The step-through type passive avoidance task was used, as described by Katoh *et al*. (1992), with some modifications. Animals were allowed to adapt for 10 min before the training, and retention was checked 24 h after the training. For the adaptation a gerbil was placed in the light compartment, and allowed to explore both compartments by leaving the guillotine door open for 5 min. After 10 min, the training period was started during which each gerbil was placed in the light compartment and allowed to enter the dark. When the gerbil entered the dark compartment, the door automatically closed and an unavoidable scrambled foot shock (1.5 mA) was delivered for 5 s. Each gerbil then stayed there for 10 s. The procedure was immediately repeated twice. For the retention test, gerbils were placed in the light compartment and the latency period before they re-entered the dark compartment was recorded for up to 180 s.

Body temperature

The experiments were performed in the sound-proofed Faraday chamber. Controls, sham-operated and ischaemic gerbils were trained to accept the temperature measurements for 5 days before thermoregulator reactions were tested, as described by Sala *et al*. (1997). Briefly, after a 1-h acclimatization period in the test room, maintained at 22 °C, body temperature was monitored with a rectal thermistor probe (PRA-22002-A, Ellab, Roedovre, Denmark) inserted 3 cm into the colon. After a 30-s equilibration period, the temperature was recorded to the nearest 0.11 °C on a CTD 85-M Thermometer (Ellab). During temperature measurements, gerbils were unrestrained and were held gently by hand at the base of the tail. Temperature was measured three times (every 10 min) before induction of ischaemia (basal) and again at 30, 60, 90 and 120 min after recirculation.

Histology

Seven days after the ischaemic injury, all the gerbils were killed by an overdose of chloral hydrate 5% and transcardially perfused with 4% paraformaldehyde for determining histological data, as described previously (Braida *et al*., 2003). Brains were removed and placed in the same fixative overnight, then embedded in paraffin wax. Five serial 5-μm coronal hippocampal sections for each animal were cut at 1.5, 1.7 and 1.9 mm caudal to the bregma, using a microtome (Leica, Mod. RM2125RT, Solms, Germany), and stained with cresyl violet. Neurons with a normal appearance in the pyramidal cell layer of the CA_1 sector were counted blind (from coded slides) in each section for each group.

Experimental design

Gerbils submitted to ischaemia were divided into different groups, receiving acutely: vehicle + vehicle; vehicle + AM 404 (0.015, 0.03, 0.1, 0.5, 1, 2 mg kg^{-1}); AM 251 (1 mg kg^{-1}) + vehicle; AM 251 + AM 404 (2 mg kg^{-1}); capsazepine (0.01 mg kg^{-1}) + vehicle; capsazepine + AM 404 (2 mg kg^{-1}); naloxone (2 mg kg^{-1}) + vehicle; naloxone + AM 404 (2 mg kg^{-1}); vehicle + THC (0.05, 0.1, 0.5, 1, 2 mg kg^{-1}); AM 251 + THC (1 mg kg^{-1}); capsazepine + THC (1 mg kg^{-1}) and naloxone + THC (1 mg kg^{-1}). AM 404 and THC were injected i.p. 5 min after recirculation; capsazepine was given s.c. 15 min before bilateral carotid occlusion, while AM 251 and naloxone were given i.p. 5 min before. Vehicle was given 15 or 5 min before or 5 min after ischaemia, either s.c. or i.p. Drugs were dissolved in an appropriate vehicle (90% saline—10% (dimethylsulphoxide) for AM 404 and AM 251; cremophor, ethanol, saline 1:1:18 for THC; Tween-80,

ethanol, saline, 1:1:8 for capsazepine; saline for naloxone) and injected in a volume of 1 ml kg^{-1} s.c. and 5 ml kg^{-1} i.p. The sham-operated group received the same volume of vehicle. The dose of capsazepine used was chosen on the basis of our previous work (Pegorini *et al*., 2006), where it reversed the neuroprotection induced by capsaicin. The dose of naloxone used was able to antagonize the rewarding effects produced by heroin (Braida *et al*., 2001) and THC (Braida *et al*., 2004), while that of AM 251 reversed the anxiolytic effect induced by THC and AM 404 (unpublished data).

Statistical analysis

Data are presented as mean (± s.e.m.). Vehicle groups (s.c. and i.p) were pooled for analysis. Total EEG spectral data are expressed as the mean difference (%) from the pre-ischaemic values. Total spectral power, motor activity, latency time and neuronal counts were analysed by one- or two-way analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's or Student's *t*-test. The accepted level of significance was $P<0.05$. All statistical analyses were carried out using Prism, version 4 software (GraphPad, USA).

