Beneficial properties of maraviroc on neuropathic pain development and opioid effectiveness in rats

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ABSTRACT

Targeting chemokine signaling pathways is crucial in neuropathy development. In this study, we investigated the influence of chronic administration of maraviroc (CCR5 antagonist) on nociception and opioid effectiveness during neuropathy, which develops as a result of chronic constriction injury (CCI) of the sciatic nerve. To investigate the mechanism of action of maraviroc, we measured the expression of glial cell markers, CCR5 and certain CCR5 ligands (CCL3, CCL4, CCL5, CCL7, CCL11), in the spinal cord and dorsal root ganglia (DRG) of vehicle- and maraviroc-treated, CCI-exposed rats. Our results demonstrate that chronic intrathecal administration of maraviroc diminished neuropathic pain symptoms on day 7 post-CCI. Western blot analysis showed that maraviroc diminished protein level of Iba-1 and GFAP and reversed the up-regulated CCR5 expression observed in spinal cord and DRG after CCI. Additionally, using qRT-PCR, we demonstrated that CCR5 and some of its pronociceptive ligands (CCL3, CCL4, CCL5) increased in the spinal cord after nerve injury, and maraviroc effectively diminished those changes. However, CCL11 spinal expression was undetectable, even after injury. In vitro primary culture studies showed that CCL3, CCL4, CCL5 and CCL7 (but not CCL11) were of microglial and astroglial origin and were up-regulated after LPS stimulation. Our results indicate that maraviroc not only attenuated the development of neuropathic pain symptoms due to significant modulation of neuroimmune interactions but also intensified the analgesic properties of morphine and buprenorphine. In sum, our results suggest the pharmacological modulation of CCR5 by maraviroc as a novel therapeutic approach for co-treatment of patients receiving opioid therapy for neuropathy.

1. Introduction

The treatment of neuropathic pain is a serious clinical problem because of poor response and undesired adverse effects. Effective and long-lasting pharmacological therapies are needed. Neuroimmune interactions are an essential element of nociceptive processing (Matsushita et al., 2014; Mika et al., 2007, 2013). Given the important role of chemokine receptors in nociceptive transmission, neuronal-glial interactions and inflammatory processes regulation, targeting chemokine signaling pathways is an attractive novel approach for neuropathic pain treatment (Abbadie et al., 2009; Gao and Ji, 2010; Julius and Basbaum, 2001; Woolf and Salter, 2000). One interesting example of a chemokine receptor involved in nociception is CCR5, which is up-regulated in the spinal cord after peripheral nerve injury (Matsushita et al., 2014).

CCR5 belongs to the CC chemokine receptor family, a category of integral membrane proteins that represent G protein-coupled receptors (GPCR) (Charo and Ransohoff, 2006; Palmqvist et al., 2007). Some in vitro studies have demonstrated that CCR5 and the mu-opioid receptor, both members of the GPCR family, may engage in cross-talk through dimerization (Chen et al., 2004; Yuan et al., 2013). These results suggest that CCR5 might be involved in the development of opioid effectiveness, which, to the best of our knowledge, has not been studied in neuropathic pain. The weakening of morphine efficacy and development of opioid tolerance is a major problem in neuropathic pain treatment. CCR5 is located in spinal primary and secondary afferent neurons; thus, its endogenous ligands (CCL3, CCL5) can modify pain transmission through the direct excitation of these cells (Bajetto et al., 1999; Oh et al., 2001; Zhang et al., 2004). Intrathecal (i.t.) administration of both CCL3 and

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CCL5 induces pain symptoms (Kiguchi et al., 2012). Additionally, intraperitoneal (i.p.) administration of these two chemokines induces pain behaviors, and this effect might be evoked by CCR5 activation in the dorsal root ganglia (DRG) (Liou et al., 2013). Lee et al. (2013) described that CCR5-knockout mice respond less to noxious stimuli than wild type mice. Together, these studies strongly suggest that CCR5 plays a crucial role in the development of neuropathic pain symptoms. Until now, only one chemokine receptor antagonist, maraviroc (MVC), has received accelerated approval from the Food and Drug Administration for clinical use. MVC is used for the treatment of HIV-infected patients who are resistant to other antiretroviral drugs and are infected by the R5-tropic virus (Lieberman-Blum et al., 2008; Lisi et al., 2012; Pease and Horuk, 2009a,b). However, still little is known about the influence of MVC on the nociceptive processes during neuropathy.

