The role of the cannabinoid receptor 2 in the modulation of neuropathic pain

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Summary

Neuropathic pain is a debilitating, chronic pain condition associated with inflammation or injury of peripheral and central nerves (Milligan and Watkins, 2009). The existing pharmacotherapies either lead to an inadequate pain relief or have psychomimetic side effects. Therefore, selective agonists targeting the peripherally expressed cannabinoid receptor 2 (CB2) came into the focus for the treatment of neuropathic pain and a number of studies have shown that CB2 is critically involved in the modulation of neuropathic pain responses. Recently, the widespread plant volatile ß-caryophyllene (BCP) was identified as a natural, selective CB2 receptors agonist, which is found in relatively high concentrations in many spice and food plants.

The present study demonstrates that orally administered BCP ameliorated thermal hyperalgesia and mechanical allodynia in mice suffering from neuropathic pain with no cannabinoid-induced psychomimetic side effects. The pharmacological effects of BCP were mediated by CB2 receptors. Similarly, histological analyses revealed that BCP reduced the inflammatory response (glia activation) in the dorsal horns of lumbar spinal cord. Thus, the natural plant product BCP may be highly effective in the treatment of long lasting, debilitating pain states and highlights the role of dietary factors in the development and modulation of chronic pain conditions.

Further, a second model for peripheral nerve injury was established. Repeated treatment with the chemotherapeutic agent paclitaxel induced a robust decrease of mechanical withdrawal latencies in CB2 receptor knockout mice. Wild type littermates in contrast showed less signs of neuropathic pain. These results suggest that CB2 receptor activation might protect against chemotherapeutic agent-induced neuropathic pain.

Taken together, both experimental approaches emphasize the important role of CB2 receptors in the control of neuropathic pain and their relevance as a possible therapeutic target to improve current neuropathic pain therapies.

Abbreviations

2-AG	2-arachidonoylglycerol
AEA	anandamide/N-arachidonoylethanolamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
B	basal
BCP	β-caryophyllene
BDNF	brain-derived neurotrophic factor
bsa	body surface area
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CB	cannabinoid
CGRP	calcitonin gene-related peptide
CNS	central nervous system
COX	cyclooxygenase
DABCO	1,4-diazabicyclo[2.2.2]octane
DAGL	diacylglycerol lipase
DEPC	diethyl pyrocarbonate
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleoside triphosphate
DTT	dithiothreitol
e.g.	for example
EAA	excitatory amino acid
ERK	extracellular signal-related kinase
etc.	et cetera
FAAH	fatty acid amide hydrolase
FAK	focal adhesion kinase
Fisher-LSD	Fisher's Least Significant Difference
GABA	γ-amino butyric acid
GFAP	glial fibrillary acidic protein
HCL	hydrochloric acid
HED	human equivalent dose
i.p.	intraperitoneal

I.R.	infrared
Iba1	ionized calcium-binding adapter molecule 1
IHC	immunohistochemistry
IL	interleukin
INF	interferon
iNOS	inducible nitric oxide synthase
JNK	Jun N-terminal kinase
kg	kilogram
ko	knock-out
LPS	lipopolysaccharide
LTP	long term potentiation
M	molar
MAGL	monoacylglycerol
MAPK	mitogen-activated protein kinase
mg	milligram
mGluR	metabotropic glutamate receptor
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
n	sample size
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
NF-κB	nuclear factor-κB
NK	neurokinin
NMDA	N-methyl-D-aspartate
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyd
PG	prostaglandin
PKC	protein kinase C
PNL	partial nerve ligation
PTN	pain transmission neuron
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
sec	seconds
SDS	sodium dodecyl sulfate

SEM	standard error of the mean
T	temperature
TLR	toll-like receptor
TNF	tumor necrosis factor
TRIS	tris (hydroxymethyl) aminomethane
TrkB	tyrosinekinase receptor B
WT	wild type
Δ^9-THC	Δ^9-tetrahydrocannabinol

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1 INTRODUCTION

Under normal conditions, the sensation of pain serves as a warning signal for the body to alert us to real or potential tissue damage. It triggers withdrawal responses and helps to protect the injured body part, which prevents from further tissue damage and facilitates healing. Under certain conditions pain can become a chronic state. Along with this, its warning function is lost and pain becomes a disease itself. If chronic pain is “caused by a lesion or disease of the somatosensory nervous system” (definition of the *International Association for the Study of Pain*; http://www.iasp-pain.org/AM/Template.cfm?Section=Pain_Definitions#Nociceptive neuron) either in the periphery or at central sides it is referred to as neuropathic pain. As neuropathic pain can arise after any condition, which is associated with nerve injury, the causes are wide spread and include mechanical injury or surgery, diabetes, infection (Herpes zoster, HIV) and cancer; or pain can occur as a side effect of chemotherapeutic treatment (Costigan et al., 2009). According to the *German Research Network on Neuropathic pain* (http://www.neuro.med.tu-muenchen.de/dfns/uns/e_portrait.html) about 5 million people in Germany are suffering from neuropathic pain. These patients often describe the quality of their pain as burning, needle prick or electric shock-like or like walking on glass (*American chronic pain association*; http://www.theacpa.org/conditionDetail.aspx?id=29). Furthermore, this pain can occur spontaneous without obvious external reason, as the nerve injury leads to a pain facilitating state at peripheral, spinal and supraspinal sites. Due to a modified skin sensitivity normally innocuous stimuli like pressure and cold can lead to a pain sensation (mechanical or cold allodynia). Noxious stimuli (e.g. heat) in turn lead to an exaggerated pain response (hypersensitivity) (Costigan et al., 2009).

The treatment of neuropathic pain is still a challenging issue as the common painkillers (ibuprofen, diclofenac, or paracetamol) are ineffective. Pharmacological treatments commonly comprise tricyclic antidepressants, anticonvulsants, opioids and *N*-methyl-D-aspartate (NMDA) receptor antagonists. Often combination therapies are used to target different receptors and thereby increase the analgesic effects (Gilron et al., 2006). But still, a realistic goal for the treatment of neuropathic pain is to archive a pain relieve of about 30-50 % (*German Research Network on Neuropathic pain*; http://www.neuro.med.tu-muenchen.de/ dfns/patienten /Def_NeP.html#the). Thus, the need for the development of new, effective drugs is immense.

1.1 Pain processing

Acute pain transmission is initiated by the activation of specialized receptors at the peripheral nerve terminal of primary nociceptive neurons (nociceptors) by specific stimuli like heat, pressure or chemicals. Nociceptors convert the stimulus into an electrical potential, which is transmitted through the axon from the periphery to the dorsal horn of the spinal cord (see Figure 1a; Milligan and Watkins, 2009).

There are three major groups of primary sensory neurons: large, myelinated Aα and Aβ fibers, lightly myelinated Aδ fibers and unmyelinated C fibers. Large diameter Aα and Aβ fibers are fast-conducting and mainly respond to innocuous stimuli like vibration and light touch, but during disease condition they can also transmit pain signals (Julius and Basbaum, 2001). Medium diameter Aδ and small diameter C fibers respond to noxious mechanical, thermal and chemical stimuli and project to second-order pain-projection neurons and nociceptive interneurons in lamina I, IV and V in the dorsal horn. Second-order pain-projection neurons either ascend on the ipsilateral side or decussate to the contralateral side at the spinal cord level and join the ascending anterolateral system to the brain stem and brain, e.g. thalamic nuclei, primary and secondary somatosensory cortex and association cortex (Milligan and Watkins, 2009).

1.2 Pathological pain processing: neuropathic pain

After lesion or disease of the somatosensory system, a sustained nociceptive input can trigger a maladaptive plasticity in the nervous system that leads to the development of chronic neuropathic pain (Costigan et al., 2009). Peripheral nerve injury induces several pathological changes in the injured and neighboring neurons at peripheral and central sides.

In the periphery, the injured tissue releases several inflammatory mediators. This “inflammatory soup” includes bradykinin, serotonin, prostaglandins, adenosine triphosphate (ATP) and protons, which increase the sensitivity and excitability of the nociceptive neurons either directly or by activation of intracellular signaling pathways (Julius and Basbaum, 2001; Ji et al., 2003). Calcitonin gene-related peptide (CGRP) and substance P induce hyperaemia and swelling. This facilitates the invasion of immune cells guided by chemokines to the side of injury. Immune cells in turn release pro-inflammatory mediators like prostaglandins and cytokines, e.g. interleukin (IL)-1β, IL-6, interferon-γ (INF-γ) and tumor necrosis factor α (TNFα) (Scholz and Woolf, 2007). The pro-inflammatory mediators account for hypernociception by either neurotoxic effects (Keswani et al., 2003) or by increasing spontaneous fiber activity and lowering activation thresholds (Schäfers et al., 2003; Cunha et al., 2005).

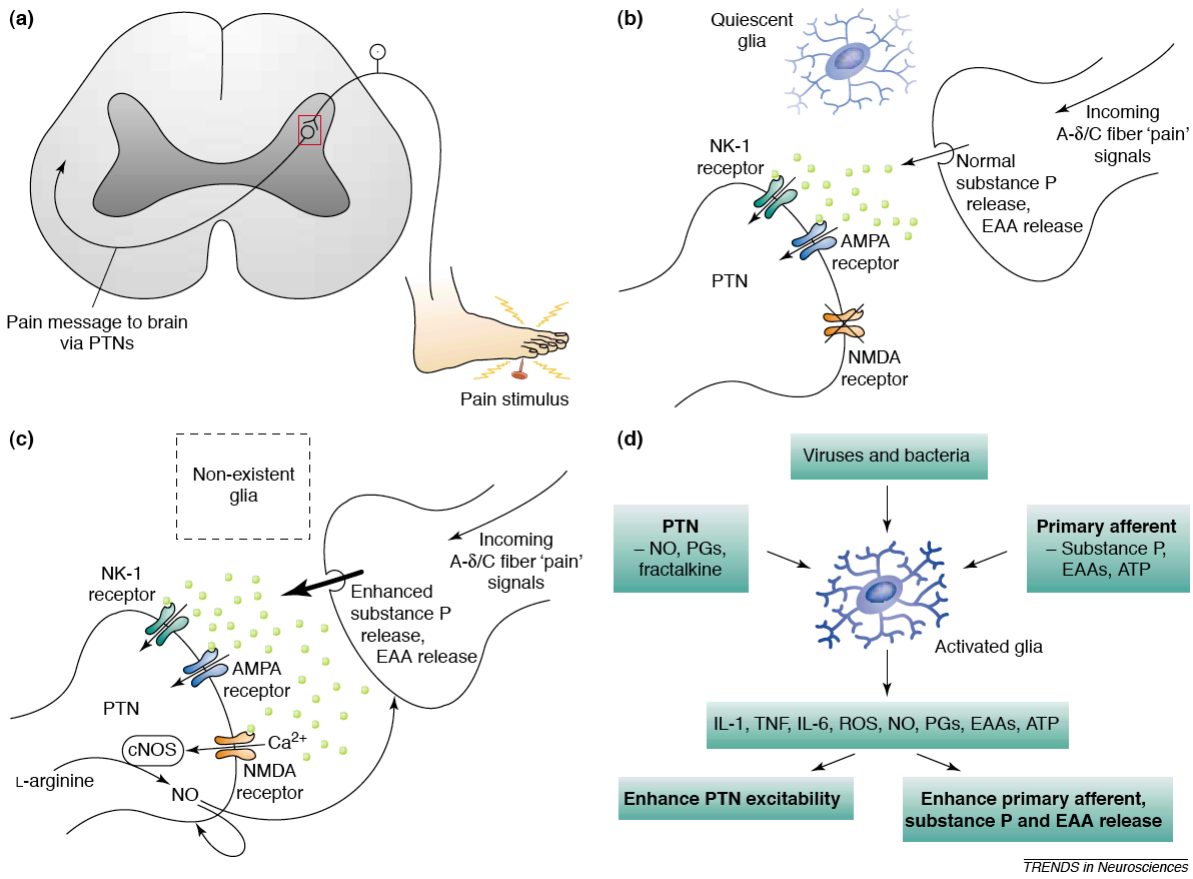


Figure 1 **Normal and pathological pain processing. (a)** Under normal conditions an acute pain stimulus, e.g. a pin prick, evokes an electrical signal that is transmitted by A-δ and C fibers to pain transmission neurons (PTNs) in the dorsal horn of the spinal cord, which then project to their supraspinal targets. **(b)** At spinal cord level, incoming signals from the periphery lead to a moderate release of excitatory amino acids (EAAs) and substance P from the presynaptic neuron. EAAs and substance P open α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and neurokinin receptor 1 (NK-1) receptor ion channels leading to a depolarization of the postsynaptic neuron. **(c)** Under pathological conditions a sustained input from the periphery leads to an enhanced excitatory amino acid (EAAs) and substance P release and the pain transmission neuron (PTN) is strongly depolarized. This unplugs magnesium (Mg^{2+}) from NMDA receptors and allows calcium (Ca^{2+}) to enter the neuron. An increase in intracellular calcium activates the production of nitric oxide (NO) by nitric oxide synthase. NO acts as a retrograde messenger and leads to a further release of EAAs from the presynaptic neuron. **(d)** Glia cells are activated by ATP, prostaglandins (PGs), NO, substance P and EAAs. They contribute to the development of neuropathic pain by the release of pro-inflammatory cytokines and further NO, prostaglandins, EAAs and ATP, which enhance the PTN excitability and the presynaptic release of EAAs. - Reprinted from Trends in Neurosciences, Volume 24, Linda R. Watkins, Erin D. Milligan, Steven F. Maier, Glial activation: a driving force for pathological pain, 450-455, Copyright (2001), with permission from Elsevier.

In the spinal cord, repetitive synaptic input from the periphery leads to alterations in the neuronal and biochemical processing, which leads to a pain-facilitating state in the dorsal horn referred to as central sensitization (Woolf, 1983). Central sensitization shares striking similarities with the long-term potentiation (LTP) in the hippocampus during learning and memory formation (Ji et al., 2003). Therefore, it is colloquially also named as the “pain memory”.

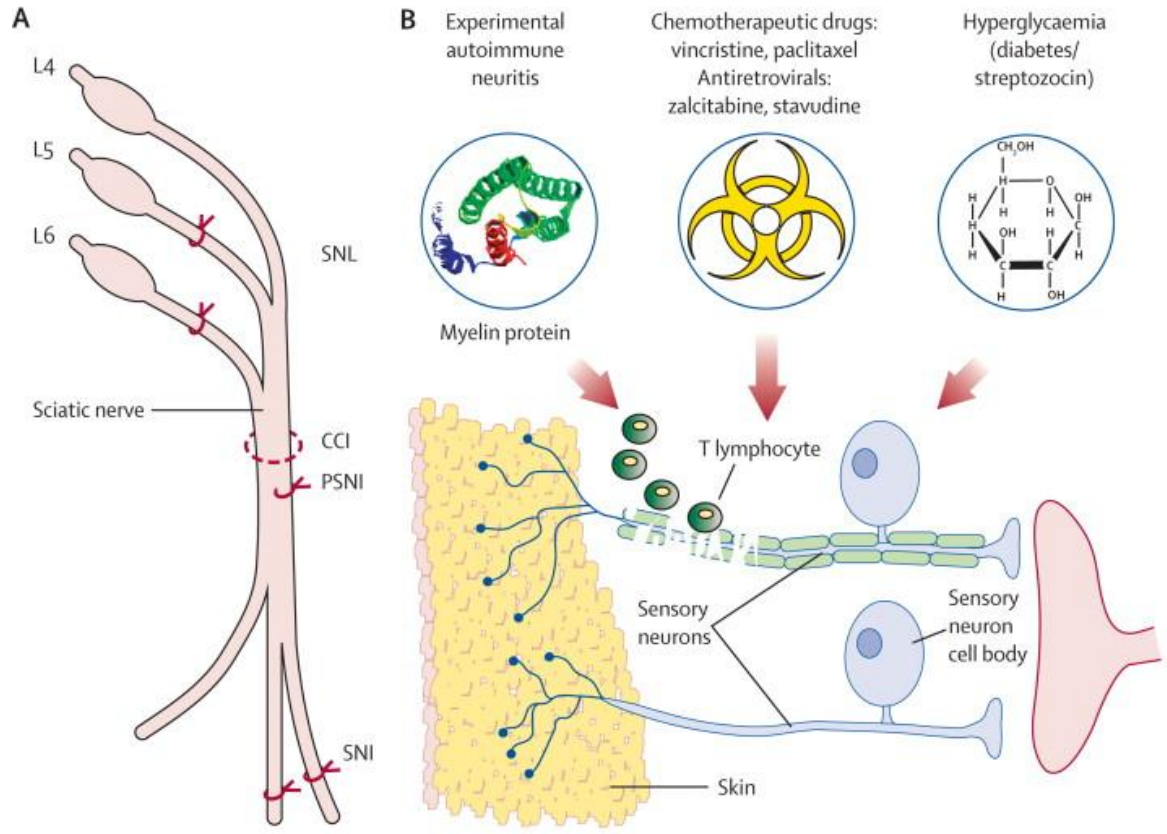
The early phase of central sensitization is characterized by activity-dependent plasticity. A co release of glutamate, substance P and CGRP leads to a strong and sustaining depolarization of the postsynaptic terminal, magnesium is removed from voltage-dependent NMDA receptors and allows a calcium influx into the neuron (see Figure 1c; Milligan and Watkins, 2009). Activation of NMDA receptors plays a key role in central sensitization, e.g. shown by a reduction of injury-induced pain in mice with a deletion of the NMDAR NR1 subunit (South et al., 2003). The increase in intracellular calcium activates e.g. the production of NO by nitric oxide synthase. NO acts as a retrograde messenger and leads to a further release of transmitter from the presynaptic neuron (Watkins et al., 2001). High nociceptive input through the multiple receptors expressed on dorsal horn neurons (AMPA, NK1, tyrosinkinase receptor B (TrkB), metabotropic glutamate receptors (mGluR)) results in the activation of intracellular kinases, like protein kinase C (PKC), extracellular signal-related kinase (ERK) and p38 that alter ion channel kinetics and properties (Ji and Woolf, 2001; Ji et al., 2003).

Secondary, a loss of inhibitory transmission is also contributing to the development of chronic pain. Brain-derived neurotrophic factor (BDNF), binding to TrkB receptors, acts on dorsal horn lamina I neurons causing a shift of the anion reversal potential. After this, γ-amino butyric acid (GABA), released by inhibitory interneurons, leads to a paradoxical excitation and spontaneous activity of lamina I neurons (Coull et al., 2005, Keller et al., 2007). Similarly, a degeneration of inhibitory GABAergic interneurons contributes to the development of pain (Scholz et al., 2005).

Late-onset changes involve the activation of transcription factors and transcription of several genes, leading to structural changes. The receptor density, synthesis of ion channels and the production of prostaglandin E_2 (PGE_2) through cyclooxygenase 2 (COX2) is increased (Woolf and Salter, 2000; Ji and Woolf, 2001; Ji et al., 2003). PGE_2 enhances the excitability of neurons to incoming stimuli and stimulate a further release of neurotransmitter (Ji et al., 2003). At this stage originally non-nociceptive Aβ fibers can also signal pain after mechanical stimulation, possibly also due to sprouting of these fibers to lamina II (Costigan et al., 2009).

Taken together, these events manifest in symptoms like spontaneous pain, hypersensitivity and mechanical and cold allodynia (Costigan et al., 2009).

Box 1 Animal models of neuropathic pain



A Traumatic nerve injury models: SNL (spinal nerve ligation), where the L5 and L6 nerves are ligated; CCI (chronic constriction injury), where several loose ligations of the sciatic nerve are made; PSNL or PNL (partial sciatic nerve ligation), where 1/3 to 1/2 of the sciatic nerve is lightly ligated; SNI (spared nerve injury), where the common peroneal and tibial branches of the sciatic nerve are tightly ligated. **B** Disease or treatment-induced neuropathic pain models: Experimental autoimmune neuritis, where nerves are sensitized after immunisation with myelin or myelin proteins; Chemotherapeutic or antiretroviral drug treatment, which is neurotoxic for sensory nerves; diabetes-associated neuropathic pain, where diabetes is induced by streptozocin administration. - Reprinted from The Lancet, Volume 11, Margarita Calvo, John M Dawes, David LH Bennett, The role of the immune system in the generation of neuropathic pain, 629-642, Copyright (2012), with permission from Elsevier.

1.3 The dual role of glia cells in neuropathic pain

More evidence is arising that glia cells contribute to the development of neuropathic pain, as activation and proliferation of glia cells in the dorsal horn of the spinal cord parallels the symptoms of neuropathic pain (Coyle, 1998).

Glia cells are non-conducting, supporting cells and constitute 70 % of total cell population in the central nervous system (CNS) (Vallejo et al., 2010). They are divided into three subtypes: oligodendrocytes, astrocytes and microglia (Vallejo et al., 2010). Oligodendrocytes are responsible for the myelination of neuronal axons, while astrocytes encapsulate the synapses and stay in close contact with several neurons (Milligan and Watkins, 2009). Similar to neurons, astrocytes express several neurotransmitter receptors and voltage-gated ion channels and modulate synaptic neurotransmission by the release of neurotransmitters (e.g. glutamate and ATP) (Hald, 2009). Additionally, they maintain glutamate homeostasis by clearing glutamate from the synaptic cleft through glutamate transporter 1 and glutamate-aspartate transporter (De Leo et al., 2006). Resident microglia, which are bone marrow-derived haematopoietic cells, are the primary immune cells of the CNS and ubiquitously distributed throughout the brain and spinal cord (Inoue and Tsuda, 2009). While the cell bodies of microglia remain quiescent, the cell processes are highly motile while surveying the CNS for pathogens, damage or injury (Nimmerjahn et al., 2005). Microglia also express several neurotransmitter receptors and can be activated by ATP, NO, substance P and pro-inflammatory cytokines (Milligan and Watkins, 2009). Due to their immune cell function microglia are also activated by the chemokine fractalkine (CX_3CL_1) through chemokine receptor CX_3CR_1 and Toll-like receptors (TLR) that are fundamental in recognizing lipids, carbohydrates, peptides and nucleic acids of pathogens (Scholz and Woolf, 2007; Nicotra et al., 2012). Especially TLR 4 and TLR 2 are key mediators of glia activation in the CNS after nerve injury (Tanga et al., 2005). Thus, it is not astonishing that activated microglia are early hallmarks of any disease in the CNS (Kreutzberg, 1996).