Drugs, materials and sources

AM 404, AM 251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide), chloral hydrate, 2,2,2-tribromoethanol, paraformaldehyde, cresyl violet, naloxone and THC (Sigma Aldrich, St Louis, MO, USA); capsazepine (Tocris Bioscience, Bristol, UK); acrylic cement (Palavit, New Galetti and Rossi, Milan, Italy).

Results

Physiological parameters—food and water consumption and body weight (76.20 ± 0.38 g)—were stable throughout the study in all groups (data not shown).

EEG recordings

Seven days after a 10-min period of carotid occlusion and treatment with AM 404 or THC 5 min after recirculation, a quantitative EEG analysis was performed in the ischaemic and sham-operated gerbils (Figures 1a and b). Significant between-group differences were observed in terms of the percentages of the pre-ischaemic values of mean total spectral power, evaluated on day 7 after AM 404 ($F_{(12,52)}=28.27$, $P<0.0001$, ANOVA) and THC ($F_{(12,52)}=11.49$, $P<0.0001$, ANOVA) treatment. *Post hoc* analysis revealed that, in comparison with sham-operated values, the vehicle group had a decrease of EEG power of about 80%. AM404, at all the doses tested, while THC, in a range between 0.1 and 1 mg kg^{-1}, significantly antagonized the ischaemia-induced EEG flattening. Pretreatment with naloxone (2 mg kg^{-1}) completely, while pretreatment with AM 251 (1 mg kg^{-1}) or capsazepine (0.01 mg kg^{-1}) only slightly, blocked the protective effect of AM404 obtained at 2 mg kg^{-1}. *Post hoc* test showed that both AM 251 and naloxone, but not capsazepine, reversed the protective effect of THC. The three antagonists *per se* did not induce any changes, when compared with the vehicle group.

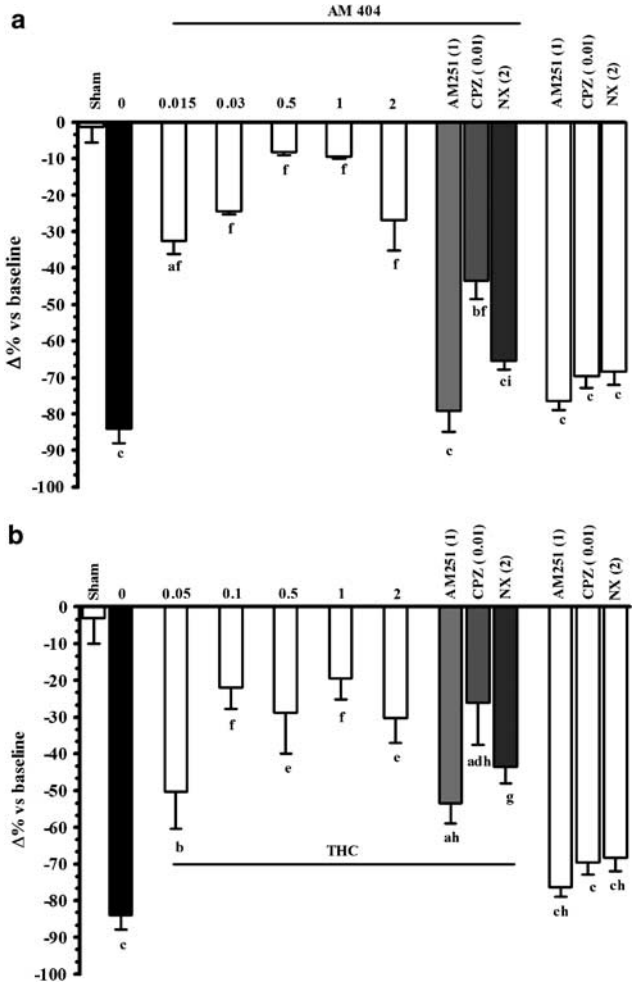

Figure 1 Cortically derived EEG total spectral power evaluated on day 7, as the difference (Δ%) from the pre-ischaemic value in freely moving, awake gerbils given increasing doses (mg kg^{-1}) of AM 404 (**a**) and THC (**b**), 5 min after recirculation. AM 251 or naloxone (NX) was given, i.p., 5 min before bilateral carotid artery occlusion. Capsazepine (CPZ) was administered, s.c., 15 min before ischaemia. The doses of AM 404 and THC combined with the antagonists were 2 and 1 mg kg^{-1}, respectively. Each column represents the mean (± s.e.m.) of five animals. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ compared with sham-operated animals; $^{d}P<0.05$, $^{e}P<0.01$, $^{f}P<0.001$ compared with vehicle; $^{g}P<0.05$, $^{h}P<0.01$, $^{i}P<0.001$ compared with AM 404 (2 mg kg^{-1}) or THC (1 mg kg^{-1}) alone (one-way ANOVA followed by Tukey's test). THC, tetraydrocannabinol; ANOVA, analysis of variance.

