

# The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain

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## Abstract

Cannabidiol, the major psycho-inactive component of cannabis, has substantial anti-inflammatory and immunomodulatory effects. This study investigated its therapeutic potential on neuropathic (sciatic nerve chronic constriction) and inflammatory pain (complete Freund's adjuvant intraplantar injection) in rats. In both models, daily oral treatment with cannabidiol (2.5–20 mg/kg to neuropathic and 20 mg/kg to adjuvant-injected rats) from day 7 to day 14 after the injury, or intraplantar injection, reduced hyperalgesia to thermal and mechanical stimuli. In the neuropathic animals, the anti-hyperalgesic effect of cannabidiol (20 mg/kg) was prevented by the vanilloid antagonist capsazepine (10 mg/kg, i.p.), but not by cannabinoid receptor antagonists. Cannabidiol's activity was associated with a reduction in the content of several mediators, such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), lipid peroxide and nitric oxide (NO), and in the over-activity of glutathione-related enzymes. Cannabidiol only reduced the over-expression of constitutive endothelial NO synthase (NOS), without significantly affecting the inducible form (iNOS) in inflamed paw tissues. Cannabidiol had no effect on neuronal and iNOS isoforms in injured sciatic nerve. The compound's efficacy on neuropathic pain was not accompanied by any reduction in nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) content. The results indicate a potential for therapeutic use of cannabidiol in chronic painful states.

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**Keywords:** Cannabidiol; Cannabinoid; Chronic pain; TRPV1; Nitric oxide; Cytokine

## 1. Introduction

The pharmacological profile of cannabidiol, the major psycho-inactive component of cannabis, which is a physiologically important isomer of delta9-tetrahydrocannabinol, the main psychoactive constituent of the plant, has received excellent reviews recently (Pertwee, 2004; Russo and Guy, 2006). Cannabidiol has anti-anxiety, anti-psychotic, neuroprotective and anticonvulsant effects, influencing the metabolism of delta9-tetrahydrocannabinol by blocking its conversion to the more psychoactive 11-hydroxy tetrahydrocannabinol for direct interaction with cytochrome P450 enzymes; it is a powerful anti-oxidant and has noteworthy anti-inflammatory and immunomodulatory effects (Malfait et al., 2000). We recently showed its therapeutic efficacy in a rat model of acute inflammation

induced by intraplantar (i.pl.) injection of carrageenan, where cannabidiol had a time- and dose-dependent anti-hyperalgesic effect when given orally 2 h after the induction of inflammation (Costa et al., 2004a).

Cannabidiol has recently been shown to act as a transient receptor potential vanilloid 1 (TRPV1) agonist with potency equivalent to capsaicin, while also inhibiting the reuptake and hydrolysis of anandamide (Bisogno et al., 2001). Last year, Health Canada approved Sativex (*Cannabis sativa* L. extract; the ratio of delta9-tetrahydrocannabinol to cannabidiol is 2.7 mg: 2.5 mg per spray); this new drug, developed by GW Pharmaceutical, proved successful as adjunctive treatment for the symptomatic relief of neuropathic pain in adults with multiple sclerosis (Rog et al., 2005) and rheumatoid arthritis (Blake et al., 2006).

The pathogenesis in nociceptive and inflammatory reactions is complex and multifunctional and is triggered and maintained by various intracellular mediators. One of these, tumor necrosis

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factor  $\alpha$  (TNF $\alpha$ ), is particularly important in triggering a cascade of other cytokines and induces the up-regulation of cyclooxygenase 2 protein while also increasing prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels, probably through the transcription nuclear factor- $\kappa$ B (NF- $\kappa$ B), inducing the activation of genes encoding for both cyclooxygenase and inducible nitric-oxide synthase (iNOS) (Schafers et al., 2004). Stimulation of TNF $\alpha$  causes a respiratory burst in phagocytes, characterized by a sharp increase in oxygen uptake; so reactive oxygen intermediates are formed (Murray and Cohn, 1980). Nitric oxide (NO) is an endogenous modulator whose diverse biological functions include acting as a neurotransmitter in the brain and other parts of the body; it may have pro-inflammatory effects including vasodilatation and edema (Clancy and Abramson, 1995). High levels of TNF $\alpha$ , reactive oxygen intermediates and NO can cause inflammation, damage cells and tissues, and contribute to hypersensitivity to pain.

The main aim of this work was therefore to evaluate the therapeutic efficacy of prolonged treatment with cannabidiol on pain in models of neuropathy and chronic inflammation. We also examined whether cannabidiol inhibited the production of nociceptive and inflammatory mediators involved in development and maintenance of these chronic painful states.

## 2. Methods

### 2.1. Animals

Male Wistar rats (200–220 g, Harlan, Milan, Italy) were housed five per cage at constant temperature (22±2 °C), with a 12:12 h light/dark cycle, and free access to food and water at all times for at least a week before being used. The experiments were carried out in accordance with current guidelines for the care of laboratory animals (Permit no. 94/2000A) and ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). For all studies animals were randomly assigned to treatment groups and the behavioural measurements were made by a single observer blind to the experimental conditions.

