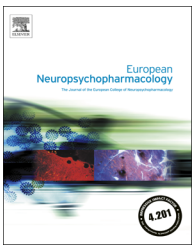
European Neuropsychopharmacology (2014) 24, 608–620



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The cannabinoid CB_2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain

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Received 23 April 2013; received in revised form 31 August 2013; accepted 12 October 2013

KEYWORDS
Inflammatory pain;
Neuropathic pain;
CB_2;
Beta-caryophyllene;
Dietary cannabinoid

Abstract
The widespread plant volatile beta-caryophyllene (BCP) was recently identified as a natural selective agonist of the peripherally expressed cannabinoid receptor 2 (CB_2). It is found in relatively high concentrations in many spices and food plants. A number of studies have shown that CB_2 is critically involved in the modulation of inflammatory and neuropathic pain responses. In this study, we have investigated the analgesic effects of BCP in animal models of inflammatory and neuropathic pain. We demonstrate that orally administered BCP reduced inflammatory (late phase) pain responses in the formalin test in a CB_2 receptor-dependent manner, while it had no effect on acute (early phase) responses. In a neuropathic pain model the chronic oral administration of BCP attenuated thermal hyperalgesia and mechanical allodynia, and reduced spinal neuroinflammation. Importantly, we found no signs of tolerance to the anti-hyperalgesic effects of BCP after prolonged treatment. Oral BCP was more effective than the subcutaneously injected synthetic CB_2 agonist JWH-133. Thus, the natural plant product BCP may be highly effective in the treatment of long lasting, debilitating pain states. Our results have important implications for the role of dietary factors in the development and modulation of chronic pain conditions.

1. Introduction

Pain is an important sensory signal indicating the occurrence or danger of tissue damage. The associated negative emotional

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0924-977X
http://dx.doi.org/10.1016/j.euroneuro.2013.10.008

effect of pain stimulates avoidance behaviors and thus helps to protect the organism from future dangerous situations (Basbaum et al., 2009; Woolf and Ma, 2007). Tissue inflammation can enhance pain sensation through the sensitization of nociceptors, peripheral neurons responding to painful stimuli, and through a sensitization of spinal neurons leading to an enhanced transmission of nociceptive signals. The resulting allodynia and hyperalgesia of the inflamed tissue also contributes to the recuperative process (Hauser et al., 2013). Pain sensation typically reverts to normal levels as the inflammatory response abates. Neuropathic pain conditions on the other hand do not serve any useful purpose. They arise from lesion or injury of peripheral nerves, sensory ganglia, spinal roots or central structures, by trauma, vascular or metabolic disorders, viral infection, neuroinflammation or autoimmune responses. These lesions trigger inflammatory responses at the site of the nerve injury and in the spinal projection area, characterized by an activation of microglia and astrocytes, and an increased production of pro-inflammatory cytokines. These responses lead to long lasting molecular and cellular changes in the spinal cord that account for many aspects of the chronic pain, as well as hyperalgesia and allodynia reported by patients suffering from neuropathic pain (Costigan et al., 2009). Neuropathic pain is often persistent and difficult to treat with current medications.

Cannabis preparations, which have been used since thousands of years for the treatment of pain (Zias et al., 1993) have recently come again into the focus as potential therapeutics for inflammatory and neuropathic pain conditions. Currently, cannabis extracts and synthetic preparations of the psychoactive cannabis compound Δ^9-tetrahydrocannabinol (THC) have been approved in many countries for clinical pain management at doses and formulations that show on only minor central side effects (Ben Amar, 2006; Iskedjian et al., 2007; Lynch and Campbell, 2011). Nevertheless, psychoactive effects of THC-containing medications are an important consideration for many clinicians and their patients. These effects are caused by the activation of the brain cannabinoid receptor 1 (CB_1), which also mediates many of the acute analgesic effects of THC (Hohmann and Herkenham, 1999). Recent interest has therefore focused on the cannabinoid receptor 2 (CB_2), which is primarily expressed in non-neuronal immune cells (Facci et al., 1995; Maresz et al., 2005). CB_2 expression is induced in many tissues and cells, including the CNS (Benito et al., 2007), under inflammatory conditions and has therefore been implicated as a potential therapeutic target for inflammatory disorders including chronic pain. CB_2-selective agonists should have an excellent safety profile like THC, while lacking its psychoactive effects. Indeed, CB_2 receptor agonists showed good anti-nociceptive effects in several animal models of pain (Guindon and Hohmann, 2008).

A natural selective agonist for CB_2 receptors is the plant volatile BCP, which represents a dietary phytocannabinoid (Gertsch et al., 2008, 2010). BCP is found in large amounts in the essential oils of many common spices and food plants, such as oregano (*Origanum vulgare* L.), cinnamon (*Cinnamomum* spp.), clove (*Syzygium aromaticum*), rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus serpyllum*), black pepper (*Piper nigrum* L.) (Bernardes et al., 2010; Hudaib et al., 2002; Jayaprakasha et al., 2003; Mockute et al., 2001; Orav et al., 2004; Zheng et al., 1992). Several health effects have been attributed to BCP or medicinal plants containing BCP (Russo, 2011), including anti-inflammatory (Gertsch et al., 2008), local anesthetic (Ghelardini et al., 2001), anti-carcinogenic (Legault and Pichette, 2007; Loizzo et al., 2008), anti-fibrotic (Calleja et al., 2012) and anxiolytic-like (Galdino et al., 2012) activity. Analgesic effects of BCP were also observed after local intraplantar application (Katsuyama et al., 2012). There is even some clinical evidence that aromatic essential oil massage containing BCP has a beneficial effect on menstrual cramps (Ou et al., 2012). We have previously shown that BCP exerts an anti-inflammatory effect in the carrageenan-induced edema test (Gertsch et al., 2008). More recently, it was shown that BCP also inhibits inflammation and tissue damage in models of colitis and nephrotoxicity in a CB_2 receptor-dependent manner (Bento et al., 2011; Horváth et al., 2012).

In the present study, we investigated the analgesic effects of BCP in formalin-induced inflammation model and in a model of neuropathic pain, which involves the partial ligation of the sciatic nerve. To assess the contribution of CB_2 receptors in the analgesic action of BCP we also carried out experiments in mice lacking CB_2 receptors.

2. Experimental procedures

2.1. Animals

Mice with a genetic deletion of the CB_2 receptor ($CB_2^{-/-}$) have been described previously (Buckley et al., 2000). Mutant mice were crossed for more than 10 generations to C57BL/6J animals and are thus considered to be congenic for this background. Animals were housed in groups of three to five mice per cage under controlled illumination (light dark cycle 12:12 h) and environmental conditions. All animals were bred in our animal facility, and had free access to water and food. Male mice were used in this study in the age of 3–4 months old. Experimental procedures complied with all regulations for animal experimentation in Germany and were approved by Landesamt für Natur, Umwelt und Verbraucherschutz in Nordrhein-Westfalen, Germany (AZ: 9.93.210.35.07.310).

2.2. Drugs

Beta-caryophyllene (5:1 isomer mixture of (E)- and (Z)-BCP)) was purified from a commercially available 70% pure preparation (TCI America) by HPLC and analyzed in GC/MS. The 95% pure BCP contained residual amounts of alpha-humulene and traces of the BCP oxidation product BCP oxide. BCP was dissolved in olive oil (Fluka) at a concentration of 0.02, 0.2, 1 and 2 mg ml^{-1} and frozen in aliquots at −20 °C. On every treatment day aliquots were defrosted and gavage-fed at a volume of 5 ml kg^{-1} with the help of a feeding-needle, thus resulting in doses of 0.1, 1, 5 or 10 mg kg^{-1} respectively. The synthetic CB_2 agonist JWH-133 was purchased in a solution, dissolved in TocrisolveTM 100, (Tocris Bioscience) and was further diluted to a concentration of 0.1, 0.5, and 1 mg ml^{-1}, and injected subcutaneously in a volume of 10 ml kg^{-1}. The CB_2 antagonist SR 144528 (kindly provided by NIH) was dissolved in ethanol: chremophor: water (1:1:18) solution at a concentration of 0.1 mg ml^{-1} and was injected in a volume of 10 ml kg^{-1} intraperitoneally (i.p.). Morphine (Merck KGaA, Darmstadt, Germany) was dissolved in saline and was injected i.p. in a doses of 5 mg kg^{-1}.