Spontaneous locomotor activity

There were significant between-group changes in spontaneous locomotor activity 1 day after ischaemia in gerbils treated with AM 404 or THC (Figures 2a and b). ANOVA revealed significant differences between groups after AM404 ($F_{(12,52)}=35.32$, $P<0.0001$) and THC ($F_{(12,52)}=17.95$, $P<0.0001$) treatment. *Post hoc* comparison revealed an increase in locomotor activity in the vehicle group compared

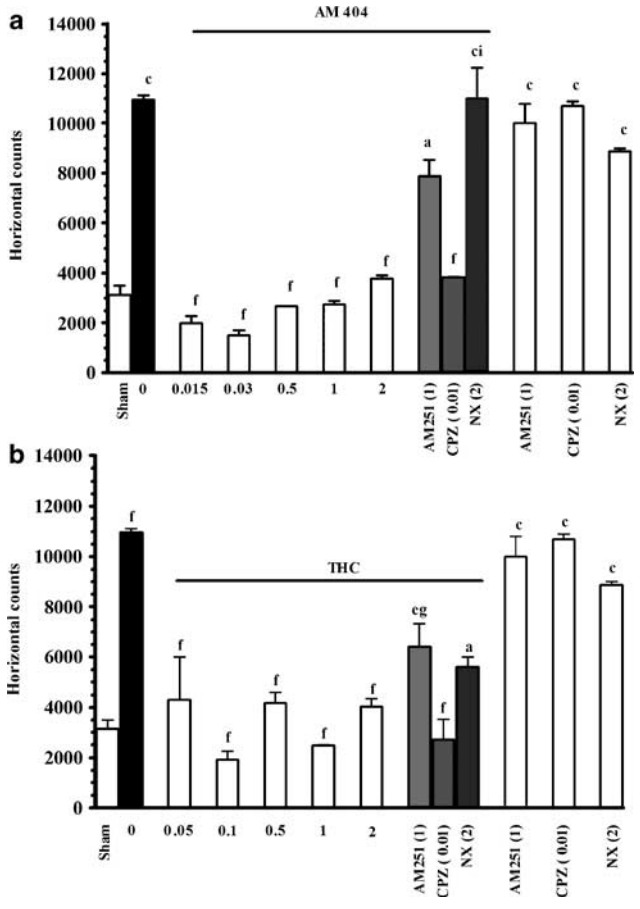

Figure 2 Effect of increasing doses (mg kg^{-1}) of AM 404 (**a**) and Δ^9-THC (**b**) given i.p. 5 min after recirculation on spontaneous motor activity evaluated for 30 min, 1 day after ischaemia in gerbils. AM 251 or naloxone (NX) was administered, i.p., 5 min before bilateral carotid occlusion. Capsazepine (CPZ) was given, s.c., 15 min before ischaemia. The doses of AM 404 and THC combined with the antagonists were 2 and 1 mg kg^{-1}, respectively. Each column represents the total horizontal counts (mean ± s.e.m.) of five animals. $^{a}P<0.05$, $^{c}P<0.001$ compared with sham-operated animals; $^{e}P<0.01$, $^{f}P<0.001$ compared with vehicle; $^{g}P<0.05$, $^{i}P<0.001$ compared with AM 404 (2 mg kg^{-1}) or THC (1 mg kg^{-1}) alone (one-way ANOVA followed by Tukey's test). THC, tetraydrocannabinol; ANOVA, analysis of variance.