Here, we explored the influences of MVC on neuropathic pain symptoms, such as allodynia and hyperalgesia, which develop after chronic constriction injury (CCI) of the sciatic nerve (CCI model). To understand the CCR5 antagonist’s mechanism of action, we measured the expression of microglia (C1q, Iba-1) and astroglia (GFAP) cell markers, CCR5 and certain CCR5 ligands (CCL3, CCL4, CCL5, CCL7 and CCL11), in the spinal cord and DRG in vehicle- and MVC-treated, CCI-exposed rats. Furthermore, we assessed the potential of MVC to improve the effectiveness of opioids (morphine and buprenorphine) in neuropathic pain. To verify our in vivo data, we evaluated the expression of chemokines in primary microglial and astroglial cultures in inflammatory conditions.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (250–300 g) from Charles River Laboratories International, Inc. (Germany) were used in this study. The animals were housed in cages lined with sawdust under a standard 12/12-h light/dark cycle (lights on at 8:00 a.m.), with food and water available ad libitum. Animals before behavioral test were allowed to acclimate to the environment for approximately 5 min prior to testing. All experiments were performed according to the recommendations of IASP (Zimmermann, 1983) and the NIH Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the II Local Bioethics Committee branch of the National Ethics Committee for Experiments on Animals based at the Institute of Pharmacology, Polish Academy of Sciences (Cracow, Poland).

2.2. Chronic constriction injury (CCI) in rats

CCI to the sciatic nerve in rats was performed under sodium pentobarbital anesthesia (60 mg/kg, i.p.). An incision was made below the hipbone, and the biceps femoris and gluteus superficialis were separated. The right sciatic nerve was exposed, and four ligatures (4/0 silk) were tied loosely around the nerve distal to the sciatic notch with 1-mm spacing until they elicited a brief twitch in the respective hind limb according to the procedure described by Bennett and Xie (1988). After the surgery, the rats developed long-lasting neuropathic pain symptoms, such as allodynia and hyperalgesia. Because we have shown in earlier studies that there are no differences between the nociceptive responses of naive and sham rats, we used naive animals for the behavioral experiments in the current study.

2.3. Intrathecal injection (i.t.)

The rats were prepared for i.t. injection under sodium pentobarbital anesthesia (60 mg/kg, i.p.) by implanting catheters according to the method of Yalksh and Rudy (1976). The i.t. catheter consisted of polyethylene tubing 13-cm long (PE 10, Intramedic; Clay Adams, Parsippany, NJ, USA) with dead space of 10 μl that had been sterilized by immersion in 70% (v/v) ethanol and fully flushed with sterile water before insertion. The catheter (7.8 cm of its length) was carefully introduced through the atlanto-occipital membrane and advanced into the subarachnoid space at the rostral level of the spinal cord lumbar enlargement (L4–L5). After implantation, the first injection of water was slowly performed (10 μl), and the catheter was tightened. Before the experiment, the rats were allowed to recover from surgery for one week.

2.4. Behavioral tests

2.4.1. Mechanical allodynia (von Frey test)

Allodynia in rats with CCI was measured using an automatic von Frey apparatus (Dynamic Plantar Aesthesiometer Cat. No. 37400, Ugo Basile, Italy). The animals were placed in plastic cages with a wire net floor 5 min before the experiment. The von Frey filament was applied in increasing values (stimuli up to 26 g) to the midplantar surface of the hind paw, and measurements were taken automatically as described previously (Makuch et al., 2013; Mika et al., 2010). The ipsilateral paw was tested twice in 2-min intervals, and the mean value was calculated.

2.4.2. Thermal hyperalgesia (cold plate test)

Hyperalgesia was assessed using a cold plate apparatus (Cold/Hot Plate Analgesia Meter No. 05044, Columbus Instruments, USA). The animals were placed on the cold plate, and the latency to lift the hind paw was recorded. The temperature of the plate was kept at 5 °C, and the cut-off latency was 30 s. In all cases, the injured paw reacted first (Makuch et al., 2013; Mika et al., 2007).

2.5. Microglial and astroglial cell cultures

Primary cultures of microglial and astroglial cells were used in our in vitro studies. Both types of cell cultures were prepared from Wistar rat pups (1 day old) according to the procedure described by Zawadzka and Kaminska (2005). The cells were isolated from the cerebral cortex and then plated at a density of 3 × 10^5 cells/cm² in a culture medium comprised of DMEM/Glutamax/high glucose (Gibco, New York, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum, 0.1 mg/ml streptomycin and 100 U/ml penicillin (Gibco, New York, NY, USA). The cultures were maintained in poly-l-lysine-coated 75-cm² culture flasks at 37 °C and 5% CO₂. After four days, the culture medium was changed. The next step was to recover the loosely adherent microglial cells by gentle shaking and centrifugation at 37 °C for 24 h (200 rpm) at day 9 and after replacing the medium at day 12. The medium was removed, and the astrocytes were replated in culture dishes, which were maintained for 3 days. Then, the astrocytes were trypsinized (0.005% Trypsin EDTA solution, Sigma-Aldrich, Saint Louis, MO, USA). The microglial/astroglial cells were resuspended in culture medium and then plated at a final density of 2 × 10^5 cells on 24-well plates for mRNA analysis. Finally, the adherent cells were incubated for 48 h in culture medium before being analyzed. Primary microglial and astroglial cell cultures were treated with LPS (100 ng/ml; Sigma-Aldrich) for 24 h. To identify microglial and astrocyte cells in vitro, we stained cultures for glial fibrillary acidic protein (GFAP; mouse anti-GFAP, 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, USA) and ionized calcium-binding adaptor molecule 1 (Iba-1; rabbit anti-Iba-1, 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, USA). Cultured primary microglia and astroglia were more than 95% positive for IBA1 and GFAP, respectively. The homogeneity of our cultures was similar to that obtained by Zawadzka and Kaminska (2005).

