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ORIGINAL ARTICLE

Analgesic and antiinflammatory effects of cannabinoid receptor agonists in a rat model of neuropathic pain

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Abstract Cannabinoid receptor (CB) agonists are known to attenuate allodynia in a range of pain models, but their long-term effects and their mechanisms of action are controversial. The present study compares the antiallodynic effects of long-term treatment with a mixed CB1/CB2 (WIN55,212-2) and a selective CB2 (GW405833) cannabinoid receptor agonist and correlates these effects with their influences on spinal cord (SC) glial activation. The substances were applied daily in a rat neuropathic pain model. Tactile allodynia was assessed, and the development of gliosis was illustrated with immunohistochemical methods. Both substances reduced mechanical allodynia. Their analgesic effect was accompanied by a significant reduction in reactive gliosis and cathepsins (CAT) X and S expression. A daily injection of either substance for 8 days was sufficient to induce a sustained antiallodynic effect, which persisted up to 6 days after the last injection. The re-appearance of mechanical allodynia after this period was associated with a breakout of a strong gliotic response in the lumbar SC. Our results emphasize the therapeutic efficacy of cannabinoid receptor agonists and their inhibitory effects on the formation of gliosis.

Keywords Allodynia · Chronic treatment · Gliosis · WIN55,212-2 · GW405833 · Spinal cord

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Introduction

Cannabinoid receptor agonists are implicated in a wide variety of central nervous system processes, including learning and memory, motor coordination, neuroprotection, control of appetite, and even development (Harkany et al. 2007; Ameri 1999; Grotenhermen 2004). In addition, a large body of evidence from preclinical studies has shown the significant analgesic efficacy of these compounds in acute as well as in chronic neuropathic pain (for review, see Lever and Rice 2007). For example a single injection (s.c. or i.v.) of the synthetic agonist WIN55,212-2 that binds to both CB1 and CB2 receptors attenuates allodynia (cold and mechanical) and hyperalgesia (thermal and mechanical) in rodent models of neuropathic pain (Bridges et al. 2001; Fox et al. 2001; Herzberg et al. 1997; LaBuda and Little 2005; Liu and Walker 2006). In animals, antinociceptive efficiency of cannabinoids is comparable to that of morphine (Bloom and Dewey 1978; LaBuda and Little 2005). Data about the effectiveness of cannabinoids in humans suffering from different types of pain are conflicting (Campbell et al. 2001; Frank et al. 2008) and hampered by the undesired psychotropic side effects (Ashton 1999; Wang et al. 2008), but there is growing evidence that especially in chronic neuropathic pain conditions, cannabinoids are a valuable treatment opportunity for patients (Beaulieu and Ware 2007). The side effects derive largely, if not exclusively, from the activation of the CB1 receptor, which is strongly expressed in the brain (Herkenham et al. 1990; Matsuda et al. 1993). In order to avoid these central CB1 effects, research efforts have been focused on highly selective agonists of the CB2 receptor that is located primarily on immune tissue (Onaivi et al. 2006; Van et al. 2005). Over the years, a number of CB2 agonists have emerged (Cheng and Hitchcock 2007; Guindon and Hohmann 2008) and were shown to exhibit

analgesic, antihyperalgesic, and antiallodynic activity (Beltramo et al. 2006; Elmes et al. 2004; Sagar et al. 2005; Valenzano et al. 2005; Whiteside et al. 2005).

Although the CB2 agonists offer promise in neuropathic pain management, a number of questions remain unanswered. Among others, it is not known where the cannabinoids exert their antinociceptive effects, on neurons and/or immune cells and in the central and/or the peripheral nervous system. Moreover, there are only insufficient comparative data about the efficacy of selective CB2 and non-selective CB1/CB2 agonists administered by a practical route of administration in the same neuropathic pain model. Finally, several studies have shown that the analgesic effect of cannabinoids have a short-lasting effect (Herzberg et al. 1997; Valenzano et al. 2005; Whiteside et al. 2005), implicating that a long-term treatment is required for an effective enduring relief of neuropathic pain. However, in contrast to CB1/CB2 cannabinoids (Costa et al. 2004; De Vry et al. 2004; Mao et al. 2000; Marchalant et al. 2007), the impact of long-term treatment with CB2 agonists has not been studied so far.

The aim of the present study was therefore to analyze and compare the effects of long-term treatments with a non-psychotropic dose of a mixed CB1/CB2 agonist WIN55,212-2 and a CB2 specific agonist GW405833 on mechanical allodynia and on mediators involved in the development and maintenance of neuropathic pain, such as gliosis and cathepsins, in a rat model of neuropathic pain.

Materials and methods

Animals

Male Wistar rats (Janvier, Le Genest Saint Isle, France) with a weight of 200–250 g were used in these experiments. Animals were housed in a climate-controlled room on a 12–12 light–dark cycle. Food and water were available ad libitum. All procedures were approved by the local animal usage committees according to German guidelines on animal care and use.