2.3. Inflammatory pain model: formalin test

Male wild type ($CB_2^{+/+}$) and $CB_2^{-/-}$ mice were injected with 20 μl of 5% formalin into the plantar surface of the right hindpaw. The left paw served as control. Formalin injection produces a biphasic pain

response. The early phase (1–5 min) is an immediate pain response caused by the formalin, while inflammatory processes and central sensitization contribute to late phase (15–30 min) pain. The number of pain responses such as paw licking, shaking and lifting were evaluated every 10 s on the ipsilateral and on contralateral sides by an observer who was blind to experimental groups. Thirty minutes before the formalin injection, the mice were gavage-fed with BCP, or vehicle. Other groups of mice were injected subcutaneously with a reference compound, JWH-133 or vehicle 30 min before the formalin treatment. As a CB_2 antagonist SR 144528 was used and administered i.p. 15 min before the agonists. As positive control for analgesia, we treated $CB_2^{-/-}$ animals with 5 mg kg^{-1} morphine i.p.

2.4. Neuropathic pain model: partial sciatic nerve ligation (PNL)

To induce neuropathic pain, a partial ligation of the right sciatic nerve was performed as described (Racz et al., 2008). Male wild type $CB_2^{+/+}$ and $CB_2^{-/-}$ mice were operated under isoflurane (2–3% in 95% O_2 for induction and 1.5–2% in 95% O_2 for maintenance) anesthesia. The duration of surgery was no longer than 10–15 min. One half to one-third of the sciatic nerve at midthigh level was tightly ligated with a polypropylene thread (9–0). In sham operated mice the nerve was exposed without ligation.

Mechanical allodynia and thermal hyperalgesia were tested as an indicator for the development of neuropathic pain by an observer who was blind to the treatment strategy. Mice were habituated to the experimental setup for 1 h on three consecutive days before the behavioral testing started. First, baseline nociceptive responses were determined. The partial nerve ligation was performed 1 day later. To assess the neuropathic pain outcome the animals were then tested on days 3, 6, 8, 10 and 14 after the partial nerve ligation.

Mechanical allodynia was assessed with a dynamic aesthesiometer (Ugo Basile Biological Research Apparatus), which consists of an electronically controlled mobile pressure-actuator that is able to exert a continuously increasing force. Mice were placed in a transparent Plexiglas chamber with a metal grid floor. The tip of the actuator (diameter: 0.5 mm) was applied to the middle of the plantar surface of the hind paw. The forces necessary to trigger withdrawal responses of the ipsilateral or contralateral hind paws were recorded. Means were determined by averaging three to five trials per paw.

Thermal hyperalgesia was evaluated using a Hargreaves apparatus (Ugo Basile Biological Research Apparatus, I.R. intensity: 40). The mean withdrawal latencies were determined by averaging three to five separate trials. Both mechanical allodynia and thermal hyperalgesia were evaluated in the same group of animals: the tests were separated at least by 1 h.

Mice received daily either BCP or vehicle by gavage, or the reference compound JWH-133 or vehicle by subcutaneous injections starting 1 day after the partial nerve ligation. On the test days, drugs were administered directly after the experiments.

2.5. Immunohistochemistry

At the end of the behavioral experiments, 4–5 mice per group were anesthetized using a Ketavet/Rompun (10 mg kg^{-1}/0.1 mg kg^{-1}) mixture and intracardially perfused with ice cold phosphate buffer (PBS) for 5 min followed by 4% PFA (paraformaldehyde) solution for 10 min. The tissue samples was quickly frozen in isopentane on dry ice and stored at −80 °C.

The section from L4–L6 of the spinal cord was dissected, embedded in O.C.T. compound (Tissue Tek®, Sakura), and sliced in 16 μm sections on a cryostat (Leica CM 3050; Leica Microsystems). Six sections per mice from all treatment groups were mounted on Star frost-coated slides. The slides were permeabilized for 1 h in 0.5% Triton X-100 (Sigma). After blocking in PBS containing 3% bovine serum albumine and 10% donkey serum, the slides were incubated for 42 h at 4 °C with primary antibody against glial fibrillary acidic protein (GFAP, goat polyclonal, 1:500, Santa Cruz Biotechnology) or ionized calcium-binding adapter molecule 1 (Iba1, rabbit polyclonal, 1:1000, Wako). A red fluorescent Cy3 anti-goat secondary antibody (1:1000, Jackson ImmunoReasearch) or green fluorescent Alexa Fluor 488 anti-rabbit secondary antibody (1:1000, Life Technologies) were used to visualize the signals. The slides were mounted with Mowiol 4–88 (Roth).

Pictures were acquired using a Zeiss Imager M2 fluorescent microscope (Carl Zeiss Microimaging). To evaluate microgliosis and astrogliosis after peripheral nerve injury, pictures of the Iba1 and GFAP double stained sections were taken with the multichannel mosaic function of the AxioVision software (Carl Zeiss Microimaging) in the 20× magnification. The mosaics were converted to one picture before quantitative analysis. To improve the overlay of the mosaic pictures the stitching function of the software was used for representative pictures. An observer, who was blind to experimental groups, analyzed four to five animals per treatment group using a macro routine in the Image J 1.42q software. The percentage of stained area in the dorsal horn compared to whole area was analyzed. Data were expressed as the difference of the ipsilateral staining compared to the contralateral staining (% stained area ipsilateral side − % stained area contralateral side).

2.6. Quantitative real time-PCR

To analyze the expression level of CB_2 receptor gene after PNL, TaqMan® gene expression assays were used. Total RNA was isolated according to manufacturer´s instruction and RNA concentrations were measured using a spectrophotometer (NanoDrop). mRNA was transcribed into double-stranded cDNA using Reverse Transcriptase II and Oligo(dt)12-18 primer (Life Technologies). For each sample 50 ng of cDNA were mixed with gene expression master mix (containing AmpliTaq Gold® DNA Polymerase, Life Technologies) and with the CB_2 gene specific assay. Expression data were then normalized to a housekeeping gene (β-actin) and analyzed using the 2-ΔΔCt method as described previously (Livak and Schmittgen, 2001).

2.7. Tetrad test

To exclude that BCP produces the psychoactive side effects known from CB_1 receptor agonists, the classical tetrad test was performed (Martin et al., 1991).

Mice were tested 45 min after an acute oral administration of BCP (1 mg kg^{-1} and 10 mg kg^{-1}) for motor activity in the open-field, immobility (catalepsy) on a ring, anti-nociception in the tail flick test and the rectal temperature was measured. The control group of mice was injected intraperitoneally (i.p.) with a dose of 10 mg kg^{-1} THC (1:1:18 ethanol, cremophor, saline) and tested 20 min after the injection. An observer, unaware of the treatments, recorded the values.

2.7.1. Open-field test

To assess motor activity mice were placed in the center of an open-field apparatus (44 × 40 × 30 cm^3) in a dimly illuminated (20 lux at the ground) room and their activity was tracked by an automatic monitoring system during 10 min (TSE Systems). Horizontal motor activity was evaluated by calculating the total distance (m) traveled by the animals.