2.6. Drug administration

The chemicals used in this study and their sources were as follows: maraviroc (MVC; Tocris, Warsaw, Poland), morphine hydrochloride (M; TEVA, Kutno, Poland), and buprenorphine (B; Polfa Warszawa S.A., Warsaw, Poland). MVC (20 μg/5 μl) was dissolved in 12% DMSO and administered preemptively via i.t. injection 16 h and 1 h before...
CCI and then once daily for 7 days. The control groups received vehicle (V; 12% DMSO) according to the same schedule. Respective drugs or 12% DMSO was delivered slowly in a volume of 5 μl through the i.t. catheter, followed by 10 μl of water for injection, which flushed the catheter. Behavioral tests were performed 30 min (von Frey) and 35 min (cold plate) after MVC administration on day 7 post-CCI. V-treated and MVC-treated rats received a single, i.t. injection of morphine or buprenorphine (4 μg/5 μl) on the 7th day after CCI (30 min after maraviroc/vehicle injection), and then, the von Frey test (30 min) and the cold plate test (35 min) were repeated (Scheme 1).

2.7. Biochemical tests

2.7.1. qRT-PCR analysis of gene expression

Ipsilateral fragments of the dorsal part of the lumbar (L4–L6) spinal cord and the DRG (L4–L6) were removed immediately after decapitation on day 7 following CCI, 4 h after MVC administration. Total RNA was extracted using the TRIzol reagent (Invitrogen), according to the methods described by Chomczynski and Sacchi (1987). A NanoDrop ND-1000 Spectrometer (NanoDrop Technologies) was used to measure the RNA concentration. Reverse transcription was performed on 2 μg of total RNA for tissue and 500 ng of total RNA for cultured cells using Omniscript Reverse Transcriptase (Qiagen Inc.) at 37 °C for 60 min. RT reactions were performed in the presence of an RNase inhibitor (rRNAsin, Promega) and oligo (dt16) primer (Qiagen Inc.). The resulting cDNA was diluted 1:10 with H2O, and approximately 50 ng of cDNA from each individual animal was used for each quantitative real-time PCR (qRT-PCR) reaction. qRT-PCR was performed using Assay-On-Demand TaqMan probes, according to the manufacturer’s protocol (Applied Biosystems) and were run in an iCycler device (Bio-Rad, Hercules). A standard dilution curve determined the amplification efficiency for each assay. The following TaqMan primers and probes were used: Rn01527838_g1 (HPRT, hypoxanthine–guanine phosphoribosyltransferase); Rn00570480_m1 (Clq, complement component 1q; microglia/macrophages marker); Rn01253033_m1 (GFAP, astroglia and satellite cells marker); Rn02132969_s1 (CCR5); Rn01464736_g1 (CCL3); Rn00671924_m1 (CCL4); Rn005795590_m1 (CCL5); Rn01467286_m1 (CCL7) and Rn00569995_m1 (CCL11). A standard dilution curve was used to determine the amplification efficiency for each assay (between 1.7 and 2). The cycle threshold values were calculated automatically by iCycler IQ 3.0 software using the default parameters. RNA abundance was calculated as 2−threshold cycle. HPRT transcript levels do not significantly change in rats exposed to CCI (Mika et al., 2010), and therefore, this transcript served as an adequate housekeeping gene.

2.7.2. Western blot

Tissue for Western blot analysis was collected from the ipsilateral, dorsal part of the spinal cord (L4–L6) 6 h after the last MVC injection on day 7 post-CCI. Tissue lysates were collected in RIPA buffer with a protease inhibitor cocktail. Then, the reaction mixtures were cleared by centrifugation (14,000 × g for 30 min at 4 °C). Samples containing 20 μg of protein were heated in a loading buffer (4 × Laemmli Buffer, Bio-Rad, Warsaw, Poland) for 5 min at 98 °C. Afterwards, all samples were resolved on 4–15% Criterion™ TGX™ pre-cast polyacrylamide gels (Bio-Rad, Warsaw, Poland). The proteins were transferred to Immune-Blot PVDF membranes (Bio-Rad, Warsaw, Poland) with semi-dry transfer (30 min, 25 V). The membranes were blocked for 1 h at RT using 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline with 0.1% Tween 20 (TBST), washed in TBST, incubated overnight at 4 °C with primary antibodies (rabbit polyclonal anti-ιba1, 1:1000, Proteintech, Chicago, USA; rabbit polyclonal anti-GAPDH, 1:5000; Novus Biologicals, Cambridge, UK; rabbit polyclonal anti-CR5, 1:500, Proteintech; mouse polyclonal anti-GAPDH, 1:5000: as a loading control, Merck Millipore Darmstadt, Germany) and incubated for 1 h at RT with a secondary goat polyclonal antibody conjugated to horseradish peroxidase (goat anti-rabbit IgG, 1:5000, Bio-Rad; mouse anti-rabbit IgG, 1:5000, Merck Millipore Darmstadt, Germany). Solutions from the SignalBoost™ Immunoreaction Enhancer Kit (Merck Millipore Darmstadt, Germany) were used to dilute the primary and secondary antibodies. The membranes were washed 2 times for 2 min each and 3 times for 5 min each with TBST. The Clarity™ Western ECL Substrate (Bio-Rad, Warsaw, Poland) was used to detect immunocomplexes, which were then visualized using a Fujifilm LAS–4000 FluorImager system. The Fujifilm Multi Gauge software was used to quantify the relative levels of immunoreactivity.