### 2.2. Drugs

Cannabidiol, dissolved in methanol, was kindly supplied by GW Pharmaceuticals (Salisbury, England); after drying off the methanol under speed-vacuum, the pure cannabidiol residue was emulsified in vehicle: cremophor, ethanol and saline (1:1:18). For *in vitro* studies, the pure cannabidiol residue was dissolved in ethanol as vehicle. *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (rimonabant) and *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo (2.2.1) heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) were kindly supplied by Sanofi-Aventis (Montpellier, France) and were dissolved in a mixture of Tween 80: dimethyl sulfoxide: distilled water (1:2:7). Capsazepine was purchased from Sigma-Aldrich (Milan, Italy) and dissolved in a 1:1:8 mixture of ethanol: Tween 80:saline.

### 2.3. Chronic constriction injury of the sciatic nerve model

Painful unilateral neuropathy was induced by chronic constriction injury of the sciatic nerve in the right hind paw, according to Bennett and Xie (1988). Briefly, the animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The right sciatic nerve was exposed at mid-thigh level through a small incision, and one-third to one half of the nerve thickness was loosely ligated with four silk threads. The wound was closed with muscle suture and skin clips and dusted with streptomycin powder. In parallel surgery, the nerve was exposed but not ligated (sham-operated rats).

### 2.4. Complete Freund's adjuvant model

We injected 0.1 ml complete Freund's adjuvant containing 0.1 mg of *Mycobacterium tuberculosis* heat killed, 0.085 ml paraffin oil and 0.015 ml mannide monooleate (Sigma Aldrich, Milan, Italy) s.c. into the plantar side of right hind paw. Control rats received an intraplantar (i.pl.) injection of the same volume of saline.

### 2.5. Pharmacological treatment

Neuropathic and inflamed rats received oral cannabidiol or its vehicle; neuropathic rats received different cannabidiol doses (2.5, 5, 10 and 20 mg/kg), while inflamed rats were given only the dose relieving the neuropathic pain (20 mg/kg) (5 ml/kg body weight). The treatment was given once a day for a week, starting from day 7 after the surgical procedure or i.pl. injection of the adjuvant. Non-inflamed and sham-operated animals received oral doses of vehicle for one week from day 7 after the i.pl. injection or surgical procedure.

The consequence of the acute dose of cannabidiol 20 mg/kg to neuropathic and inflamed rats was evaluated in rats chronically treated with vehicle for one week, and challenged with the drug 24 h before the behavioral evaluations.

To study how the cannabinoid and/or vanilloid receptor was involved in the effects of cannabidiol, we tested the ability of specific cannabinoid CB<sub>1</sub>, CB<sub>2</sub> and TRPV1 receptor antagonists to reverse these effects. On the last day of cannabidiol dosing, the cannabinoid CB<sub>1</sub> receptor-specific antagonist rimonabant (0.5 mg/kg, i.p.) or the vanilloid TRPV1-specific antagonist capsazepine (10 mg/kg, i.p.) was co-administered with cannabidiol (20 mg/kg) or its vehicle; the cannabinoid CB<sub>2</sub> receptor-selective antagonist SR144528 (3 mg/kg) was administered p.o., since its known oral bioavailability (Rinaldi-Carmona et al., 1998), 1 h before cannabidiol or vehicle. Sham-operated animals received the vehicles only. Nociceptive behavior was assessed 1 h after cannabidiol administration. The doses of SR144528 and rimonabant were chosen since they antagonize the anti-hyperalgesic effects of the respective agonists in rodents (Bridges et al., 2001; Scott et al., 2004). The dose of capsazepine was shown by Di Marzo et al. (2001) to antagonize the effects of the selective TRPV1 receptor agonist capsaicin in rats, and by us (Costa et al., 2004b) to reverse the anti-hyperalgesic effect of cannabidiol in the model of carrageenan-induced acute inflammation in rats.

## 2.6. Mechanical hyperalgesia

We measured mechanical hyperalgesia using a Randall-Selitto analgesimeter (Ugo Basile, Varese, Italy). Latencies to withdrawal in response to a calibrated pressure were assessed on the ligated/inflamed and contralateral hind paws on day 0 (before surgery and i.pl. injection) and again on day 7 (before starting the drug treatment) and on day 14 (24 h after the last dose). Cut-off was set at 150 g.

## 2.7. Thermal hyperalgesia

We measured thermal hyperalgesia using a Hargreaves apparatus (Ugo Basile, Varese, Italy). Before the experiments the animals were placed in a transparent Perspex box with a thin glass floor and allowed to acclimatize for 10–15 min. A focused beam of radiant heat was applied to the plantar surface and latencies to withdrawal were assessed on the ligated/inflamed and contralateral hind paws on day 0 (before surgery and i.pl. injection) and again on day 7 (before starting the drug treatment) and on day 14 (24 h after the last dose). Cut-off was set at 33 s.

## 2.8. Biochemical tests

Biochemical tests were done on animals given 20 mg/kg cannabidiol or its vehicle for seven days. Fourteen days after the surgical procedure or i.pl. injection, 24 h after the last dose, nociceptive behavior was evaluated and rats were then sacrificed by decapitation.

### 2.8.1. PGE<sub>2</sub> in plasma

Blood was collected in a tube containing 6.7 mM indomethacin and a buffered solution (pH 7.4) of 0.04 mM disodium EDTA in 0.08% NaCl, as anticoagulant, and immediately centrifuged at 1250 ×g for 10 min. The upper plasma layer was removed, rapidly frozen in liquid nitrogen and stored at –20 °C until the assay. PGE<sub>2</sub> in plasma was measured with an enzyme immunoassay (EIA), employing a commercial kit from Amersham Pharmacia Biotech (Milan, Italy).