2.7.2. Catalepsy

Mice were placed on a vertical tube (diameter: 5.5 cm, heights: 16 cm). The immobility time was recorded for 5 min. Animals that fell down or

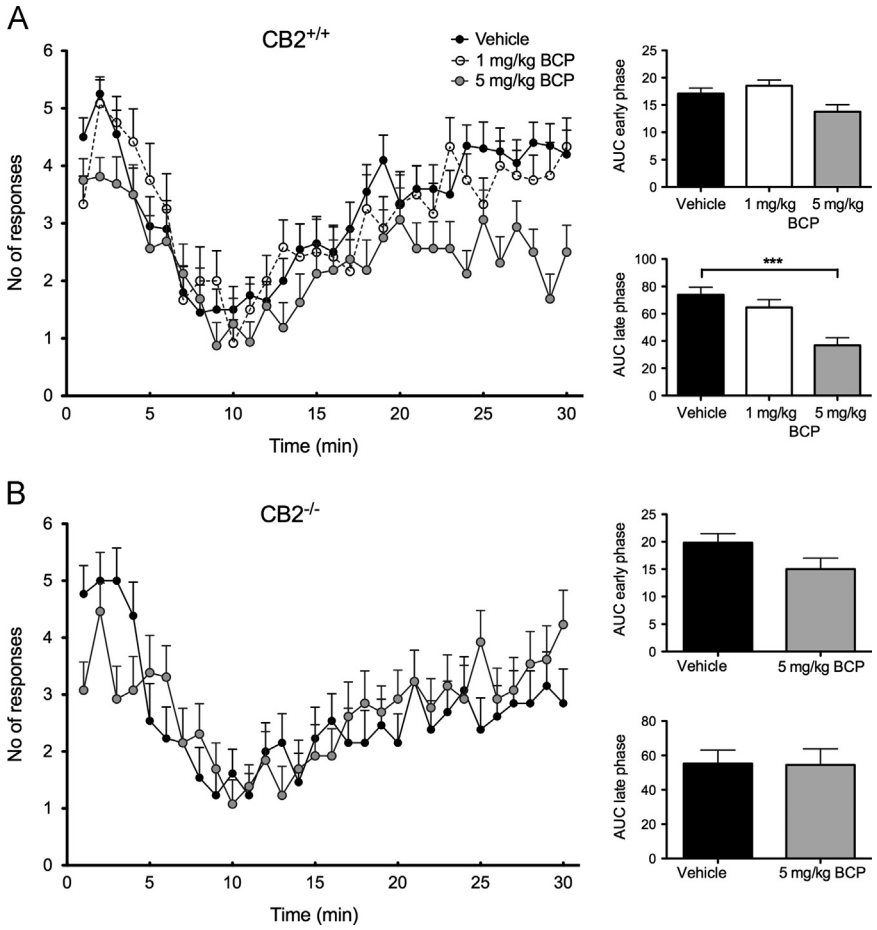

Figure 1 BCP significantly decreased the pain responses in the late phase of the formalin test in $CB_2^{+/+}$, but not $CB_2^{-/-}$ mice. A Pain responses of $CB_2^{+/+}$ mice after 1 and 5 mg kg^{-1} BCP treatment compared to vehicle treated mice in the formalin test (vehicle group: $n=12$; BCP groups: $n=12$). Analysis of the area under the curve (AUC) showed that late phase responses (15–30 min after formalin injection) were significantly reduced after oral administration of 5 mg kg^{-1} BCP ($p<0.00001$). (B) $CB_2^{-/-}$ mice did not show any significant changes in pain responses after 5 mg kg^{-1} BCP treatment compared to the vehicle group (vehicle group: $n=10$; 5 mg kg^{-1} BCP group: $n=10$). ***, treatment effect $p<0.0001$. Means $\pm$ SEM are indicated.

jumped off the ring were allowed five more trials. One animal was excluded from the experiments after five jumps off the ring.

2.7.3.Tail flick

Tail flick latencies were determined using a tail flick apparatus (Columbus Instruments, OH, USA). Mice were manually restraint and the tail was placed over a radiant heat source. We measured the tail withdrawal latency setting the cut-off time at 12 s.

2.7.4.Rectal body temperature

We assessed the body temperature using a rectal thermometer (BAT-12, Harvard Instruments, Millis, Massachusetts). Temperatures were recorded before and 1 h after the drug administration. In case of THC treatment the second measurement was performed 40 min after the injection. Data are expressed as the difference between the two body temperatures ($\Delta T = T$ after treatment $- T$ before treatment).

The results of the cannabinoid tetrad were analyzed by one-way ANOVA or repeated measurement ANOVA (body temperature)

2.8. Statistical analysis

For all experimental results mean values and the standard error of the means (SEM) were calculated. The statistical analysis was carried out using Statistika 7.1 (StatSoft Inc.). In case of the neuropathic pain experiments repeated measurements ANOVA was used to analyze the treatment effects (categorical factors: nerve injury and treatment. In the formalin test, repeated measurements ANOVA was used to analyze the time course of pain reactions. For the analysis of the BCP effect and the JWH-133 treatment, an area under curve (AUC) was calculated and compared using factorial ANOVA. The results of the cannabinoid tetrad were analyzed by one-way ANOVA or repeated measurement ANOVA (body temperature). In the histological analysis of the astrocyte density the Mann-Whitney U test was performed. In all tests the level of significance was set at $p \leq 0.05$.

3. Results

3.1. BCP reduced inflammatory pain responses in the late phase of the formalin test

To assess the effect of oral BCP on formalin-induced pain responses, we administered BCP by gavage 30 min before the formalin injection. In wild type mice, BCP significantly altered formalin-induced pain reactions when administered at a dose of 5 mg kg^{-1} (Figure 1A, $F_{1,34}=7.06$, $p=0.012$). By

analyzing the area under the curve (AUC), we found no significant treatment effect for the early phase (0–5 min), but a significant attenuation of pain responses during the late phase (15–30 min; $F_{1,34}=24.41$, $p<0.0001$). BCP at a dose of 1 mg kg^{-1} was ineffective (Figure 1 A). The synthetic CB_2 agonist JWH-133 was also effective ($F_{1,34}=6.66$, $p=0.014$) at a relatively high dose of 10 mg kg^{-1} (Figure 2A). After calculating the early and late phase AUC, JWH-133 similarly showed a significant analgesic effect only in the late phase ($F_{1,35}=4.74$, $p=0.037$). Both substances were completely ineffective in $CB_2^{-/-}$ animals (BCP treatment: $F_{1,23}=0.87$ p=n.s. JWH-133 treatment: $F_{1,14}=0.109$ p=n.s.), thus indicating that the antinociceptive effects are indeed mediated by CB_2.

To further substantiate the involvement of CB_2 receptors, we also blocked CB_2 receptor signaling with the selective antagonist SR 144528. The injection of SR 144528 15 min before JWH-133 at a dose of 1 mg kg^{-1} (Figure 3A) blocked JWH-133 analgesia during the late phase ($F_{1,28}=2.67$ p=n.s.). Interestingly, a dose of 3 mg kg^{-1} SR 144528 was necessary to antagonize the analgesic effect of BCP (Figure 3B, $F_{1,28}=3.53$, p=n.s.). A dose of 1 mg kg^{-1} was not effective (data not shown). The late phase AUC also did not differ significantly in the BCP-SR 144528 treated group compared to the vehicle treated mice ($F_{1,28}=2.67$, p=n.s.). Additionally, we treated $CB_2^{-/-}$ mice with morphine (5 mg kg^{-1}) as a reference compound, which exerts its effect through the non-cannabinoid μ opioid receptors. Morphine significantly reduced the animal's nociceptive response (Figure 4, $F_{1,21}=4.96$, $p=0.037$). AUC analysis showed that morphine exerted a highly significant analgesic effect in the early phase ($F_{1,21}=17.67$, $p<0.001$), but not in the late phase ($F_{1,21}=3.39$, p=n.s.).