2.8. Statistical analyses

The behavioral data (Figs. 1 and 2) are presented in grams and seconds as the mean ± SEM. One-way analysis of variance (ANOVA) was used to evaluate the experimental results. Differences between groups were analyzed with Bonferroni’s post-hoc test. ***p < 0.001 indicates differences between naive rats and V-treated CCI-exposed rats; ###p < 0.001 indicates differences between V-treated CCI-exposed rats and MVC-treated CCI-exposed rats; **p < 0.01 and +++p < 0.001 indicate differences vs. V + treated CCI-exposed rats; *&p < 0.05 and & & & & &p < 0.001 indicate differences vs. MVC + V-treated CCI-exposed rats; & & & & &p < 0.001 indicate differences vs. MVC + B-treated CCI-exposed rats; ^p < 0.05, ^ & p < 0.01 and ^ ^ & p < 0.001 indicate differences between M- or B-treated vs. MVC + M- or MVC + B-treated, CCI-exposed rats. The results of the qRT-PCR (Figs. 3, 5) and Western blot (Fig. 4) analyses are presented as the fold change compared with naive rats on the

![Scheme 1](image-url)
ipsilateral side of the dorsal lumbar spinal cord and DRG. The results were obtained from three groups of rats: naive; V-treated CCI-exposed; and MVC-treated CCI-exposed. The qRT-PCR analysis data are presented as the mean ± SEM, which represent normalized averages that were derived from the threshold cycles in qRT-PCR from 4 to 15 samples for each group. For the Western blot analysis, the groups contained from 4 to 10 samples for each group. Differences between groups were analyzed using ANOVA and Bonferroni’s multiple comparisons test. *p < 0.05, **p < 0.01 and ***p < 0.001 indicate differences between naive rats and V-treated CCI-exposed rats; **p < 0.01 and ***p < 0.001 indicate differences between V-treated CCI-exposed rats and MVC-treated CCI-exposed rats.

The results of the primary microglial and astroglial cultures (Table 1) are presented as the fold change of control compared with the untreated control cells. The data are presented as the mean ± SEM. The differences between groups were analyzed with Student’s t-test. **p < 0.01 and ***p < 0.001 indicate differences between the untreated and LPS-treated cells.

All graphs and analyses were prepared using GraphPad Prism5.

3. Results

3.1. The effect of repeated administration of MVC on the development of neuropathic pain symptoms on day 7 after CCI

In the von Frey test, strong mechanical allodynia was observed on day seven after CCI (Fig. 1A). The ipsilateral paw responded to a stimulation of 13.1 ± 1.0 g on day seven, and naïve rats responded to 26.0 ± 0.0 g, and sham rats responded to 25.5 g ± 0.4 (data not shown on the graphs). MVC significantly attenuated the mechanical allodynia from 13.1 ± 1.0 g to 19.4 ± 1.2 g on day seven 30 min after the last injection (Fig. 1A).

In the cold plate test, strong thermal hyperalgesia was observed on day seven after CCI (Fig. 1B). The ipsilateral paw responded to stimulation after 7.2 ± 1.9 s on day seven, while naïve rats responded after 29.4 ± 0.3 s, and sham rats responded after 28.5 s ± 0.9 (data not shown on the graphs). MVC significantly attenuated the thermal hyperalgesia, with latency times increasing from 7.2 ± 1.9 s to 17.7 ± 1.9 s on day seven 30 min after the last injection (Fig. 1B).

3.2. The effect of repeated administration of MVC on the analgesic effects of morphine and buprenorphine in CCI-exposed rats

MVC (20 μg/5 μl) significantly attenuated the CCI-induced mechanical allodynia and thermal hyperalgesia on day seven 30 min after the last injection (Fig. 2A and B, respectively). The analgesic effects of morphine and buprenorphine themselves were similar to the administration of MVC alone, but in combination (4 μg of morphine or buprenorphine 60 min after the administration of MVC), the observed analgesia was substantially more effective, as measured by the von Frey test (Fig. 2A) and the cold plate test (Fig. 2B).