Surgery

A modified spinal nerve ligation model (Kim and Chung 1992) was used in this study. The left L5 spinal nerve was transected (L5T) according to the procedure described by Ringkamp et al. (1999). Control rats (sham) underwent a surgery with exposure of L5, but no transection.

Behavioral test

Tactile allodynia was assessed by measuring paw withdrawal thresholds to an increasing pressure stimulus using a dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy). Testing was conducted on both the ipsi- (transected) and contralateral paw prior to (habituation phase, hab) and after surgery or 1 h after drug injection. For the measurements, the animals were placed into raised plexiglass boxes with mesh flooring and allowed to acclimatize for at least 15 min until exploratory behavior ceased. Sampling was conducted by a metal filament, which was applied manually to the ventral mid-plantar hind paw. The force raised (0–50 g) with time (20 s) until the rat lifted its paw. The mean withdrawal threshold for both hind paws was taken from a set of three applications, not less than 2 min apart.

Animals were only selected for pharmacological treatment in the present study if they developed an ipsilateral threshold difference of at least 5 g between pre- and postsurgery measurements and a DiffScore (contralateral threshold minus ipsilateral threshold) of a minimum of 5 g.

Drug applications

Baseline mechanical allodynia was checked in operated rats at day 1 after surgery, and drugs were administered daily from day 2 after surgery onward up to day 8 (transient) or day 15 (chronic; Fig. 1). Each group of animals was used for only one drug administration protocol.

WIN55,212-2 (ratCB1 2.4±0.3 nM; ratCB2 35.6 nM, McPartland et al. 2007) was supplied by Tocris Bioscience (Ellisville, USA) and was administered intraperitoneal (i.p.) in ethanol, Cremophor (Sigma-Aldrich, Taufkirchen, Germany) and saline (1:1:18) at a concentration of 0.1 mg/kg.

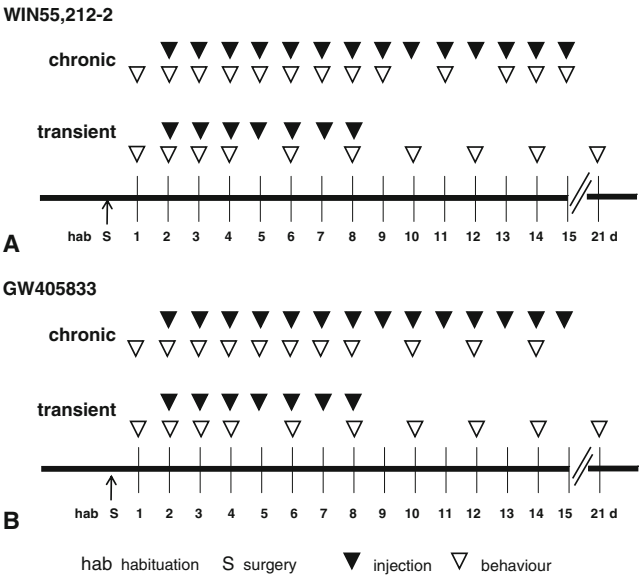

Fig. 1 **a**, **b** Injection and behavior analysis schedules. L5T-operated animals were treated with WIN55,212-2 (**a**), GW405833 (**b**), or vehicle for 14 days (chronic) or 7 days (transient), and mechanical allodynia was monitored for up to day 20/21 after surgery

GW405833 (ratCB1 273±42.6 nM; rat CB2 3.6±1.1 nM, Valenzano et al. 2005) was obtained from Tocris Bioscience made up in ethanol, Cremophor (Sigma-Aldrich) and saline (5:1:94) and injected i.p. at a concentration of 0.1 mg/kg. The doses of both compounds were chosen to avoid side effects like catalepsy, motor impairment, or sedation (Bridges et al., 2001; Valenzano et al., 2005; Wang et al., 2008). Control animals (vehicle) received vehicle only. Dosing volumes for all animals were 0.5 ml/kg.

Immunohistochemistry

Animals were transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cord (SC) was excised and postfixed for 24 h in the perfusion fixative. The lumbar SC was embedded in paraffin. Serial, transversal 18-µm-thick sections were cut throughout the SC segments. Sections were mounted on Superfrost slides (Carl Roth, Karlsruhe, Germany).

Single immunohistochemical stainings were performed using the following primary antibodies: goat anti-rat cathepsin S (anti-CATS; 1:100-200, Santa Cruz Biotechnology, Santa Cruz, USA; suitable for detection of prepro-, pro-, and mature form of CATS), goat anti-mouse cathepsin X (anti-CATX; 1:100-200; R&D systems, Abingdon, UK; suitable for detection of prepro-, pro-, and mature form of CATX), the microglia/macrophage marker mouse anti-phosphotyrosine (anti-PT66; 1:1,000; Sigma), and the astrocyte marker mouse antiglial fibrillary acidic protein (anti-GFAP; 1:5,000; Chemicon, Hampshire UK). Primary antibodies were detected by the avidin–biotin peroxidase system as described (Leichsenring et al. 2008). Specificity of the staining was either confirmed by omitting primary antibodies or by preabsorption with a fivefold (by weight) excess of specific blocking peptides for 2 h at RT (for anti-CATS and anti-CATX).