3.2. Neuropathic pain symptoms are diminished in BCP treated mice

We next measured mechanical allodynia (von Frey test) and thermal hyperalgesia (Hargreaves test) in BCP treated versus vehicle treated wild type mice after partial sciatic nerve ligation, an animal model of neuropathic pain. Baseline responses were similar in all groups for both tests.

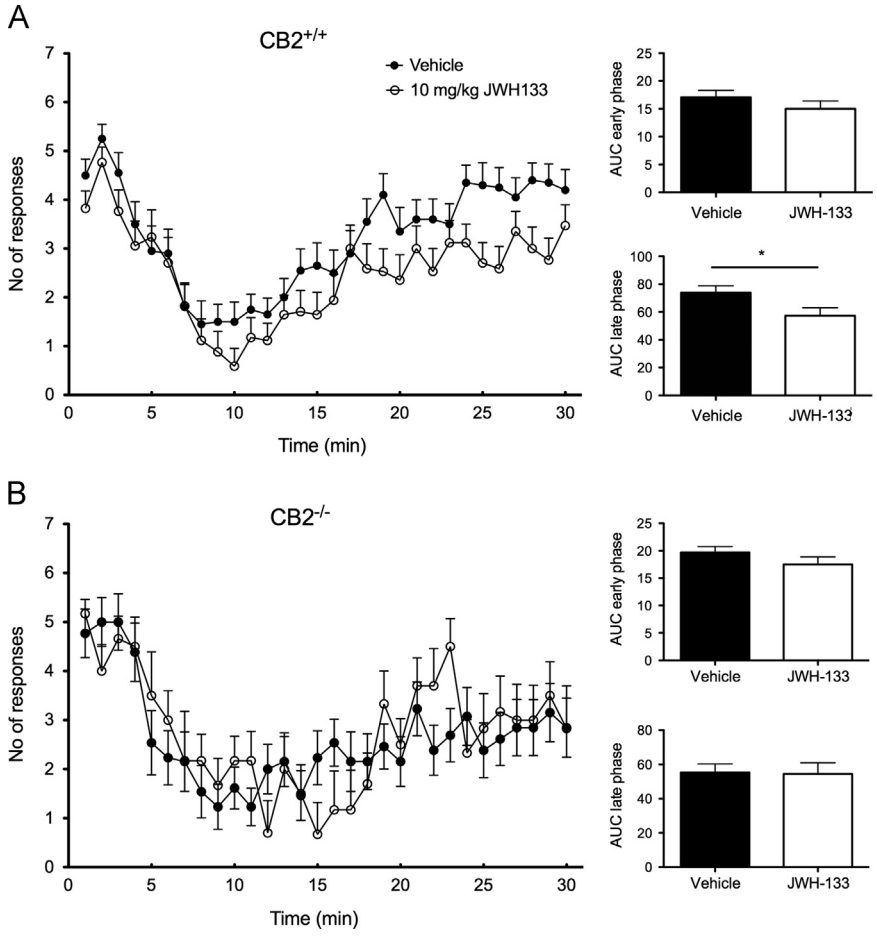

Figure 2 JWH-133 significantly decreased the pain responses in $CB_2^{+/+}$ but not $CB_2^{-/-}$ mice. A JWH-133 treatment (10 mg kg^{-1}) slightly but not significantly decreased the pain responses in $CB_2^{+/+}$ mice compared to vehicle treated mice in the formalin test (vehicle group: $n=12$; JWH-133 group: $n=10$). Analysis of the area under the curve (AUC) showed that late phase responses (15–30 min after formalin injection) differed significantly between the JWH-133 and vehicle treated groups ($p=0.037$). (B) $CB_2^{-/-}$ mice did not show any significant changes in pain responses after 10 mg kg^{-1} JWH-133 treatment (vehicle group: $n=10$; JWH-133 treated group: $n=10$). Calculation of area under the curve revealed the same results. Means ± SEM are indicated.

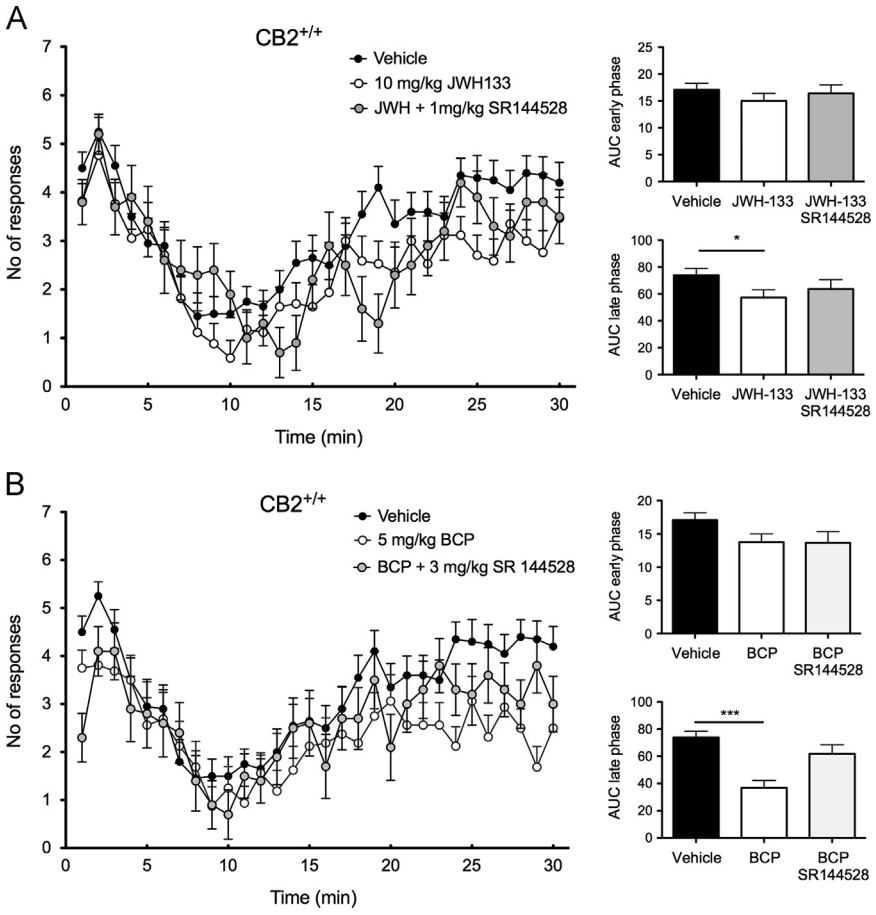

Figure 3 Pretreatment with a selective CB_2 receptor antagonist, SR 144528 blocked the analgesic effects of both JWH-133 and BCP in the formalin test. (A) After 1 mg kg^{-1} SR 144528 treatment the synthetic CB_2 receptor agonist, JWH-133 did not show any analgesic effect in the late phase of formalin test. (B) Interestingly, the analgesic effect of BCP could be antagonized only with a higher dose, 3 mg kg^{-1} of SR 144528. (JWH 133+1 mg kg^{-1} SR 144528 treated group $n=10$, BCP+3 mg kg^{-1} SR 144528 treated group $n=10$). Means±SEM are indicated.