Activated glia cells contribute to the development of neuropathic pain by the release of pro-inflammatory cytokines like TNFα, IL-1β, IL-6 as well as BDNF and nuclear factor-κB (NF-κB), which are released after intracellular p38 and ERK MAPK activation (Cao and Zhang, 2008; Ji et al., 2009). Pro-inflammatory cytokines like TNFα, IL-1β and IL-6 either mediate their action through direct modulation of neurons or indirect through activation of immune cell signaling pathways (Thacker et al., 2007). BDNF acts on dorsal horn lamina I neurons leading to an inversion of inhibitory GABA currents (Coull et al., 2005), while NFκB activation induces the synthesis of NO and PGE_2, which further sensitize the dorsal horn neurons (Cao and Zhang, 2008). In astrocytes, the glutamate transporters become dysregulated and a decrease in glutamate reuptake could contribute to an increased synaptic

input (Ji et al., 2006). Consequently, inhibition of glia metabolism was shown to attenuate pain-related behavior in mouse models of neuropathic pain (Scholz and Woolf, 2007).
Analysis of the time course of glia activation revealed that the activation of microglia precedes and most likely initiates astrogliosis. Thus, it was suggested that microglia are crucial for the development of neuropathic pain, while astrocytes maintain the prolonged pain state (Hald et al., 2009).

However, the contribution of glia cells is more complex than being just the “bad guys”. It was also shown that glia cells have protective roles in neurodegenerative diseases, although their function here is still not fully understood. As immunocompetent cells they are responsible for clearing debris, for fighting infections by phagocytosis as well as for repairing processes (Streit, 2009). Weather glia activation becomes dysfunctional or beneficial, seems to depend on the context of activation. Studies with TNFα knockout mice showed that a lack of TNFα produces a late, exaggerated microglia response in mice exposed to acute nitric oxide excitotoxicity (Turrin, 2006). Thus, depending on the timing and context of TNFα action it may also be crucial for neuroprotection. Next, appropriate phagocytosis of apoptotic cells by glia was shown to suppress lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines, suggesting that phagocytosis and clearance are beneficial (Tassiulas, 2009). Finally, glia cells also release some cytokines, which are associated with beneficial actions during pathological pain conditions. IL-4 and IL-10 are known to reverse pathological pain and promote neuronal survival (Milligan and Watkins, 2009).

Due to their critical role in the development and maintenance, but also clearance of chronic pain states, glia cells display an interesting target for the treatment of pain. Recent evidences verified that ligands of the endocannabinoid system can modulate glia activation and in parallel decrease pain (see next chapters 1.4 and 1.5; Cabral and Marciano-Cabral, 2005; Cabral and Griffin-Thomas, 2009).

1.4 The endocannabinoid system

The discovery of Δ^9-tetrahydrocannabinol (Δ^9-THC) as the major psychoactive compound in the essential oils of *Cannabis Sativa L.* in 1964 by Raphael Mechoulam and colleagues (Gaoni and Mechoulam, 1964) and finally the discovery and cloning of the cannabinoid receptor 1 (CB1) in 1990 (Matsuda et al., 1990) followed by the cannabinoid receptor 2 (CB2) in 1993 (Munro et al., 1993) triggered important advances in endocannabinoid research.

The endocannabinoid system comprises a signaling system including receptors, endogenous ligands and enzymes for synthesis and degradation of the endogenous ligands (see Figure 2). It is involved in the “fine tuning” of several processes in the body including

memory, movement, food intake, nociception and inflammation (Di Marzo and Matias, 2005; Hohmann and Suplita, 2006; Burstein and Zurier, 2009; Marsicano and Lafenêtre, 2009; El Manira and Kyriakatos, 2010).

The two cannabinoid receptors are $G_{i/o}$-protein coupled receptors with seven transmembrane-domains. The CB1 receptor is one of the most abundant neuromodulatory receptors in the brain. It is expressed presynaptically on peripheral and central nerve terminals, especially in hippocampus, cortex, cerebellum, basal ganglia and brainstem (Wilson and Nicoll, 2002). The CB2 receptor is mainly expressed on peripheral immune cells, but was also identified on microglia in the CNS (Facci et al., 1995; Howlett et al., 2004; Maresz et al., 2005; Kleyer et al., 2012). CB2 receptor expression was also reported to be up regulated in astrocytes under pathological conditions (Benito et al., 2007; Garcia-Ovejero et al., 2009).

In 1992 the arachidonic acid-derived lipid N-arachidonoylethanolamide (AEA; anandamide) was identified as the first endogenous ligand (endocannabinoid) for cannabinoid receptors (Devane, 1992). AEA is found in low amounts in the brain and is only a partial agonist for both receptors (Katona and Freund, 2012). Thus, it was likely that there should be another endogenous ligand with full agonist activity. Indeed, in 1995 2-arachidonoylglycerol (2-AG) was identified as the second endogenous ligand, which is a full agonist for CB1 and CB2 receptors (Mechoulam et al., 1995). Moreover, 2-AG occurs in larger amounts in the brain and is now postulated to be the key endocannabinoid at central synapses (Katona and Freund, 2012). Both endocannabinoids are produced on demand, triggered by an increase in intracellular Ca^{2+} and activation of mGluRs in postsynaptic neurons (Straiker and Mackie, 2006; Katona and Freund, 2008). AEA is synthesized by a Ca^{2+} sensitive N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), while 2-AG is synthesized by the the diacylglycerol lipases (DAGL) α and β (Piomelli, 2003). Endocannabinoids then travel retrogradely to activate presynaptically located CB1 receptors. As retrograde messengers they act as synaptic circuit breaker by closing calcium channels and opening potassium channels and thereby decreasing the presynaptic neurotransmitter release (Katona and Freund, 2008). For CB2 receptors the picture is yet not that clear if they modulate calcium transients (Beltramo, 2009), but recent reports suggest that CB2 receptor activation increases intracellular calcium (Shoemker et al., 2005; Gertsch et al., 2008).

Typical for $G_{i/o}$-protein coupled receptors, CB1 and CB2 receptor activation leads to an inhibition of adenylate cyclase activity and subsequent inhibition of cyclic adenosine monophosphate (cAMP) accumulation. Additionally, cannabinoid receptor activation induces MAPK signaling pathways like ERK and FAK (focal adhesion kinase) in the case of CB1 receptor activation and ERK1/2 and p38 in the case of CB2 receptor activation (Piomelli, 2003; Beltramo, 2009).

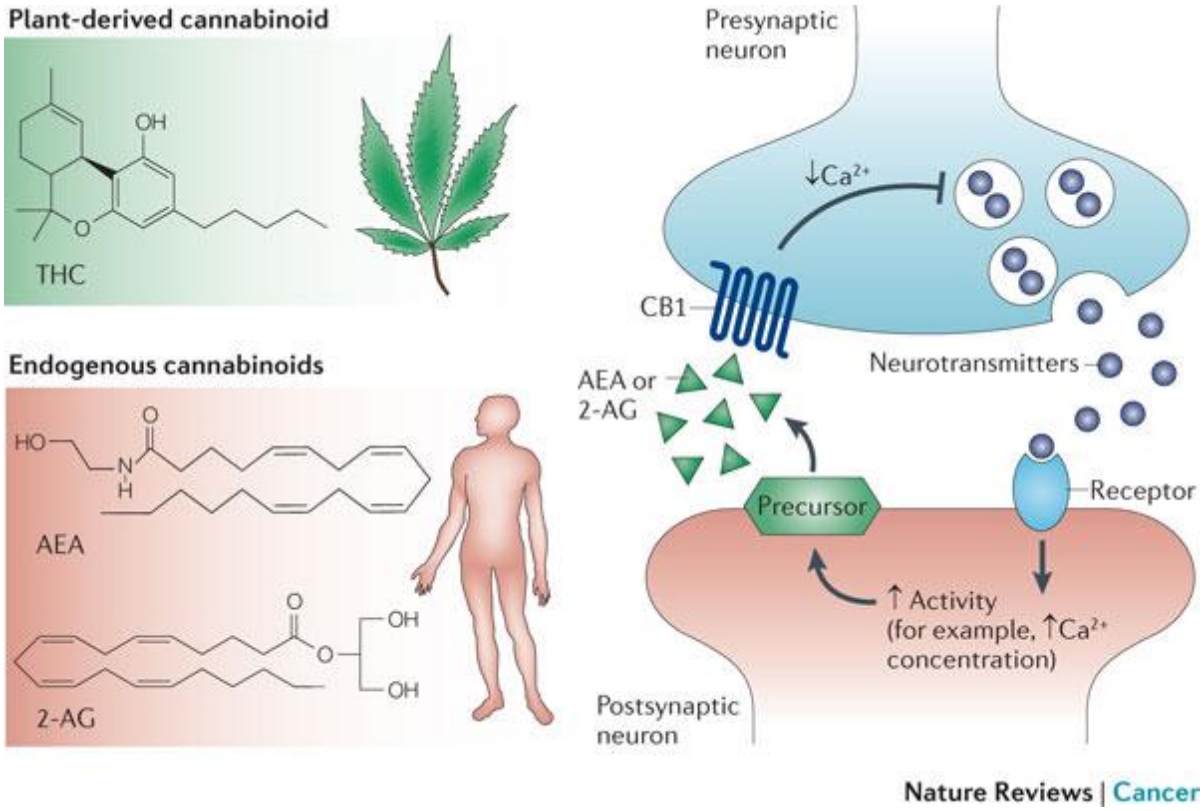


Figure 2 The endocannabinoid system. Plant-derived cannabinoids like Δ^9-tetrahydrocannabinol (Δ^9-THC) and endogenous cannabinoids like AEA and 2-AG bind to cannabinoid receptors 1 and 2 on neurons and immune cells (not depicted here). Both endogenous cannabinoids are produced after an increase in intracellular Ca^{2+} in postsynaptic neurons. As retrograde messengers, endocannabinoids travel to presynaptically expressed cannabinoid receptors to activate them. Activation of cannabinoid receptors leads to an inhibition of voltage-gated Ca^{2+} channels and the activation of K^+ channels. The depolarization of the presynaptic neuron is thereby diminished and consequently also the release of neurotransmitter. After clearance of endocannabinoids from the synaptic cleft, they are degraded by fatty acid amide hydrolase or monoacylglycerol to arachidonic acid and ethanolamine (not depicted here). - With permission from Macmillan Publishers Ltd: Nature Reviews Cancer, Volume 12, Guillermo Velasco, Cristina Sánchez and Manuel Guzman, Towards the use of cannabinoids as antitumour agents, 436-444, Copyright (2012).

Endocannabinoid signaling is terminated by the endocannabinoid degrading enzymes monoacylglycerol (MAGL) and fatty acid amide hydrolase (FAAH) (Piomelli, 2003).

Due to its central expression, CB1 receptor activation accounts for the psychomimetic side effects of cannabinoid ligands like Δ^9-THC on cognition, motor activity and memory (Wilson and Nicoll, 2002). The four major behavioral effects of cannabinoid intoxication, hypomotility, catalepsy on a ring, hypothermia and anti-nociception, are measured in the tetrad test (Martin et al., 1991).

In marked contrast, activation of CB2 receptors alone is devoid of psychomimetic side effects, while it similarly produces strong anti-nociceptive and anti-inflammatory effects under pathological conditions (Kinsey et al., 2011; Rahn et al., 2011).

1.5 The endocannabinoid system and pain

The analgesic properties of cannabis preparations are known since antiquity and were used in the treatment of various pain conditions (Zias et al., 1993). Recently, the endocannabinoid system came again into the focus for the treatment of pain. The localization of its receptors on peripheral nociceptive neurons and in several brain regions involved in pain signaling (Sañudo-Peña et al., 1999), as well as on immune cells (Maresz et al., 2005) made it an attractive therapeutic target. Clinical trials have already demonstrated beneficial effects of cannabis extracts and synthetic Δ^9-THC preparations in pain patients. Some of these substances have already been approved in selected countries for clinical pain management at doses and formulations that show only minor central side effects (Ben Amar, 2006; Iskedjian et al., 2007; Karst and Wippermann, 2009; Lynch and Campbell, 2011). Nevertheless, the potential for psychomimetic effects of Δ^9-THC containing medications is an important consideration that limits their clinical use. Thus, several approaches were exploited to decrease psychomimetic side effects in cannabinoid-based therapies:

Firstly, peripherally restricted cannabinoid agonists that only marginally cross the blood-brain-barrier were developed to circumvent central CB1 receptor activation. These compounds were shown to similarly exert anti-hyperalgesic effects in animal models of pain than common cannabinoid ligands (Dyson et al., 2005; Dziadulewicz et al., 2007; Wei et al., 2012). Secondly, inhibitors of the endocannabinoid degrading enzymes FAAH and MAGL were used to increase the endocannabinoid tone and thereby induce anti-nociception with no or minimal psychomimetic side effects (Jhaveri et al., 2006; Kinsey et al., 2009; Clapper et al., 2010; Schlosburg et al., 2010). Both approaches still do not fully exclude the risk of central mediated side effect. Finally, effort was put on the identification of selective agonists for the peripheral CB2 receptor, which completely lacks the liability of potential psychomimetic side effects. Indeed, reports that synthetic CB2 receptor agonists produce anti-nociceptive effects in animal models of acute and chronic pain are rising. Synthetic CB2 agonists like JWH-133, AM1241 and GW405833 among several others were shown to exert beneficial effects on both mechanical allodynia and thermal hyperalgesia in animal models of inflammatory and neuropathic pain. The specificity of these effects for CB2 receptors was confirmed with the use of CB2 receptor antagonists like SR144528 and AM630 (Guindon and Hohmann, 2008; Beltramo, 2009).

It has been also shown that CB2 receptor expression is up regulated in dorsal root ganglia and spinal cord tissue under neuropathic pain conditions, paralleling the activation of glia cells (Zhang et al., 2003; Hsieh et al., 2011). Activation of CB2 receptors in turn attenuates glia responses in the dorsal horn of the spinal cord (Romero Sandoval and Eisenach, 2007, Luongo et al., 2010 Leichsenring et al., 2009), which supports a immunomodulatory role of

CB2 receptors in pain. The use of genetically modified mice (Buckley et al., 2000; Rácz et al., 2008b) gave further insights into the role of CB2 receptors during the development of neuropathic pain. Thus, constitutive CB2 knockout mice with a partial ligation of the sciatic nerve exhibited pain not only in the ipsilateral paw, but also developed a mirror image of pain in the contralateral paw. This was accompanied by an exaggerated microglia and astrocyte response in the dorsal horn, spreading from the ipsilateral side to the contralateral side. Mice overexpressing the CB2 receptor in contrast showed a decreased manifestation of neuropathic pain (Rácz et al., 2008b). Consequently, evidence is growing that CB2 receptors are crucial mediators in the control of inflammatory responses during pathological pain. The same authors found that interferon-γ, expressed by neurons and astrocytes, is critically involved in this process, as CB2/interferon-γ double knockout mice lacked the mirror image of pain found in the CB2 knockout mice. The authors stated that CB2 receptor activation possibly diminishes the interferon-γ-induced neuroinflammatory processes, involving iNOS and chemokine receptor 2 expression in microglia (Rácz et al., 2008a).
Given the anti-nociceptive and immunomodulatory properties of CB2 receptors and their mainly peripheral distribution, they clearly form a promising target for the development of new drugs in the treatment of neuropathic pain.

1.6 The plant volatile β-caryophyllene

Some of the most widespread medicines, recreational drugs or poisons, most of them belonging to the group of alkaloids, are secondary metabolites from plants: morphine, nicotine, caffeine, cocaine, quinine, atropine, strychnine, cardiac glycosides, paclitaxel and Δ^9-THC among many others (Lüttge et al., 2005). However, beside these compounds with obvious pharmacological activity, evidence is rising that also secondary metabolites, which are so far mainly known for their aromatic or chromophore properties, are pharmacologically interesting (Kris-Etherton et al., 2002; Tapsell et al., 2006). *Cannabis sativa L.* plants are a rich source for such plant metabolites. Besides the well-known Δ^9-THC, the essential oil of cannabis comprises a variety of around 400 other secondary plant metabolites, including over 65 other cannabinoid-like components (Gertsch et al., 2008). So far, only three classical cannabinoids with a terpenophelic structure have been shown to directly act on CB1 or CB2 receptors (Pertwee, 2008), while other, structurally different metabolites (referred to as “phytocannabinoids”) were also shown to act on the endocannabinoid system, e.g. fatty acid derivatives and polyphenols (Gertsch et al., 2010). Still, there might be a variety of unexplored phytocannabinoids with therapeutic potential present in the essential oils of cannabis or other metabolite-rich plants. Indeed, it was found that the essential oil of

cannabis lacking the classical cannabinoids still presented cannabinoid receptor binding. Screening of the isolated constituents identified the sesquiterpene β-caryophyllene (BCP; see Figure 3) as the first natural, selective agonist for CB2 receptors with K_i values of the (E)-BCP isomer in the nM range (K_i = 155 ± 4 nM). The (Z)-BCP isomer also showed a slight binding affinity (K_i = 485 ± 36 nM), while the oxidation product BCP oxide and the opened-ring isomer α-humulene did not show binding affinities to CB2 receptors (Gertsch et al., 2008). Besides its occurrence in cannabis, BCP is among the most widespread plant volatiles and is also found in large amounts in the essential oils of different spice and food plants, such as oregano (*Origanum vulgare L*.), cinnamon (*Cinnamomum spp*.), clove (*Syzygium aromaticum*), rosemary (*Rosmarinus officinalis L*.), thyme (*Thymus serpyllum*) and black pepper (*Piper nigrum L*.) among many others (Zheng et al., 1992; Mockute et al., 2001; Hudaib et al., 2002; Jayaprakasha et al., 2003; Orav et al., 2004; Bernardes et al., 2010). Thus, it represents a novel dietary phytocannabinoid. BCP and related sesquiterpenes have insecticidal and anti-viral properties, and constitute part of the plant defense system against pathogens (Dunkić et al., 2011; Maffei et al., 2011; Huang et al., 2012). Due to its weak aromatic taste, it is also commercially used as a food additive (Gertsch et al., 2008). As such, it is approved by several independent agencies with no known toxicity (US Food and Drug Administration (FDA), US Flavor and Extract Manufacturers Association (FEMA), Joint FAO/WHO Expert Committee on Food Additives (JECFA)). Several health effects have been attributed to BCP or plants containing BCP, including anti-inflammatory (Gertsch et al., 2008), local anaesthetic (Ghelardini et al., 2001) and anti-carcinogenic (Legault and Pichette, 2007; Di Sotto et al., 2010) activity. The natural source of BCP makes it surely an interesting compound, also for the treatment of chronic pain conditions.

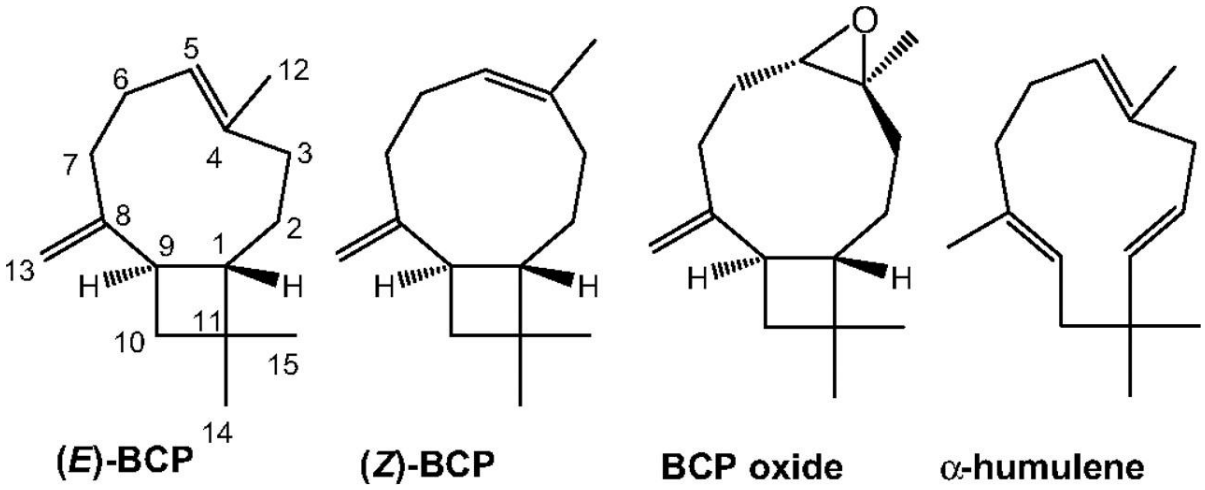


Figure 3 Chemical structure of the sesquiterpene BCP isomers: (E)-BCP and (Z)-BCP, their oxidation product BCP oxide and the opened-ring isomer α-humulene. From ©Gertsch et al., 2008 (PNAS).

1.7 Aims of the study

The recent years of research clearly indicated that the endocannabinoid system is critically involved in the modulation of chronic pain. However, cannabinoid-based therapies are so far often associated with central-mediated, adverse side effects and therefore constrain their therapeutic use. The plant volatile BCP was identified as the first natural, selective CB2 receptor agonist, which lacks the potential of side effects due to its mainly peripheral-located target. Earlier studies also verified its anti-inflammatory properties. Because there is especially a need for the evaluation of new and effective drugs for the treatment of neuropathic pain, one aim of this study was to evaluate the potential of BCP to exert anti-nociceptive effects in a model of neuropathic pain. Here, a mouse model of partial nerve ligation-induced neuropathic pain was used and the development of mechanical allodynia and thermal hyperalgesia after chronic treatment with different doses of BCP was analyzed. To exclude that BCP produces similar psychoactive side effects known from CB1 receptor agonists, the cannabinoid tetrad test was performed.

It is known that the appearance of activated glia cells in the spinal cord is associated with the occurrence of pain. Therefore, a further purpose of the study was to investigate the effect of BCP on the activation of glia cells in the spinal cord by immunohistochemistry. To get insights into signaling pathways possibly involved in chronic pain modulation by BCP, quantitative real-time PCR for several candidate molecules was performed.

In the next part of the study a second animal model for neuropathic pain was established. It is known that chemotherapeutic agents like cisplatin, vincristine or paclitaxel induce neuropathic pain symptoms in cancer patients. The mechanisms underlying the development of chemotherapeutic drug-induced neuropathic pain differ from those in traumatic nerve injury models. Therefore, a further aim of the study was to investigate the role of CB2 receptors in this neuropathic pain model. For the establishment of chemotherapeutic drug-induced neuropathic pain mice received injections of paclitaxel and the development of mechanical allodynia, thermal hyperalgesia and cold allodynia was measured in wild type and CB2 receptor knockout mice.