3.3. The effect of repeated administration of MVC on C1q, GFAP and CCR5 mRNA in the spinal cord and DRG 7 days after CCI

In the spinal cord, C1q mRNA levels were strongly up-regulated in CCI-exposed rats compared with naïve rats (1.0 ± 0.1 vs. 14.7 ± 1.6; Fig. 3A). MVC diminished the spinal level of C1q mRNA in CCI-exposed rats from 14.7 ± 1.6 to 11.4 ± 0.5 (Fig. 3A). In the DRG, the C1q mRNA level was elevated from 1.0 ± 0.1 to 5.8 ± 0.5 in CCI-exposed rats compared to naïve rats (Fig. 3B). Repeated treatment with MVC significantly decreased the level of C1q in the DRG from 5.8 ± 0.5 to 3.2 ± 0.2 (Fig. 3B).

In the spinal cord, increased up-regulation of GFAP mRNA expression was observed in CCI-exposed rats compared with naïve rats (1.0 ± 0.1 vs. 2.5 ± 0.3; Fig. 3C). Repeated administration of MVC significantly diminished (from 2.5 ± 0.3 to 1.8 ± 0.3) the level of GFAP mRNA in the spinal cord (Fig. 3C). Additionally, in the DRG, the GFAP mRNA level was up-regulated from 1.0 ± 0.1 to 5.0 ± 1.1 (Fig. 3D), and MVC did not influence these changes.

CCR5 mRNA was enhanced in CCI-exposed rats in the spinal cord from 1.0 ± 0.1 to 3.28 ± 0.3 (Fig. 3E) and in the DRG from 1.0 ± 0.1 to 7.1 ± 1.4 (Fig. 3F) compared with naïve rats. MVC decreased the spinal level of CCR5 mRNA from 3.28 ± 0.3 to 2.2 ± 0.3 but an even stronger down-regulation was observed in the DRG from 7.1 ± 1.4 to 3.4 ± 0.4 (Fig. 3E and F, respectively).

3.4. The effect of repeated administration of MVC on Iba-1, GFAP and CCR5 protein levels in the spinal cord and DRG 7 days after CCI

Changes in the protein levels corresponded well with the up-regulation of C1q, GFAP and CCR5 mRNA. In the spinal cord, the Iba-1

Fig. 1. The influence of maraviroc (MVC; 20 μg/5 μl; i.t.; 16 h and 1 h before CCI and then once daily for 7 days) on the development of mechanical allodynia (A; von Frey test) and thermal hyperalgesia (B; cold plate test) on day 7 after CCI. Allodynia and hyperalgesia were assessed 30 and 35 min after the last drug administration. The data are presented as the mean ± SEM, and the horizontal dotted line shows the cut-off value. The intergroup differences were analyzed using ANOVA with Bonferroni’s multiple comparisons test. ***p < 0.001 indicates differences between naïve rats and V-treated CCI-exposed rats; ###p < 0.001 indicates differences between V-treated CCI-exposed rats and MVC-treated CCI-exposed rats. V, vehicle; MVC, maraviroc.
decreased the level of the astroglial activation marker in the spinal cord (Fig. 4D), but to a lesser extent than Iba-1 protein. MVC also significantly decreased the level of CCR5 protein in the spinal cord from 1.4 ± 0.1 to 0.9 ± 0.1 and in the DRG from 1.3 ± 0.1 to 0.9 ± 0.1 in CCI-exposed rats (Fig. 4E and F, respectively).

3.5. The effect of repeated administration of MVC on CCL3, CCL4, CCL5, CCL7 and CCL11 mRNA expression level in the spinal cord and DRG 7 days after CCI

CCL3 mRNA was up-regulated in the spinal cord from 1.0 ± 0.1 to 3.0 ± 0.3 (Fig. 5A) and in the DRG from 1.0 ± 0.1 to 7.4 ± 1.0 (Fig. 5B) in CCI-exposed rats compared with naive rats. MVC diminished the level of CCL3 mRNA from 3.0 ± 0.3 to 2.2 ± 0.1 in the spinal cord (Fig. 5A) but did not influence the level of CCL3 mRNA in the DRG (Fig. 5B).

CCL4 mRNA was strongly up-regulated in CCI-exposed rats compared with naive rats in the spinal cord (7.2 ± 0.8 vs. 1.0 ± 0.1, respectively; Fig. 5C) and in the DRG (6.5 ± 1.5 vs. 1.0 ± 0.1, respectively; Fig. 5D). MVC strongly decreased the level of CCL4 mRNA from 7.2 ± 0.8 to 3.6 ± 0.5 in the spinal cord (Fig. 5C) but did not influence the observed level of CCL4 mRNA in the DRG (Fig. 5D).

CCL5 mRNA was up-regulated in CCI-exposed rats compared with naive rats in the spinal cord (1.5 ± 0.2 vs. 1.0 ± 0.2, respectively; Fig. 5E) and in the DRG (2.0 ± 0.6 vs. 1.0 ± 0.5, respectively; Fig. 5F). MVC decreased the level of CCL5 mRNA in the spinal cord from 1.5 ± 0.2 to 0.7 ± 0.2 (Fig. 5E) and in the DRG from 2.0 ± 0.6 to 0.9 ± 0.1 (Fig. 5F).