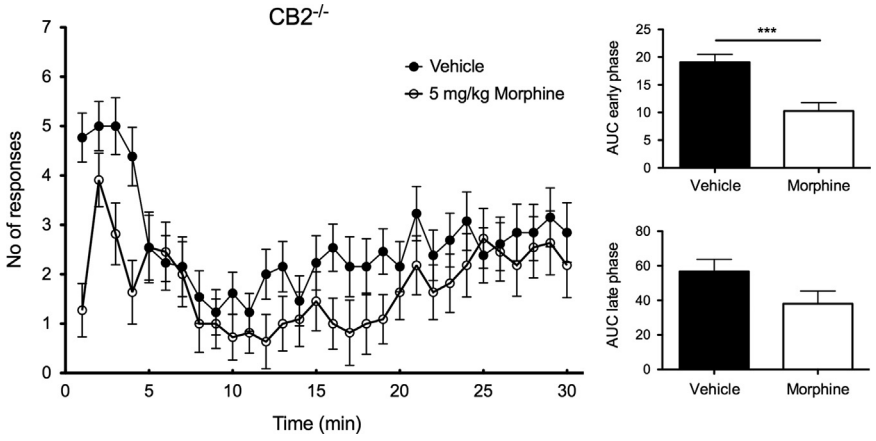

Figure 4 Morphine significantly decreased the pain responses in the formalin test in $CB_2^{-/-}$ mice. 5 mg kg^{-1} morphine significantly reduced the pain reaction in the formalin test ($p=0.037$). AUC analysis showed that early phase responses were significantly reduced after morphine treatment ($p<0.001$). (vehicle group: $n=10$; morphine group: $n=10$) ***, treatment effect $p<0.0001$. Means±SEM are indicated.

The partial ligation of the right sciatic nerve induced mechanical allodynia (Figure 5 A, $F_{1,18}=387.34$, $p<0.001$) and thermal hyperalgesia (Figure 5B, $F_{1,18}=20.40$, $p<0.001$) on the ipsilateral, but not the contralateral hind paw. Sham operated animals showed no alterations in thermal or mechanical pain responses (Supplementary Figure 1).

The animals received daily doses of BCP (0.1, 1, 5 or 10 mg kg^{-1}) by gavage, starting 1 day after the surgery. The doses of 1 mg kg^{-1} ($F_{1,18}$=20.66, p<0.001) and 5 mg kg^{-1} ($F_{1,19}$=20.47, p<0.0001) BCP increased mechanical withdrawal thresholds of ipsilateral hind paws (Figure 5A). Treatment with 10 mg kg^{-1} BCP directly exhibited a robust effect on day 3, which then persisted over the testing period ($F_{1,18}$=33, p<0.001). In the Hargreaves test only 1 mg kg^{-1} BCP, but not other doses, reduced thermal hyperalgesia gradually over the 2-week testing period even up to the basal level ($F_{1,18}$=9.12, p=0.007; time × treatment: $F_{4,72}$=2.93, p=0.026). Administration of the synthetic CB_2 agonist, JWH-133 at the doses of 1 and 5 mg kg^{-1} significantly increased mechanical withdrawal thresholds (Figure 5A; 1 mg kg^{-1}: $F_{1,16}$=9.59, p=0.0069; 5 mg kg^{-1}: $F_{1,18}$=30,71, p<0.001), but failed to diminish thermal hyperalgesia in the Hargreaves test (Figure 5B). These results show that CB_2 agonists are effective in reducing mechanical allodynia, but not thermal hyperalgesia, after sciatic nerve injury.

3.3. BCP inhibits neuropathic pain through CB_2 receptor activation

To determine if BCP exerts its effects on the development of neuropathic pain via CB_2 receptor activation, the experiments were repeated in $CB_2^{-/-}$ mice (Figure 6). We have previously shown that baseline responses of $CB_2^{-/-}$ mice were similar to $CB_2^{+/+}$ animals (Racz et al., 2008). Furthermore, the sham surgery did not influence the pain responses to mechanical and thermal stimuli (Figure 6C and D). After the partial nerve ligation vehicle treated $CB_2^{-/-}$ mice developed mechanical allodynia ($F_{1,14}$=25.97, p<0.001) and thermal hyperalgesia in the ipsilateral side ($F_{1,19}$= 4.97, p=0.038) and a mirror image of mechanical hyperalgesia in the contralateral side ($F_{1,14}$=8.39, p=0.012) as reported previously (Racz et al., 2008). In these mice 1 mg kg^{-1} BCP failed to show any effects on mechanical allodynia or thermal hyperalgesia (Figure 6A and B). Also, BCP treatment did not influence the responses of sham-operated $CB_2^{-/-}$ mice (Figure 6C and D).

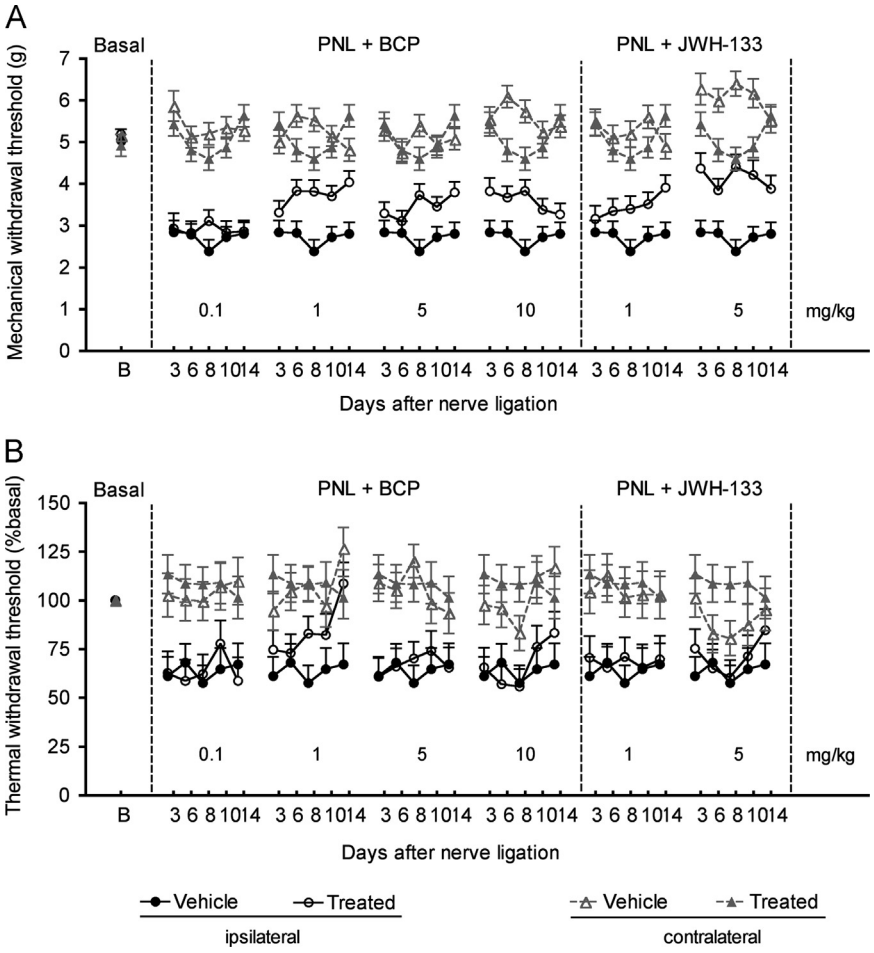

Figure 5 Effect of different doses (0.1, 1, 5 and 10 mg kg^{-1}) of BCP and JWH-133 (1 and 5 mg kg^{-1}) treatment on mechanical allodynia and thermal withdrawal latencies in $CB_2^{+/+}$ mice. A Chronic treatment with 1, 5 and 10 mg kg^{-1} BCP and both doses of JWH-133 significantly rescued PNL induced mechanical allodynia in the ipsilateral side. (B) Chronic treatment with 1 mg kg^{-1} BCP significantly rescued PNL induced thermal hyperalgesia in the ipsilateral side, while 1 and 5 mg kg^{-1} JWH-133 treatment showed no effect. The mice were tested at day 3, 6, 8, 10 and 14 after surgery. Vehicle group: n=10; 0.1 mg kg^{-1} BCP group: n=8; 1 mg kg^{-1} BCP group: n=10; 5 mg kg^{-1} BCP group: n=11; 10 mg kg^{-1} BCP group: n=10; 1 mg kg^{-1} JWH-133 group: n=8; 5 mg kg^{-1} JWH-133 group: n=10. **, treatment effect p<0.01; ***, treatment effect p<0.001. Means±SEM are indicated.