2 MATERIAL

2.1 Equipment

Equipment	Company
Open field arena	TSE Systems
Balance	BP 121S, Sartorius
Camera	AxioCam MRm, Zeiss
Catalepsy ring	In-house workshop
Centrifuges	Galaxy mini, VWR
	Biofuge fresco, Heraeus
	Multifuge 2 S-R, Heraeus
Cryostate	CM3050S, Leica
Vaporizer for isofluorane	ISOFLO, Eickmeyer
Hargreaves' apparatus	Ugo Basile
Hot/Cold plate	Ugo Basile
Incubator	BE-ED-FD, Binder
Magnetic stirrer	MR 3001K, Heidolph
Microscope	Axioplan 2, Zeiss
	Imager M2, Zeiss
PCR machine	iCycler, Bio-Rad Laboratories
pH-Meter	pH 515i, WTW
Rectal thermometer	Portable Thermometer BAT-12, Harvard Apparatus
Animal shaver	Isis GT420, Aesculap
Spectral photometer	91-ND-1000 UV/Vis, Nanodrop
Tail flick analgesia meter	Columbus Instruments, OH., USA
Fast real-time PCR system	7900HT, Applied Biosystems
Vortexer	Vortex Genie 2, Scientific Industries
Von Frey apparatus	Ugo Basile

2.1 Chemicals and Reagents

Substance	Company
0,9 % Saline	Braun
0.1 M DTT	Life Technologies
10 mM dNTP Mix	Life Technologies
5 x First Strand buffer	Life Technologies
Betaisodona	Mundipharma
Bromochloropropanol	Sigma-Aldrich
Bovine serum albumin	PAA
Cooling spray	Roth
Cremophor	Sigma-Aldrich
DABCO	Roth
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich
Ethanol	VWR
Fluoromount G	SouthernBiotech
Glycerol	Sigma-Aldrich
Guanidine HCL	Sigma-Aldrich
Hydrochloric acid	Sigma-Aldrich
Isofluorane	Abott GmbH
Isopentane	Sigma-Aldrich
Isopropyl alcohol	Roth/VWR
Mowiol	Roth
OCT Compound	Sakura Finetek
Oligo$(dT)_{12\text{-}18}$ Primer	Life Technologies
Olive oil	Sigma-Aldrich
Oxygen	Linde
PapPen	Vector Laboratories
Paraformaldehyd	Sigma-Aldrich
PBS tablets	Life Technologies
RNase free water	Life Technologies
RNAse OFF	Applichem
SDS	Sigma-Aldrich
Sodium hydroxide	Sigma-Aldrich
Superscript II Reverse Transcriptase	Life Technologies
TRIS-HCL	Roth

Tocrisolve 100	Tocris
Triton X-100	Sigma-Aldrich
Trizol	Life Technology

2.2 Solutions

2.2.1 Histological experiments

PFA/PBS solution		
Paraformaldehyde		4 % (w/v)
PBS		

Sucrose		
Sucrose		10 % (w/v)
	or	20 % (w/v)
PBS		

Permeabilization solution	
Triton X-100	0.5 % (v/v)
PBS	

Blocking solution	
Normal donkey serum	10 % (v/v)
Bovine serum albumin (BSA)	3 % (w/v)
PBS	

Mowiol – embedding medium	
Mowiol 4-88	9 % (w/v)
Glycerol	22 % (w/v)
DABCO	2.4 % (w/v)
TRIS-HCl	0.1 M
Distilled water	

2.2.2 Biomolecular experiments

Ethanol	
Ethanol	75 % (v/v)
RNase free water	

DEPC water	
DEPC	0.1 % (v/v)
RNase free water	

2.3 Software

Software	Company
ActiMot	TSE Systems
AxioVision	Carl Zeiss, Version 4.8.0.0
Image J	by Wayne Rasband, Version 1.42q
Office 2008	Microsoft Corporation
Prism 4	GraphPad Software, Inc, Version 4.0a (2003)
SDS 2.2	Applied Biosystems
Statistika	StatSoft, Inc., Version 7.1 (2005)

2.4 Drugs

Drug	Vehicle	Doses (mg/kg)	Company
β-caryophyllene (BCP)	Olive oil	0.1, 1, 5, 10	provided by Jürg Gertsch (Institute of Biochemistry and Molecular Medicine, University of Bern, Switzerland)
JWH-133	Tocrisolve 100	1, 5	Tocris
Δ^9-THC	Cremophor, Saline	5, 10	THC Pharm
Paclitaxel	Saline (0.9%)	4	HEXAL

2.5 Antibodies

Antigen	Species	Fluorochrome	Company
Antibodies			
GFAP	goat	-	Santa Cruz
Iba1	rabbit	-	Wako
goat IgG	donkey	Alexa 488	Life Technologies
goat IgG	donkey	Cy3	Life Technologies
rabbit IgG	donkey	Alexa 488	Life Technologies
Antiserum			
donkey IgG			Sigma-Aldrich

2.6 Gene expression assays

All gene expression assays and the gene expression master mix used in the experiments were purchased from Applied Biosystems (Life Technologies). Following assays were used:

Target gene		ID
Actb	beta-actin	Mm01205647_g1
Igtp	interferon gamma induced GTPase	Mm00497611_m1
Tgtp1	T-cell specific GTPase 1	Mm01295120_m1
Cnr2	cannabinoid receptor 2	Mm00438286_m1
Nos2	inducible nitric oxide synthase 2	Mm00440502_m1
IL-1β	interleukin-1β	Mm01336189_m1

2.7 Mouse strains

Wild type and CB2 knock-out mice on a C57BL/6J background were used. Mice with a genetic deletion of the CB2 receptor have been described previously (Buckley et al., 2000). Mutant mice were crossed for more than 10 generations to C57BL/6J mice and thus are considered to be congenic for this background.

3 METHODS

3.1 Behavioral experiments

Animal husbandry and all behavioral experiments took place in the S1 (Sicherheitsstufe 1, §§ 4-7 GenTSV) animal facility of the university clinics in Bonn („Haus für experimentelle Therapy", HET). Experimental procedures complied with all regulations for animal experimentation in Germany and were approved by local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW; AZ: 9.93.2.10.35.07.310 and 87-51.04.2010.A393).

3.1.1 Animals

For the neuropathic pain experiments 12 to 16 weeks old, for the cannabinoid tetrad nine to ten weeks old male and female mice were used. Mice were either taken from breeding colonies of our own animal facility or purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in groups of three to five mice per cage under controlled illumination (light-dark cycle: 12:12 h) and environmental conditions (temperature: 22 ± 2 °C; humidity: 55 ± 5 %). All mice had free access to water and food pellets.

Mice, treated with paclitaxel, were cleaned separately and the bedding was disposed of as cytotoxic waste.

3.1.2 Experimental design

3.1.2.1 Partial nerve ligation and BCP treatment

All experiments were performed using the following protocol: Mice were habituated to the experimental setup for $\geq$ 1h during three consecutive days. After habituation, baseline behavioral measurements were conducted. One day later the partial nerve ligation was performed. On the next day the daily BCP, JWH-133 or vehicle treatment started for a period of two weeks (see also 3.1.3). To assess the development of pain and the effect of treatment, mice were regularly tested in the behavioral tests (von Frey test, 3.1.6;

Hargreaves' test, 3.1.7) on day 3, 6, 8, 10 and 14 after the partial nerve ligation (Figure 4). On the test-days drugs were administered after finishing the experiments.

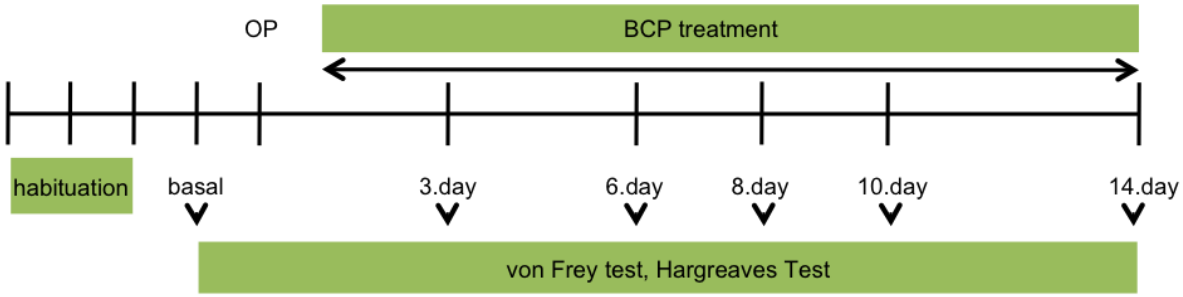


Figure 4 Time schedule of the BCP experiments. BCP treatment started one day after the surgery (OP). The following behavioral tests (von Frey test and Hargreaves' test) were conducted at day 3, 6, 8, 10 and 14.

3.1.2.2 Paclitaxel-induced neuropathic pain model

Habituation and baseline measurements were performed as described above. After baseline measurements the first paclitaxel injection was administered to CB2 knock-out and wild type mice. Behavioral measurements (von Frey test, 3.1.6; Hargreaves' test, 3.1.7; Cold plate test, 3.1.8) were performed on days 4, 7, 11, 15 and 22 after the first injection (Figure 5).

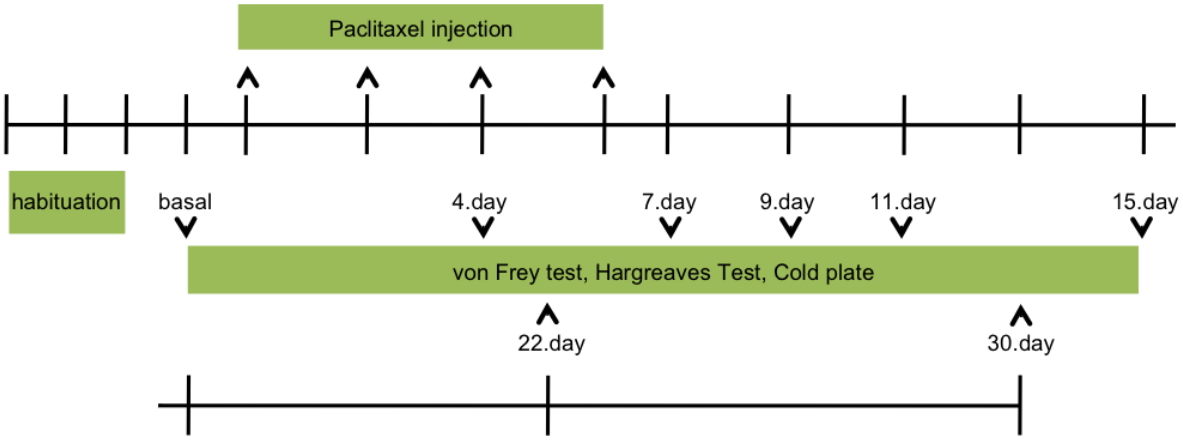


Figure 5 Time schedule of the paclitaxel experiments. First paclitaxel injection was administered one day after the basal measurements followed by injections on day 2, 4 and 6 after the first injection. Behavioral tests (von Frey test, Hargreaves' test and Cold plate test) were conducted at day 3, 6, 8, 10 and 14.

3.1.3 Cannabinoid treatment

Concentrated BCP (5:1 isomer mixture of (*E*)- and (*Z*)-BCP; purified from a commercially available 70 % pure preparation (TCI) by HPLC and analysis in GC/MS) was kindly provided by Jürg Gertsch. BCP (95 % purity) contained residual amounts of α-humulene and the BCP oxidation product BCP-oxide (see Figure 3). The BCP stock solution was dissolved in olive oil (Sigma-Aldrich) at a concentration of 0.02, 0.2, 1 and 2 mg/ml and frozen in 2 ml aliquots at -20 °C. Aliquots were defrosted on each treatment day and administered by gavage at a volume of 5 ml/kg with the help of a feeding-needle, thus resulting in doses of 0.1, 1, 5 or 10 mg/kg respectively.

JWH-133 was dissolved in Tocrisolve 100 (both Tocris Bioscience) at a concentration of 0.1 and 0.5 mg/ml and mice received subcutaneous injections in a volume of 10 ml/kg, thus resulting in doses of 1 and 5 mg/kg.

Δ^9-THC, in a stock solution with ethanol (THC Pharm), was dissolved in Cremophor (Sigma-Aldrich) in a 1:1 mixture prior to dilution with saline to reach a final mixture of 1:1:18 at a Δ^9-THC-concentration of 0.5 and 1 mg/ml. Mice received intraperitoneal (i.p.) injections in a volume of 10 ml/kg, thus resulting in doss of 5 and 10 mg/kg.

3.1.4 Partial sciatic nerve ligation

A partial ligation of the right sciatic nerve was performed to induce neuropathic pain. The partial nerve ligation (PNL) model is a reliable model to investigate neuropathic pain and was first described in mice by Malmberg and Basbaum (1998). Briefly, mice were initially anesthetized in a box with an oxygen/isoflurane mixture (2 – 2.5 % in 95 % O_2), fixed on the surgery table and kept under a constant stream of isoflurane (1,5 – 2 % in 95 % O_2) to maintain anesthesia. The right thigh and hip were shaved and disinfected with betaisodona (Mundipharma GmbH). After a small incision to the skin using a scalpel the underlying muscle was punctured with sharp scissors and the sciatic nerve exposed with thin tweezers. One half to one third of the nerve was ligated with a medical polypropylene thread (9-0). In sham-operated mice the nerve was exposed, but not ligated. Finally, muscle and skin were tightly sutured with polypropylene threads (7-0; 5-0) and the lesion site disinfected.

3.1.5 Paclitaxel-induced neuropathic pain

Smith et al. (2004) introduced the paclitaxel-induced neuropathic pain model for mice. Paclitaxel produces a robust bilateral neuropathy without the need of an invasive surgery. To establish this model and to study gene effects between CB2 knock-out and wild type mice, the protocol of Smith et al. (2004) was slightly adjusted: On each injection day, paclitaxel

(HEXAL) was freshly diluted in physiological saline to a final concentration of 0.4 mg/ml. Four injections of 4 mg/kg paclitaxel were administered i.p. on alternating successive days (days 0, 2, 4 and 6) to induce neuropathic pain. On day 4, on which also behavioral measurements were conducted, paclitaxel was administered after finishing the experiments to avoid behavioral disturbances in the tests. All equipment used in direct contact with paclitaxel, solutions and paclitaxel stock solutions were disposed of as cytotoxic waste.

3.1.6 Von Frey test

Mice developing neuropathic pain present an increased sensitivity towards normally non-noxious mechanical stimuli (allodynia) (Costigan et al., 2009). This mechanical allodynia was assessed with a dynamic plantar aesthesiometer (Figure 6, Ugo Basile Biological Research Apparatus; maximum force: 15 g, ramp: 20 s). It consists of an electronically controlled mobile pressure-actuator that is able to exert a continuously increasing force.

Individual mice were placed in elevated transparent Plexiglas chambers (9 x 9 x 13 cm) with a metal mesh floor. After cessation of exploratory behavior (≤ 1h), experiments were started. The metal filament of the actuator (diameter: 0,5 mm) was applied to the middle of the plantar surface of the hind paw and automatically lifted with increasing force until a clear withdrawal response of the ipsilateral or contralateral hind paw was triggered. The withdrawal responses were automatically recorded and given as the withdrawal-triggering force in grams. Measurements were repeated three times for each paw, separated by > 3 min to avoid habituation, and means were calculated. Mice were not tested while being very active or sleeping. Testing chambers were cleaned with a 70 % ethanol solution between mice.

3.1.7 Hargreaves' test

Neuropathic pain also induces diminished withdrawal thresholds towards noxious heat stimuli (thermal hyperalgesia) (Costigan et al., 2009). This thermal hyperalgesia was evaluated using a Hargreaves' apparatus with a mobile I.R. (infrared) heat source (Figure 6, Ugo Basile Biological Research Apparatus, I.R. intensity: 40).

Mice were placed in elevated transparent Plexiglas chambers (10 x 10 x 13 cm) with glass floor. After cessation of exploratory behavior (≤ 1 h), the experiments were started. The radiant I.R. source of the apparatus was placed under the plantar surface of the hind paws and the heat stimuli activated. A clear withdrawal, shaking or licking reaction of the mice switched off the I.R. source and the apparatus automatically displayed the withdrawal latency in seconds. Mean withdrawal latencies were determined by averaging three to five trials

separated by > 5 min to avoid habituation. Mice were not tested if they were very active or sleeping. Testing chambers were cleaned with a 70 % ethanol solution between mice.

Von Frey test and Hargreaves' test were performed with the same group of mice, with a minimum intertest interval of 1 hour.

3.1.8 Cold plate test

The Cold plate test was used to measure cold allodynia, which is also frequently observed in mice suffering from neuropathic pain (Choi et al., 1994). For this test a commercially available Hot/Cold plate apparatus (Figure 6, Ugo Basile) was used. It consists of a round plate surrounded by a cylindrical Plexiglas restrainer. The temperature of the plate is adjustable and was maintained at 5 ± 1 °C for cold allodynia measurement. Individual mice were placed on the plate and the mean number of right and left hind paw elevations was assessed during 5 min. The Cold plate was cleaned with a 70 % ethanol solution between mice. The Cold plate test and von Frey test were performed with the same group of mice, with a minimum intertest interval of 1 hour.

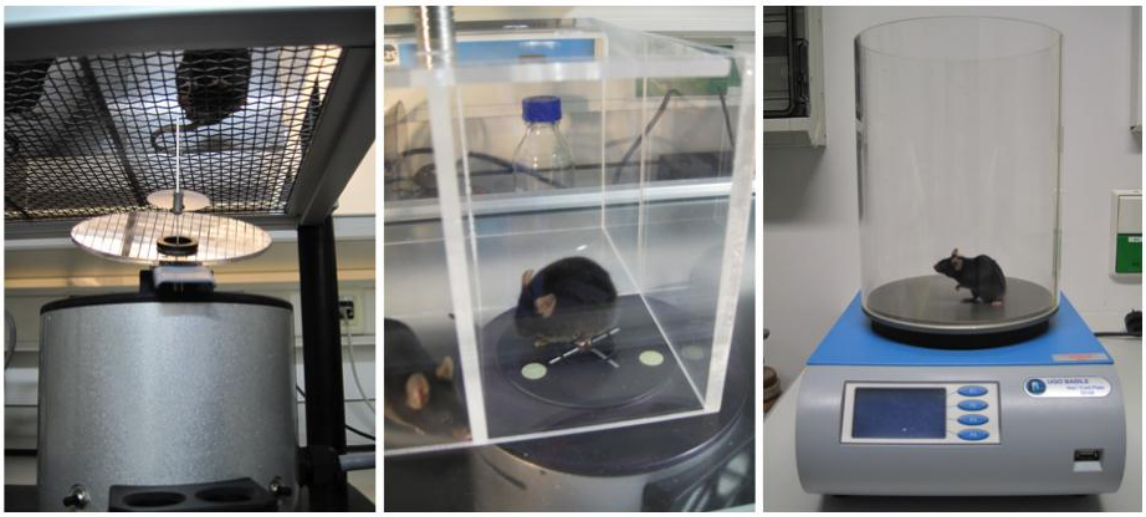


Figure 6 Experimental set ups for behavioral measurements. Mice were tested in the von Frey test (left panel), Hargreaves' test (middle panel) and on a Cold plate (right panel).

3.1.9 Tetrad test

To exclude that BCP produces the psychoactive side effects known from CB1 receptor activation (Martin et al., 1991), the tetrad test was performed. Motor activity in the open field, immobility (catalepsy) on a ring, anti-nociception in the tail flick test and rectal temperature were measured 45 min after an acute oral administration of BCP in different doses (1 mg/kg and 10 mg/kg) or vehicle (olive oil), as well as after an i.p. injection of Δ^9-THC (5 mg/kg) as

positive control. To similarly measure peak cannabinoid-induced effects, other groups of control mice were injected i.p. with a dose of 10 mg/kg Δ^9-THC or vehicle (ethanol/Cremophor/saline) and were tested 20 min after the injection (peak of i.p. Δ^9-THC metabolism). All drug solutions were freshly prepared before the experiments. An additional observer, who was unaware of the treatments, assisted to record the test values.

3.1.9.1 Open-field test

To evaluate locomotor activity, mice were placed individually in the center of an open-field arena (45 x 45 x 23 cm) in a dark and soundproof room. An automatic monitoring system with an infrared light beam frame surrounding the arena (ActiMot, TSE Systems, Bad Homburg, Germany) tracked the light beam breaks (XY location) of the mice during 10 min. The motor activity was then evaluated by calculating the total distance that the mice travelled during that time. The testing arena was cleaned with a 70 % ethanol solution between mice.

3.1.9.2 Catalepsy

Mice were placed on a vertical tube (diameter: 5.5 cm; height: 16 cm). The time that the mice remained immobile during the 5 min observation period was recorded. Mice that fell down or jumped off the ring were immediately placed back on the ring. One animal was excluded from the experiments after five jumps off the ring.

3.1.9.3 Tail flick test

The tail flick latencies were determined with a tail-flick apparatus (Columbus Instruments, OH., USA). Mice were manually restraint and the tail was placed over a radiant I.R. heat source. The latency until mice withdrew their tail was measured. The I.R. intensity was adjusted so that the mean tail withdrawal latency was around 4-5 s. The cut-off time was set at 12 s to prevent tissue damage.

3.1.9.4 Rectal temperature

Body temperature of mice was measured using a rectal thermometer. Temperatures were recorded immediately and 1 h after the administration of BCP (1 and 10 mg/kg), Δ^9-THC (5 mg/kg) or olive oil. The groups receiving 10 mg/kg Δ^9-THC and Cremophor were measured 35 minutes after administration, according to the earlier start of behavioral measurements. Data are expressed as the change in body temperature (ΔT = T after treatment – T before treatment).

3.1.10 Statistical analysis of behavioral data

For all data mean values and the standard error of the mean (SEM) were calculated. Statistical analyses were carried out using Statistika 7.1 (StatSoft Inc.). If not stated otherwise, differences between groups in the behavioral experiments were analyzed by repeated measures analysis of variance (ANOVA). The categorical factors *side* (ipsilateral side vs. contralateral side), *surgery* (sham mice vs. PNL mice) and *treatment* (drug-treated mice vs. vehicle-treated mice) were analyzed and *time* was used as within-effect. Significant interactions in the repeated measures ANOVA were followed by Fisher's Least Significant Difference (Fisher-LSD) *post hoc* test.

Results of the cannabinoid tetrad were analyzed by one-way ANOVA or repeated measures ANOVA (body temperature) using *treatment* as main factor.

The level of significance was set at $p \leq 0.05$.

3.2 Histological experiments

3.2.1 Tissue preparation

At the end of the behavioral experiments mice were sacrificed by cervical dislocation. The lumbar region of the spine was cut off and the spinal cord was flushed out with the help of a syringe filled with 0.9 % physiological saline solution. The tissue samples were quickly frozen in isopentane on dry ice.

To improve microglia staining we performed a second IHC series using pre-fixed tissues. Therefore, mice were anesthetized using a Ketavet/Rompun mixture (10 mg/kg / 0.1 mg/kg) and were intracardially perfused with phosphate buffered saline (PBS) on dry ice for 5 min followed by 4 % paraformaldehyde (PFA) solution for 10 min. Subsequently the spinal cord was harvested as described before and kept in 10 % saccharose solution overnight. Next, the tissue samples were transferred to 20 % saccharose solution for 24 h and finally shock-frozen in isopentane on dry ice.