CCL7 mRNA was strongly increased in CCI-exposed rats compared with naive rats in the spinal cord (37.4 ± 7.7 vs. 1.0 ± 0.2, respectively; Fig. 5G) and in the DRG (46.1 ± 1.1 vs. 1.0 ± 0.5, respectively; Fig. 5H). MVC did not influence the level of CCL7 mRNA observed in the spinal cord, but the drug significantly increased the level of CCL7 mRNA in the DRG from 4.6 ± 1.1 to 6.4 ± 0.6 (Fig. 5G and H, respectively).

CCL11 mRNA was undetectable in the spinal cord but was up-regulated in the DRG in CCI-exposed rats compared with naive rats (3.7 ± 0.5 vs. 1.0 ± 0.2, respectively; Fig. 5I). MVC did not influence these changes (Fig. 5J).

3.6. The effect of LPS stimulation on CCL3, CCL4, CCL5, CCL7 and CCL11 mRNA levels in microglial and astroglial cells — in vitro studies

Our in vitro study found a strong up-regulation of CCL3, CCL4 and CCL7 mRNA after 24 h of LPS treatment (Table 1) in both microglia as well as astroglia cells. Additionally, CCL5 expression was extremely up-regulated after LPS treatment in microglial (1.0 ± 0.2 vs. 1108.9 ± 125.79) and astroglial cells (1.0 ± 0.1 vs. 859.911 ± 30.96) as compared to CCL3, CCL4, and CCL7. CCL11 mRNA was undetectable in both cell cultures (Table 1).

4. Discussion

Our study demonstrated that chronic i.t. administration of the selective CCR5 antagonist MVC significantly reduced neuropathic pain symptoms, such as allodynia and hyperalgesia, which are observed after CCI. These findings are in line with previous research suggesting that CCR5 plays a crucial role in the development of neuropathic pain. In 2012, Padi et al. (2012) showed that oral administration of RAP-103, an antagonist of CCR5 and CR2, inhibited the development of hypersensitivity after partial sciatic nerve ligation. Further supporting our results, Matsushita et al. (2014) demonstrated that MVC effectively diminished neuropathic pain symptoms after L4–L5 spinal nerve injury. Interestingly, our results for the first time indicate that MVC not only diminished the protein concentration was up-regulated in CCI-exposed rats compared with naive rats (16.3 ± 2.9 vs. 1.0 ± 0.2, respectively; Fig. 4A). MVC significantly attenuated the level of microglial activation marker from 16.3 ± 2.9 to 8.8 ± 1.1 (Fig. 4A). In the DRG, the Iba-1 level was also increased in CCI-exposed rats compared with naive rats (6.0 ± 0.7 vs. 1.0 ± 0.3, respectively; Fig. 4B). Repeated administration of MVC significantly decreased the level of Iba-1 in the DRG from 6.0 ± 0.7 to 3.9 ± 0.5 (Fig. 4B).

The GFAP level was also higher in CCI-exposed rats compared with naive rats in the spinal cord (1.9 ± 0.06 vs. 1.0 ± 0.04, respectively; Fig. 4C) and in the DRG (1.4 ± 0.06 vs. 1.0 ± 0.06, respectively; Fig. 4D), but to a lesser extent than Iba-1 protein. MVC also significantly decreased the level of the astroglial activation marker in the spinal cord from 1.9 ± 0.06 to 1.5 ± 0.1 and in the DRG from 1.4 ± 0.06 to 1.2 ± 0.03 in CCI-exposed rats (Fig. 4C and D, respectively). The CCR5 protein level was significantly up-regulated in the spinal cord in CCI-exposed rats compared with naive rats (1.4 ± 0.1 vs. 1.0 ± 0.1; Fig. 4E) and weakly in the DRG (Fig. 4F). MVC significantly decreased the level of CCR5 protein in the spinal cord from 1.4 ± 0.1 to 0.9 ± 0.1 and in the DRG from 1.3 ± 0.1 to 0.9 ± 0.1 in CCI-exposed rats (Fig. 4E and F, respectively).