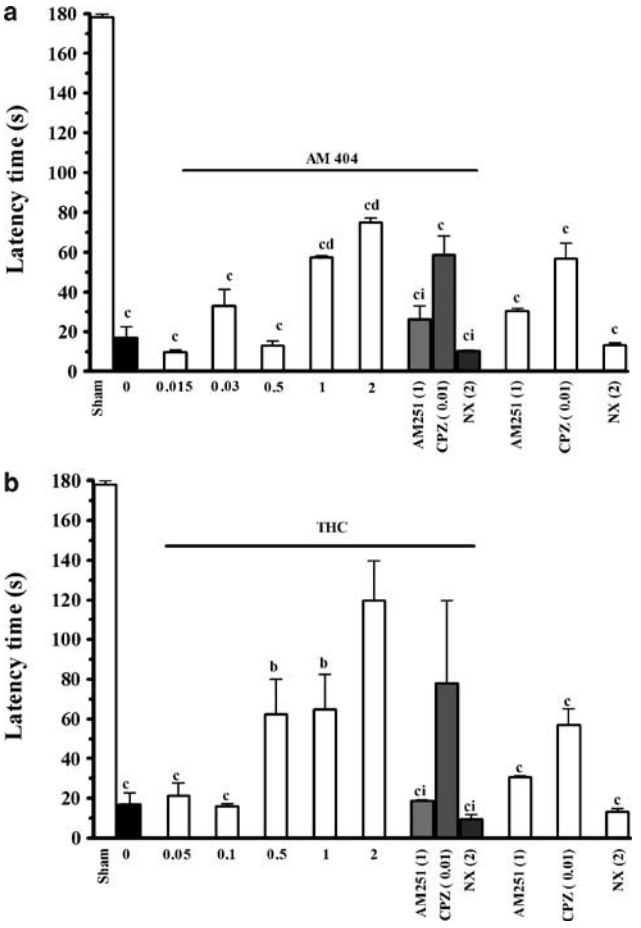

Figure 3 Mean (± s.e.m.) escape latency in the passive avoidance task 3 days after 10-min carotid occlusion. Increasing doses (mg kg^{-1}) of AM 404 (**a**) and THC (**b**) given 5 min after recirculation. AM 251 or naloxone (NX) was administered, i.p., 5 min before bilateral carotid occlusion. Capsazepine (CPZ) was given, s.c., 15 min before ischaemia. The doses of AM 404 and THC combined with the antagonists were 2 and 1 mg kg^{-1}, respectively. Each column represents the mean (± s.e.m.) of five animals. $^{b}P<0.01$, $^{c}P<0.001$ compared with sham-operated animals; $^{d}P<0.05$ compared with vehicle; $^{i}P<0.001$ compared with AM 404 (2 mg kg^{-1}) or THC (1 mg kg^{-1}) alone (one-way ANOVA followed by Tukey's test). THC, tetraydrocannabinol; ANOVA, analysis of variance.

to the sham-operated group, whereas a complete recovery in the activity levels was observed, at all the tested doses, in the AM 404 and THC-treated animals. Naloxone and AM 251 reversed the protective effect of AM 404 (2 mg kg^{-1}) but capsazepine had no effect. When combined with THC (1 mg kg^{-1}), AM 251 induced a partial but significant antagonism of the effects of THC. Capsazepine and naloxone failed to block the protective effect of THC. The three antagonists *per se* did not affect hyperlocomotion.

Passive avoidance

Figure 3 shows the mean latency time measured 3 days after recirculation in ischaemic gerbils treated with increasing doses of AM 404 or THC (Figures 3a and b). There were significant between-group changes in mean latency time in AM 404 ($F_{(12,52)}=6.18$, $P<0.0001$, ANOVA) and THC ($F_{(12,52)}=143.9$, $P<0.0001$, ANOVA) groups. *Post hoc* analysis indicated that vehicle-treated gerbils experienced significant impairment, as shown by the decrease in mean escape latency in comparison with the sham-operated group. AM404 and THC progressively improved, even if only slightly, this memory deficit. Pretreatment with AM 251 and capsazepine did not affect the cognitive improvement induced by AM 404 (2 mg kg^{-1}), whereas it was completely antagonized by naloxone (Tukey's test). When AM 251 and naloxone were given in combination with THC (1 mg kg^{-1}), complete antagonism of the effect of THC was observed (Tukey's test). Capsazepine failed to block the protective effect of THC.

Body temperature

Changes in body temperature were only seen at 30 min after the period of ischaemia (2-way ANOVA, followed by

Bonferroni's test). Thus, in Table 1 only findings obtained at 30 min after ischaemia are presented. Treatment with AM404 did not affect the mean rectal temperature at any dose tested, but THC did ($F_{(12,52)} = 10.77$, $P < 0.0001$, ANOVA). *Post hoc* analysis revealed that in THC groups treated with 1 and 2 mg kg^{-1} there was a decrease of body temperature. Pretreatment with AM 251 antagonized THC (1 mg kg^{-1})-induced hypothermic effect while capsazepine and naloxone, which *per se* did not affect this parameter, were ineffective.

Histology

Histological examination of the hippocampus at 7 days after recirculation, following 10 min of occlusion and 5 min of treatment, showed that severe alterations in the CA_1 region occurred in the ischaemic group compared to the sham-operated animals (Figure 4). Most of the pyramidal cells were darkly stained and shrunken. A comparison of the number of neuronal cells counted (using ANOVA) revealed significant differences between the groups ($F_{(8,36)} = 23.78$, $P < 0.0001$ for AM 404 (2 mg kg^{-1}); $F_{(8,36)} = 6.85$, $P < 0.0001$ for THC (1 mg kg^{-1})). A *post hoc* analysis revealed a decreased number of neuronal cells in the vehicle-treated gerbils compared with the sham-operated group and both drugs completely prevented this loss of cells (Figure 5). Pretreatment with naloxone significantly antagonized AM 404-induced neuroprotective effect, whereas AM 251 and capsazepine failed to block it. The three antagonists *per se* caused a mean decrease in the number of neuronal cells similar to that seen in the vehicle-treated group. Only pretreatment with AM 251 significantly antagonized the neuroprotective affect of THC.