All tissue samples were stored at -80 °C until further use.

3.2.2 Preparation of tissue sections

The lumbar part from L4 to L6 of the spinal cord was selected, embedded in O.C.T. compound (Tissue Tek®, Sakura) and quickly cooled down with cooling spray (Roth) to prevent thawing.

For the non-perfused tissue 12 µm thick, transversal sections were prepared on a cryostat (Leica CM 3050; Leica Microsystems) at -20 °C. Tissue of one mouse per treatment group with four to six sections each mouse was mounted on Star frost-coated slides.

For the perfused tissue 16 µm thick, transversal sections were prepared at -18 °C. Tissue of one mouse per treatment group with six sections each mouse was mounted on Star frost-coated slides.

Sections were dried on a heating plate and finally stored at -80 ° C.

3.2.3 Immunofluorescent staining

Frozen slides were put on a heating plate at 38 °C for drying (30 min) and the sections were surrounded with a hydrophobic barrier with a PapPen. All steps were performed at room temperature unless otherwise stated.

Two series of immunohistochemistry (IHC) were performed. In the first IHC series cryosections of non-perfused tissue were used. Here, the sections were fixed for 20 min in

4 % PFA solution, and washed three times for 10 min in PBS. For permeabilization, sections were incubated in 0.5 % Triton X-100 (Sigma-Aldrich) for 1 h. After three further washing steps a blocking solution containing 3 % BSA (PAA) and 10 % donkey serum (Abcam) in PBS was applied and the sections were incubated in a humid chamber for 2 h. After removal of the blocking solution the sections were incubated with polyclonal primary antibody against astrocytic glial fibrillary acidic protein (GFAP, goat polyclonal, 1:500, Santa Cruz Biotechnology) at 4 °C overnight. On the next day, the sections were kept at 37 °C in an incubator for 2 h. After removal of the antibody solution the sections were washed three times in PBS, shortly placed in 0.5 % BSA in PBS and incubated with green fluorescent Alexa Fluor 488 anti-goat secondary antibody (Life Technologies). The slices were mounted with Fluoromount G (Southern Biotech) and fixed with nail polish to prevent drying of the mounting fluid.

To improve our staining strategy for the microglia staining a second IHC series with cyrosections of perfused tissue was performed. The tissue sections were shortly placed in PBS and incubated in 0.5 % Triton X-100 (Sigma-Aldrich) for 1 h to permeabilize the cell membranes. After three washing steps a blocking solution containing 3 % BSA and 10 % donkey serum in PBS was applied and the sections were incubated in a humid chamber for 2 h. After removal of the blocking solution, the sections were incubated with a primary antibody against astrocytic GFAP (goat polyclonal, 1:500, Santa Cruz Biotechnology) and microglial ionized calcium-binding adapter molecule 1 (Iba1, rabbit polyclonal, 1:1000, Wako) at 4 °C for 42 h. On the second staining day the sections were put at 37 °C in an incubator for 2 h. After removal of the antibody solution, the sections were washed three times in PBS, shortly placed in 0.5 % BSA in PBS and incubated with red fluorescent Cy3 anti-goat secondary antibody (1:1000, Jackson ImmunoReasearch) and green fluorescent Alexa Fluor 488 anti-rabbit secondary antibody (1:1000, Life Technologies). The slices were mounted with Mowiol 4-88 (Roth).

3.2.4 Image acquisition

For the first IHC series (non-perfused tissue) images were acquired using a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss Microimaging). Images of GFAP-stained ipsilateral and contralateral dorsal horns were taken with a 40x, 1.3 NA oil-immersion objective and constant exposure time (340 ms) and settings (80 %) for quantitative analysis. Representative images were taken with a 20x, and a 40x 1.3 NA oil-immersion objective. All images were taken with the same exposure time (representative pictures: 370 and 200 ms) and settings (intensity: 80 %).

For the second IHC series (perfused tissue) pictures were acquired using a Zeiss Imager M2 fluorescent microscope (Carl Zeiss Microimaging), which was newly installed in the laboratory. Therefore, images of the Iba1 and GFAP double-stained, spinal cord sections were taken this time with the multichannel mosaic function of the new AxioVision software (Carl Zeiss Microimaging) at 20x magnification (constant exposure time, Iba1: 430 ms, GFAP: 170 ms; intensity: 80 %). The mosaics were converted to one image before quantitative analysis. To improve the overlay of the mosaic images the “stitching” function of the software was used for representative pictures, which correctly aligns the single images to each other.

3.2.5 Semiquantitative analysis of glia staining

The method of Romero-Sandoval and Eisenach (2007) was modified to evaluate microgliosis and astrogliosis after peripheral nerve injury. Here, an increase in fluorescent GFAP or Iba1 signals above a defined intensity threshold is interpreted as an increase in cell numbers (proliferation) and is therefore a sign for glial activation. A change in morphology (hypertrophy, retracted and thickened processes) can also lead to an increase in fluorescent signal.

In the first IHC series (non-perfused tissue) whole images of ipsilateral or contralateral dorsal horns (40x magnification) of three mice per treatment group were analyzed. Using a macro routine in Image-J 1.42q (NIH) the pictures were pre-processed before the analysis to scale (conversion of pixel to micrometer) and sharpen the images. Afterwards the color was inverted to facilitate the subsequent analysis. In a second macro the stained area whose intensity lay above a defined intensity threshold was compared to whole image area (percent stained area). Data were expressed as the difference between the signals in the ipsilateral compared to the contralateral side (percent stained area ipsilateral side - percent stained area contralateral side) to normalize data.

In the second IHC series (perfused tissue) ipsilateral or contralateral dorsal horns (20x magnification) of four to five mice per treatment group were analyzed using a similar macro routine in Image-J 1.42q (NIH) as in the first staining series. Due to the different image acquisition (mosaic function, see also 3.2.4) with the new microscope, here an observer blind to the experimental groups manually surrounded the dorsal horns to define the region of interest. The pictures were pre-processed before analysis to scale (conversion of pixel to micrometer) and sharpen the pictures as well as invert the color. The stained area whose intensity lay above a defined intensity threshold was compared to whole region of interest (percent stained area; see also Figure 7). Data were expressed as the difference between

the signals in the ipsilateral compared to the contralateral side (percent stained area ipsilateral side - percent stained area contralateral side) to normalize data.

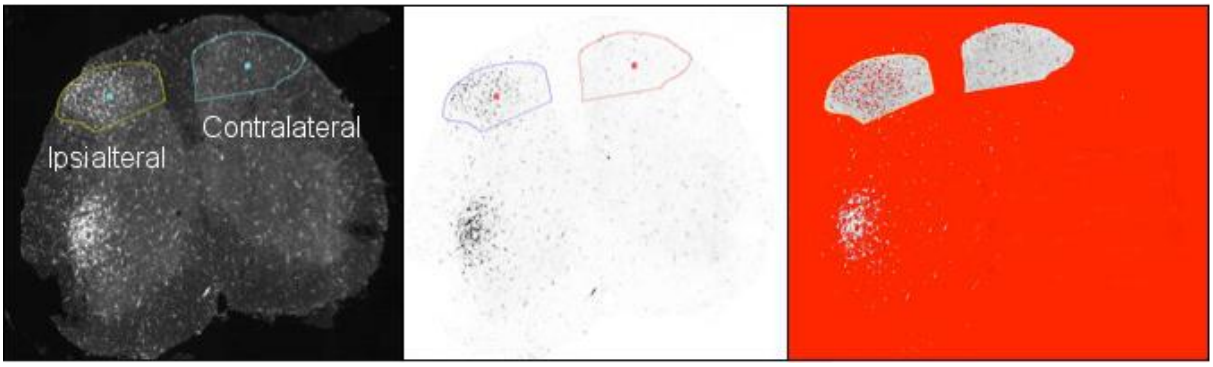


Figure 7 Example for quantitative analysis with ImageJ. The ipsilateral and contralateral dorsal horns were manually surrounded (left panel) and the colors of the image were inverted (middle panel). The area of stained signals, which lay above the pre-defined threshold (here marked as red dots in the dorsal horns; right panel) was measured.

3.2.6 Statistical analysis of histological data

For the analysis of histological data the Mann-Whitney-U test was performed. The level of significance was set at $p \leq 0.05$.

3.3 Biomolecular experiments

3.3.1 Tissue preparation

Mice were sacrificed by cervical dislocation and the lumbar region of the spine was cut off. The spinal cord was flushed out with the help of a syringe filled with 0.9 % physiological saline solution and maintained on ice while ipsilateral and contralateral sides were carefully separated with a scalpel. The tissue samples were quickly frozen in isopentane on dry ice and stored at -80 °C.

3.3.2 RNA isolation

Total RNA of the ipsilateral and contralateral sides of the spinal cord was extracted using Trizol reagent. All steps were performed at room temperature, exceptions are mentioned below.

For isolation, 0.3-0.5 ml Trizol reagent was added to 30-50 mg spinal cord tissue and homogenized using a pistil. After centrifugation at 12000 g for 10 min at 4 °C to remove insoluble material, the supernatant was transferred to a fresh 1.5 ml Eppendorf tube. 1-Bromo-3-chloropropane (30-50 µl) was added to the samples and mixed. For phase separation, samples were incubated for 3 min and centrifuged at 12000 g for 10 min at 4 °C. The aqueous phase was transferred to a fresh 1.5 ml tube.

To precipitate RNA, 250 µl isopropanol was added and samples were incubated for 10 min. After 10 min centrifugation at 12000 g at 4 °C the supernatant was discarded and the RNA pellet washed with 1 ml 75 % ethanol. After repeated centrifugation the supernatant was discarded and the RNA pellet was air-dried for ≈ 30 min. The RNA was then redissovled in 20 µl DEPC water and incubated for 10 min and additional 15 min at 55 °C. For some samples, where the A_{260}/A_{280} ratio was very low or high (see 3.3.3), an additional cleaning step was performed. Therefore, the samples were reprecipitated with 250 µl isopropanol and repeatedly treated following the protocol above.

3.3.3 Determination of RNA concentration

RNA concentrations can be determined by measuring the sample absorbance at 260 nm (A_{260}) using a spectrophotometer (NanoDrop, ND 1000). An absorbance of 1 unit at 260 nm thereby corresponds to 40 µg of RNA per milliliter. The ratio of the sample absorbance at 260 nm and 280 nm (A_{260}/A_{280}) provides similarly an estimate of the purity of RNA regarding

contaminants such as protein. Pure RNA has an A_{260}/A_{280} ratio of 1.8 to 2.0. After determination of RNA concentration, samples were stored at -80 °C.

3.3.4 Reverse transcription

mRNA was transcribed into double-stranded cDNA using Reverse Transcriptase II and $Oligo(dt)_{12-18}$ primer (Life Technologies). In each reaction sample a total of 1 µg RNA was used for transcription. The PCR machine was set up as follows:

Temperature	Duration
70 °C	10 min
4 °C	< 3 min
First strand buffer, dNTPs and DTT were added	
42 °C	2 min
4 °C	< 3 min
Reverse Transcriptase II was added	
42 °C	60 min
70 °C	15 min
4 °C	< 10 min

After the reverse transcription all samples were filled up to 40 µl with RNAse free water to reach a final cDNA concentration of 25 ng/µl and were stored at –20 °C.

3.3.5 Quantitative real time-PCR

For the gene expression analysis TaqMan® gene expression assays were used. TaqMan assays base on a gene specific probe coupled to a fluorophore and a quencher. After binding to the specific cDNA sequence during amplification the fluorophore is separated from the quencher and emits a fluorescence signal. The number of amplification cycles needed to detect a sufficient fluorescence signal gives information about the relative amount of specific cDNA in the sample.

For each sample 50 ng of cDNA were mixed with gene expression master mix (containing AmpliTaq Gold® DNA Polymerase, Life Technologies) and with one of the gene specific assays (CB2, Nos2, igtp, tgtp, IL-1β or Actb; see 2.7; Life Technologies), centrifuged and stored in the dark on ice until the start of the measurement. The thermal cycling in the Taqman 7900HT was proceeded as the follows:

Step	Temperature	Duration	Number of cycles
Initial denaturation	95 °C	10 min	1 cycle
Denaturation	95 °C	15 s	
Annealing/extension	60 °C	1 min	40 cycles

Data displayed by the SDS analyzing software (Applied Biosystems; SDS 2.2.2) were normalized to a housekeeping gene (β-actin) and analyzed with the $2^{-\Delta\Delta Ct}$ method as described previously (Livak and Schmittgen, 2001). β-actin (Actb) was chosen as housekeeping gene because it was shown to be stably expressed during neuropathic pain conditions (Wan et al., 2010).

3.3.6 Statistical analysis of biomolecular data

For biomolecular analyses a factorial ANOVA was applied. Categorical factors were *surgery*, *side* and *treatment*. For the CB2 expression analysis, we used the Fisher-LSD *post-hoc* test for the interaction of *surgery* and *side* followed the analysis. The level of significance was set at $p \leq 0.05$.

4 RESULTS

The present data were divided into two main parts to investigate the role of CB2 receptors in the modulation of chronic neuropathic pain. In the first part, the therapeutic potential of the natural CB2 receptor agonist BCP was evaluated in the treatment of neuropathic pain. Here, we used the partial sciatic nerve ligation as a model for neuropathic pain.

In the second part, the role of CB2 receptors for the development of chemotherapy-induced neuropathic pain was evaluated. Here, a mouse model of paclitaxel-induced neuropathic pain was established and genotype effects between wild type and CB2 knockout mice were studied.

4.1 The natural CB2 receptor agonist BCP in the treatment of neuropathic pain

4.1.1 Neuropathic pain symptoms were diminished in BCP treated wild type mice

Painful responses towards normally innocuous mechanical stimuli (allodynia) and decreased thresholds towards noxious thermal stimuli (hyperalgesia) are indicators for the development of neuropathic pain (Costigan et al., 2009). Therefore, mice were tested in the von Frey test for mechanical allodynia and in the Hargreaves' test for thermal hyperalgesia.

Baseline responses of male mice in the von Frey test were similar between the different treatment groups. As baseline responses in the Hargreaves' test differed between some groups, data were here expressed as percent of baseline.

The partial ligation of the right sciatic nerve induced mechanical allodynia and thermal hyperalgesia in ipsilateral hind paws, revealed by significantly decreased mechanical withdrawal thresholds compared to contralateral hind paws in the von Frey test (Figure 8 A; *side*; $F_{1,18} = 387.34$, $p < 0.001$) and thermal withdrawal latencies in the Hargreaves' test (Figure 8 B; $F_{1,18} = 20.40$, $p < 0.001$). The mechanical ($F_{1,22} = 0.44$, $p > 0.05$) or thermal withdrawal responses ($F_{1,22} = 0.58$, $p > 0.05$) in sham-operated mice remained unaffected (Figure 9 A and B).

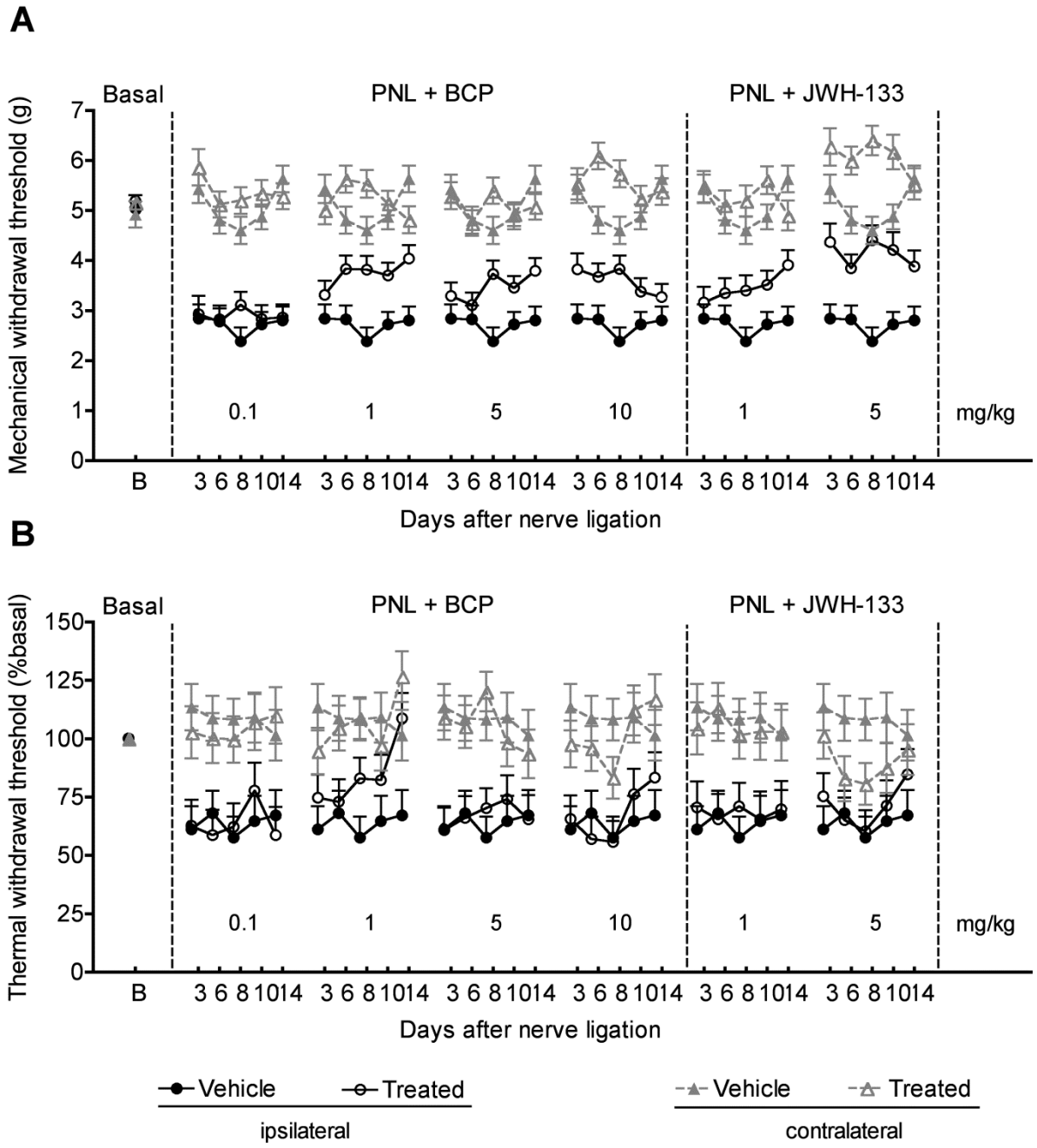


Figure 8 Effect of different doses (0.1, 1, 5 and 10 mg/kg) of BCP and JWH-133 (1 and 5 mg/kg) treatment on mechanical allodynia and thermal hyperalgesia in PNL wild type mice.
A 1, 5 and 10 mg/kg BCP and 1 and 5 mg/kg JWH-133 treatment significantly reduced PNL-induced mechanical allodynia in the ipsilateral side. **B** Only 1 mg/kg BCP treatment significantly reduced PNL-induced thermal hyperalgesia in the ipsilateral side, while treatment with 1 and 5 mg/kg JWH-133 showed no effect. Ipsilateral and contralateral hind paws of male mice were tested in the von Frey test and Hargreaves' test to evaluate mechanical allodynia and thermal hyperalgesia. Mice were tested at day 3, 6, 8, 10 and 14 after surgery (vehicle: n= 10; 0.1 mg/kg BCP: n = 8; 1 mg/kg BCP: n = 10; 5 mg/kg BCP: n = 11; 10 mg/kg BCP: n = 10; 1mg/kg JWH-133: n = 8; 5 mg/kg JWH-133: n = 10). Thermal withdrawal latencies are expressed as percent of basal responses. B = basal responses. **, *treatment* $p < 0.01$, ***, *treatment* $p < 0.001$. Means $\pm$ SEM are indicated.

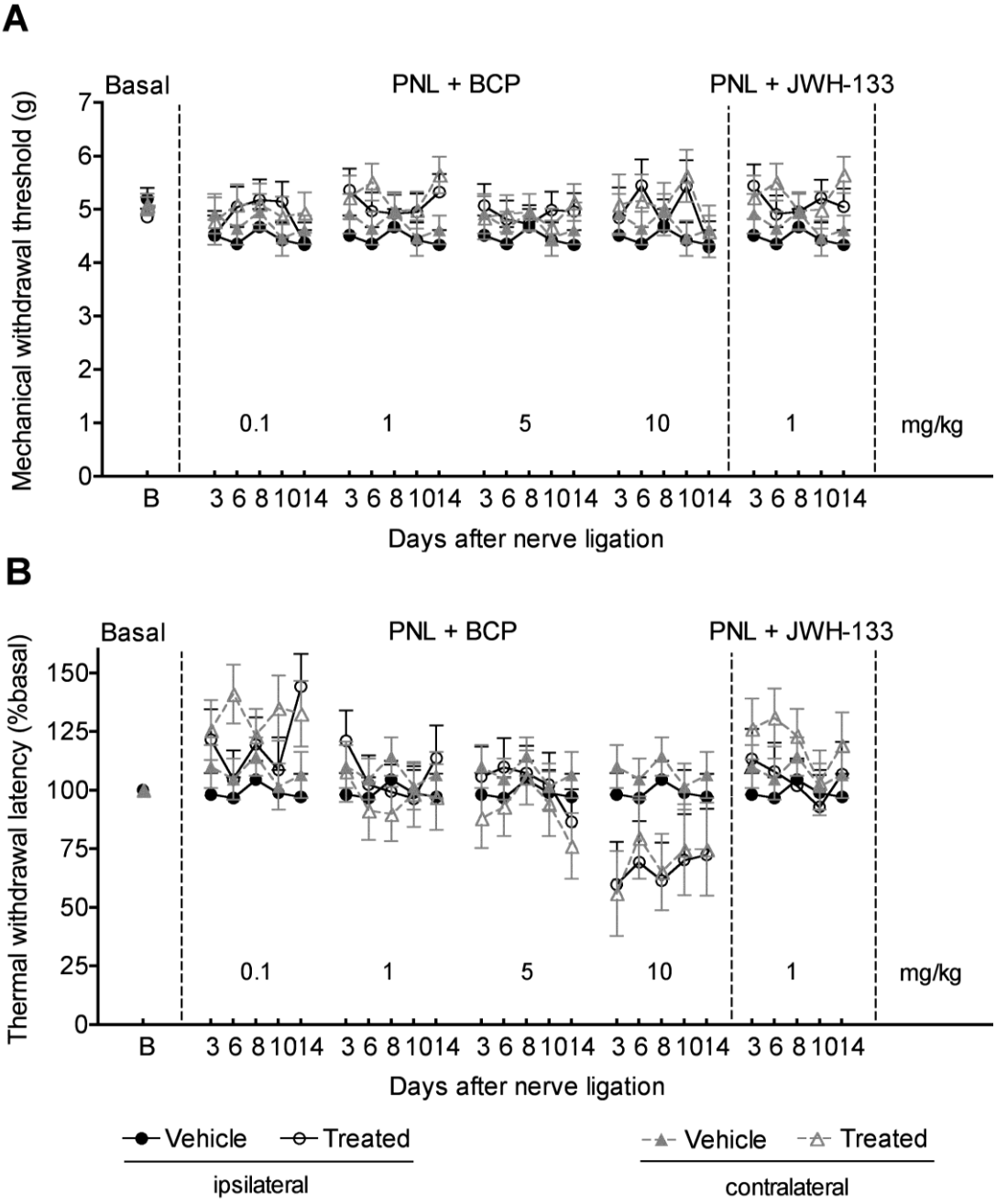


Figure 9 Effect of different doses (0.1, 1, 5 and 10 mg/kg) of BCP and 1 and 5 mg/kg JWH-133 treatment on mechanical allodynia and thermal hyperalgesia in sham-operated wild type mice. A Mechanical withdrawal thresholds of sham operated mice were not affected by BCP or JWH-133 treatment. **B** Also thermal withdrawal latencies of sham-operated mice were not altered by the BCP or JWH-133 treatment. Ipsilateral and contralateral hind paws of male mice were tested in the von Frey test and Hargreaves' test to evaluate mechanical allodynia and thermal hyperalgesia. Mice were tested at day 3, 6, 8, 10 and 14 after surgery (vehicle: n= 12; 0.1 mg/kg BCP: n = 5; 1 mg/kg BCP: n = 6; 5 mg/kg BCP: n = 6; 10 mg/kg BCP: n = 3; 1 mg/kg JWH-133: n = 6). Thermal withdrawal latencies are expressed as percent of basal responses. It has to be considered that replicate number of sham animals was low in some groups as they accompanied experiments as controls but were not part of the main investigation. B = basal responses. Means ± SEM are indicated.