Our study found a strong up-regulation of CCL3, CCL4 and CCL7 mRNA after 24 h of LPS treatment (Table 1) in both microglia as well as astroglia cells. Additionally, CCL5 expression was extremely up-regulated after LPS treatment in microglial (1.0 ± 0.2 vs. 1108.9 ± 125.79) and astroglial cells (1.0 ± 0.1 vs. 859.911 ± 30.96) as compared to CCL3, CCL4, and CCL7. CCL11 mRNA was undetectable in both cell cultures (Table 1).
The influence of maraviroc (MVC) on mRNA levels of microglial and astroglial markers and CCR5 in the spinal cord (A, C, E) and DRG (B, D, F) during neuropathic pain. Maraviroc (20 μg/5 μl; i.t.; 16 h and 1 h before CCI and then once daily for 7 days) diminished the mRNA expression of C1q (A, B) and CCR5 (E, F) in the dorsal part of the lumbar spinal cord and in the DRG 7 days post-CCI. MVC also influenced the level of GFAP mRNA (C, D) in the spinal cord. The data are presented as the mean ± SEM. The intergroup differences were analyzed using ANOVA with Bonferroni’s multiple comparisons test. ***p < 0.001 indicates differences between naive rats and V-treated CCI-exposed rats. #p < 0.05 and ###p < 0.001 indicate differences between V-treated CCI-exposed rats and MVC-treated CCI-exposed rats. N, naive; V, vehicle; MVC, maraviroc.
Fig. 4. The influence of maraviroc (MVC) on protein levels of Iba-1, GFAP and CCR5 in the spinal cord (A, C, E) and DRG (B, D, F) during neuropathic pain. Maraviroc (20 μg/5 μl; i.t.; 16 h and 1 h before CCI and then once daily for 7 days) diminished the protein level of Iba-1 (A, B), GFAP (C, D) and CCR5 (E, F) in the dorsal part of the lumbar spinal cord and in the DRG 7 days post-CCI. The data are presented as the mean ± SEM. The intergroup differences were analyzed using ANOVA with Bonferroni’s multiple comparisons test. *p < 0.05 and ***p < 0.001 indicate differences between naive rats and V-treated CCI-exposed rats. #p < 0.05 and ##p < 0.01 indicate differences between V-treated CCI-exposed rats and MVC-treated CCI-exposed rats. N, naive; V, vehicle; MVC, maraviroc.
the development of neuropathic pain but also intensified both morphine and buprenorphine analgesia. It is well accepted that subcutaneous injection of buprenorphine has better analgesic effects than morphine (Kouya et al., 2002). In 2013 Sukhtankar et al. (2013) has shown that the analgesic properties of intrathecally administrated buprenorphine (0.01–3 μg) are better than morphine in neuropathic and inflammatory pain in mice. However, in our study in rats we demonstrated that intrathecal injection of morphine and buprenorphine (4 μg) does not differ in analgesic properties in CCI-exposed animals. Based on the abovementioned results the crucial role of CCR5 in antinociceptive MVC properties may be postulated.

CCR5 expression has been demonstrated in a variety of cell types, including granulocyte precursors, primary monocytes/macrophages, primary T cells, and CD4+ and CD8+ lymphocytes (Rottman et al., 1997). In the central nervous system, CCR5 is expressed in neurons, astrocytes and microglia (Kaul et al., 2007; Rottman et al., 1997). Increasing evidence indicates a crucial role of microglial signaling in the pathogenesis of neuropathic pain. Microglial cells display the capability to change morphology rapidly in response to injury. After peripheral nerve injury, microglial cells transform to a reactive form in response to the expression of various genes, such as cell-surface receptors and nociceptive factors, which enhance synaptic transmissions in the spinal cord (Garden and Moller, 2006; Hanisch, 2002; Mika et al., 2013). Furthermore, in response to nerve injury, microglial cells migrate toward injured regions. TAK-779, a specific CCR5 antagonist, reduces the microglial migration rate, indicating that CCR5 is important for this phenomenon (Carbonell et al., 2005; Marella and Chabry, 2004). In the current study, we provide evidence that the CCR5 transcript and protein levels are strongly up-regulated in the ipsilateral dorsal spinal cord and DRG after CCI, and MVC prevents these changes.

These observations suggest that by reducing the level of CCR5, MVC can affect the degree of activation and migration of microglia. Among the CCR5 ligands, CCL3 and CCL4 appear to be important in neuropathic pain development. Using neutralizing antibodies, Saika et al. (2012) showed that CCL3 contributes to the progress of thermal hyperalgesia and tactile allodynia, unlike CCL4, which contributes mainly to tactile allodynia. In 2010, the up-regulation of CCL3 in macrophages and Schwann cells in a murine partial sciatic nerve ligation model and the parallel development of pain was documented (Riguchi et al., 2010). In the current study, we establish that the mRNA expression of CCL3 and CCL4 was enhanced in the injured side of the spinal cord after CCI, paralleling the activation of C1q- and GFAP-positive cells. Furthermore, MVC significantly diminished these spinal changes. Our in vitro primary cell culture studies confirmed that CCL3 and CCL4 mRNA levels in microglial and astroglial cells were increased after LPS stimulation. Thus, we hypothesize that spinal microglia and astroglia might be some of the CCL3- and CCL4-producing cells that are involved in establishing allodynia and hyperalgesia.