### 2.8.2. NO production

NO production was assessed on the basis of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>, which are the NO oxidation end products. The paw tissue was homogenized and centrifuged; NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> concentrations were assayed fluorimetrically as previously described (Costa et al., 2002). Nitrite/nitrate content in the paw was calculated using a standard curve and expressed as nmol/g tissue.

### 2.8.3. NOS Western immunoblot analysis

Inflamed and non-inflamed paw tissues were homogenized in 1:4 (w/v) Tris–HCl (50 mM)–EDTA (0.1 mM) buffer, pH 7.4, containing a protease inhibitor cocktail (one tablet per 10 ml; Roche Diagnostics, Milan, Italy), the homogenate was centrifuged at 4 °C for 10 min at 9000 ×g. Nerves from ligated and sham-operated rats were homogenized in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate,

1% Nonidet P-40, 0.5% sodium deoxycholate, 0.02% sodium azide, 1 mM phenylmethylsulphonyl fluoride, 10 mM leupeptin). The nerve homogenate and the supernatant of paw tissue homogenate were ultracentrifuged at 4 °C for 1 h at 100,000 ×g. The microsomal and cytosolic fractions were stored at –80 °C until eNOS, nNOS and iNOS assay. iNOS and nNOS were measured in the cytosolic fraction, eNOS in the microsomal fraction according to Costa et al. (2002, 2005), employing polyclonal anti-eNOS antibody from Santa Cruz Biotechnology Inc. (CA, U.S.A.) and polyclonal anti-iNOS and anti-nNOS antibodies from Cayman Chemical (Ann Arbor, MI, U.S.A.). The grey level of the bands was quantified by image analysis software (ImageJ package for Windows, Scion Corporation, Frederick, MD, U.S.A.).

### 2.8.4. Lipid peroxide

Lipid peroxides were assayed spectrophotometrically, as malondialdehyde (MDA) content, in hind paw tissue homogenate prepared with a T25, 18N Ultra-Turrax in a ratio of 1 g wet tissue weight to 9 ml potassium phosphate (50 mM)–EDTA (0.1 mM) buffer pH 7.4 (Costa et al., 2005).

### 2.8.5. Se-dependent glutathione peroxidase and glutathione-S-reductase activity

Paw tissue was homogenized 1:4 (w/v) in Tris–HCl buffer (20 mM, pH 7.6). The homogenate was centrifuged at 4 °C for 10 min at 9000 ×g and the supernatant was ultracentrifuged at 4 °C for 1 h at 100,000 ×g to obtain the cytosolic fraction. The specific activity of the cytosolic glutathione peroxidase was assayed spectrophotometrically at 37 °C according to Wendel (1981) and that of glutathione reductase at 30 °C according to Colman (1971). The activity of both enzymes was expressed in oxidized NADPH nmol/min/g of wet tissue weight.

### 2.8.6. TNFα

TNFα protein was measured using an enzyme-linked immunosorbent assay (ELISA) on the dorsal root ganglia, ipsilateral to surgery, corresponding to L4–L6 spinal cord and on the sciatic nerve, ipsilateral to surgery. Sciatic nerve and ipsilateral dorsal root ganglia were weighed and homogenized in phosphate-buffered saline (PBS), pH 7.4, containing a mix of protease inhibitors, 20 μl of PBS/mg of tissue. After centrifugation at 10,000 ×g at 4 °C for 10 min, the supernatant was removed and assayed in duplicate by the rat TNFα ultrasensitive ELISA kit (Biosource International, Camarillo, CA, U.S.A.), according to the manufacturer's instruction.

### 2.8.7. Transcription factor NF-κB

Transcription factor was assayed with an ELISA kit (Active Motif, Rixensart, Belgium), for the detection of NF-κB activation by a combination of NF-κB-specific oligonucleotide binding and subsequent detection of the p65 subunit of NF-κB with specific antibody. Sciatic nerves were homogenized in 100 μl ice-cold hypotonic lysis buffer (supplied with the nuclear extract kit, Active Motif, Rixensart, Belgium) per mg of tissue. After centrifugation at 850 ×g for 10 min, 500 μl of hypotonic buffer supplemented with 25 μl of Nonidet P-40 was added to

the pellet and the mixture was centrifuged at 14,000 ×g for 2 min at 4 °C. Pellets were suspended in 50 µl of hypertonic lysis buffer and incubated with shaking for 30 min at 4 °C. Samples were then centrifuged at 14,000 ×g for 10 min at 4 °C, and the supernatant, containing nuclear extracts, was stored at –80 °C until use. Nuclear protein extract (10 µg) was added on the oligonucleotide-coated ELISA plate and incubated for 1 h at room temperature. Primary antibody recognizing an epitope on p65, which is accessible only when NF-κB is activated and bound to its target DNA, was added to wells and incubated for 1 h. This was followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody and, after 1 h, by the HRP substrate. The reaction was stopped after 5–10 min and the absorbance was measured on a spectrophotometer (Multiskan® EX, ThermolabSystem) at 450 nm. Jurkat cell nuclear extracts were used as an activated NF-κB positive control. NF-κB wild type and mutated consensus oligonucleotides were used to monitor the specificity of the assay: a wild type oligonucleotide should compete with NF-κB for binding, while the mutated consensus oligonucleotide should have no such effect.