BCP treatment in male mice

To evaluate the effect of orally administered BCP on the development of neuropathic pain, mice received daily BCP-treatment (0.1, 1, 5 or 10 mg/kg dissolved in olive oil) by gavage.

The doses of 1 and 5 mg/kg BCP increased mechanical withdrawal thresholds of ipsilateral hind paws gradually over the testing period of two weeks, starting from 3.31 g at day 3 to 4.04 g at day 14 (1 mg/kg) and from 3.29 g at day 3 to 3.79 g at day 14 (5 mg/kg), respectively (Figure 8 A). Treatment with 10 mg/kg exhibited directly a robust effect on day 3 (3.81 g), which then slightly decreased over the testing period to 3.26 g. When comparing these results to the vehicle treated groups, a significant *treatment* effect for all three doses was revealed (1 mg/kg: $F_{1,18} = 20.66$, $p > 0.001$; 5 mg/kg: $F_{1,19} = 20.47$, $p < 0.0001$; 10 mg/kg: $F_{1,18} = 33$, $p < 0.001$), while the treatment with 0.1 mg/kg BCP produced no significant effect (0.1 mg/kg: $F_{1,16} = 2.57$, $p > 0.05$).

Only treatment with 1 mg/kg BCP also decreased thermal hyperalgesia in the Hargreaves' test. This dose even rescued the thermal withdrawal latencies to basal levels after two weeks, from 74.69 % of basal at day 3 to 108.69 % of basal at day 14 (Figure 8 B). Statistical analysis again revealed a significant *treatment* effect for this dose ($F_{1,18} = 9.12$, $p = 0.007$) and a significant *time* and *treatment* interaction ($F_{4,72} = 2.93$, $p = 0.026$). The doses of 0.1, 5 and 10 mg/kg BCP did not affect thermal withdrawal latencies (0.1 mg/kg: $F_{1,16} = 0.00$, $p > 0.05$; 5 mg/kg: $F_{1,19} = 0.24$, $p > 0.05$; 10 mg/kg: $F_{1,18} = 0.29$, $p > 0.05$). The dose of 1 mg/kg BCP thus seemed to be the most effective.

BCP treatment did not influence mechanical (0.1 mg/kg BCP: $F_{1,15} = 0,04$, $p > 0.05$; 1 mg/kg BCP: $F_{1,16} = 1.09$, $p > 0.05$; 5 mg/kg BCP: $F_{1,16} = 0.01$, $p > 0.05$; 10 mg/kg BCP: $F_{1,13} = 0.03$, $p > 0.05$) or thermal withdrawal responses (0.1 mg/kg BCP: $F_{1,16} = 2.95$, $p > 0.05$; 1 mg/kg BCP: $F_{1,16} = 0.34$, $p > 0.05$; 5 mg/kg BCP: $F_{1,16} = 0.08$, $p > 0.05$; 10 mg/kg BCP: $F_{1,13} = 4.18$, $p > 0.05$) of ipsilateral hind paws in sham-operated mice (Figure 9, A and B).

BCP treatment in female mice

To evaluate if BCP treatment similarly induces anti-nociceptive effects in female mice, experiments were repeated in female wild-type mice. The partial ligation of the right sciatic nerve induced mechanical allodynia (Figure 10, A; *side*; $F_{1,18} = 173.66$, $p < 0.001$) and thermal hyperalgesia (Figure 10, B; $F_{1,18} = 10.99$, $p < 0.003$) in the ipsilateral hind paw of female mice. The sham operation had no effect on mechanical ($F_{1,4} = 0.59$, $p > 0.05$) or thermal withdrawal responses ($F_{1,4} = 0.13$, $p > 0.05$). Here, it has to be considered that replicate number for female sham animals was low as they accompanied experiments as controls but were not part of the main investigation.

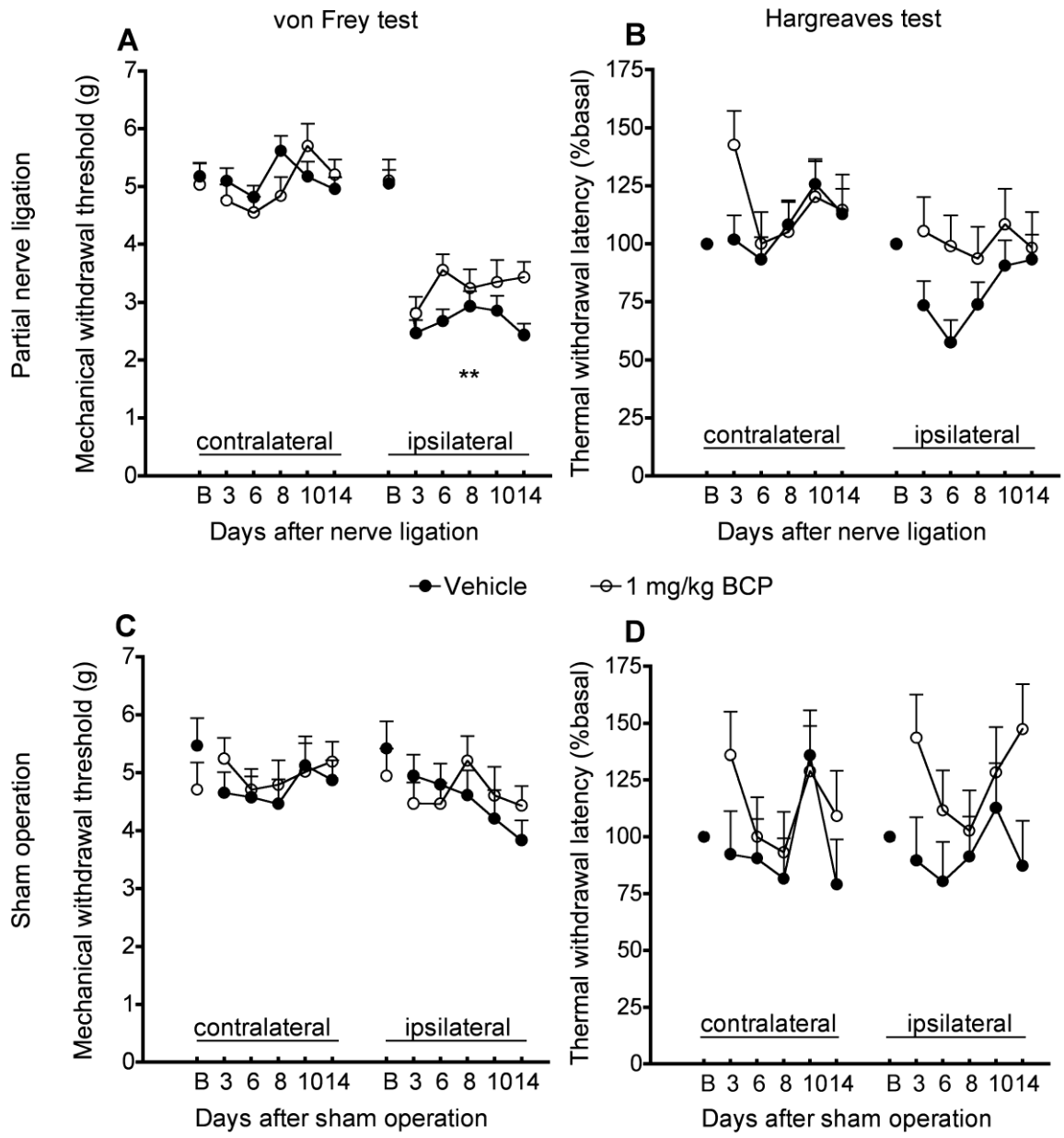


Figure 10 BCP treatment attenuated PNL-induced mechanical allodynia and thermal hyperalgesia in female wild type mice. A Treatment with 1 mg/kg BCP significantly attenuated mechanical allodynia in ipsilateral hind paws of female mice. **B** Treatment with 1 mg/kg BCP showed no significant reduction of thermal hyperalgesia in female mice, although their mean withdrawal latencies were higher compared to vehicle animals. **C** Mechanical withdrawal thresholds of sham operated mice. **D** Thermal withdrawal latencies of sham-operated mice. Ipsilateral and contralateral hind paws of female mice were tested in the von Frey test and Hargreaves' test to evaluate mechanical allodynia and thermal hyperalgesia. Mice were tested at day 3, 6, 8, 10 and 14 after surgery (PNL + vehicle: n = 10; PNL + 1mg/kg BCP: n = 5, Sham + vehicle: n = 3; Sham + 1mg/kg BCP: n = 3). Thermal withdrawal latencies are expressed as percent of basal responses. B = basal responses. **, *treatment* $p < 0.01$. Means $\pm$ SEM are indicated.

The dose of 1 mg/kg of BCP was chosen for the treatment of female wild type mice. Similar to male mice, chronic treatment with 1 mg/kg BCP significantly decreased mechanical allodynia (Figure 10, A; *treatment*: $F_{1,13} = 10.49$, $p = 0.006$) in ipsilateral sides. The *treatment* effect for thermal hyperalgesia was not significant (Figure 10, B; $F_{1,13} = 3.30$, $p = 0.093$). BCP treatment did not influence mechanical ($F_{1,4} = 0.14$, $p > 0.05$) or thermal withdrawal thresholds ($F_{1,4} = 1.92$, $p > 0.05$) of sham-operated mice (Figure 10, C + D).

Treatment with the synthetic CB2 agonist JWH-133

As a reference, daily subcutaneous injections of the synthetic CB2 receptor agonist JWH-133 in the doses of 1 and 5 mg/kg were used. This treatment significantly increased the mechanical withdrawal thresholds at both doses compared to vehicle-treated mice (Figure 8; *treatment*: 1 mg/kg: $F_{1,16} = 9.59$, $p = 0.0069$; 5 mg/kg: $F_{1,18} < 0.001$), but failed to diminish thermal hyperalgesia in the Hargreaves' test (Figure 9; 1 mg/kg: $F_{1,16} = 0.54$, $p > 0.05$; 5 mg/kg: $F_{1,18} = 1.53$, $p > 0.05$).

4.1.2 BCP treatment inhibited neuropathic pain through CB2 receptor activation

To determine if BCP exerts its analgesic effects in neuropathic pain via CB2 receptor activation, the experiments were repeated in CB2 knock-out mice.

As it was shown previously (Rácz et al., 2008b), baseline responses of CB2 knock-out mice were similar to wild type mice. After the partial nerve ligation vehicle treated CB2 knock-out mice developed mechanical allodynia and thermal hyperalgesia in the ipsilateral side. This was revealed by significantly decreased mechanical withdrawal thresholds in the von Frey test (Figure 11, A; *surgery*; $F_{1,14} = 25.97$, $p < 0.001$) and thermal withdrawal latencies in the Hargreaves' test (Figure 11, B; $F_{1,19} = 4.97$, $p = 0.038$). In the contralateral side CB2 knock-out mice additionally developed a mirror image of mechanical hyperalgesia ($F_{1,14} = 8.39$, $p = 0.012$) as reported previously (Rácz et al., 2008b), but no thermal hyperalgesia ($F_{1,19} = 0.34$, $p > 0.05$). The sham operation did not influence the pain responses to mechanical (*side*; $F_{1,14} = 0.13$, $p > 0.05$) and thermal stimuli ($F_{1,20} = 2.27$, $p > 0.05$).

Previously, we identified that the dose of 1 mg/kg BCP was the most effective dose in the neuropathic pain model in wild type mice. Therefore, this dose was chosen for the treatment of the CB2 knock-out mice. Here, 1 mg/kg BCP failed to show any effects on mechanical allodynia in the ipsilateral side (Figure 11, A; *treatment*: $F_{1,15} = 0.83$, $p > 0.05$) or contralateral side ($F_{1,15} = 1.19$, $p > 0.05$). Similarly, thermal hyperalgesia in ipsilateral hind paws was also not affected by BCP treatment (Figure 11, B; $F_{1,20} = 0.08$, $p > 0.05$). BCP treatment similarly did not influence the responses of ipsilateral hind paws in sham-operated CB2 knock-out mice (Figure 11, C + D; von Frey: $F_{1,17} = 0.01$, $p > 0.05$; Hargreaves': $F_{1,23} = 0.72$, $p > 0.05$).

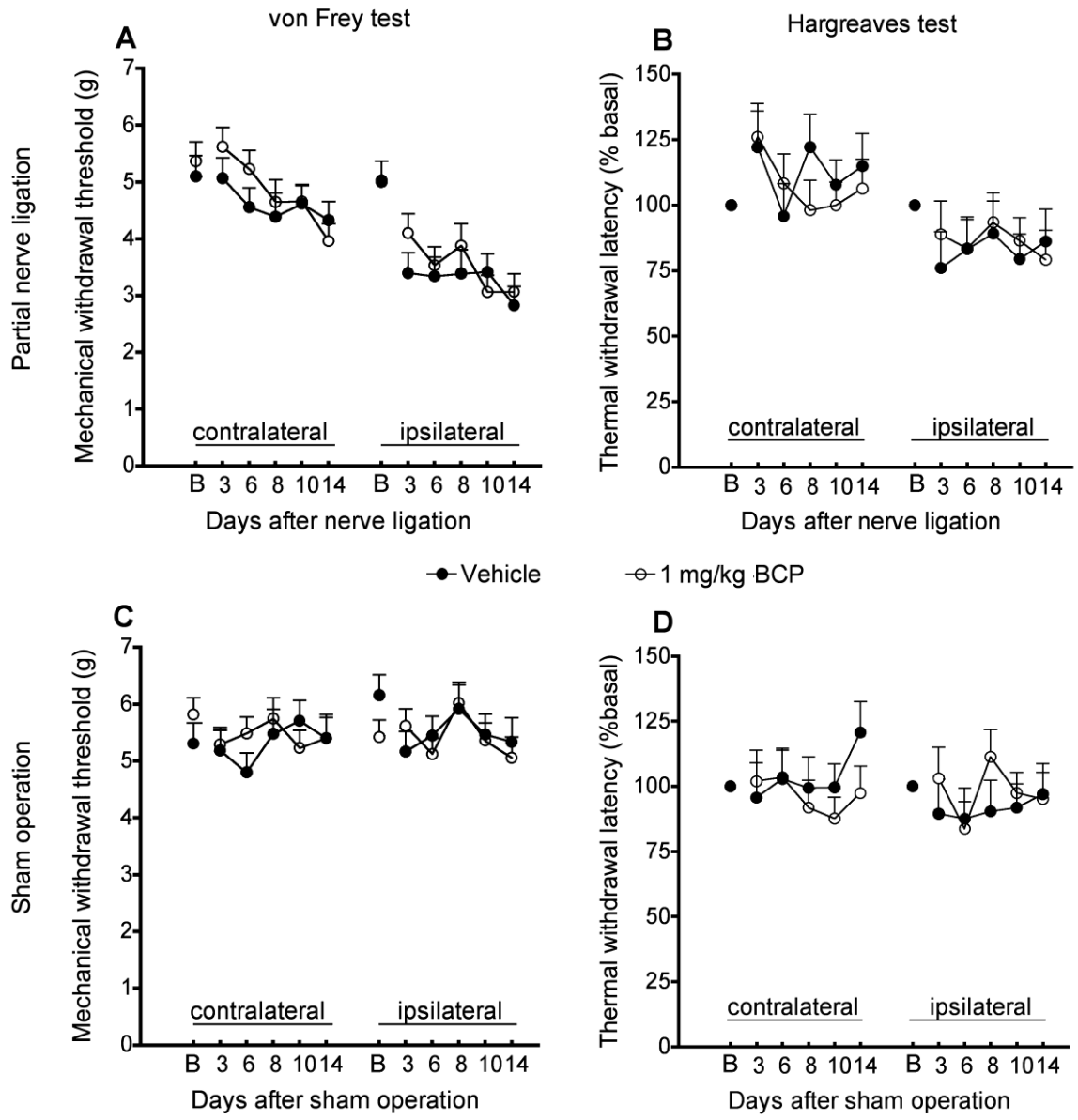


Figure 11 BCP treatment did not attenuate PNL-induced mechanical allodynia and thermal hyperalgesia in CB2 knock-out mice. A Treatment with 1 mg/kg BCP did not alter mechanical allodynia in ipsilateral hind paws of CB2 knock-out mice (vehicle: n = 8; 1mg/kg BCP: n = 9). Additionally to the ipsilateral side, CB2 knock-out mice developed a mirror image of pain at the contralateral side after surgery (Rácz et al., 2008) **B** Treatment with 1 mg/kg BCP also had no influence on thermal hyperalgesia in CB2 knock-out mice (vehicle: n = 10; 1mg/kg BCP: n = 12). **C** Mechanical withdrawal thresholds of sham operated mice (vehicle: n = 8; 1mg/kg BCP: n = 11). **D** Thermal withdrawal latencies of sham operated mice (vehicle: n = 11; 1mg/kg BCP: n = 14). Ipsilateral and contralateral hind paws of CB2 knock-out mice were tested in the von Frey test and Hargreaves' test to evaluate mechanical allodynia and thermal hyperalgesia. Mice were tested at day 3, 6, 8, 10 and 14 after surgery. Thermal withdrawal latencies are expressed as percent of basal responses. B = basal responses. Means ± SEM are indicated.

4.1.3 BCP elicited no psychomimetic effects in the tetrad test

In earlier studies (Gertsch et al., 2008) and in the present study, it was shown that BCP exerts its anti-nociceptive effects through specific CB2 receptor activation. To determine if BCP treatment is devoid of CB1 mediated psychomimetic side effects, wild type mice were tested in the tetrad test. CB1 receptor activation leads to distinct behavioral changes, like hypomotility, hypothermia, catalepsy and analgesia (Martin et al., 1991). The tetrad test, therefore, includes the open field test, catalepsy on a ring, tail flick test and the measurement of body temperature. The doses of 1 and 10 mg/kg BCP were used for acute treatment in the tetrad test as these doses represent the most effective (1 mg/kg) and the highest dose (10 mg/kg) used in the neuropathic pain model.

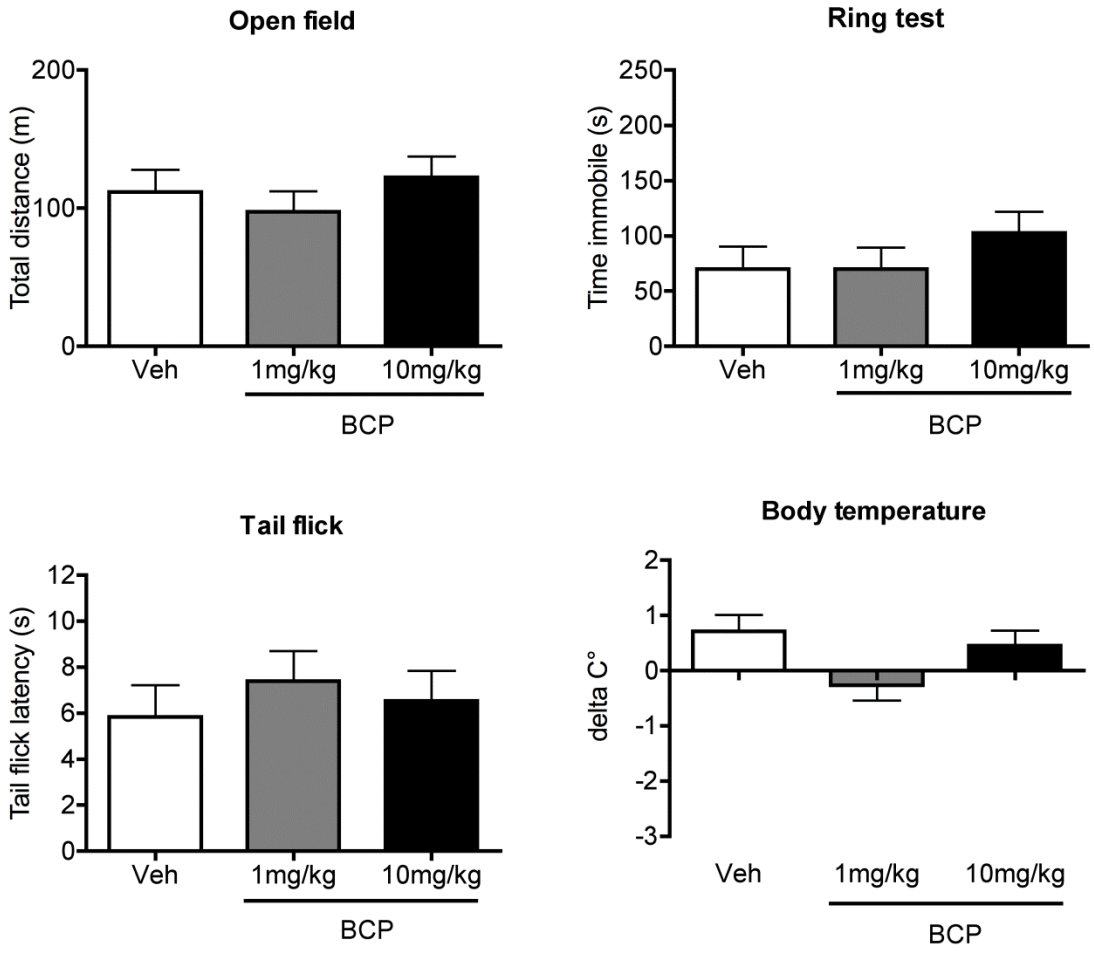


Figure 12 Treatment with BCP did not elicit psychomimetic side effects in wild type mice. Mice were tested 45 min after an acute oral administration of 1 and 10 mg/kg BCP or vehicle (Veh; olive oil) in the tetrad test (vehicle: n = 7; 1 mg/kg BCP: n = 8; 10 mg/kg BCP: n = 8): Motor activity in the open field, immobility on a ring (catalepsy), anti-nociception in the tail flick test and hypothermia. Means ± SEM are indicated

Body weights were similar between the groups. Mice treated with 1 and 10 mg/kg BCP were tested 45 min after the drug administration. Oral treatment with 1 and 10 mg/kg BCP did not induce CB1 mediated psychomimetic side effects (Figure 12). One-way ANOVA revealed that there were no significant activity changes in the open field (*treatment*: $F_{2,20} = 0.59$, $p > 0.05$), no ring catalepsy ($F_{2,20} = 1.14$, $p > 0.05$) and no anti-nociception in the tail flick test ($F_{2,20} = 0.33$, $p > 0.05$). Similarly, no significant change in body temperature could be observed (Repeated measures ANOVA; *treatment*: $F_{2,20} = 1.3$, $p > 0.05$; *time* x *treatment*: $F_{2,20} = 2.8$, $p > 0.05$).