Data analysis

Behavior

All data are expressed as means±SEM. Changes in nociceptive behavior were analyzed by means of two-way ANOVA to determine statistical significance. All pairwise multiple comparisons were performed by Holm–Sidak's post hoc test.

Immunohistochemistry

Immunostained sections were visualized at the microscopic level (Axioskop 2; Zeiss, Oberkochen, Germany) under brightfield illumination and Nomarski optics. Images were captured with an imaging system (JVC, KY-F75U camera) connected to a computer equipped with an image program (Diskus 4.50, Hilgers, Königswinter, Germany).

Alterations of staining intensity or distribution of stained structures of each animal were independently analyzed by two examiners blind with respect to the treatment of the animals.

Results

Development of mechanical allodynia in L5T rats

All experimental animals (L5T and sham) maintained good health throughout the experimental period. They exhibited normal weight gain and normal level of general activity. Furthermore, autotomy or spontaneous pain behavior was not observed. From the first day after surgery, the ipsilateral paw of the L5T rats became markedly sensitive to mechanical stimuli. Testing mechanical allodynia using the dynamic plantar aesthesiometer revealed a significant decrease in paw withdrawal threshold of the ipsilateral hindpaw compared to the contralateral side and sham-operated animals. The mean decrease of the ipsilateral threshold was 10 g, whereas the DiffScore increased by 12 g (Fig. 2a, b). This marked increase lasted for at least 1 month (end of observation period).

The effects of repeated intraperitoneal injection of WIN55,212-2 or GW405833 on mechanical allodynia

The main limitation of cannabinoids for clinical uses has been their psychoactive side effects. In our studies, systemic daily treatment of animals with WIN55,212-2 or GW405833 was well tolerated by all rats. Although not formally measured, we did not observe any overt motor deficits or sedation and catalepsy. Moreover, in an independent experiment, we found that a single injection of the applied dose of 0.1 mg/kg WIN55,212-2 failed to ameliorate acute mechanical allodynia. In addition, there was no change in contralateral thresholds throughout the treatment period for both cannabinoid agonists (data not shown).

In contrast, both drugs produced a marked attenuation of mechanical allodynia throughout the observation period of 2 weeks (chronic group, Fig. 3a, b). This amelioration was significant for the treatment period for both drugs (Fig. 3a, b). The mean percent reversal for the treatment period days 3–15 was 42.5% for WIN55,212-2 and 45.5% for GW405833. Thus, neither the time course nor the efficacy differed substantially between WIN55,212-2 and GW405833.

In a second set of experiments, we investigated whether repeated injections of cannabinoids produce effective long-term reversal of allodynia that outlasts the injection period

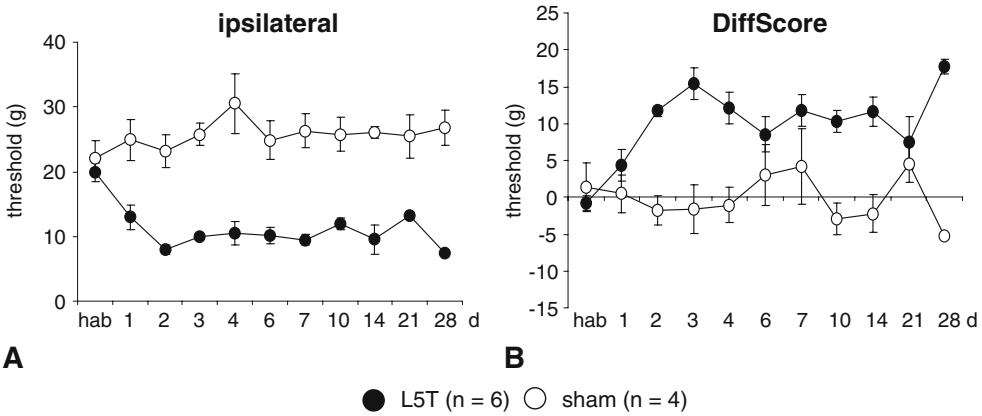

Fig. 2 **a**, **b** Mechanical allodynia after L5 transection. The responses to an increasing pressure stimulus in animals with (*L5T*) and without (*sham*) transection of L5 expressed as ipsilateral threshold (**a**) and DiffScore (**b**). The L5T animals exhibited a pronounced mechanical allodynia up to 28 days. *hab* habituation phase. Data shown are the mean ± SEM

(transient group, Fig. 3c, d). Therefore, we applied daily injections of the drugs for a period of 7 days starting at 2 days after surgery and monitored the paw withdrawal thresholds up to day 20/21 after surgery. During the injection period, both drugs, WIN55,212-2 and GW405833, attenuated the mechanical allodynia (Fig. 3c, d), similar to the effects described for the chronically treated animals (Fig. 3a–b). After the injection period, the antiallodynic effect of both substances persisted but differed slightly in potency and duration. While GW405833 was more potent than