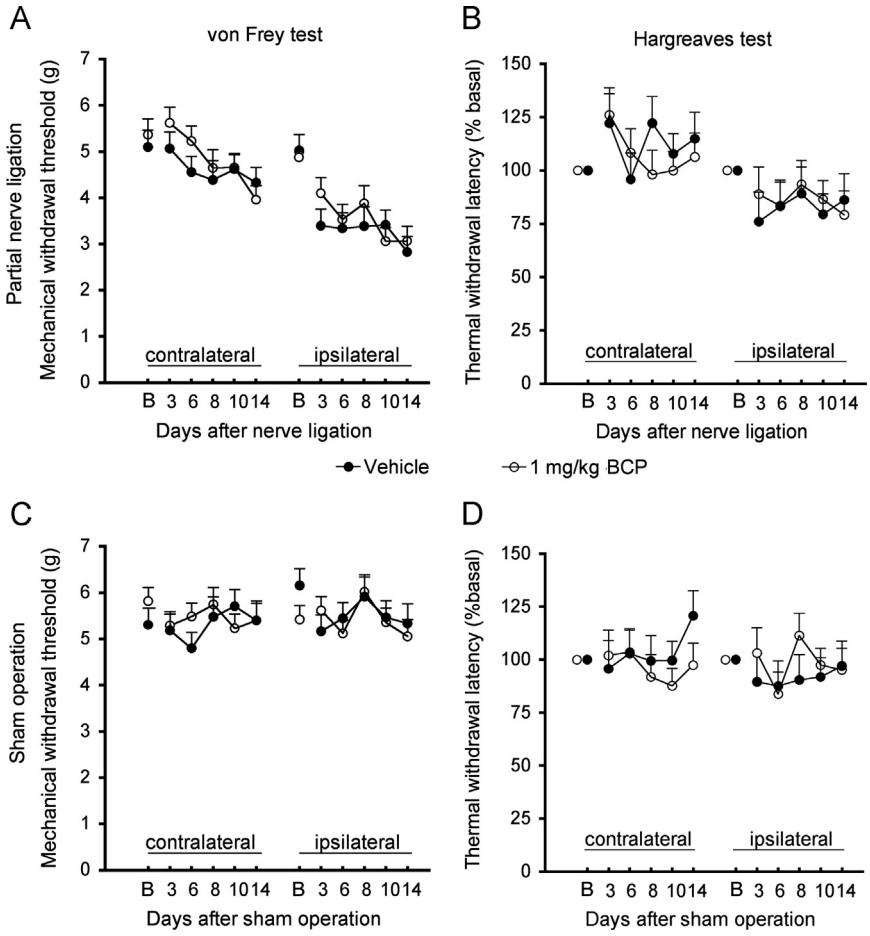

Figure 6 Chronic BCP treatment did not attenuate PNL induced mechanical allodynia (A) and thermal hyperalgesia (B) in $CB_2^{-/-}$ mice. Additionally to the ipsilateral side, $CB_2^{-/-}$ mice developed a mirror image of pain at the contralateral side after surgery (Racz et al., 2008). Ipsilateral and contralateral hindpaws of mice were tested in the von Frey test and Hargreaves test to evaluate mechanical allodynia (vehicle group: $n=8$; 1 mg kg^{-1} BCP group: $n=9$) and thermal hyperalgesia (vehicle group: $n=10$; 1 mg kg^{-1} BCP group: $n=12$). (C) Mechanical withdrawal thresholds of sham operated animals (vehicle group: $n=8$; 1 mg kg^{-1} BCP group: $n=11$). (D) Thermal withdrawal latencies of sham operated animals (vehicle group: $n=11$; 1 mg kg^{-1} BCP group: $n=14$). $CB_2^{-/-}$ mice were tested at day 3, 6, 8, 10 and 14 after surgery. Means $\pm$ SEM are indicated. B = basal responses.

3.4. BCP treatment reduces the density of spinal cord glia cell-markers after peripheral nerve injury

Development of neuropathic pain is accompanied by the activation and proliferation of glia cells in the spinal cord (Scholz and Woolf, 2007). Therefore, we performed histological stainings with the microglia marker ionized calcium-binding adapter molecule 1 (Iba1) and astrocyte marker glial fibrillary acidic protein (GFAP).

The partial nerve ligation strongly increased microglia density in the ipsilateral dorsal horns of lumbar spinal cord sections (Figure 7A and B). Thus, the stained area in vehicle-treated mice was significantly larger compared to sham operated animals (surgery effect: $U=25$, $p<0.001$). We also observed a microgliosis after BCP treatment (surgery effect: $U=23$, $p<0.001$), but at a significantly lower level when compared to vehicle controls (treatment effect: $U=164$, $p=0.006$). The partial nerve ligation induced a significant increase in density of astrocytes in the ipsilateral dorsal horn of the lumbar spinal sections compared to sham operation (Figure 7C and D; surgery effect: $U=164$; $p=0.003$) in vehicle controls, but not in BCP-treated animals ($U=188$, $p=$n.s). Together, these results show that BCP treatment prevented astrocytosis and reduced microgliosis.

3.5. PNL induced the CB_2 gene expression in the spinal cord

To analyze the expression pattern of CB_2 receptors after PNL and BCP treatment, we performed quantitative real-time PCR. The partial ligation of the sciatic nerve induced an increase in CB_2 receptor mRNA in the ipsilateral side, but not in the contralateral side, as revealed by a significant interaction of surgery and side in the factorial ANOVA ($F_{1,29}=4.63$, $p=0.03$). The following Fisher-LSD post-hoc test revealed that the expression of CB_2 in the ipsilateral