2.9. Statistics

The results are expressed as the mean±standard error of the mean (S.E.M.). Data were analyzed by one-way repeated

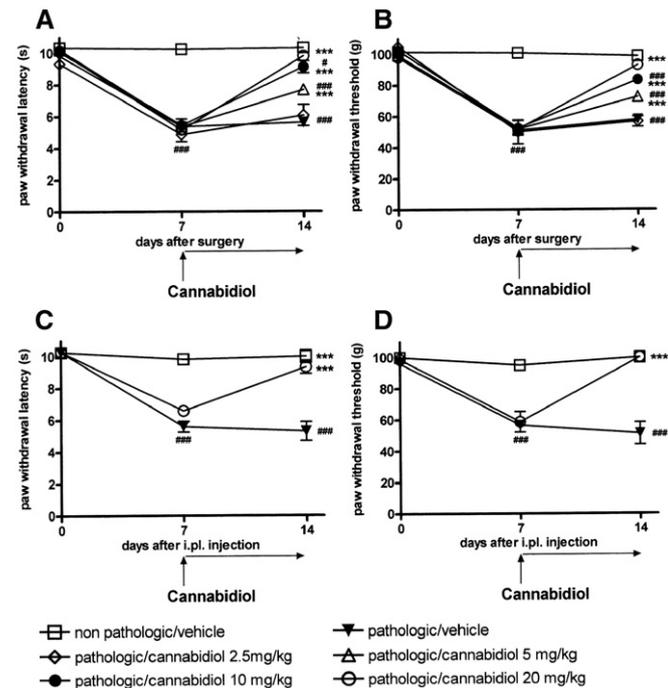


Fig. 1. Anti-hyperalgesic effect of cannabidiol in the rat model of neuropathic pain (A and B) and in the rat model of chronic inflammation induced by the complete Freund’s adjuvant (C and D). Effects of repeated cannabidiol (2.5–20 mg/kg, p.o.), once daily, for seven days, starting one week after sciatic nerve injury or adjuvant i.p. injection on thermal (A, C) and mechanical (B, D) hyperalgesia. Rats were evaluated 24 h after the last administration. Results are expressed as the mean±S.E.M. of 4–7 rats. #P<0.05, ###P<0.001, compared with the non-pathologic/vehicle treatment group; \*\*\*P<0.001, compared with the pathologic/vehicle treatment group.

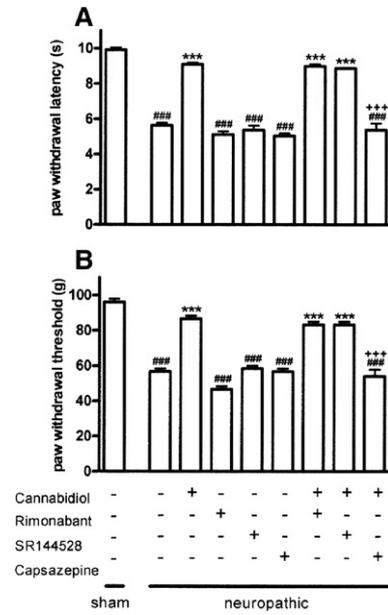


Fig. 2. The TRPV1 antagonist capsazepine prevents the anti-hyperalgesic effect of cannabidiol in the rat model of neuropathic pain. Effect of a single dose of rimonabant (0.5 mg/kg, i.p.), SR144528 (3 mg/kg, p.o.) and capsazepine (10 mg/kg, i.p.) on the anti-hyperalgesic effect of repeated cannabidiol (20 mg/kg, p.o.), once daily for seven days, starting one week after sciatic nerve injury, on thermal (A) and mechanical (B) hyperalgesia. Results are expressed as the mean±S.E.M. of 3–5 rats. ###P<0.001, compared with the sham-operated/vehicle treatment group; \*\*\*P<0.001, compared with the neuropathic/vehicle treatment group; +++P<0.001, compared with neuropathic/cannabidiol treatment group.

measures analysis of variance (ANOVA). When ANOVA showed significant differences, pair-wise comparisons between means were tested by the post hoc Tukey’s method. Significance was set at P<0.05. Non-parametric data (the relative densitometric values) were analyzed with Kruskal–Wallis ANOVA followed by Dunn’s test. All statistical analyses were done using the statistical GraphPad Software package (San Diego, CA, U.S.A.).

3. Results

3.1. Anti-hyperalgesic effect of cannabidiol in neuropathic and inflamed rats

The scores for withdrawal latency for both thermal and mechanical stimuli were tested 7 and 14 days after surgery (Fig. 1A and B) or i.p. injection (Fig. 1C and D). Before surgery or injection, rats withdrew their left and right hind paws from radiant heat with a latency of about 10 s and sustained a mechanical force of about 100 g. Seven days after sciatic nerve ligation or adjuvant injection, there was a significant decrease (about 50%) in both thermal and mechanical withdrawal latency in the ipsilateral paw, but no significant changes on the contralateral side. Thermal and mechanical hyperalgesia was still present in neuropathic and inflamed rats treated for the subsequent seven days with vehicle.

Repeated administration of cannabidiol attenuated both thermal and mechanical hyperalgesia of neuropathic rats in a

dose-dependent manner ( $r^2=0.9720$ ,  $F=69.39$ ,  $P=0.0141$ ;  $r^2=0.9853$ ,  $F=134.4$ ,  $P=0.0074$ ); the highest dose (20 mg/kg), in particular, abolished hyperalgesia, restoring the physiological threshold. This dose also induced full pain relief in inflamed rats, restoring their physiological thresholds. The nociceptive thresholds of the contralateral paws were not affected by repeated treatment with this dose (data not shown). An acute dose of 20 mg/kg cannabidiol did not affect the nociceptive thresholds of either the neuropathic or the inflamed rats after thermal and mechanical stimuli (data not shown).