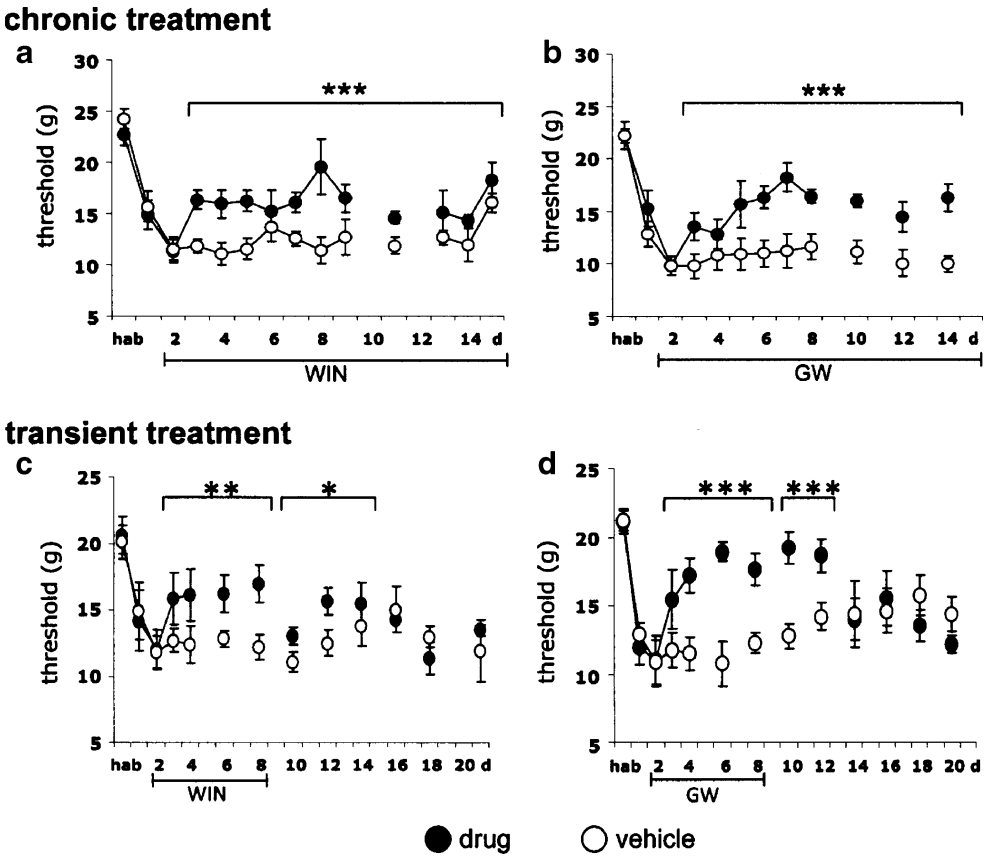

Fig. 3 Effects of chronic and transient treatment with the CB1/CB2 agonist WIN55,212-2 or the selective CB2 agonist GW405833 on mechanical allodynia. Daily intraperitoneal injection of WIN55,212-2 (**a**; *WIN*, n=10; *Veh*, n=9) or GW405833 (**b**; *GW*, n=8; *Veh*, n=8), from day 2 after surgery for 2 weeks reduced tactile allodynia associated with L5 transection. Ipsilateral values were significantly reversed when compared to vehicle-treated animals. WIN55,212-2 (**c**; *WIN*, n=5; *Veh*, n=4) or GW405833 (**d**; *GW*, n=4; *Veh*, n=4) injected once daily for 8 days starting 2 days after L5 transection reversed tactile allodynia during and after the injection period. The antiallodynic effect of GW405833 persisted up to 4 days; that of WIN55,212-2 persisted up to day 6 after the last injection. Data shown are the mean ± SEM. Data were statistically compared using two-way ANOVA. All pairwise multiple comparisons were performed by Holm–Sidak's post hoc test. *hab* habituation phase; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

WIN55,212-2 showing 100% of the maximal possible effect of the injection period in the early postinjection period, the antiallodynic effect of WIN55,212-2 outlasted the injection period for 6 days in contrast to 4 days for GW405833. After this time period, the withdrawal thresholds reached vehicle level (Fig. 3c, d).

The effects of repeated intraperitoneal injection of WIN55,212-2 or GW405833 on glial activation and cathepsin expression

There is considerable evidence that CNS inflammation plays a crucial role in the initiation and maintenance of neuropathic pain (for recent reviews, see McMahon et al. 2005; Moalem and Tracey 2006; Omoigui 2007) and that the cathepsins, lysosomal cysteine proteases, are major components of this inflammatory machinery (Barclay et al. 2007; Clark et al. 2007; Leichsenring et al. 2008). Since all major types of glial cells express cannabinoid receptors, we analyzed whether repeated injections of WIN55,212-2 or GW405833 promote their antiallodynic effects by interfering with the inflammatory processes in the SC.