Discussion

In the present study, using a well-established animal model of ischaemia, we showed that both the exogenous cannabinoid agonist, THC, and the anandamide transporter inhibitor, AM 404, given i.p. after bilateral carotid occlusion, fully protected the animals from the deleterious effects of the ischaemic insult. This neuroprotection was quantified in terms of complete recovery of all the behavioural and EEG parameters. Among them, both cognitive and sensorimotor deficits can be found in some human pathological conditions, such as heart attack and coronary artery bypass surgery (Hunter *et al*., 1998). Furthermore, the histopathology seen in the gerbil is similar to that observed in the hippocampal CA_1 region of human brain following cardiac arrest (Hunter *et al*., 1995).

Results obtained from EEG mean total spectral analysis indicated that both THC and AM 404 reversed EEG flattening effect (isoelectric electroencephalogram), induced by bilateral carotid occlusion on day 7 after ischaemia. It is known that after transient global ischaemia, EEG drop is indicative of a severe depression of electrophysiological activity and reflects an impairment of cerebral blood flow. Such a decrease has been related to the severe and selective

Table 1 Effect of AM 404 and THC given alone or in combination with different antagonists on rectal temperature (°C) in gerbils subjected to cerebral ischaemia[a]

Pretreatment	*Dose (mg kg^{-1})*	*Treatment*	*Dose (mg kg^{-1})*	*Rectal temperature (mean ± s.e.m.)*
Sham	—	Vehicle	—	−0.30 ± 0.19
Vehicle[b]	—	Vehicle	—	−0.55 ± 0.16
AM 251	1	Vehicle	—	0.00 ± 0.16
Capsazepine	0.01	Vehicle	—	0.25 ± 0.08
Naloxone	2	Vehicle	—	0.15 ± 0.13
Vehicle	—	AM 404[c]	0.015	0 ± 0.41
Vehicle	—	AM 404[c]	0.03	0.25 ± 0.24
Vehicle	—	AM 404[c]	0.5	−0.50 ± 0.32
Vehicle	—	AM 404[c]	1	−0.75 ± 0.08
Vehicle	—	AM 404[c]	2	−0.37 ± 0.46
Vehicle	—	THC[c]	0.05	−0.67 ± 0.47
Vehicle	—	THC[c]	0.1	0.17 ± 0.24
Vehicle	—	THC[c]	0.5	0.20 ± 0.20
Vehicle	—	THC[c]	1	−2.90 ± 0.51***
Vehicle	—	THC[c]	2	−2.50 ± 0.16**
AM 251[d]	1	AM 404[c]	2	−0.25 ± 0.32
Capsazepine[e]	0.01	AM 404[c]	2	−1.50 ± 1.00
Naloxone[d]	2	AM 404[c]	2	−1.00 ± 0.76
AM 251[d]	1	THC[c]	1	−1.00 ± 0$^{###}$
Capsazepine[e]	0.01	THC[c]	1	−2.50 ± 0.44
Naloxone[e]	2	THC[c]	1	−2.16 ± 0.60

Abbreviation: THC, tetraydrocannabinol.
Each column represents the mean (± s.e.m.) of five gerbils.
[a]Data were measured at peak effect (30 min) after recirculation.
[b]Pool of animals given, i.p., 5 and 15 min before carotid occlusion.
[c]Given 5 min after recirculation.
[d]Given, i.p., 5 min before carotid occlusion.
[e]Given, s.c., 15 min before carotid occlusion.
***$P < 0.001$; **$P < 0.01$ vs sham and vehicle group; $^{###}P < 0.001$ compared with THC (1 mg kg^{-1}) alone group (one-way ANOVA followed by Tukey's test).