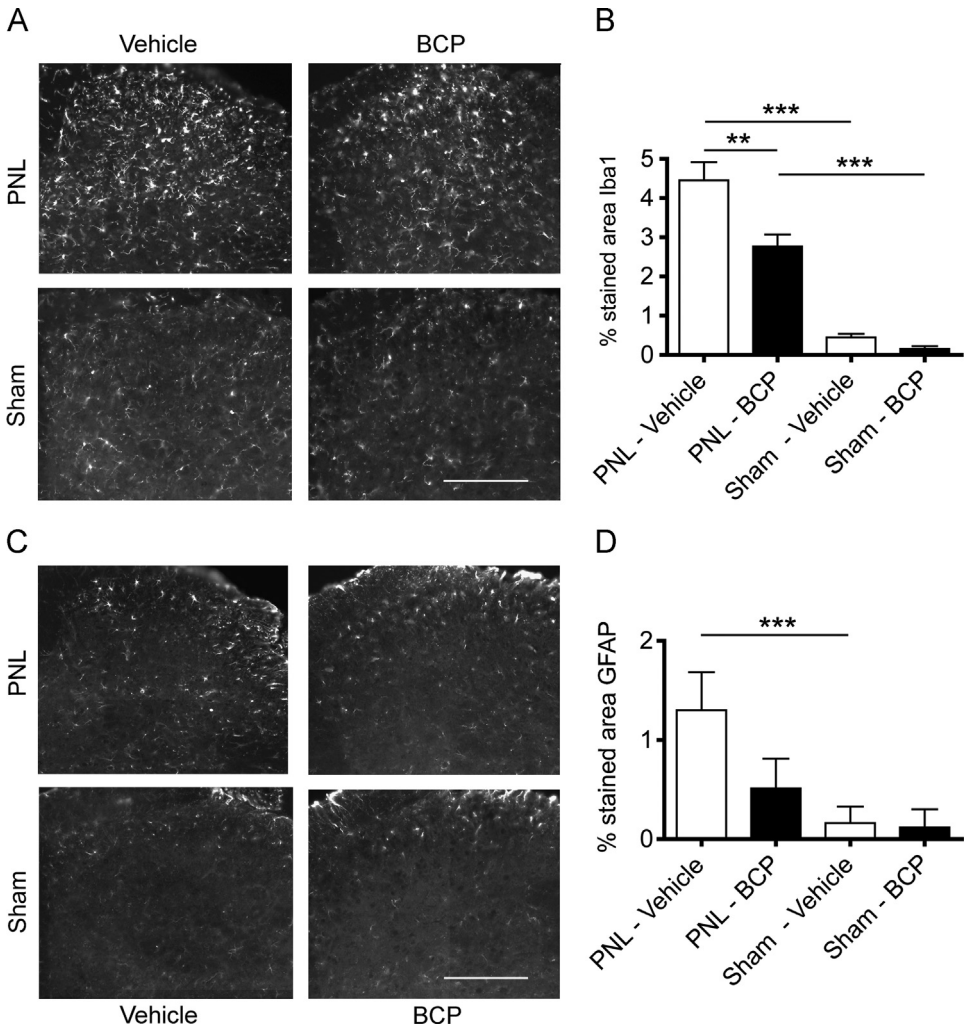

Figure 7 Chronic treatment with 1 mg kg^{-1} BCP reduced the density of microglia and astrocytes in the dorsal horn of spinal cord. Representative pictures of the Iba1 (A) and GFAP (C) immunostaining in the ipsilateral dorsal horns of the lumbal spinal cord sections recorded with the 20 × magnification objective. Quantitative analysis revealed a reduction of microglia (B) and astrocyte (D) signals in the ipsilateral dorsal horn after 2 weeks of 1 mg kg^{-1} BCP treatment (n=4-5 per group). **, treatment effect $p<0.01$; ***, surgery effect $p<0.001$. Means ± SEM are indicated.

side was significantly increased compared to ipsilateral sham values (Figure 8; p=0.0096). BCP treatment did not influenced the CB_2 receptor expression (treatment: $F_{1,29}$= 1.51; p=n.s).

3.6. Absence of psychoactive effects after BCP treatment

To determine if BCP exerts acute psychomimetic effects similar to CB_1 receptor agonists, we tested BCP-treated mice in the classical cannabinoid tetrad of tests (Figure 9). Oral administration of 1 and 10 mg kg^{-1} BCP did not produce any significant changes in the open-field activity ($F_{2,20}$=0.59, p=n.s.) or body temperature ($F_{2,20}$=1.3, p=n.s). It also produced no ring catalepsy ($F_{2,20}$=1.14, p=n.s.) and had no analgesic effect in the tail flick test ($F_{2,20}$=0.33, p=n.s.). In contrast, our reference compound Δ^9-THC elicited the typical cannabinoid-induced hypomotility ($F_{1,11}$=29.03, $p<0.001$), catalepsy ($F_{1,11}$=24.13, $p<0.001$) and hypothermia ($F_{1,10}$= 54.7, $p>0.0001$), although tail flick analgesia just failed to reach significance ($F_{1,11}$=4.51, p=0.057; data not shown).

4. Discussion

Here we demonstrate that the phytocannabinoid BCP, administered orally, exerts analgesic effects in animal models of inflammatory and neuropathic pain. The effects were absent in $CB_2^{-/-}$ mice and blocked by the CB_2 antagonist SR 144528. They were thus dependent on CB_2 receptor signaling. To our knowledge, this is the first demonstration that a common food ingredient is highly effective in animal models of chronic pain at physiologically relevant doses.

The plant volatile and food additive BCP is a selective natural CB_2 receptor agonist with no significant affinity for CB_1 receptors (Gertsch et al., 2008, 2010) Acute BCP treatment significantly reduced carrageenan-induced edema formation,

demonstrating that BCP is able to suppress inflammation via activation of CB_2 receptors. Our present results show that BCP is also efficacious in another inflammatory pain model, the formalin test, which is considered as a model of central pain sensitization (Beltramo et al., 2006). BCP had an analgesic effect in the late phase of formalin test after acute treatment. Anti-inflammatory and analgesic effects were similarly reported for other synthetic CB_2 receptor agonists like GW405833 (Clayton et al., 2002) and AM1241(Beltramo et al., 2006) AM1241 also produced analgesia in different chemical-induced (capsaicin, formalin) pain models and substance P-induced extravasation, when treated locally or systemically (Hohmann et al., 2004; Ibrahim et al., 2003). The CB_2-selective agonist O-3223 reduced inflammatory and neuropathic pain without any CB_1-dependent psychomimetic side effect (Kinsey et al., 2011). Our reference compound JWH-133 is a highly selective synthetic CB_2 agonist with full efficacy on rodent CB_2 receptor (Beltramo, 2009). However, there are several discrepancies in the literature considering its anti-inflammatory effect, which may depend on its poor bioavailability and overall pharmacokinetic properties. In this study, JWH-133, injected subcutaneously at a relatively high dose presented a significant analgesic effect in the formalin test during the 30 min observation period. This effect was restricted to the late phase and could be reversed by treatment of CB_2 receptor antagonist, SR 144528. Similar effects were observed with BCP, which in our hands showed a very pronounced analgesic effect and interestingly, we also needed higher SR 144528 concentration to antagonize the BCP effect than in case of our reference compound, JWH-133. A possible explanation could be that a natural agonist has a higher affinity to the CB_2 receptors and might improve the efficiency of CB_2 signaling (Shoemaker et al., 2005). We suppose that reduction of inflammatory processes by BCP and other CB_2 agonists directly contribute to their anti-nociceptive effects in different inflammatory pain models.

Neuropathic pain produces similar symptoms of hyperalgesia, allodynia, and spontaneous pain as peripheral tissue inflammation, but the underlying pathological processes are induced by nerve injury (Devor, 2006). Pharmacotherapies for neuropathic pain using morphine, THC, or a combination

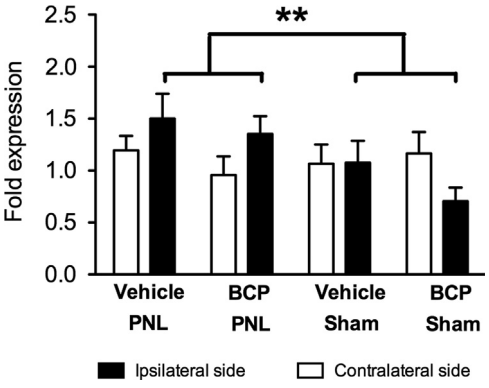

Figure 8 PNL induced a significant up-regulation of the CB_2 mRNA level. Expression of the CB_2 receptor gene was determined by quantitative RT-PCR in the lumbar spinal cord dorsal horn. CB_2 expression increased in the ipsilateral side after PNL, but was not affected by BCP treatment. **, PNL effect $p=0.0096$, $n=4$–5 per group, means+SEM are indicated.