Two weeks after surgery, nontreated L5T (Fig. 4) and vehicle-treated L5T animals (Fig. 5) exhibited a strong gliosis in the ipsilateral SC. Immunostaining for GFAP and PT66 revealed numerous highly activated astrocytes and microglia distributed in the ipsilateral ventral and dorsal horns (Figs. 4 and 5). Concurrent with these cellular changes, we noted an upregulation of cathepsin expression. A large number of CATS- and CATX-immunopositive cells were scattered throughout the gliotic regions (Fig. 4). These cells were of small size and exhibited glial-cell-like morphology (Fig. 4). The cathepsin immunoreactivities were found as spherical granules distributed within the cytoplasm.

Chronic treatment with either WIN55,212-2 or GW405833 for 14 days reduced the L5T-induced astrocytic and microglial activation in the SC (Fig. 5). In comparison to vehicle-treated animals immunostainings for GFAP, PT66, CATS, and CATX exhibited reduced intensity per cell and a decreased number of immunopositive cells in the ventral and dorsal horns (Fig. 5). The immunostaining patterns of drug-treated animals resembled those observed in sham animals (Figs. 4 and 5). Aside from these areas, immunoreactivities were unaffected (data not shown).

In addition, we investigated the glial reactions in the SC of animals treated transiently for 8 days with either drug. We selected a time point, at which the antiallodynic effect of the drugs had ceased and the animals re-developed significant mechanical allodynia, which is 13–15 days after the last drug injection (Fig. 3). At this time point, all transiently treated animals re-displayed a prominent gliotic reaction in the ventral and dorsal horns, which was as strong as that of vehicle-treated animals (Fig. 6) and different to the pattern observed in the chronically treated animals (Fig. 5).

Discussion

Neuropathic pain is one of the most difficult types of pain to treat. Mixed CB1/CB2 cannabinoids have been demonstrated to provide analgesia, but their high incidence of adverse effects outweighs their effectiveness. Cannabinoids acting selectively on CB2 receptors, which lack the drawbacks of central side effects, offer a promising alternative for future analgesic therapy. Previous reports supporting the analgesic effects of CB2 agonists applied single injections, but studies analyzing their long-term effectiveness and their influence/s on central pain-related cellular processes in the SC are missing. Hence, in the present work, we analyzed the effect of repeated administration of the CB2 agonist GW405833 on mechanical allodynia and on pain-induced gliosis in the SC and compared them with the CB1/CB2 cannabinoid WIN55,212-2.

Our results show that both substances, applied daily in a low non-psychotropic dosage, are equally effective in reducing mechanical allodynia induced by L5 transection. The analgesic effect of both substances is accompanied by a significant reduction in reactive gliosis and in the expression of the cathepsins X and S in the lumbar SC. Furthermore, a daily injection of either substance for 8 days is sufficient to induce a sustained antiallodynic effect that persists up to 6 days after the last injection. The re-appearance of mechanical allodynia after this period is associated with a recurring strong gliotic response in the lumbar SC.

This is the first study analyzing the effect of a daily applied specific CB2 agonist, GW405833 (Valenzano et al. 2005; Yao et al. 2007), on long-term mechanical allodynia in a neuropathic pain model. GW405833 exhibited robust antiallodynic effects. Thus, our results are consistent with previous acute studies in neuropathic pain models using multiple CB2 agonists—AM1241, JWH-133, and L768242 (GW405833)—suggesting that CB2 receptors are implicated in mechanical allodynia (Beltramo et al. 2006; Elmes et al. 2004; Ibrahim et al. 2003; Sagar et al. 2005). These acute effects of CB2 agonists on neuropathic pain symptoms were achieved by using higher doses than 0.1 mg/kg.

However, all these studies show that the effect of these agonists diminish over time. Therefore, we extend these data and tested a chronic application scheme with a very low dose (0.1 mg/kg) that does not produce side effects, neither in the CNS nor on sensory thresholds (Valenzano et al. 2005). In our experiments, we could demonstrate that GW405833 applied daily during the development of neuropathy effectively and stably reverses the allodynia associated