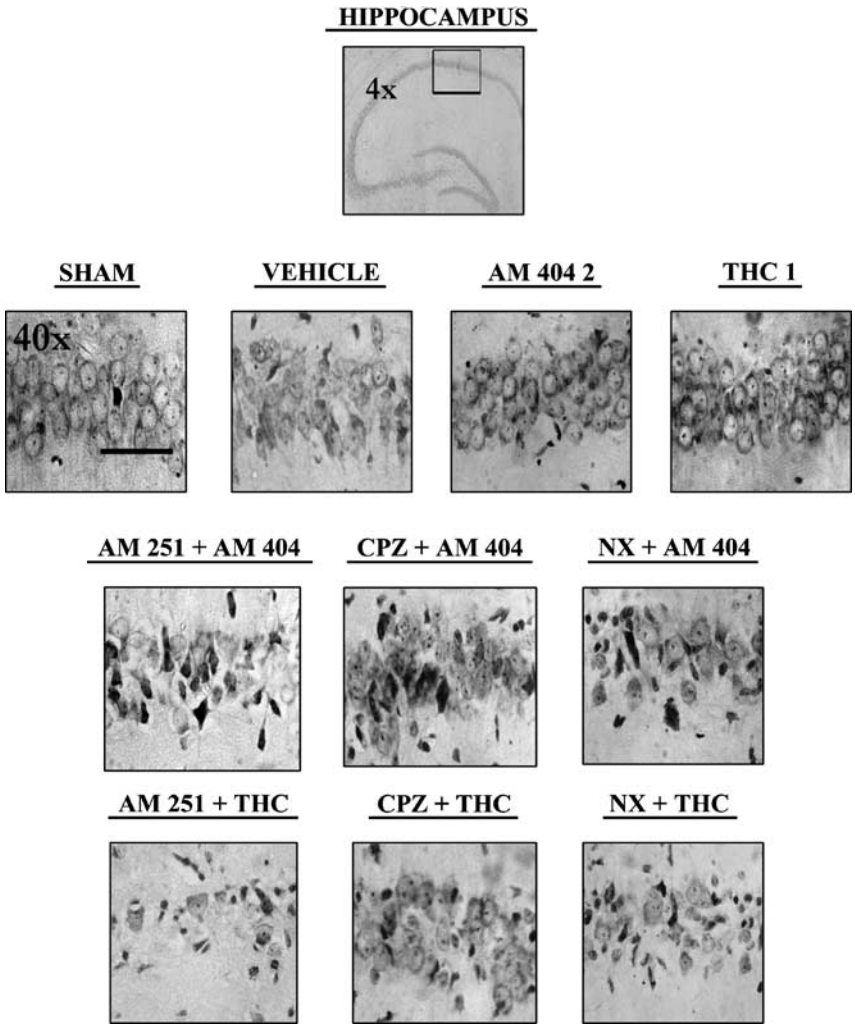

Figure 4 Photomicrographs of the hippocampal CA_1 region of gerbils with or without 10 min ischaemia, 7 days after recirculation. AM 251 and naloxone (NX) were given, i.p., 5 min before bilateral carotid occlusion; capsazepine (CPZ) was given, s.c., 15 min before ischaemia. Doses are expressed as mg kg^{-1}. Bar = 50 μm.

neuronal death appearing in the CA_1 layer of the hippocampal formation following an impairment of synaptic activity (Kirino and Sano, 1984; Smith *et al*., 1984; Zola-Morgan *et al*., 1986; Schmidt-Kastner and Hossmann, 1988).

Vasodilatation may be involved in the protection against neuronal death on day 7 effected by THC (1 mg kg^{-1}) and AM 404 (2 mg kg^{-1}). Cannabinoid perfusion increases the diameter of cerebral arterioles and cerebral arteries (Hillard, 2000) in a CB_1 receptor-dependent fashion, indicating that the main cerebrovascular effect of cannabinoids is vasodilatation. This accords with findings that marijuana smoking in humans is accompanied by an increase in cerebral blood flow (Mathew *et al*., 2003). Furthermore, AM 404 has been found to be a vasodilator and activator of $TRPV_1$ receptors expressed in *Xenopus* oocytes (Zygmunt *et al*., 2000) and in the rat hepatic artery (Zygmunt *et al*., 1999).

The full protection against motor hyperactivity seen in both THC and AM 404-treated gerbils indicates that these drugs induce a recovery of the ability of animals to form spatial maps, initially suppressed due to the loss of CA_1 neurons, as suggested by O'Neill and Clemens (2000). Any intrinsic effects of the drugs can be excluded, since the evaluation was carried out 24 h after treatment.

Both THC and AM 404 dose-dependently prevented ischaemia-induced memory impairment. This condition has been clinically demonstrated during cardiac arrest, revealed as amnesia and characterized by impaired learning and memory of events after the injury (Volpe and Hirst, 1983). The deficits in learning and memory induced by global cerebral ischaemia showed a close correlation with the neuronal death in the hippocampal CA_1 region, as suggested by Block (1999). An increase in number of intact CA_1 neurons (about 75%) that are most vulnerable to hypoxic insult and involved in learning and memory was observed in THC- and AM 404-treated gerbils subjected to ischaemic insult.