### 3.2. Effects of cannabinoid $CB_1$ , $CB_2$ and TRPV1 receptor antagonists on cannabidiol-induced anti-hyperalgesia

The ability of specific cannabinoid  $CB_1$ ,  $CB_2$  and TRPV1 receptor antagonists to reverse the anti-hyperalgesic effect of 20 mg/kg cannabidiol in neuropathic rats was tested the last day of cannabidiol dosing (Fig. 2A and B), 60 min after cannabidiol administration. Rimonabant, the cannabinoid  $CB_1$  receptor-specific antagonist (0.5 mg/kg, i.p.), and SR144528, a cannabinoid  $CB_2$ -receptor specific antagonist (3 mg/kg, p.o.), did not modify the withdrawal threshold of ipsilateral paws either when given singly or co-administered with cannabidiol; the simultaneous administration of cannabidiol and the TRPV1 receptor antagonist capsazepine (10 mg/kg, i.p.) did reverse the anti-hyperalgesic action of cannabidiol. When administered alone to neuropathic rats, capsazepine did not affect their withdrawal thresholds. Similar results were obtained when the behavioural evaluations were made 24 h after the last dose (data not shown).

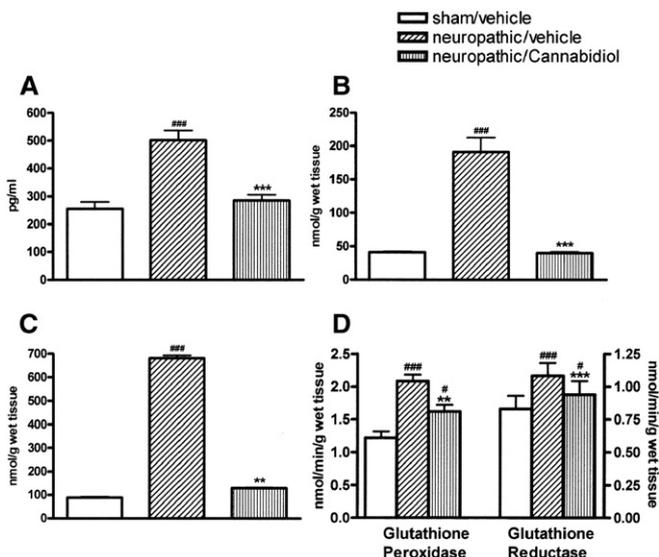


Fig. 3. Effect of cannabidiol on some mediators in the rat model of neuropathic pain. Effect of repeated cannabidiol (20 mg/kg, p.o.) once daily for seven days, starting one week after sciatic nerve injury on (A)  $PGE_2$  plasma level, paw tissue content of (B) malondialdehyde and (C) nitric oxide, and (D) enzymatic activity of Se-dependent glutathione peroxidase and glutathione reductase. Results are expressed as the mean  $\pm$  S.E.M. of 4–7 rats.  $^{\#}P<0.05$ ,  $^{###}P<0.001$ , compared with the sham-operated/vehicle treatment group;  $^{**}P<0.01$ ,  $^{***}P<0.001$ , compared with the neuropathic/vehicle treatment group.

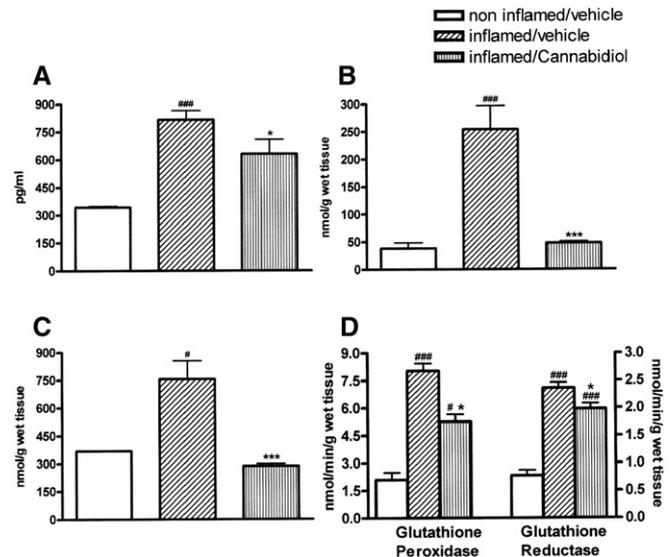


Fig. 4. Effect of cannabidiol on some mediators in the rat model of chronic inflammation. Effect of repeated cannabidiol (20 mg/kg, p.o.), once daily for seven days, starting one week after complete Freund's adjuvant i.pl. injection, on (A) plasma  $PGE_2$ , paw tissue content of (B) malondialdehyde, (C) nitric oxide, and (D) enzymatic activity of Se-dependent glutathione peroxidase and glutathione reductase. Results are expressed as the mean  $\pm$  S.E.M. of 3–4 rats.  $^{\#}P<0.05$ ,  $^{###}P<0.001$ , compared with the non-inflamed/vehicle treatment group;  $^{*}P<0.05$ ,  $^{***}P<0.001$ , compared with the inflamed/vehicle treatment group.