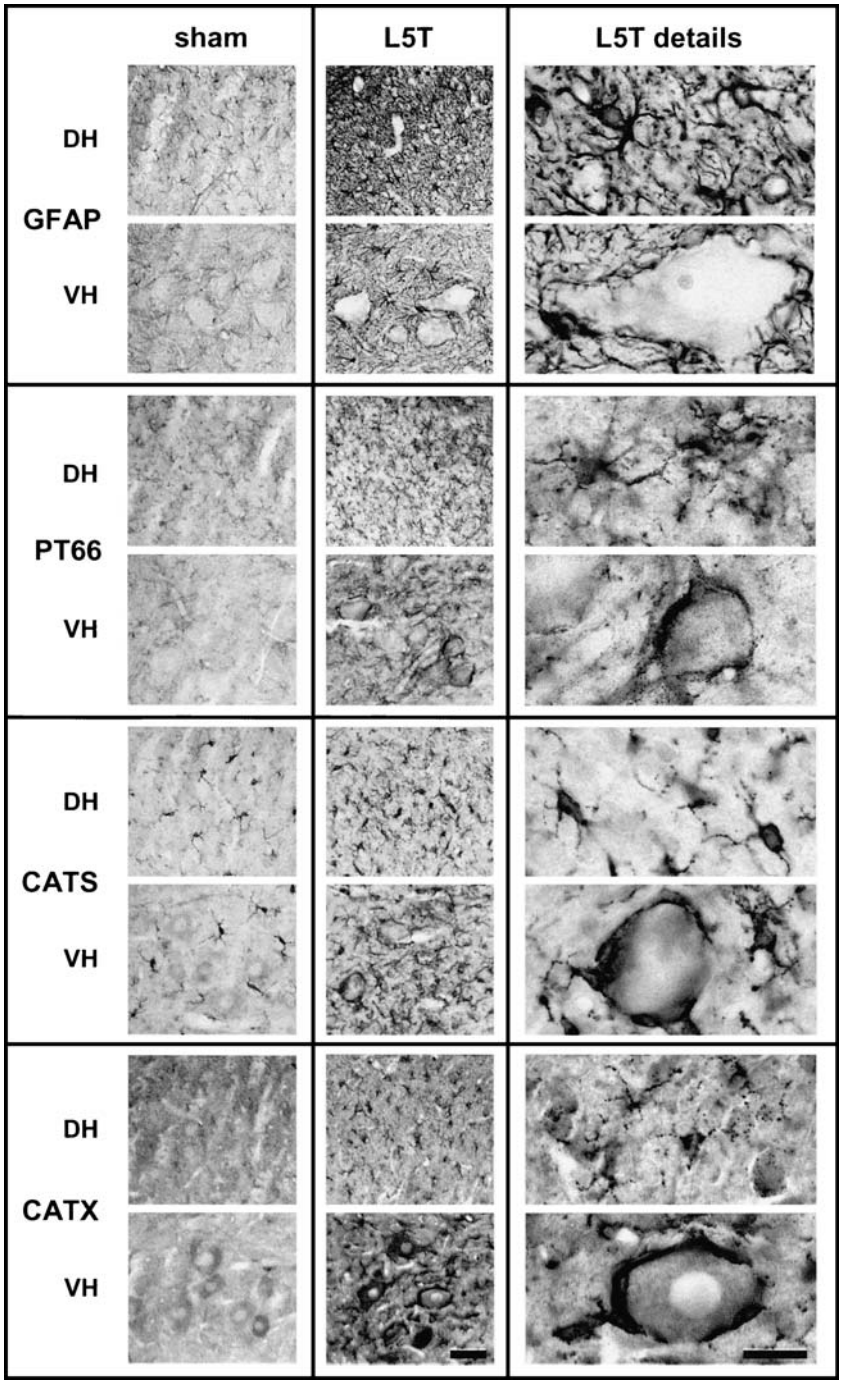

Fig. 4 Immunohistochemical stainings of the lumbar spinal cord of L5T and sham animals. Representative examples of immunostainings (*L5T*, $n=5$; *sham*, $n=5$) for astrocytes (*GFAP*), microglia (*PT66*), and the cathepsins S (*CATS*) and X (*CATX*) in the ipsilateral dorsal (*DH*) and ventral horn (*VH*) of the lumbar SC at day 14 after L5 transection or sham operation. Note the intensive astro- and microgliosis and the upregulation of CATS and CATX in both SC regions of L5T-treated animals. Scale bars, 50 and 20 μm (details)

with the nerve injury. The antiallodynic efficacy of GW405833 was similar to that of WIN55,212-2 applied under identical conditions. The latter is in agreement with the study of Costa et al. (2004) using chronic application of WIN55,212-2 in the chronic constriction injury model of neuropathy.

We then stopped the treatment after an injection period of 8 days and followed the withdrawal thresholds over the following 2 weeks. The antiallodynic effect of both substances persisted up to 6 days after the last injection. Thus, in addition to a strong antiallodynic effect on long-term mechanical allodynia induced by nerve injury, we have shown that repetitive application of either cannabinoid receptor agonist induced a prolonged alleviation of mechanical allodynia beyond the application period. This is the first evidence for a long-term effect of cannabinoid receptor agonists. One reason for this prolonged effect might be the downregulation of the pain-inducing mechanisms, but on the other hand, we cannot exclude a slow clearance rate of the cannabinoids.