Two groups of mice were treated with our positive-control compound Δ^9-THC. One group was tested, similar to BCP-treated mice, 45 min after the treatment with Δ^9-THC (5 mg/kg) and one group was tested after a shorter period of time and with a higher dose of Δ^9-THC

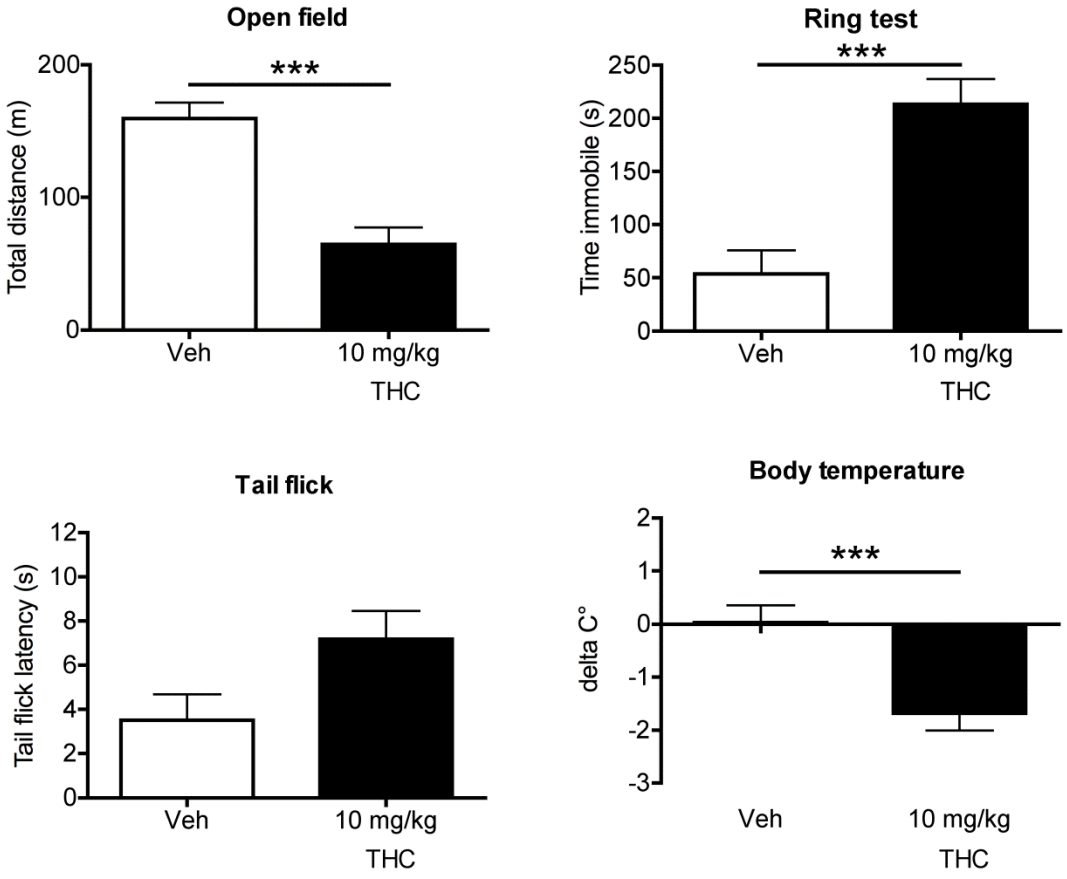


Figure 13 Treatment with the reference compound Δ^9-THC (10 mg/kg) produced typical CB1 receptor-mediated behavioral changes in the tetrad test: Hypomotility, catalepsy, anti-nociception and hypothermia. Wild type mice were tested 20 min after an acute intraperitoneal injection of Δ^9-THC or vehicle (Veh; Cremophor) in the tetrad test (vehicle: n = 7; 10 mg/kg Δ^9-THC: n = 6): Motor activity in the open field, immobility on a ring (catalepsy), antinociception in the tail flick test and hypothermia. ***, *treatment* $p < 0.001$. Means + SEM are indicated.

(20 min after treatment with 10 mg/kg) to archive a robust effect. Treatment with Δ^9-THC elicited typical cannabinoid-induced behavioral changes. Mice treated with 10 mg/kg Δ^9-THC were tested 20 min after the drug administration (Figure 13). They were less active in the open field (One-was ANOVA: $F_{1,11} = 29.03$, $p < 0.001$) and remained for a longer period immobile on the ring in the catalepsy test ($F_{1,11} = 24.13$, $p < 0.001$). Repeated measures ANOVA also revealed a significant drop in body temperature 35 min after the Δ^9-THC treatment (*treatment*: $F_{1,10} = 54.7$, $p > 0.001$; *time* x *treatment*: $F_{1,10} = 14.1$, $p > 0.004$). Tail flick analgesia just failed to reach the level of significance (One-was ANOVA; $F_{1,11} = 4.51$, $p = 0.057$). Mice treated with 5 mg/kg Δ^9-THC were tested 45 min after drug administration (Figure 14). The dose of 5 mg/kg Δ^9-THC also induced hypomotility ($F_{1,11} = 6.80$, $p = 0.024$),

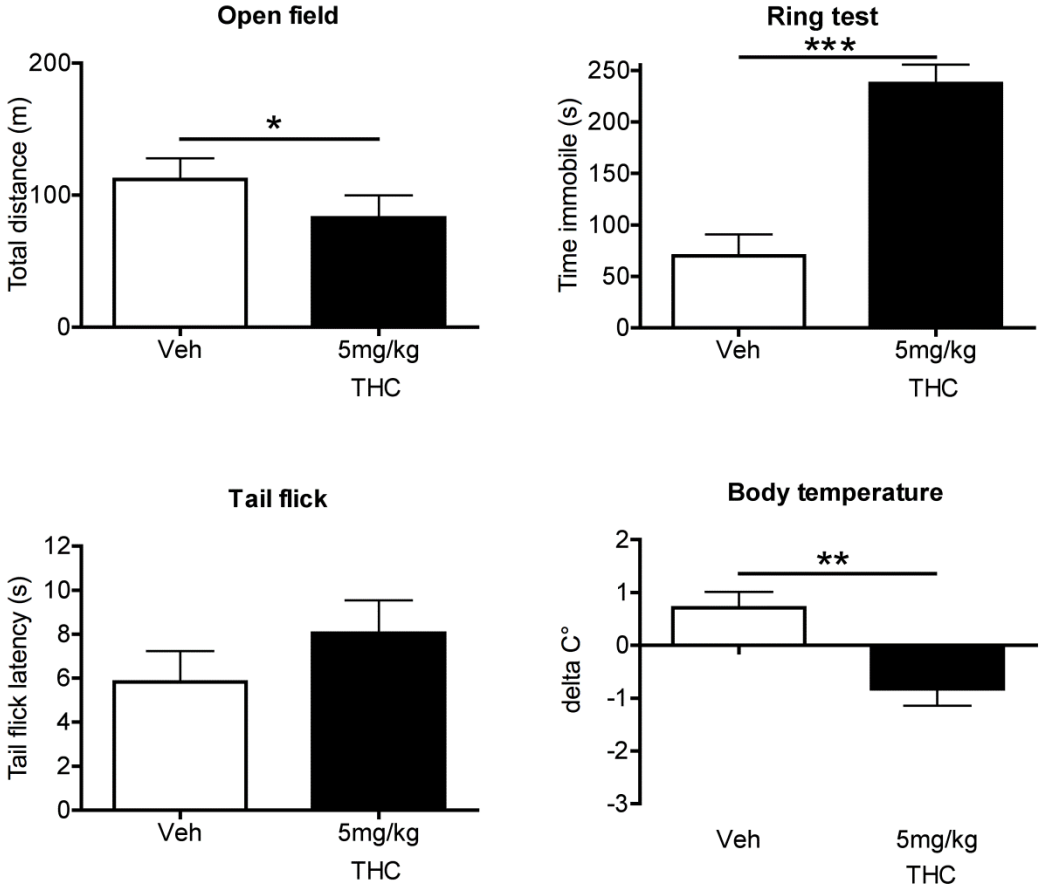


Figure 14 Treatment with the reference compound Δ^9-THC (5 mg/kg) produced typical CB1 receptor-mediated behavioral changes in the tetrad test: Hypomotility, catalepsy, anti-nociception and hypothermia. Mice were tested 45 min after an acute intraperitoneal injection of Δ^9-THC or oral administration of vehicle (Veh; Olive oil) in the tetrad test (vehicle: n = 7; 5 mg/kg Δ^9-THC n = 6): Motor activity in the open field, immobility on a ring (catalepsy), antinociception in the tail flick test and hypothermia. *, *treatment* $p < 0.05$; **, *treatment* $p < 0.01$; ***, *treatment* $p < 0.001$. Means $\pm$ SEM are indicated.

catalepsy ($F_{1,11}$ = 50.59, p < 0.001) and hypothermia (Repeated measures ANOVA; *time* x *treatment*; $F_{1,11}$ = 14.19, p = 0.0037). Solely in the tail flick test no significant analgesic effects could be observed ($F_{1,11}$ = 1.04, p = 0.328).

4.1.4 BCP reduced the density of glia cell immunoreactivity in the lumbar spinal cord

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

In the first staining series using cryosections of non-perfused tissue, the density of astrocytes signals in the ipsilateral dorsal horns of lumbar spinal cord was significantly increased in vehicle treated PNL mice compared to sham-operated mice (Figure 15, A, B; Mann-Whitney U-test; U = 56; p = 0.019). Treatment with BCP strongly reduced astrocyte signals in the dorsal horn of PNL mice in comparison to the vehicle control mice (U = 58; p = 0.023) and normalized astrocyte levels to that of sham-operated mice (U = 59; p > 0.05).

As the microglia staining in the first staining series was too faint for quantification, a second staining series was performed using cryosections of perfused tissue.

The results of the second GFAP staining were in accordance with the findings of the first series. The partial nerve ligation induced an increase in astrocyte signals in the ipsilateral dorsal horns compared to sham operation (Figure 17; U = 164; p = 0.003). Treatment with BCP reduced the mean percentage of stained area in PNL mice, but this effect failed to reach significance (U = 226, p > 0.05). Nevertheless, the density of astrocyte signals in the BCP treated PNL group was not significantly different from that of sham-operated BCP mice (U = 188, p > 0.05).

Similarly, the partial nerve ligation strongly increased microglia signals in the ipsilateral dorsal horns of lumbar spinal cord sections (Figure 16). Quantitative analysis revealed that this effect was highly significant, when compared to sham operated vehicle-treated mice (U = 25, p < 0.001). After treatment with BCP, a robust decrease in the density of microglia signals in dorsal horns compared to vehicle control mice was observed (U = 164, p = 0.006). However, the density of microglia in PNL mice was still significantly higher than in sham animals (U = 23, p < 0.001).

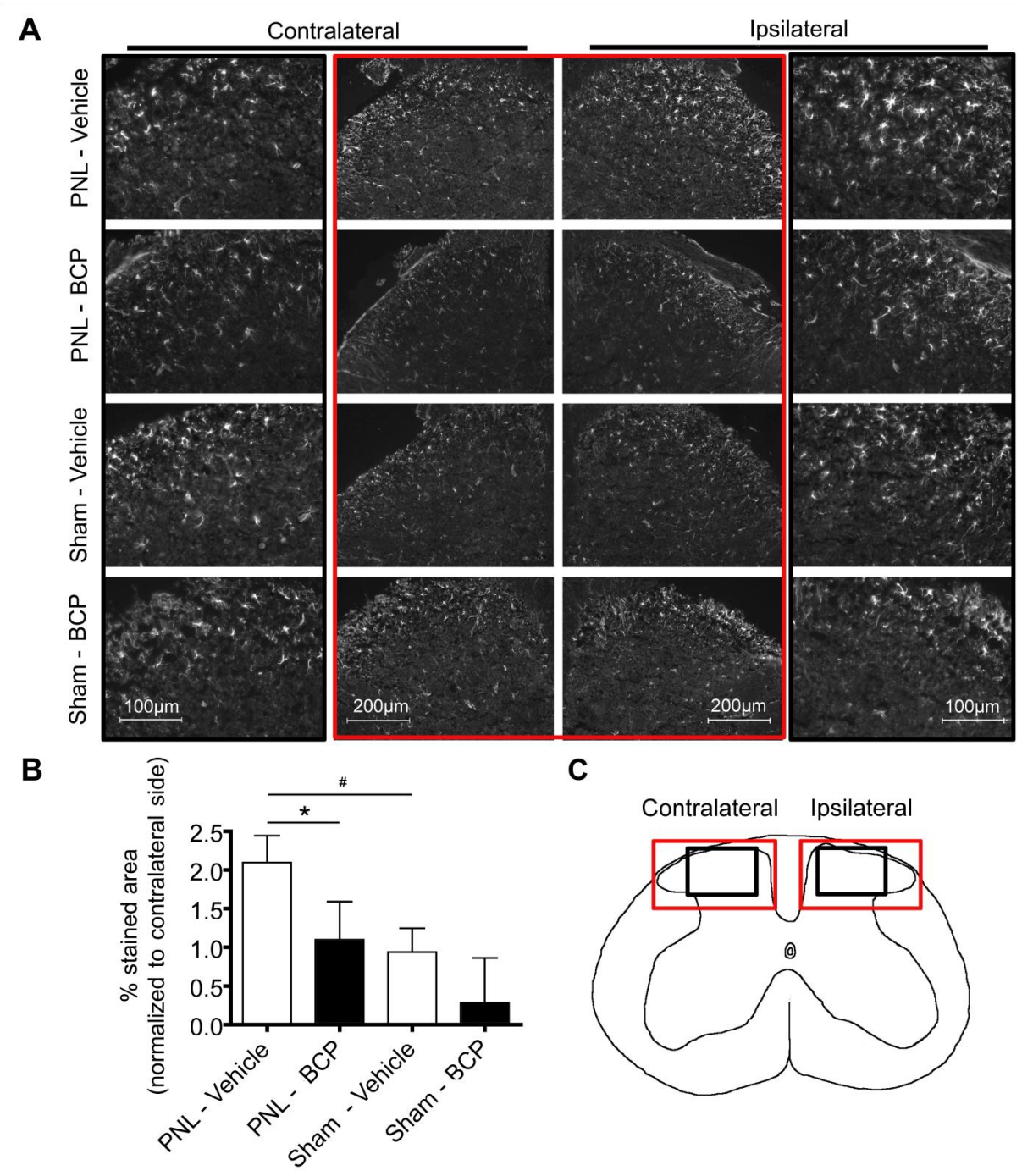


Figure 15 Chronic treatment with 1 mg/kg BCP reduced the mean density of astrocyte signals in the ipsilateral dorsal horn of the lumbar spinal cord. A Representative pictures of the glial fibrillary acidic protein (GFAP) immunostaining in the contralateral and ipsilateral dorsal horns of the lumbal spinal cord recorded with the 20x (red frame) and 40x (black frame) magnification objective. **B** Quantitative analyses revealed a reduction of astrocyte signals in the ipsilateral dorsal horn in 1 mg/kg BCP treated mice (n = 3 per group). **C** Schematic picture of a transverse lumbar spinal cord section. *, *treatment* $p < 0.05$; #, *surgery* $p < 0.05$. Means ± SEM are indicated.

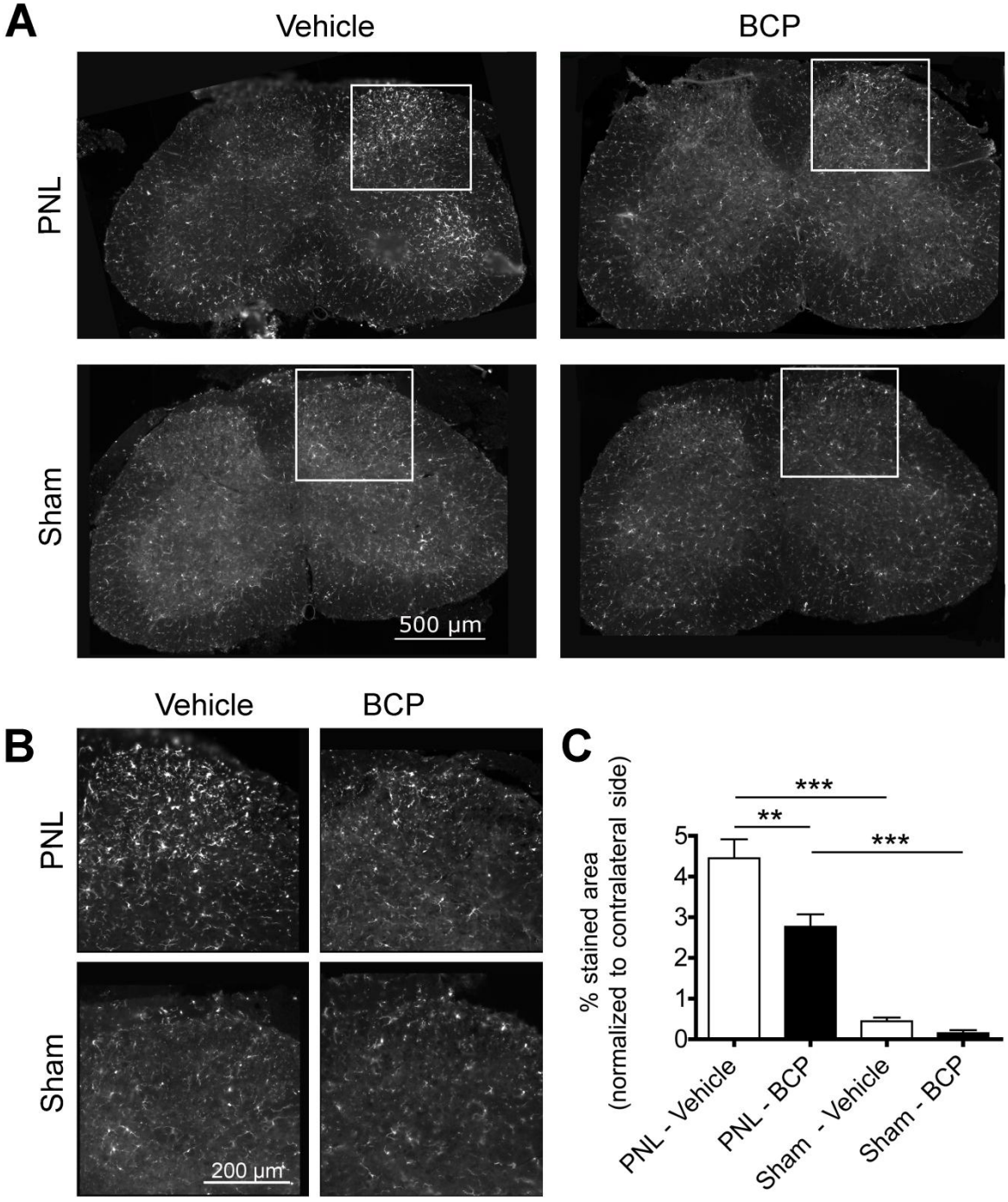


Figure 16 Chronic treatment with 1 mg/kg BCP reduced the mean density of microglia signals in the ipsilateral dorsal horn of the lumbar spinal cord. A Representative pictures of the Iba1 immunostaining in whole lumbar spinal cord sections recorded with the 20x magnification objective. Ipsilateral dorsal horns are located on the right, contralateral dorsal horns accordingly on the left. **B** Magnification of Iba1 stained ipsilateral dorsal horns recorded with the 20x magnification objective (see white frame in A). **C** Quantitative analyses (see also Figure 7) revealed a reduction of microglia signals in the ipsilateral dorsal horn (normalized to contralateral dorsal horn) after two weeks of 1 mg/kg^{-} BCP treatment (n = 4-5 per group). **, *treatment* $p < 0.01$; ***, *surgery* $p < 0.001$. Means $\pm$ SEM are indicated.