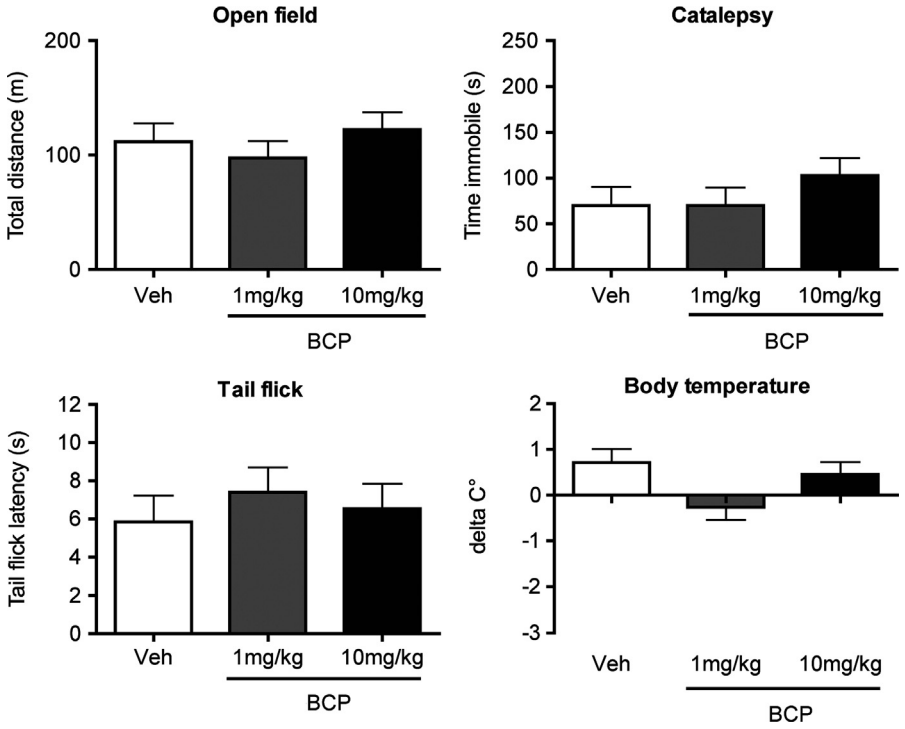

Figure 9 Treatment with BCP did not elicit psychoactive side effects in $CB_2^{+/+}$ mice. Mice were tested 45 min after an acute oral administration of 1 and 10 mg kg^{-1} BCP or vehicle (olive oil) in the classical cannabinoid tetrad for motor activity in the open-field, immobility on a ring (catalepsy), anti-nociception in the tail flick test and hypothermia ($n=7$–8 per group). Means+SEM are indicated.

of THC and cannabidiol (Sativex) produce inadequate pain relief and at higher doses they have unwanted psychoactive side effects (Berman et al., 2004; Naef et al., 2003). It is now well-documented that peripherally expressed CB_2 receptors play an important role in the development of neuropathic pain and can be targeted to reduce chronic pain associated with nerve injury (Beltramo, 2009; Guindon and Hohmann, 2008). Our data demonstrate that chronic BCP treatment reduces mechanical allodynia and thermal hyperalgesia in a model of neuropathic pain. Importantly, we found no signs of tolerance during the 2-week administration period. On the contrary, the BCP effect became stronger during the treatment period. As reported for other CB_2 agonists (Kinsey et al., 2011; Rahn et al., 2011), BCP did not induce any of the CB_1 receptor-specific psychomimetic responses, like hypomotility, catalepsy, hypothermia, or tail flick analgesia.

The synthetic CB_2 selective agonists NESS400, AM1241 and GW405833 dose-dependently attenuated tactile and thermal hypersensitivity produced by spinal nerve ligation and a spared nerve injury model (Beltramo et al., 2006; Ibrahim et al., 2003). Similar to our results, NESS400 diminished the nerve injury induced inflammatory processes in the spinal projection area, like microglia and astroglia activation, further increased expression of anti-inflammatory interleukin (IL)-10 and reduced expression of IL-1ß and interferon (IFN)-γ (Luongo et al., 2010). Intrathecal administration of JWH-133 reversed partial sciatic nerve ligation-induced mechanical allodynia and this effect was completely abolished in $CB_2^{-/-}$ animals (Yamamoto et al., 2008). Together these findings clearly demonstrated that CB_2 receptor activation alone is sufficient for attenuation the neuropathic pain symptoms and suggest a role of spinal CB_2 receptors for amelioration the neuropathic pain symptoms.

Microglia are thought to initiate the neuropathic pain processes by the release of proinflammatory chemokines and cytokines that in turn activates astrocytes and leads to further microglia activation (Tanga et al., 2004). In an earlier study we could show that CB_2 activation decreases spinal microglia and astrocyte activation in the spinal cord after nerve injury (Racz et al., 2008). Latest studies have shown that in the neuropathic pain processes, microglia activation decreases to baseline after three weeks, though astrocytes activation and hypersensitivity remains (Hald et al., 2009; Tanga et al., 2004). In contrast, $CB_2^{-/-}$ mice showed an exaggerated glial response that spread to the contralateral dorsal horn (Racz et al., 2008). BCP treatment now reduced expression of microglia and astrocytes markers in the dorsal horn of lumbar spinal cord, which is in line with the reduced hypersensitivity detected in the behavioral tests. These findings support our hypothesis that BCP exerts its anti-inflammatory properties through attenuation of the spinal glia activation. It was previously shown that BCP inhibits TH1 cytokines in LPS stimulated whole blood[18].

For thermal hyperalgesia, a low dose of BCP, 1 mg kg^{-1} was more efficacious than higher doses, thus probably reflecting a bell shaped dose-response curve, which is commonly observed with cannabinoid receptor agonists (Calabrese, 2008; Malfait et al., 2000; Sulcova et al., 1998). The mechanisms underlying this phenomenon are still not clarified. It is possible that dose-dependent changes in receptor occupation result in a differential activation of intracellular signaling cascades and thus in distinct physiological outcomes (Beltramo et al., 2006; Sulcova et al., 1998). Indeed, there is evidence that 2-AG induced ERK-MAPK phosphorylation with low ED_{50}, stimulated Ca^{2+} transients with a higher ED_{50}, and inhibited adenylate cyclase with highest ED_{50} (Shoemaker et al., 2005).Mechanical hyperalgesia, which is clinically more relevant, did not show such a pronounced dose dependency. It is thus conceivable that thermal hyperalgesia and mechanical allodynia, which are mediated by different nociceptive neurons, are differently affected by CB_2 agonism.

BCP is the first natural CB_2 receptor agonist, which could orally reduce inflammatory responses in different animal models of pain. Recently it was shown that BCP inhibits colon inflammation and protects from cisplatin-induced nephrotoxicity via CB_2 receptors (Bento et al., 2011). An important question to be considered now is whether or not this dose is relevant to humans? It has been suggested to calculate the human dose equivalent based on normalization of the body surface areas (Reagan-Shaw et al., 2008), which means that an effective dose in mice has to be divided by 12.3 in order to estimate the human equivalent dose of a 60 kg adult. Thus, a human daily dose of 0.08–0.41 mg/kg BCP would correspond to the optimal dose in mice. We have previously estimated an average daily BCP intake in the range of 10–200 mg, which corresponds to a dose of 0.16–3.3 mg/kg for a 60 kg human. This dose would certainly be sufficient for significant CB_2 cannabinoid receptor activation. Thus, it is likely that BCP belongs to a group of common plant natural products with major potential impact on human health. The oral intake of this dietary cannabinoid with vegetable food could be advantageous in the daily routine clinical practice over synthetic cannabinoid agonists.

Role of funding source

This work was supported by Grants from the Bundesministerium für Forschung und Lehre (NGFN2 FKZ 01GS0853) and the Deutsche Forschungsgemeinschaft (FOR926). The sponsors had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

IR and AZ designed the experiments. AK, IR and AZ drafted the manuscript. AK and IR performed the experiments and the data analysis. AM and BP contributed to the experiments. AK and AMZ performed tetrad test. JG provided the BCP and read critically the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank Eva Drews for critically reading the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2013.10.008.

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