### 3.3. Effect of cannabidiol on plasma $PGE_2$

In neuropathic and inflamed vehicle-treated animals, the plasma concentration of  $PGE_2$  doubled. Repeated treatment of neuropathic rats with 20 mg/kg of cannabidiol brought the  $PGE_2$  plasma content to the level of the sham-treated animals (Fig. 3A). Repeated treatment of inflamed rats with 20 mg/kg cannabidiol induced a slight, not significant reduction of  $PGE_2$  production (Fig. 4A).

### 3.4. Effects of cannabidiol on lipid peroxide production

Free radical production during the neuropathy and chronic inflammation resulted in membrane lipoperoxidation. Accordingly, the content of MDA, an indicator of lipid peroxidation, in injured and inflamed paws increased respectively five-fold and six-fold. Repeated doses of cannabidiol suppressed the lipid peroxide overproduction in paw tissue of neuropathic (Fig. 3B) and inflamed (Fig. 4B) rats.

### 3.5. Effect of cannabidiol on NO production and NOS content

In vehicle-treated animals, 14 days after nerve sciatic lesion and adjuvant injection, the levels of nitrite/nitrate, the end-products of NO oxidation, in injured (Fig. 3C) and inflamed (Fig. 4C) paws were high. The  $NO_2^-/NO_3^-$  content was seven times in injured paws and double in inflamed paws the level found in the respective control animals. Repeated 20 mg/kg doses brought the  $NO_2^-/NO_3^-$  content down to a non-pathological level. The increased production of NO in the

hind paw tissues after the chronic constriction injury was associated with increases in the levels of inducible and neuronal isoforms of sciatic nerve NOS. As shown in Fig. 5A and B, cannabidiol did not reduce the over-expression of the two proteins. The enhanced production of  $\text{NO}_2^-/\text{NO}_3^-$  in inflamed paw tissues was associated with increased levels of iNOS and eNOS. As shown in Fig. 5C and D, only over-expression of eNOS decreased after repeated treatment with cannabidiol, whereas the increase in iNOS content was not affected.

### 3.6. Effects of cannabidiol on the activity of glutathione-dependent enzymes

To compensate the oxidative damage, the glutathione system is activated in paw tissue of neuropathic and inflamed rats. The cytosolic activity of glutathione peroxidase and glutathione reductase was about double that in sham-operated animals (Fig. 3D), and four and three times higher than non-inflamed animals (Fig. 4D). Repeated doses of cannabidiol significantly inhibited this stimulation.

### 3.7. Effect of repeated cannabidiol in neuropathic rats on NF- $\kappa$ B

ELISA for activated NF- $\kappa$ B showed that the DNA-binding activity of NF- $\kappa$ B p65 increased in the sciatic nerve of

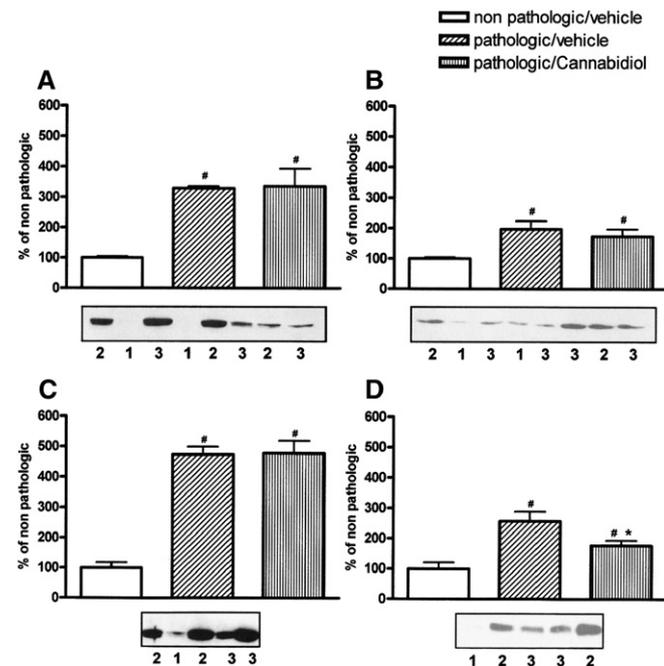


Fig. 5. Effect of cannabidiol on NOS expression in sciatic nerve of neuropathic rats (A, B) and in paw tissue of inflamed rats (C, D). Effect of repeated cannabidiol (20 mg/kg, p.o.), once daily for seven days, starting one week after sciatic nerve injury, on iNOS (A) and nNOS (B) content and after complete Freund's adjuvant i.pl. injection on iNOS (C) and eNOS (D) content. Densitometric analysis using ImageJ package for Windows, Scion Corporation, is shown. Results are expressed as mean  $\pm$  S.E.M. of 3–6 rats.  $^{\#}P < 0.05$ , as compared with sham/vehicle treatment group;  $^*P < 0.05$ , compared with pathologic/vehicle treatment group. Lane 1: non-pathologic/vehicle; Lane 2: pathologic/vehicle; Lane 3: pathologic/cannabidiol.