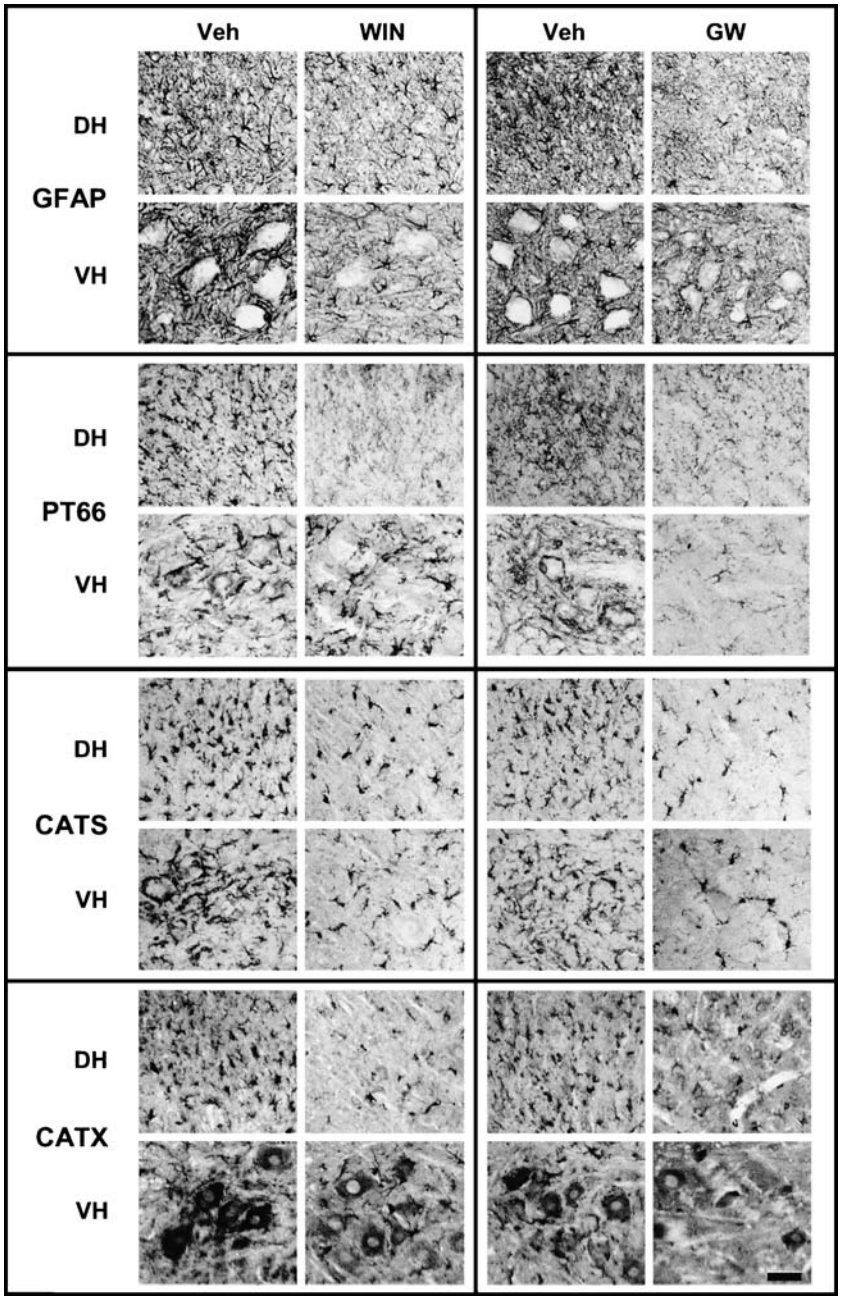

Fig. 5 Immunohistochemical stainings of the lumbar SC after chronic treatment with cannabinoid receptor agonists. Daily injections of WIN55,212-2 (*WIN*, $n=10$; *Veh*, $n=9$) or GW405833 (*GW*, $n=8$; *Veh*, $n=8$) for 2 weeks induced a remarkable reduction in L5T-induced GFAP, PT66, CATS, and CATX immunostaining in the dorsal (*DH*) and ventral horn (*VH*). Scale bar, 50 μm (all)

The cellular mechanisms by which cannabinoids mediate their analgesic effects are complex involving effects in the central nervous system and in the periphery (for review, see Pacher et al. 2006). Within the past years, glial cells and circulating immune cells have been recognized as powerful components of pain creation and maintenance (for reviews, see McMahon et al. 2005; Watkins and Maier 2003) and express cannabinoid receptors (for reviews, see Parolaro 1999; Stella 2004). Therefore, we tested whether the agonists, which showed substantial antiallodynic potential in this study, influence the formation or extent of the injury-driven gliosis in the SC. In agreement with previous studies, the transection of L5 induced a strong gliotic reaction in the SC (Blackbeard et al. 2007; Honore et al. 2000; Leichsenring et al. 2008; Znaor et al. 2007). After a 14-day period of daily GW405833 or WIN55,212-2 injection, immunostainings for GFAP, PT66, CATS, and CATX were significantly suppressed, and the staining patterns were identical to those of sham-operated animals. Thus, our data suggest that activation of cannabinoid receptors prevents or reverses an initially formed gliotic reaction in the SC. Similar histological evidence for an antiinflammatory potential of cannabinoid agonists has been reported for inflammatory and acute pain models (Costa et al. 2004; Romero-Sandoval and Eisenach