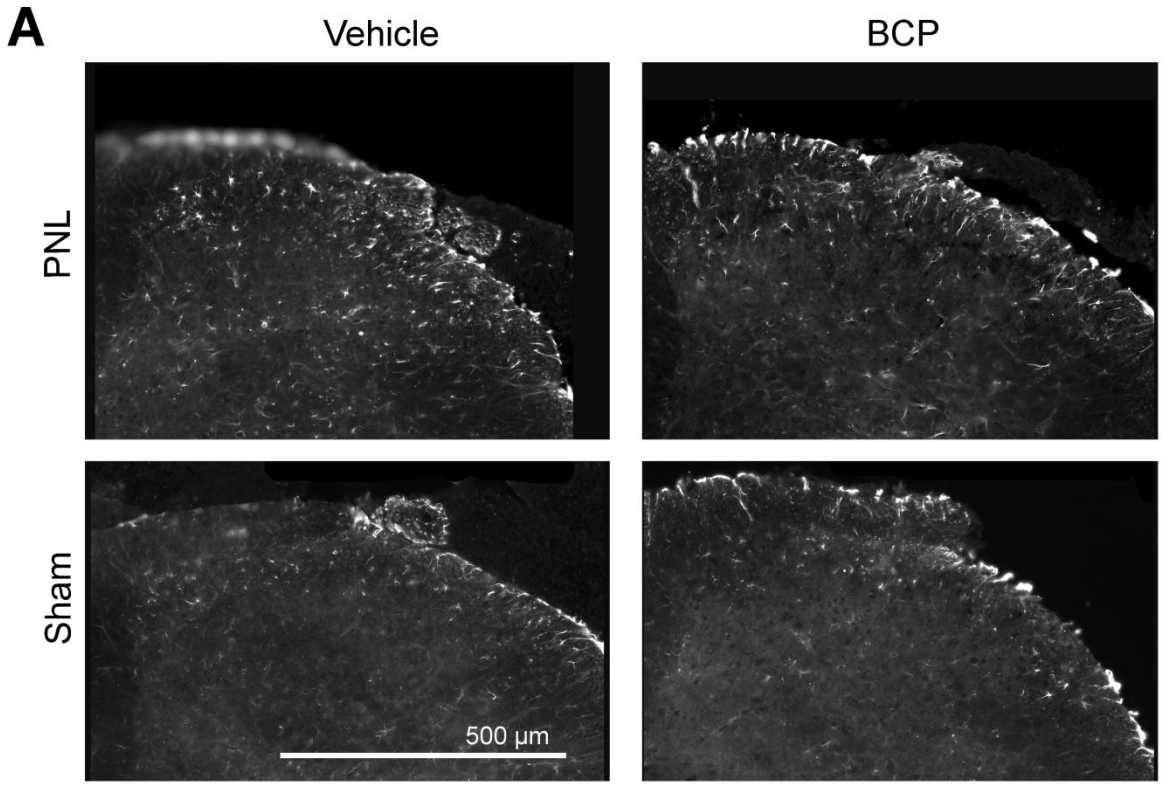


Figure 17 Chronic treatment with 1 mg/kg BCP reduced the mean density of astrocytes signals in the ipsilateral dorsal horn of the lumbar spinal cord. A Representative pictures of the GFAP immunostaining in the ipsilateral dorsal horns of lumbar spinal cord sections recorded with the 20x magnification objective. **B** Although quantitative analyses (see also Figure 7) revealed that the treatment in PNL mice did not significantly decrease astrocytes signals after two weeks of 1 mg/kg BCP treatment compared to vehicle mice, mean values here were similar to that of sham operated mice (n = 4-5 per group). ***, *surgery* $p < 0.001$. Means ± SEM are indicated.

4.1.5 Biomolecular analysis

To further study possible molecular downstream targets of the BCP treatment, quantitative real-time PCR was performed and the expression patterns of the CB2 receptor, inducible nitric oxide synthase 2 (iNOS), interferon-γ induced GTPase (igtp), T-cell specific GTPase 1 (tgtp) and interleukin-1β (IL-1β) mRNA was analyzed.

The partial ligation of the sciatic nerve induced an increase in CB2 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 ipsilateral PNL values were significantly increased compared to ipsilateral sham values (Figure 18; $p = 0.0096$). The CB2 receptor expression was not influenced by the BCP treatment (*treatment:* $F_{1,29} = 1.51$; $p > 0.05$).

iNOS expression was not significantly modulated, although mean iNOS expression was increased in the ipsilateral sides, the main effect failed to reach the level of significance (*side*: $F_{1,29} = 4.00$, $p = 0.0550$).

We found no modulation in the expression patterns of Igtp, Tgtp and Il-1β, neither by the surgery nor by the BCP treatment.

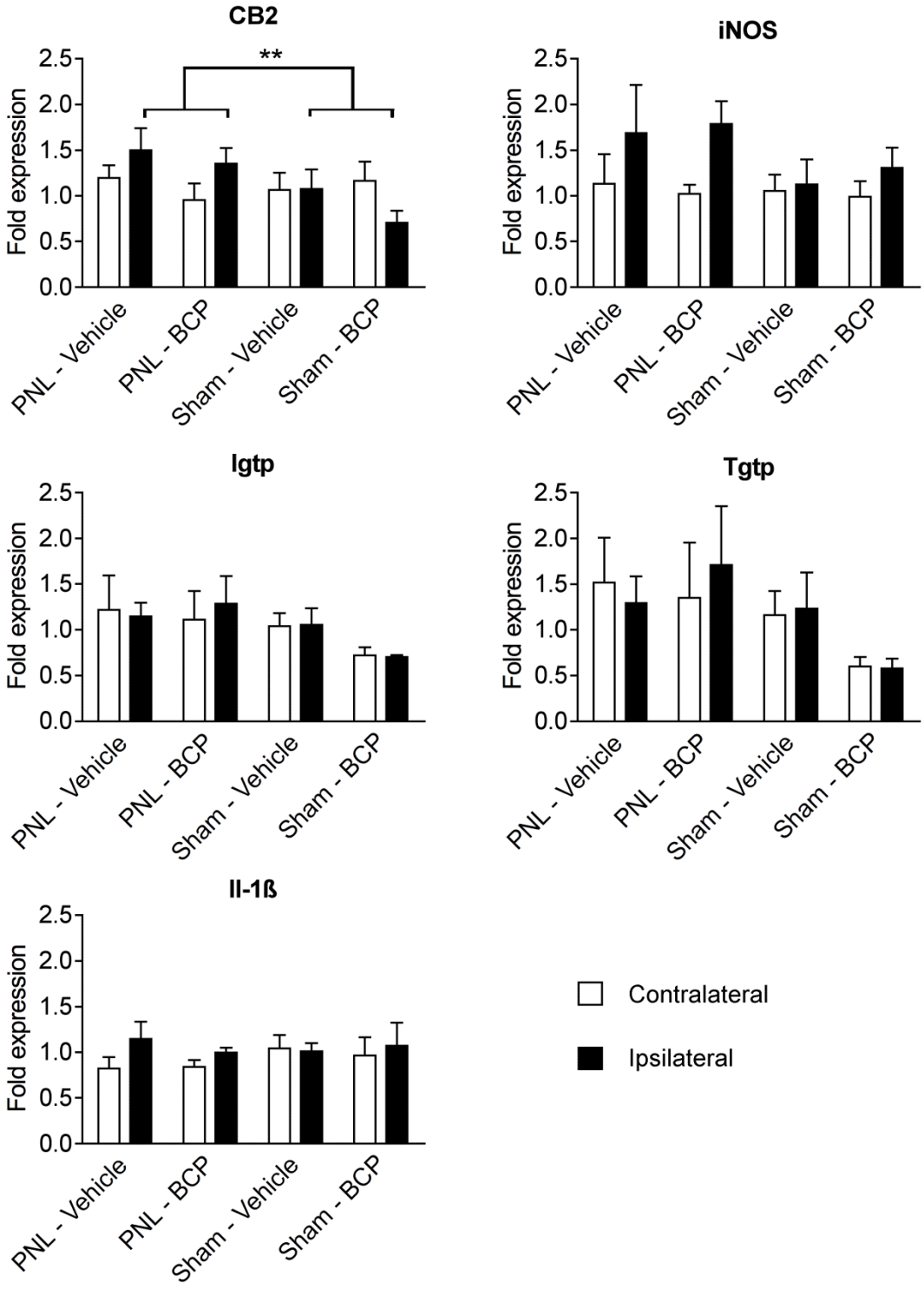


Figure 18 Quantitative analysis of mRNA levels of CB2, iNOS, Igtp, Tgtp, IL-1β in the lumbar dorsal horn of partial nerve ligated mice compared to sham operated mice (n = 4-5 per group). The partial nerve ligation induced an up-regulation of CB2 mRNA in the ipsilateral side of lumbar dorsal horn in vehicle and BCP-treated mice compared to vehicle treated sham mice. Neither the partial nerve ligation nor the BCP treatment changed the mRNA levels of iNOS, Igtp, Tgtp or IL-1β. **, Fisher-LSD *post-hoc* test for *surgery* x *side*, $p < 0.05$. Means + SEM are indicated.

4.2 Paclitaxel-induced neuropathic pain in CB2 knock-out and wild type mice

A further mouse model of neuropathic pain was established to investigate the role of the CB2 receptor during chronic pain development. Chemotherapeutic drug-induced neuropathic pain is initiated by different mechanisms from those in traumatic nerve injury models and yet not studied well. Therefore, the chemotherapeutic agent paclitaxel was administered in wild type and CB2 knock-out mice.

The treatment with paclitaxel only minimally influenced the overall health state of mice. Both, CB2 knock-out and wild type mice gained normal weight during the experiments and repeated measures ANOVA revealed no significant differences in weight between the strains (Figure 20, *strain*; $F_{1,14} = 0.21$; $p > 0.05$).

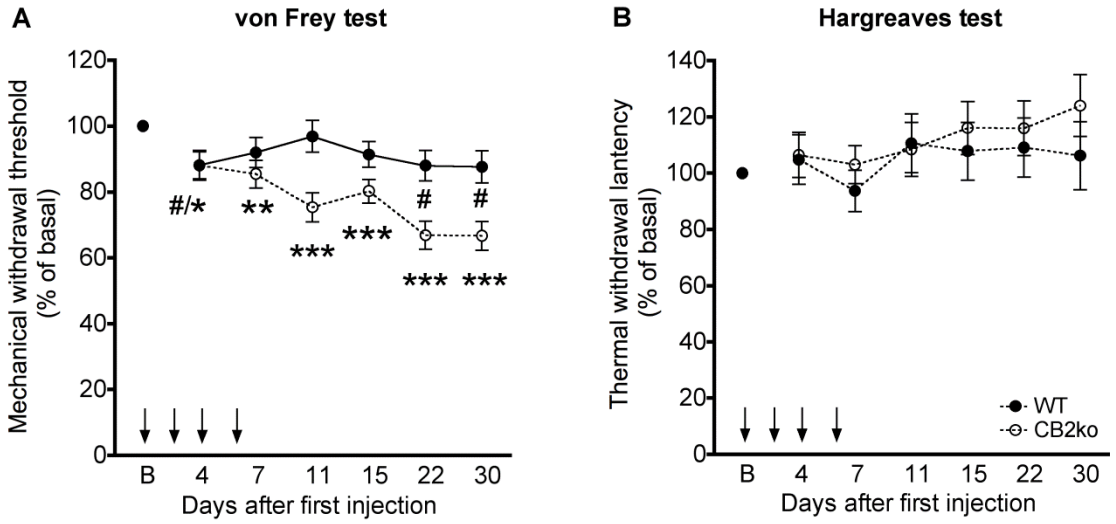


Figure 19 Development of mechanical allodynia, but not thermal hyperalgesia in CB2 knock-out and wild type mice during and after the treatment with Paclitaxel. To induce ne uropathic pain four injections of 4 mg/kg Paclitaxel were administered on alternative successive days (day 0, 2, 4 and 6; arrows). Ipsilateral and contralateral hind paws of CB2 knock-out (CB2ko) and wild type mice (WT) were tested in the von Frey test (A) and Hargreaves' test (B) to evaluate mechanical allodynia and thermal hyperalgesia (CB2 knock-out: n = 12; wild type: n = 10). Wild type and CB2 knock-out mice were tested at day 4, 7, 11, 15, 22 and 30 after the first injection. Data are expressed as percent of basal responses. Fisher-LSD *post-hoc* test for *time* x *strain*: ***, CB2ko compared to basal $p < 0.001$; **, CB2ko compared to basal $p < 0.01$; *, CB2ko compared to basal $p < 0.05$; $^{\#}$, WT compared to basal $p < 0.05$. Means $\pm$ SEM are indicated. B = basal responses.

4.2.1 CB2 knock-out mice were more susceptible to paclitaxel treatment than wild type mice

In a first testing series mechanical allodynia and thermal hyperalgesia were measured in paclitaxel-treated CB2 knock-out and wild type mice. Interestingly, wild type mice developed less signs of mechanical allodynia than CB2 knock-out mice. Thus, a significant *strain* effect and *time* x *strain* interaction in the von Frey test was observed (Figure 19, A; repeated measures ANOVA, *strain*: $F_{1, 42 =} 10.62$, $p = 0.002$; *time* x *strain*: $F_{6, 252} = 4.05$, $p < 0.001$). A following Fisher-LSD *post-hoc* test for *time* and *strain* revealed significantly decreased mechanical withdrawal thresholds in CB2 knock-out mice at all test days (day 4: $p = 0.0105$; day 7: $p = 0.0018$; day 11, 15, 22 and 30: $p < 0.001$) in comparison to base values, while mechanical withdrawal thresholds in wild type mice were solely decreased at day 4 ($p = 0.0200$), 22 ($p = 0.0186$) and 30 ($p = 0.0151$). In the Hargreaves' test neither CB2 knock-out mice nor wild type mice showed signs of thermal hyperalgesia (Figure 19, B; strain: $F_{1, 42 =} 0.43$, $p > 0.05$; *time* x *strain*: $F_{6, 252 =} 0.51$, $p > 0.05$).

4.2.2 Paclitaxel induced no signs of cold allodynia

In a second testing series the measurement of cold allodynia was performed additionally to mechanical allodynia. Because there was no effect observed in the Hargreaves' test in the first testing series, which is also in line with the literature (Smith et al., 2004), we did not determine the thermal hyperalgesia here.

Although repeated measures ANOVA showed no significant effect for the factor *strain* in the von Frey test this time (Figure 20, B; $F_{1, 32 =} 1.22$, $p > 0.05$), the factors *time* and *strain* showed a significant interaction ($F_{6, 192} = 2.27$, $p = 0.038$). Fisher LSD *post hoc* analysis revealed decreased mechanical withdrawal thresholds of CB2 knock-out mice on day 22 and 30 compared to basal level ($p = 0.002$, $p = 0.014$), but not in wild type mice ($p > 0.05$). In contrast to former reports (Smith et al., 2004), neither CB2 knock-out mice nor CB2 wild type mice developed cold allodynia during the testing period of four weeks (Figure 20, C; *strain*: $F_{1, 32 =} 0.01$, $p > 0.05$; *time* x *strain*: $F_{6, 192 =} 0.31$, $p > 0.05$).

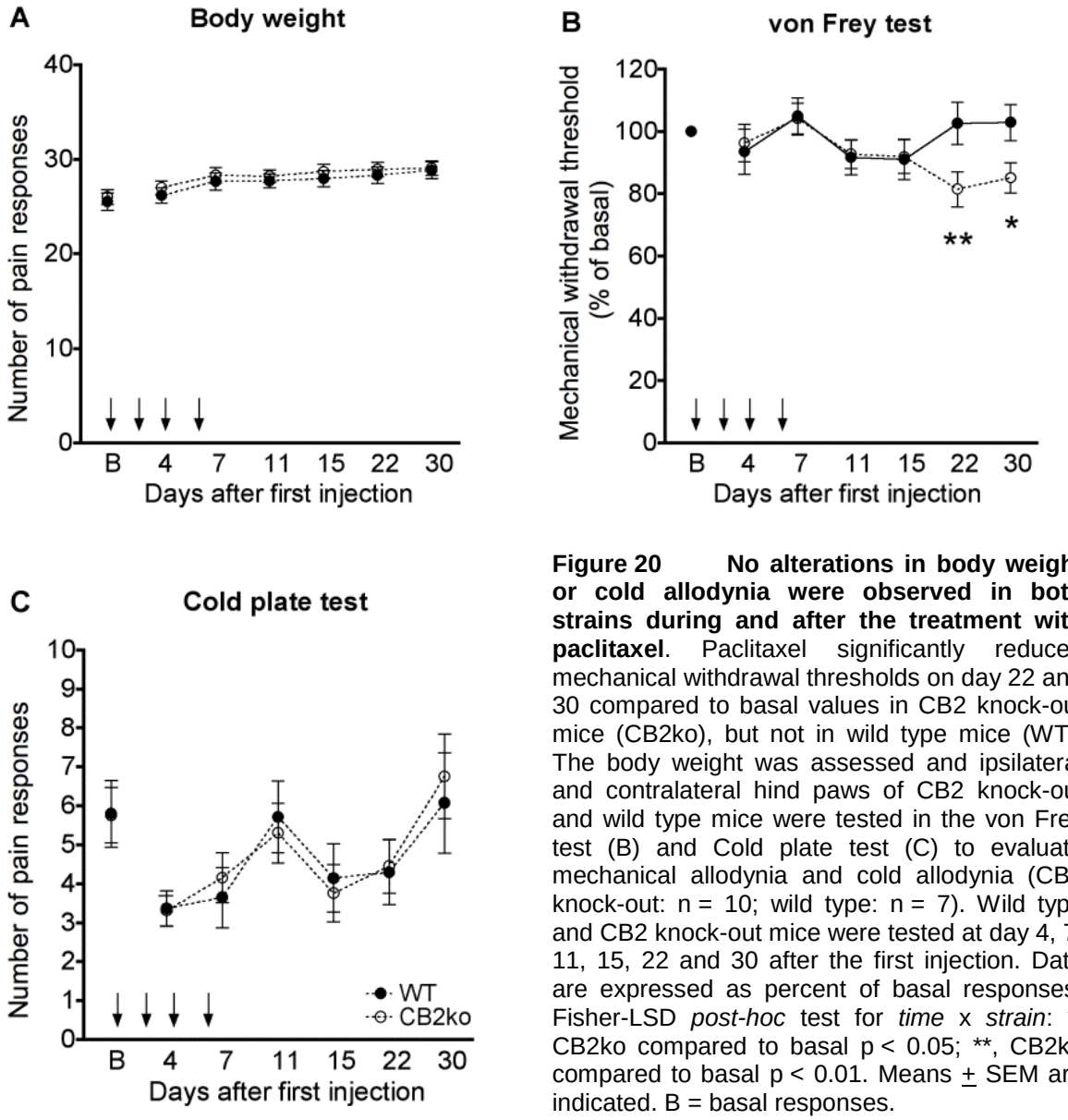


Figure 20 No alterations in body weight or cold allodynia were observed in both strains during and after the treatment with paclitaxel. Paclitaxel significantly reduced mechanical withdrawal thresholds on day 22 and 30 compared to basal values in CB2 knock-out mice (CB2ko), but not in wild type mice (WT). The body weight was assessed and ipsilateral and contralateral hind paws of CB2 knock-out and wild type mice were tested in the von Frey test (B) and Cold plate test (C) to evaluate mechanical allodynia and cold allodynia (CB2 knock-out: n = 10; wild type: n = 7). Wild type and CB2 knock-out mice were tested at day 4, 7, 11, 15, 22 and 30 after the first injection. Data are expressed as percent of basal responses. Fisher-LSD *post-hoc* test for *time* x *strain*: *, CB2ko compared to basal $p < 0.05$; **, CB2ko compared to basal $p < 0.01$. Means $\pm$ SEM are indicated. B = basal responses.

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5 DISCUSSION

Chronic neuropathic pain is a debilitating condition with strong impacts on patients' mood and daily life routine. Although a lot of effort has been put in the establishment of new and effective therapies, treatment of neuropathic pain is still a challenging issue as the whole pain processing is altered. The endocannabinoid system seems to be a promising target for the development of new pain therapies as the analgesic effects of the cannabinoid constituents in the essential oil of *Cannabis sativa* are known since thousands of years (Zias et al., 1993). However, psychoactive side effects due to central receptor activation constrain their therapeutic use and authorization (Ben Amar, 2006). It is now well-documented that the peripheral CB2 receptor plays an important role in the development of neuropathic pain and that its activation alone is sufficient to reduce chronic pain associated with nerve injury (Beltramo, 2009; Guindon and Hohmann, 2008).

The present study investigated the properties of the phytocannabinoid BCP, a selective CB2 receptor agonist, to ameliorate mechanical allodynia and thermal hyperalgesia in mice with a partial nerve ligation, measured in two behavioral tests (von Frey and Hargreaves' test). The pharmacological specificity of the anti-nociceptive effects to CB2 receptor activation was verified by the use of CB2 receptor knockout mice. Acute BCP treatment induced no possible psychomimetic side effects, which are associated with CB1 receptor activation, in the tetrad test. Furthermore, histological analyses revealed a decrease in the inflammatory response (microglia and astrocyte activation) in the dorsal horn of the lumbar spinal cord after BCP treatment, which is a hallmark after nerve injury and accompanying the occurrence of pain.

In the last part of the study a second mouse model for neuropathic pain was established. Mice were treated with the chemotherapeutic agent paclitaxel to induce neuropathic pain symptoms, which were evaluated in three behavioral tests (von Frey, Hargreaves' and Cold plate test). A comparison between CB2 knockout mice and wild type littermates revealed that the CB2 receptor knock-out mice were more susceptible to paclitaxel treatment than wild type mice.

Both experimental approaches emphasize the involvement of CB2 receptors in the development of neuropathic pain and are further discussed in the following sections.

5.1 Anti-nociceptive effects of the plant volatile BCP

The plant sesquiterpene and food additive BCP was recently found to be a highly selective, natural CB2 receptor agonist ((E)-BCP: K_i = 155 ± 4 nM) with a structure totally unrelated to classical phytocannabinoids (Gertsch, 2008; Gertsch et al., 2008; Gertsch et al., 2010). *In vitro* studies with CHO-K1 cells expressing human CB2 receptors verified that BCP activates the whole $G_{i/o}$ protein signaling machinery known from CB2 receptor activation: it inhibited cAMP production, initiated intracellular Ca^{2+} release and phosphorylation of MAPK-ERK1/2 and p38. The same authors similarly provided evidence for beneficial effects of BCP *in vivo* by using an animal model of inflammatory pain (carrageenan model). Interestingly, BCP showed a stronger anti-inflammatory effect in this model as the synthetic CB2 receptor JWH-133, although the binding affinity of JWH-133 to CB2 receptors is higher (K_i = 3.4 nM) (Gertsch et al., 2008).

As the source of chronic, inflammatory pain used in this former study is located in peripheral tissue, while the pathological processes of neuropathic pain underlie nerve injury (Devor, 2006), the present study focused on the effects of BCP in an animal model of peripheral nerve injury-induced neuropathic pain.

Chronic treatment with BCP substantially ameliorated mechanical allodynia and thermal hyperalgesia in male and female mice after the partial ligation of the right sciatic nerve. These effects were likely attributed to selective CB2 receptor activation as they were completely absent in CB2 receptor knockout mice. Acute treatment with a low and a high dose of BCP completely lacked psychomimetic side effects, like hypomotiliy, anti-nociception, hypothermia or catalepsy, seen in the tetrad test after cannabinoid intoxication. This suggests that BCP mediated no off-target effects at the tested doses by binding to central CB1 receptors, which could contribute to analgesia. The lack of psychomimetic side effects was also reported for several other synthetic CB2 agonists (Hanus et al., 1999; Kinsey et al., 2011; Rahn et al., 2011).

No signs of tolerance were seen during the experimental time of two weeks. In contrary, the beneficial effects of BCP accumulated over time, which is clearly advantageous over other analgesic drugs like morphine that known to strongly induce tolerance after chronic administration.