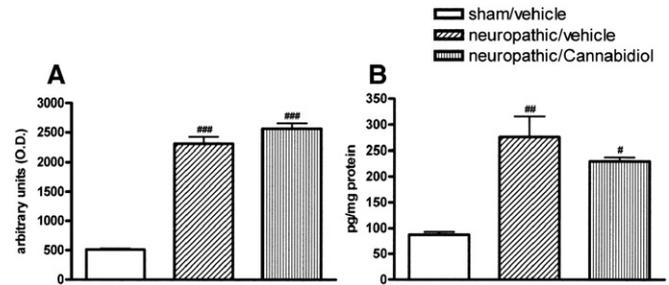


Fig. 6. Effect of cannabidiol on NF- $\kappa$ B activation in nuclear fraction of sciatic nerve, expressed as arbitrary units (optical density, O.D.) at 450 nm and on TNF $\alpha$  content in dorsal root ganglia in the rat model of neuropathic pain. Effects of repeated cannabidiol (20 mg/kg, p.o.), once daily for seven days, starting one week after sciatic nerve injury, on (A) NF- $\kappa$ B activation and (B) TNF $\alpha$  content. Results are expressed as mean  $\pm$  S.E.M. of 3–4 rats.  $^{\#}P < 0.05$ ,  $^{##}P < 0.01$ ,  $^{###}P < 0.001$ , compared with the sham/vehicle treatment group.

neuropathic rats (Fig. 6A). Repeated treatment did not significantly modify up-regulation.

### 3.8. Effect of repeated cannabidiol in neuropathic rats on TNF $\alpha$

Determination of TNF $\alpha$  by ELISA showed higher levels in lumbar L4, L5 and L6 dorsal root ganglia of neuropathic rats than in sham-operated animals and no changes in injured sciatic nerve. Repeated doses of 20 mg/kg cannabidiol did not significantly modify this increase in dorsal root ganglia (Fig. 6B).

## 4. Discussion

The main finding reported here is that the non-psychoactive cannabis constituent cannabidiol reverses both thermal and mechanical hyperalgesia after seven days of repeated oral treatment, in two different models of persistent pain, the chronic constriction injury model of neuropathic pain and the complete Freund's adjuvant-induced model of inflammatory pain. This result is noteworthy, since the treatment started on day 7 when the pathology was well established, suggesting that cannabidiol's ability to improve established pathologies may have therapeutic implications.

Thermal and mechanical hyperalgesia, which developed within one week of the sciatic nerve injury and the i.pl. injection of complete Freund's adjuvant, was still present in neuropathic and adjuvant-injected rats treated with vehicle for seven days, whereas it was significantly attenuated after repeated doses of cannabidiol. This effect was dose-dependent and was elicited by cannabidiol doses that did not alter the nociceptive response in either non-pathological animals or in the contralateral paw of pathological animals (data not shown). We have previously shown that cannabidiol can counteract acute inflammatory pain such as that induced by i.pl. injection of carrageenan, employing a therapeutic regimen (Costa et al., 2004a). The findings reported here highlight the potent anti-hyperalgesic effect of cannabidiol in persistent pain too. The effect of the highest acute dose of cannabidiol in pathological animals was evaluated in rats chronically treated with vehicle and challenged with

cannabidiol on the last day. Cannabidiol did not reverse hyperalgesia in either neuropathic or adjuvant-injected rats, suggesting that repeated doses were necessary to elicit the improvement of pain behavior. Malfait et al. (2000) already reported that only repeated treatment with cannabidiol blocked the progression of collagen-induced polyarthritis in mice and protected joints against severe damage. In addition, Finn et al. (2004) showed that pre-treatment with a single i.p. dose of 5 mg/kg cannabidiol did not reduce formalin-evoked nociceptive behavior.

Cannabidiol inhibits the cellular uptake and enzymatic hydrolysis of the endogenous cannabinoid anandamide (Bisogno et al., 2001), which is involved in pain control through activation of cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors. These two combined effects ultimately result in enhanced levels of anandamide outside and inside the cell, and this in turn might account for the anti-hyperalgesic and anti-inflammatory actions of cannabidiol, so cannabidiol may prove to be the first clinical pharmaceutical to influence endocannabinoid function. Since cannabidiol has negligible affinity for cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors (Pertwee, 1997), the therapeutic effects in neuropathy and chronic inflammation might be due to indirect activation of both these receptors, which have an important role in a number of pain models (for review see Walter and Stella, 2004). Our results show that cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors are not involved in the therapeutic efficacy of cannabidiol against neuropathic pain. In fact cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptor-specific antagonists did not reverse cannabidiol's anti-hyperalgesic effect. However, capsazepine, a selective antagonist for the vanilloid TRPV1 receptor, did reverse the anti-hyperalgesic effect, indicating TRPV1 as the molecular target of this activity. Since both anandamide and cannabidiol behave as agonists of TRPV1, we cannot say whether cannabidiol activates vanilloid receptors directly or indirectly, through anandamide. TRPV1 receptors are up-regulated after inflammation (Amaya et al., 2003) and nerve injury (Fukuoka et al., 2002) and this may contribute to inflammatory and neuropathic hyperalgesia (Kanai et al., 2005), so the therapeutic action of cannabidiol could be due to desensitization of this receptor, similarly to the natural agonist (capsaicin). Unlike capsaicin, in our hands cannabidiol did not induce pain by itself after acute administration. Thus, we can suggest that, as other TRPV1 agonists such as ricinoleic acid (Vieira et al., 2000), resiniferatoxin (Szallasi and Blumberg, 1990), scutigeral (Szallasi et al., 1999), cannabidiol differs from capsaicin in the relative ratio of initial excitation (perceived as burning pain) to subsequent desensitisation. Furthermore, we recently found that the anti-hyperalgesic activity of cannabidiol in a rat model of acute inflammation was mediated by TRPV1 (Costa et al., 2004b).