The protective effect of THC was accompanied by a clear hypothermic effect at the highest doses. At present, post-ischaemic hypothermia provides the best long-term functional and histological protection against the effects of global ischaemia (Colbourne and Corbett, 1995; Colbourne *et al*., 1999; Hickey *et al*., 2000). However, since THC

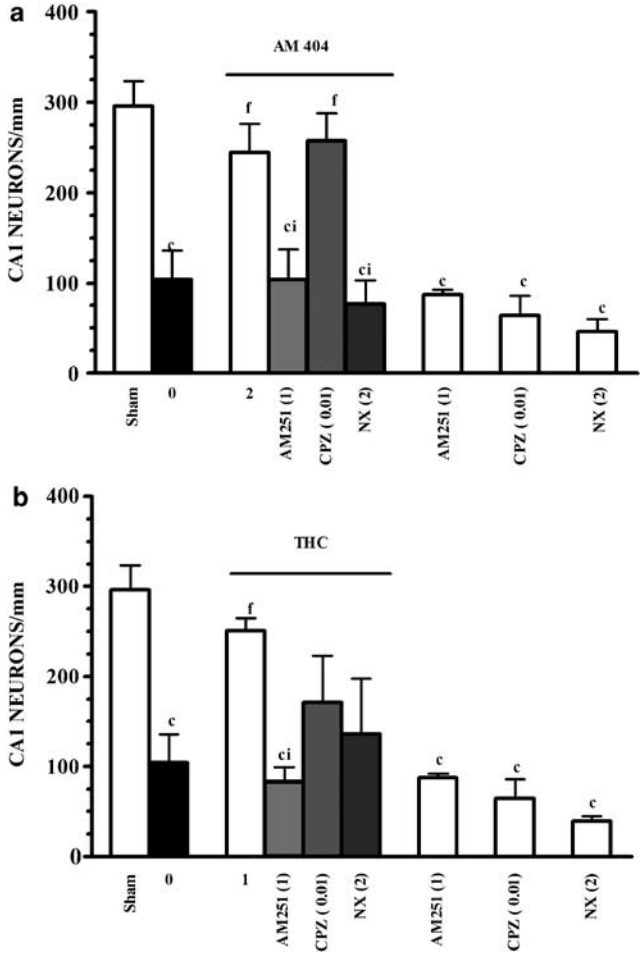

Figure 5 Effect of AM 404 (**a**) and THC (**b**) on neuronal count, performed 7 days after recirculation, in the CA_1 region of the hippocampus of sham-operated or ischaemic gerbils. AM 404 and THC were given, i.p., 5 min after recirculation. AM 251 or naloxone (NX) was given, i.p., 5 min before bilateral carotid occlusion. Capsazepine (CPZ) was administered, s.c., 15 min before ischaemia. Each column represents the mean (± s.e.m.) of five hippocampal sections from the same coronal plane for each animal. Doses are expressed as mg kg^{-1}. $^{c}P<0.001$ compared with sham; $^{f}P<0.001$ compared with vehicle; $^{i}P<0.001$ compared with AM 404 (2 mg kg^{-1}) or THC (1 mg kg^{-1}) alone (one-way ANOVA followed by Tukey's test). THC, tetraydrocannabinol; ANOVA, analysis of variance.

exhibited neuroprotective effects at both hypothermic and normothermic doses, hypothermia was not a factor in this study. In addition, Louw *et al*. (2000) found that there was significantly less neocortical injury after induction of global cerebral ischaemia in THC-treated rats when rigorous temperature control was maintained through the experiment.

No change in body temperature was observed in AM 404-treated animals. This is in accordance with the results of Rawls *et al*. (2006); they demonstrated that this endocannabinoid inhibitor transporter produced a clear hypothermic effect, which required a vanilloid $TRPV_1$ activation, only at doses higher than 10 mg kg^{-1}.

The neuroprotection obtained with THC is consistent with previous findings where other cannabinoids, like CP 55 940 (Braida *et al*., 2000) and cannabidiol (Braida *et al*., 2003), prevented ischaemia-induced damage in the same animal model of ischaemia. In addition, after repeated treatment with THC for 7 days prior to bilateral carotid artery occlusion produced a neuroprotection in rats *in vivo*, accompanied by a reduction in systolic blood pressure (Louw *et al*., 2000). In *in vitro* studies, THC protected neurons against excitotoxic insults, mediated by exposure to the glutamatergic agonists *N*-methyl-D-aspartate and KA (Hampson *et al*., 1998; El-Remessy *et al*., 2003; Chen *et al*., 2005; Gilbert *et al*., 2007; Kreutz *et al*., 2007).

The neuroprotection obtained with AM 404 is in accordance with the findings of Karanian *et al*. (2005), who reported that the anandamide transporter inhibitor enhanced the action of exogenous anandamide *in vitro* protecting against excitotoxic damage in hippocampal slice cultures by a mechanism involving CB_1 receptors. In addition, AM 404 repeatedly administered for 2 weeks produced a marked recovery of 6-OH-dopamine-induced dopamine depletion in a rat model of Parkinson's disease (Garcia-Arencibia *et al*., 2007).