Interestingly, the pharmacological profile of BCP varied between the two tested modalities. The three higher doses of BCP (1, 5 and 10 mg/kg) diminished mechanical withdrawal responses in the von Frey test by a similar extend. Although the beneficial effects of BCP seemed to be more pronounced in 1 mg/kg and 5 mg/kg compared to 10 mg/kg, the lack of a clear dose-response in this modality might reflect the high potency of BCP to reach a maximal effect at low doses.

In contrast, thermal hyperalgesia was specifically reduced by the dose of 1 mg/kg BCP and therefore clearly showed an inverted U-shaped dose response curve for thermal withdrawal thresholds.

These modality-specific differences of BCP treatment are probably underlying the different kind of nerve fibers involved in mechanical allodynia (Aβ-fibers) and thermal hyperalgesia (C-fibers), and subsequent different mechanisms of initiation. While thermal hyperalgesia underlies a peripheral facilitation of small diameter C-fiber activity, which subsequently leads to a central sensitization, mechanical allodynia is mediated by large diameter Aβ-fibers. Due to central sensitization the input from mechanosensitive Aβ-fibers can also activate second-order pain projection neurons in the spinal cord and stimuli like light touch can be perceived as painful (Baron, 2000). In line with this, studies of Curto-Reyes and colleagues (2010) suggested that only spinal CB2 receptors are involved in the anti-allodynic effects of CB2 treatment in neuropathic pain, while both spinal and peripheral CB2 receptors mediate the antihyperalgesic effects.

Although the therapeutic window of BCP in the treatment of mechanical allodynia was clearly broader, it cannot be excluded that higher doses of BCP would reveal the “missing” part of the inverted U-shaped dose-response curve for mechanical allodynia similarly to that seen in thermal hyperalgesia.

A U-shaped dose-response effect for BCP treatment was already observed in the carrageenan model, where the lowest dose tested (5 mg/kg) showed the highest efficiency to reduce paw edema (Gertsch et al., 2008). Similarly, U-shaped dose response curves are repeatedly reported for cannabinoid agonists, e.g. for cannabidiol, AM124, WIN55212-2, BAY39-7271, HU-211 as well as for the endocannabinoid AEA (Sulcova et al., 1998; Malfait et al., 2000; Calabrese, 2008; Rahn et al., 2010). The underlying mechanisms are still not clarified, but could be subjected to a concentration-dependent, differential activation of G protein subsets (G_i and G_o) leading to different or even opposing downstream effects. Shoemaker and colleagues could show in 2005 that the extend of intracellular signaling-stimulation was not only agonist-specific but also concentration-dependent, e.g. 2-AG induced ERK-MAPK phosphorylation with low ED_{50} (half maximal effective dose), stimulated Ca^{2+} transients with a medium ED_{50} and inhibited adenylate cyclase with highest ED_{50} in Chinese hamster ovary cells. In contrast, noladin ether and CP-55,940 most potently inhibited adenylate cyclase followed with less potency by ERK-MAPK phosphorylation and Ca^{2+} transient-stimulation. Additionally to this concentration dependent activation of different signaling pathways, it cannot be excluded that off-target effects at high doses of BCP might oppose the CB2-mediated anti-nociceptive effects.

The effects described above can also account for divergent results of the various CB2 receptor-selective agonists on neuropathic pain symptoms or discrepancies between

research groups testing the same compound. So far, studies of CB2 receptor mediated anti-nociceptive effects strictly used synthetic agonists with chemical structures often differing from the classical cannabinoids. Acute treatment with the aminoalkylindole AM1241dose-dependently attenuated tactile and thermal hypersensitivity produced by spinal nerve ligation in rats (Ibrahim et al., 2003), while in naïve animals AM1241 produced anti-nociceptive effects in the Hargreaves', but not the von Frey test, following here an inverted U-shaped dose-response curve similar to that found in the present study (Rahn et al., 2010). The synthetic CB2 selective, tricyclic pyrazole NESS400 was tested in a chronic treatment approach in a spared nerve injury model and exhibited modality-specific anti-nociception: it prevented the formation of thermal hyperalgesia at the highest dose tested, while mechanical allodynia was only transitorily reduced (Luongo et al., 2010). Although in the present study also a modality-specificity was found in the dose-response effects, the reduction of mechanical allodynia here remained over time. In 2008 Yamamoto and colleagues showed that a single intrathecal administration of JWH-133, a Δ^8-THC derivative, which was similarly used as a reference compound in the present study, reversed partial sciatic nerve ligation-induced mechanical allodynia. This effect was completely abolished in CB2 knockout animals. Single local or systemic administrations of JWH-133 in contrary were ineffective (Yamamoto et al., 2008). Although in the present study here a chronic treatment approach was used, these results are in line with the findings of Yamamoto et al. as JWH-133 similarly was able to reverse mechanical allodynia in the same animal model of pain. Nevertheless, under chronic treatment conditions the systemic administration (i.p.) of the drug was similarly efficient in a dose-dependent manner. Interestingly, no anti-hyperalgesic effects during the JWH-133 treatment were observed. As thermal withdrawal thresholds after JWH-133 treatment were so far not tested in other studies, the present study shows for the first time that the JWH-133-mediated anti-nociceptive effects might be modality-specific. In comparison, the natural occurring BCP produced a more global anti-nociceptive effect than the synthetic JWH-133, although JWH-133 shows a greater binding affinity towards hCB2 receptors as it was demonstrated before (Gertsch et al., 2008).

Some points may have to be considered while comparing the results of CB2 receptor agonist on neuropathic pain: Most studies only tested smaller doses of the compounds, which could lead to the assumption of clear dose-response effects, although some compounds might exhibit an inverted U-shaped dose-response curve when testing a broader dose spectrum. Secondly, as there are no clear standards for testing pain killers in animals, pain studies comprise a variety of different animal models for neuropathic pain (e.g. spinal nerve ligation, chronic constriction model, partial nerve ligation, etc.), a variety of different behavioral tests for the same modality (e.g. von Frey hairs, dynamic von Frey devices, etc.)

to measure the pain outcome, and several routes of administration for the drugs, which makes it difficult to directly compare the compounds efficacy.

Nevertheless, taken together all findings have clearly demonstrated that CB2 receptor activation alone is sufficient for attenuation of neuropathic pain symptoms and suggest an important role of peripheral and spinal CB2 receptors during neuropathic pain development. The natural source of BCP and the possibility of an oral intake as a dietary factor make BCP and interesting compound that could be clearly advantageous over other synthetic cannabinoid agonists in clinical practice.

5.2 BCP reduced glia signals in the spinal cord

Glia cells are thought to contribute to the development of neuropathic pain. Their activation and proliferation in the spinal cord parallels the development of neuropathic pain symptoms (Coyle, 1998). A fast activation and proliferation of microglia cells is seen as early as 4 hours after nerve injury in the dorsal horn of the lumbar spinal cord. This is followed by a late activation of astrocytes, which are gradually up regulated starting around postoperative day four (Tanga et al., 2004). These findings suggest that microglia are mainly involved in the initiation of neuropathic pain by the release of mediators that in turn activate astrocytes and further microglia cells. Astrocytes are thought to play a role in promoting and maintaining neuropathic pain, because it was shown that microglia activation decreases to baseline after three weeks, while astrocyte-activation and behavioral hypersensitivity remains (Tanga et al., 2004; Hald et al. 2009). Both types of activated glia cells release several pro-inflammatory factors, e.g. IL-1β and TNF-α, that are known to induce neuropathic pain symptoms after central administration (Oka et al., 1995 and 1996), while an administration of their inhibitors is capable to attenuate neuropathic pain (Sommer et al., 1999; Schäfers et al., 2003).

CB2 receptors are expressed on cells of immune origin and therefore also on microglia, the immune cells of the CNS (Facci et al., 1995; Howlett et al., 2004; Maresz et al., 2005). But CB2 receptors were also reported to be present in astrocytes under pathological conditions (Benito et al., 2007; Garcia-Ovejero, 2009). Due to their expression profile it is likely that CB2 receptors mediate their beneficial effects during neuropathic pain through a modulation of the inflammatory response.

Insights into the role of CB2 receptor activation in glia cells during the development of neuropathic pain were given by experiments using CB2 receptor knockout mice. These mice exhibited not only an exaggerated glia response compared to wild type littermates, but the inflammation also spread to the contralateral dorsal horn, which was accompanied by mechanical allodynia and thermal hyperalgesia in the uninjured paw (Rácz et al., 2008b). In

contrary, activation of the CB2 receptor by intrathecal JWH-015 treatment was shown to decrease microglia and astrocyte activation in the lumbar spinal cord after paw incision (Romero-Sandoval et al., 2007). These results were confirmed by Luongo et al. using another CB2 agonist (NESS400), which similarly diminished the number of nerve injury-induced activated microglia and astrocytes in the spinal projection area. It is hypothesized that CB2 receptor activation shifts microglia into an anti-inflammatory phenotype thereby reducing the expression of pro-inflammatory mediators like IL-1ß, IFN-γ and TNF-α, while increasing the expression of the anti-inflammatory IL-10 (Romero-Sandoval et al., 2009; Luongo et al., 2010). These results suggest that CB2 receptors are crucial key mediators in the control of the inflammatory response during pathological pain.

The present histological analysis of Iba1 (microglia marker) and GFAP (astrocyte marker) staining in the lumbar dorsal horn of spinal cord slices similarly revealed increased signals of Iba1 and GFAP signals ipsilateral to the partial nerve ligation, but not to sham operation, in vehicle-treated mice. Treatment with the natural CB2 agonist BCP diminished Iba1 and GFAP expression in the dorsal horn, which is in line with the diminished hypersensitivity seen in the behavioral tests. The BCP treatment had no effect on Iba1 or GFAP expression in BCP treated sham animals compared to vehicle treated sham animals. This could be due to less activated glia cells in the absence of neuropathic pain and therefore less amounts of CB2 receptors, which could be targeted by BCP.

Together with the behavioral data the histological analyses suggest that BCP inhibits the formation of allodynia and hyperalgesia by immunomodulatory effects, rather than directly modulating the pain transmission in peripheral or central neurons. Consequently, BCP neither increased withdrawal thresholds towards noxious heat stimuli (Hargreaves test) in general (e.g. in sham animals) nor modulated acute pain responses in the tail flick test, features which are known from analgesic drugs like morphine (Whiteside et al., 2005; Rahn et al., 2010).

5.3 CB2 receptors are up regulated in the spinal cord

It is frequently reported that CB2 receptor expression is up regulated in DRGs and lumbar dorsal horns of the spinal cord after peripheral nerve injury, which correlates with the appearance of activated microglia (Zhang et al., 2003; Beltramo, 2006;Luongo et al., 2010). In the absence of CB2 receptors, gene expression profiles revealed an enhanced interferon-γ response in lumbar spinal cords of mice suffering from neuropathic pain, which might account for the exaggerated, bilateral pain response reported for these mice (Rácz et al., 2008a). Indeed, in interferon-γ/CB2 double knockout mice the exaggerated pain response after nerve injury was lost and pain development remained similar to that of wild type mice.

Interferon-γ is a cytokine that is involved in the host defense against pathogens and tumors and plays a comprehensive role in immunomodulation, e.g. in the regulation of cytokine production (IL-12, TNF-α) (Bach et al., 1997). Additionally, it induces the transcription of several guanosine triphosphatases (GTPases) (Mertens and Howard, 2006), some of which were also up regulated in the CB2 receptor knockout mice suffering from neuropathic pain. (Rácz et al., 2008a). The authors of this study postulated that interferon-γ, which is released by astrocytes and neurons, promotes the consolidation of neuropathic pain and induces several signaling pathways in microglia resulting in iNOS and chemokine receptor 2 up regulation. This in turn would lead to a further activation of microglia and astrocytes and interferon-γ release. Activation of the CB2 receptors could dampen this formation of a “vicious circle” and therefore anticipate an uncontrolled glial activation.

To reveal if BCP mediates its effect by diminishing the interferon-γ response during neuropathic pain, expression analyses for the mRNAs of two interferon-γ-induced GTPases (igtp and tgtp), which were shown to be up regulated after nerve ligation (Rácz et al., 2008a), as well as analysis of CB2, iNOS and pro-inflammatory IL-1β mRNA were performed in the present study.

As it was reported before for other neuropathic pain models (Luongo et al., 2010; Hsieh et al., 2011), CB2 receptor mRNA was up regulated in the ipsilateral, but not contralateral sides of mice with a partial sciatic nerve ligation. In sham animals no alterations in the CB2 mRNA expression were found compared to ligated animals nor did BCP treatment influence CB2 mRNA expression. The CB2 receptor expression profile matched the sides of BCP-mediated anti-nociception: The withdrawal thresholds of ipsilateral sides were increased, but we found neither BCP-mediated alterations of the withdrawal thresholds of contralateral sides, nor that of sham animals. This is consistent with the general view that CB2 receptors only seem to play a minor role under normal, naïve conditions, but are markedly up regulated under various disease conditions at the sides involved in the disease (Pacher and Mechoulam, 2011).

In contrast to CB2 mRNA expression, no changes in the expression profile were found for iNOS, igtp, tgtp or IL-1β. The mean iNOS expression was increased in ipsilateral sides of mice with a partial nerve ligation, but this effect was not significant and clearly no treatment effect of BCP was seen in these animals. Although it was already shown that CB2 agonists are able to decrease pro-inflammatory IL-1β levels (Luongo et al., 2010), we found no treatment effect for IL-1β levels, but also no surgery effect. Eventually, here the wrong time point was chosen or an analysis of the actual protein level is a better approach for interleukin measurement and should be evaluated in future studies.

The biomolecular results suggest that BCP diminishes not directly the interferon response, but is possibly acting through other signaling pathways, which might anticipate the

downstream interferon response. On the other hand it has to be considered that the mRNA analysis here only reveals a snapshot of the processes, which are going on during neuropathic pain. Again, a systematic analysis of different time points and analyses of the actual protein levels would certainly be required to give a clear answer to this issue.

5.4 BCP: an all-round talent?

Reviewing the recent literature, it becomes clear that the beneficial effects of BCP are not only restricted to neuropathic pain treatment. There are several health effects that have been attributed to BCP treatment. Our group already demonstrated that BCP exhibited anti-inflammatory effects by inhibiting the formation of edema in the carrageenan model of inflammatory pain (Gertsch et al., 2008). Similarly, BCP reduced the pain responses in the late phase of the formalin test, a model of central sensitization, which was prevented in CB2 knockout mice (Klauke et al., in preparation). Two recent studies reported that oral BCP treatment also inhibited colon inflammation and tissue damage in dextran sulfate sodium-induced colitis, in which one study verified that CB2 and PPARγ receptor-activation, is required for these effects (Young et al., 2007; Bento et al., 2011). In a mouse model of cisplatin-induced nephrotoxicity, BCP ameliorated kidney dysfunction, renal inflammation and oxidative stress in a CB2 dependent manner (Horváth et al., 2012). Additionally, further beneficial effects of BCP have been reported that were not directly linked to CB2 receptor activation in these studies. These effects include local anaesthetic (Ghelardini et al., 2001), anti-carcinogenic (Di Sotto et al., 2010; Legault and Pichette, 2007; Loizzo et al., 2008), anti-fibrotic (Calleja et al., 2012) and anxiolytic-like activity (Galdino et al., 2012).

Besides the beneficial effects of BCP and other CB2 receptor agonists alone, cannabinoid agonist might also provide new opportunities for combination therapies. Cannabinoid and opioid receptors are co-expressed in areas, which are involved in pain processing, e.g. dorsal horn of the spinal cord (Welch and Stevens, 1992; Hohmann et al., 1999; Salio et al., 2001), the periaqueductal grey, raphe nuclei and central medial thalamic nuclei (Mansour et al., 1988; Herkenham et al., 1991; Lichtman et al., 1996). It was shown that cannabinoid agonists increase the effectiveness of μ-opioid receptor agonists (Reche et al., 1996; Yesilyurt et al., 2003; Finn et al., 2004; Tham et al., 2005; Cox et al., 2007). Additionally, evidence is raising that combination therapies with opioids and cannabinoids might reduce the development of morphine-induced tolerance. Morphine administration activates glia cells and the release of pro-inflammatory cytokines and chemokines, which counteracts the morphine-induced analgesia and enhances morphine tolerance and dependence (Watkins et al., 2009). CB2 receptor agonists were recently shown to down regulate the morphine-induced activation of glia and thereby might increase the effectiveness of opioids in long-term

use (Tumati et al., 2012; Merighi et al., 2012). Similarly, there are reports showing that CB2 receptor agonists are increasing the analgesic effects of non-steroidal anti-inflammatory drugs (NSAIDs) (Guindon et al., 2006a,b; Ulugöl et al., 2006). Finally, BCP was shown to potentiate the anticancer activity of paclitaxel (Legault and Pinchette, 2007). Taken together, combination therapies might offer the possibility of decreasing the doses of opioids, NSAIDs or paclitaxel and thereby reducing the risk of unwanted side effects.

Compared to synthetic CB2 receptor agonists, BCP provides the clear advantage of an effective oral treatment as it is readily bioavailable and metabolized with a $T_{max} > 1h$ after a single oral administration (Gertsch et al., 2010). The most efficient dose-range in mice was between 1 and 5 mg/kg. To get first hints about the amounts of BCP a human has to ingest for sufficient receptor-binding, the human equivalent dose (HED) can be estimated. The US Food and Drug Administration suggested to calculate the HED based on normalization of the body surface areas (bsa):

$$HED\ (mg/kg) = animal\ dose\ (mg/kg) \times \frac{mouse\ km}{human\ km},$$

where the k_m value for mouse ($k_m = 3$) and a 60 kg human ($k_m = 37$) is based on body weight divided by bsa (Reagan-Shaw et al., 2008). Following this, a human daily dose of 0.08-0.41 mg/kg BCP would correspond to the effective dose in mice. It was already estimated that the average daily intake of BCP with vegetable food and spices lies in the range of 10 to 200 mg (Gertsch et al., 2008), which would correspond to a dose of 0.16-3.3 mg/kg for a 60 kg human. This dose would certainly be sufficient for significant CB2 receptor activation, but further investigations have to be performed to get deeper insights about the impact of BCP on human health.

5.5 Establishing a model of paclitaxel-induced neuropathic pain

Paclitaxel, which naturally occurs in the bark of the Pacific yew (*Taxus brevifolia*), is widely used as an antineoplastic agent, specifically in ovarian and breast cancer therapy. It impedes the cell cycle in the late phase of mitosis by promoting microtubule assembly, which leads to the formation of abnormal microtubule arrays, interrupts normal cell function and finally leads to cell death (Scripture et al., 2006). Although these effects are desired for cancer therapy, it certainly also affects the healthy tissue. Paclitaxel accumulates in intermediate or low amounts in dorsal root ganglia, brain, spinal cord and sensory neurons (Cavaletti et al, 2000). In sensory neurons paclitaxel similarly promotes the formation of

microtubule aggregates, which impairs the axonal transport, results in sensory nerve demyelination and neuronal cell death (Masurovsky et al., 1981; Lipton et al., 1989; Apfel et al., 1991).

Paclitaxel-evoked neuropathy is manifested as pain in the distal extremities, forming a glove and stocking pattern. It preferentially impairs myelinated A-β and to a lesser extent A-δ fibers, which carry sensory information about mechanical and cold stimulation to the spinal cord. Accordingly, patients suffer from an impaired perception of light touch, vibration and cold. In contrast, the sensitivity towards thermal heat stimuli is not altered (Dougherty et al., 2004).

Paclitaxel was also shown to induce neuropathic pain symptoms in rodents (Polomano et al., 2001; Smith et al., 2004). In mice, several strains were tested for development of mechanical allodynia, which was strain dependent with some strains appearing to be more sensitive (DBA/2), while other were less sensitive (C3H/He, C57BL/6). Two strains were further tested for thermal hyperalgesia and cold allodynia. While thermal thresholds remained unaffected, both strains developed cold allodynia (Smith et al., 2004). In rats also thermal hyperalgesia was found (Polomano et al., 2001).

Few studies also focused on the role of CB2 receptors in paclitaxel-induced neuropathic pain. Similarly to traumatic nerve injury models, agonists like AM1241, AM1714 and MDA19 reduced mechanical allodynia in a CB2 receptor-dependent manner (Rahn et al., 2007; Rahn et al., 2008; Xu et al., 2010). But the mechanisms by which CB2 receptor agonists modulate chemotherapeutic agent-induced neuropathic pain remains unknown - especially as it was found that astrocytes, but probably not microglia are involved in neuropathic pain after paclitaxel treatment (Zhang et al., 2012).

To establish the paclitaxel-evoked neuropathic pain model in our laboratory, the protocol of Smith et al. (2004) was slightly adjusted and wild type C57BL/6J and CB2 knockout mice on the same background were tested. Although wild type mice slightly developed signs of mechanical allodynia, CB2 knockout mice exhibited a robust decrease in mechanical withdrawal latencies, while thermal withdrawal latencies remained unchanged as it was reported by Smith and colleagues. In a second round of experiments cold allodynia was also assessed. In contrast to the reports from the literature, none of the strains developed cold allodynia in these experiments. Differences between the present measurements and literature reports might arise from the different testing methods used. Smith et al. (2004) used von Frey hairs to test mechanical allodynia, while in the present study a dynamic aesthesiometer was used. Similar, Smith et al. (2004) applied the acetone method to measure cold allodynia and not the Cold plate test as used in our study.

Nevertheless, the present results suggest that CB2 receptor activation protects against paclitaxel-evoked neuropathic pain, which is in line with the results of traumatic nerve injury models (Rácz et al., 2008b; Rahn et al., 2008).

6 CONCLUSION AND OUTLOOK

Summarized, the present study is the first demonstration that a common food ingredient is highly effective in an animal model of neuropathic pain at physiologically relevant doses. BCP is the first bioactive, natural CB2 receptor agonist, which is able to reduce neuropathic pain symptoms in mice, without exerting any CB1-associated psychomimetic side effects. Thus, it is likely that BCP belongs to a group of common plant products with a major potential impact on human health. The possibility of an oral intake of this dietary cannabinoid with vegetable food could be advantageous over other synthetic cannabinoid agonists in the daily clinical routine and improve current neuropathic pain therapies.

The establishment of another animal model for neuropathic pain further stresses the crucial role of CB2 receptors in the development of neuropathic pain. Additional experiments might give deeper insights in CB2 receptor activation during paclitaxel-induced neuropathic pain by examining the immune responses in dorsal root ganglia and dorsal horns of the spinal cord. Using a more sensitive mouse strain than C57BL/6J mice, paclitaxel-induced neuropathic pain would certainly be a promising model to test the effects of BCP treatment, because BCP was also shown to potentiate the anticancer effects of paclitaxel (Legault and Pichette, 2007). A combined therapy of BCP and paclitaxel during cancer could therefore offer the opportunity of lowering the paclitaxel dose, which in turn reduces the risk of side effects (e.g. neuropathic pain symptoms) and, in parallel, may directly inhibit the development of neuropathic pain by CB2 receptor activation.

7 LITERATURE

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