Cyclooxygenase 2 and NOS are two proteins involved in neuropathy and chronic inflammation (O'Reilly and Loomis, 2006; De Alba et al., 2006). Both the models of chronic pain employed by us involve an increase in plasma PGE<sub>2</sub> and in the NO system. The anti-hyperalgesic activity of cannabidiol was associated with restoration of the physiological level of PGE<sub>2</sub> in neuropathic rats and with a slight reduction of prostanoid content in adjuvant-injected rats. This non-significant inhibition of plasma PGE<sub>2</sub> might be due to the difficulty of cannabidiol in affecting the

very marked prostanoid increase in inflamed rats. Our results also illustrate the NO involvement in the symptoms of complete Freund's adjuvant-induced chronic inflammation and of neuropathy induced by the chronic constriction injury of the sciatic nerve. After the onset of the pathologies, NO levels rose significantly in paw tissues of both groups of rats. Cannabidiol abolished this increase. The increase in NO content of neuropathic rats was due to sciatic nerve iNOS and nNOS over-expression and that in adjuvant-injected animals to over-expression of the constitutive eNOS and iNOS isoforms, as shown by Gad and Khattab (2000). This result attributes an important role to NO in pain behaviour, since hyperalgesia and the NO increase simultaneously disappeared with repeated cannabidiol treatment. However, Chou et al. (2005) have provided genetic evidence that NO evoked by complete Freund's adjuvant injection may have important implications in central sensitization. Esposito et al. (2006) showed that cannabidiol inhibits nitrite production in differentiated A $\beta$ -treated PC12 neurons, in a concentration-related manner. In our hands, cannabidiol reduced the over-expression of the eNOS constitutive isoform, without affecting iNOS up-regulation in inflamed paw tissues and did not significantly inhibit the increases in iNOS and nNOS content in injured sciatic nerve. Most NO production depends on iNOS activity, whose over-expression was not reduced by repeated cannabidiol treatment in inflamed and neuropathic rats; so the fact that repeated treatment with cannabidiol restores the physiological NO level in tissues can possibly be ascribed to cannabidiol scavenger activity against this free radical. Increases in the formation of several reactive free radicals, and consequently also in lipid peroxide levels, are implicated in the pathogenesis of many inflammatory disorders and also in neuro-inflammation after nerve injury (Naik et al., 2006). NO, which can originate locally or from cells that infiltrate the site of inflammation (Levy and Zochodne, 1998), rapidly reacts with free radicals, namely superoxide anions, to form the stable but highly toxic peroxynitrite, which can induce lipid peroxidation. The Se-dependent glutathione peroxidase and glutathione reductase enzymes play pivotal roles in the reduction of lipid peroxides and hydrogen peroxide, in a reaction involving reduced glutathione, as co-substrate. The activity and the levels of these glutathione-dependent enzymes rise during chronic inflammatory and neuropathic pathologies in rats and also in man, presumably because of the increased levels of lipid peroxides (Agha et al., 1999; Mulherin et al., 1996; Di Simplicio et al., 1995). Our results confirmed these findings: 14 days after the induction of neuropathy and chronic inflammation, levels of lipid peroxides and the activities of glutathione peroxidase and glutathione reductase were enhanced in both neuropathic and adjuvant-injected animals. Cannabidiol abolished the increases in the levels of MDA and inhibited the increase in enzymatic activity in pathological tissues. In the light of what we stated before, we cannot exclude that the anti-hyperalgesic effect of cannabidiol is due to its well-known antioxidant properties (Hampson et al., 1998; Iuvone et al., 2004) independently of vanilloid receptor activation. Hampson et al. (1998), measuring the oxidative potential of cannabidiol using a cyclic voltmeter and a Fenton-reaction based system, showed that cannabidiol was a potent antioxidant, with oxidative potential similar to the strong antioxidant butylhydroxytoluene. These

authors considered that the cannabidiol antioxidant abilities might explain its protective effect against glutamate neurotoxicity, where two well-known antioxidants such as ascorbate and  $\alpha$ -tocopherol were less protective.

We had also initially suggested that cannabidiol might inhibit cyclooxygenase and NOS protein expression by acting on TNF $\alpha$  release, since this cytokine is reported to play a pivotal role in the generation and maintenance of neuropathic pain (Sommer et al., 1998). TNF $\alpha$  can also induce the up-regulation of cyclooxygenase 2 protein and raise PGE<sub>2</sub> levels in the injured nerve, probably through the transcription factor NF- $\kappa$ B, inducing the activation of genes encoding for both cyclooxygenase and NOS (Schafers et al., 2004). Repeated cannabidiol treatment of neuropathic rats did not reverse the increase in dorsal root ganglia TNF $\alpha$  content and the NF- $\kappa$ B activation; so we can exclude involvement of the NF- $\kappa$ B cascade in cannabidiol's anti-hyperalgesic effect. It is becoming increasingly clear that reactive species may contribute to the complex processes regulating gene expression of iNOS and cyclooxygenase 2 and that antioxidants inhibit the expression of both inflammatory genes (Hecker et al., 1996; Subbaramaiah et al., 1998).

Our present findings about the anti-hyperalgesic property of this natural component of cannabis, with no psychotropic effects, show its further clinical utility. We thus believe that the anti-hyperalgesic potency of cannabidiol, which is non-toxic, could well make it useful as an oral agent to control chronic painful inflammatory diseases.

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