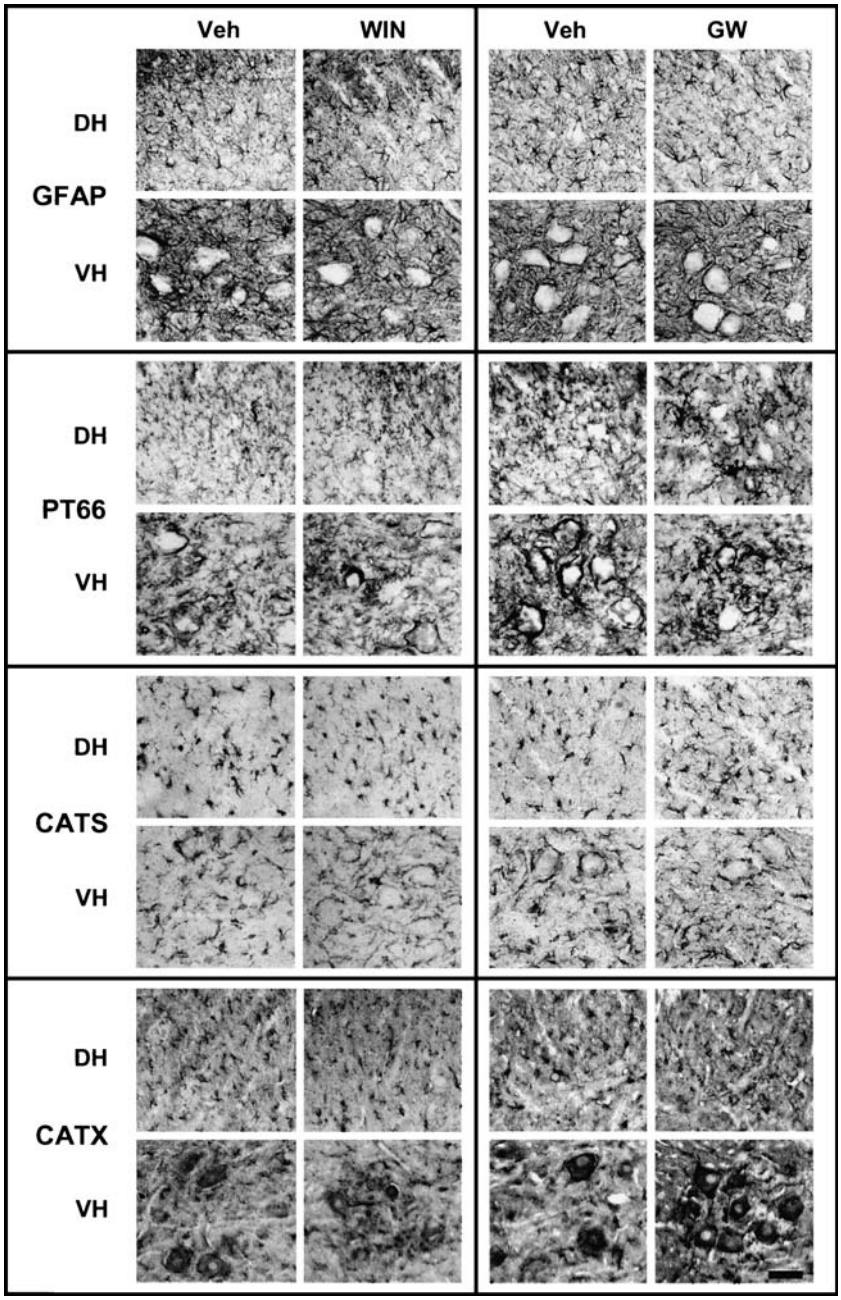

Fig. 6 Immunohistochemical stainings of the lumbar SC after transient treatment with cannabinoid receptor agonists. Animals were daily injected with WIN55,212-2 (*WIN*, *n*=5; *Veh*, *n*=4) or GW405833 (*GW*, *n*=4; *Veh*, *n*=4) for 8 and 13–15 days after the last injection immunostained for GFAP, PT66, CATS, and CATX. At this latter time point, mechanical allodynia had reappeared. Both the dorsal (*DH*) and the ventral horn (*DH*) in the lumbar SC of transiently treated animals exhibited strong gliotic changes that are similar to those of vehicle-treated animals. Scale bar, 50 μm (all)

2007) as well as for rodent models of multiple sclerosis (Arevalo-Martin et al. 2003; Croxford and Miller 2003; Ortega-Gutierrez et al. 2005b).

Because this cannabinoid-induced reduction of gliosis was accompanied by a reversal of mechanical allodynia and, on the other hand, the discontinuation of the cannabinoid application induced the re-appearance of both the gliotic reaction and the mechanical allodynia, we speculate that activated glial cells and the cathepsins S and X contribute to pain sensation. This assumption is consistent with the increasing recognition of glia as key player in the pain processes (McMahon et al. 2005; Moalem and Tracey 2006; Watkins et al. 2007). Moreover, the antiallodynic effect of cannabinoids reported in our study is supported by studies showing that cannabinoid agonists inhibit the production of proinflammatory mediators in microglia (Ehrhart et al. 2005; Walter et al. 2003) and astrocytes (Ortega-Gutierrez et al. 2005a; Sheng et al. 2005), which may regulate pain processing.

Taken together, the antiallodynic and antiinflammatory properties and the long-term effectiveness of non-psychotropic dosages of cannabinoid receptor agonists highlight the therapeutic potential of these compounds in suppressing neuropathic pain states.

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