The finding that AM 251 prevented neuroprotection induced by both THC and AM 404 indicates that CB_1 receptors are involved in this effect. The antagonism by AM 251 of the neuroprotection induced by THC does not agree with the findings of other studies, where the protective actions of THC were mediated primarily or exclusively by effects on chemical antioxidants (Chen and Buck, 2000; Hampson *et al*., 2000, Marsicano *et al*., 2002). Therefore, it appears that in some circumstances, THC exerts neuroprotective effects exclusively through non-receptor-mediated antioxidant properties, while in other models stimulation of CB_1 receptors by THC or WIN 55212-2 might produce neuroprotection. Differences between the effects observed in different models may be related to the cell type or model system, and to differences in the toxic insults employed.

As expected, capsazepine did not reverse the protective effect of THC. Accordingly, the neuroprotective effect of THC, evaluated in an *in vitro* model in which the AF5 CNS cell line was exposed to toxic levels of N-methyl-D-aspartate, was not reversed by the $TRPV_1$ antagonist (Chen *et al*., 2005). In addition, THC-induced suppression of serum interleukin-12 levels in *Legionella pneumophila*-infected cells was not attenuated by pretreatment with the $TRPV_1$ antagonist.

Although it has been shown that AM 404 binds $TRPV_1$ receptors in the rat isolated hepatic artery (Zygmunt *et al*., 2000), it is unlikely that this occurred at the doses of this endocannabinoid transporter inhibitor used in our study. Activation of $TRPV_1$ leads to an increased influx of Ca^{2+} (Szallasi and Blumberg, 1999), glutamate release (Marinelli *et al*., 2002) and substantial contribution to neuronal excitotoxicity (apoptosis) (Maccarrone *et al*., 2000; Hail, 2003; Yue *et al*., 2004). It could be argued that the dose of capsazepine used in the present study was too low to reverse any $TRPV_1$-mediated effects. However, Costa *et al*. (2006) showed that capsazepine (10 mg kg^{-1}) reversed the anti-hyperalgesic effect of AM 404 in a rat model of neuropathic pain. In addition, Tzavara *et al*. (2006) found that 5 mg kg^{-1} capsazepine were able to antagonize the normalization of hyperlocomotion induced by AM 404 (3 mg kg^{-1}) in a model

of hyperdopaminergia in knockout mice. However, in our experiments, using our model of global ischaemia, when the dose was increased (0.05 mg kg^{-1}) capsazepine exhibited *per se* a protective effect (data not shown). This latter finding is in accordance with previous results (Veldhuis *et al*., 2003) where the vanilloid antagonist *per se* reduced brain injury caused by excitotoxicity, and when given in a range of doses between 0.005 and 5 μmol kg^{-1} (Akabori *et al*., 2007) (similar to ours) it significantly improved survival rates in a rat model of haemorrhagic shock.

The opioid antagonist, naloxone, blocked the neuroprotective effect induced by THC in terms of EEG flattening and memory impairment, and that induced by AM 404 on all the parameters evaluated. An interaction between the opioid and endocannabinoid system is not surprising since cannabinoids and opioids share several pharmacological effects, including hypothermia, sedation, analgesia and the inhibition of motor activity (Bloom and Dewey, 1978; Manzanares *et al*., 1999). Systemic administration of THC increases extracellular levels of the endogenous opioid receptor ligand β-endorphin in both the ventral tegmental area and the nucleus accumbens, and injection of β-endorphin into the ventral tegmental area markedly enhances the ability of rats to discriminate the effects of experimenter-administered THC (Solinas *et al*., 2004). In addition, it has been shown that CB_1 receptors and μ- and δ-opioid receptors in the striatum are strongly linked at the intracellular level (Berrendero *et al*., 2003). Thus, on the one hand, cannabinoid receptor agonists can release endogenous opioids, and, on the other hand, stimulation of opioid receptors can facilitate the signal transduction of cannabinoid receptors. Therefore, it is likely that opioid agonists facilitate certain effects of THC by mimicking some of the effects of endogenous opioids released by THC and, at the same time, facilitating CB_1 receptor-induced effects at the intracellular level. Conversely, the reduction of the protective actions of THC, induced by opioid antagonists, is probably due to blockade of the effects of endogenous opioids released by THC and antagonism of CB_1 receptor-mediated effects at the intracellular level.

To summarize, our findings provide further evidence for the involvement of cannabinoid receptors and, for the first time, of opioid but not vanilloid receptors, in the neuroprotection, against cerebral ischaemia, induced by THC and AM 404. For these findings to be relevant to a clinical situation, further studies are needed to verify if a delayed administration of these drugs (from 30 min to 3 h after ischaemia) still produces neuroprotection.

Conflict of interest

The authors state no conflict of interest.

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