CCL5 is the major ligand of CCR5, and an increasing number of studies have confirmed its essential role in nociceptive transmission. CCL5 has been identified in injured nerves, which indicates a potential role in neuropathology (Liou et al., 2013; Oh et al., 2001). In addition, CCL5 acts as a ligand for the chemokine receptors CCR1, CCR3 and CCR5 and influences monocyte and T cell migration (Aujebor et al., 2001; Bless et al., 2000). Recent evidence indicates that CCL5 modulates the inflammatory response in different pathological conditions, such as multiple sclerosis, human immunodeficiency virus, dementia and rheumatoid arthritis (Gerard and Rollins, 2001). Some researchers have concluded that reducing CCL5 release, and thus diminishing inflammatory processes, may be a useful strategy for the treatment of neurodegenerative disorders in clinical settings. Liou et al. (2013) demonstrated that daily peritoneal injection of Met-RANTES, which antagonizes the binding of CCL5 to CCR5, significantly attenuated thermal hyperalgesia and tactile allodynia induced by partial sciatic nerve ligation in mice. These data suggest that peripheral CCL5 is implicated in the processing of pain information (Liou et al., 2013). We confirmed the up-regulation of CCL5 mRNA in the spinal cord and DRG, and we have revealed that MVC diminished these changes. Our in vitro studies showed that CCL5 is the strongest up-regulated CCR5 ligand in microglial and astroglial cell cultures after LPS stimulation. This fact stresses the importance of CCL5 not only in the inflammation but also in neuropathy and suggests that both types of glial cells may produce the factor during pathology conditions. Therefore, the inhibition of glial activation by maraviroc affects the level of this pronociceptive chemokine.

Partial sciatic nerve ligation also increased the spinal expression of CCL7, especially in astrocytes (Imai et al., 2013). Our in vitro studies confirmed the astroglial expression of CCL7, which is substantially more prominent than the microglial. Our qRT-PCR results confirmed a strong increase of CCL7 mRNA in the spinal cord and additionally showed similar changes in the DRG. Surprisingly, repeated administration of MVC even potentiated CCL7 expression in the DRG. In 2013, Imai et al. suggested that increased CCL7 expression may serve to ease the interactions between astrocytes and microglia in the spinal cord and could play an essential role in neuropathic pain, which is in agreement with our results. However, additional research is needed to explain this phenomenon.

The CCL11 is also a ligand of CCR5; however, there are no reports concerning its role in the development of neuropathic pain. In our study, CCL11 mRNA was undetectable in the spinal cord. Our in vitro primary cell culture studies showed that CCL11 mRNA was also not detectable in microglia and astroglia. Interestingly, we observed that in the DRG the CCL11 mRNA level was up-regulated after CCI; however, MVC did not influence these changes. The exact role of CCL11 in the DRG after CCI needs to be elucidated in the future.

The detailed mechanism of CCR5 action has not been fully ascertained yet; however, there is some evidence of heterologous desensitization of opioid and chemokine receptors, which suggests that in the future exogenous chemokine receptor ligands could be used as a co-analgesic drug in opioid therapy in neuropathic pain treatment (Brack et al., 2004). The mechanism of decreased analgesic potency of opioids in neuropathic pain is not fully understood. One possible reason may be an alteration of spinal mu opioid receptors. The ineffectiveness of morphine in neuropathic pain might be caused by a reduced number of presynaptic opioid receptors caused by the degeneration of primary

### Table 1

<table>
<thead>
<tr>
<th>Primary glia cell culture</th>
<th>mRNA level (fold change of control)</th>
<th>Non-stimulated</th>
<th>LPS-Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microglia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-stimulated</td>
<td>1.0 ± 0.05</td>
<td>119.7 ± 32.93**</td>
<td></td>
</tr>
<tr>
<td>LPS-Stimulated</td>
<td>1.0 ± 0.1</td>
<td>62.8 ± 5.08***</td>
<td></td>
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<tr>
<td>Astrogia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-stimulated</td>
<td>1.0 ± 0.04</td>
<td>47.8 ± 3.04***</td>
<td></td>
</tr>
<tr>
<td>LPS-Stimulated</td>
<td>1.0 ± 0.04</td>
<td>853.9 ± 4.19***</td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>1.0 ± 0.12</td>
<td>1108.9 ± 125.79***</td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>1.0 ± 0.06</td>
<td>70.7 ± 9.04***</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>1.0 ± 0.06</td>
<td>859.911 ± 30.96***</td>
<td></td>
</tr>
<tr>
<td>CCL7</td>
<td>1.0 ± 0.17</td>
<td>182.18 ± 2.67***</td>
<td></td>
</tr>
<tr>
<td>CCL11</td>
<td>Not detected</td>
<td>Not detected</td>
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</table>
afferent neurons after nerve damage (Porreca et al., 1998; Przewlocki and Przewlocka, 2001, 2005). Opioid receptors, similar to CCR5 receptors, are present on glial and neuronal cells (Bidack et al., 2000; Chuang et al., 1995; Liou et al., 2013). Several research groups have shown that opioid receptor agonists, such as morphine, can increase CCR5 expression (Miyagi et al., 2000). Increasing numbers of in vitro studies have demonstrated that CCR5 and MOR may crosstalk with each other by undergoing dimerization. These two receptors may interact favorably through interfacing transmembrane helices V and VI to form a heterodimer (Yuan et al., 2012). Suzuki et al. (2002) demonstrated for the first time that opioid receptors and CCR5 form oligomers and that this oligomerization modulates the function of that complex. Two years later, heterodimerization and cross-desensitization between MOR and the CCR5 in co-expressed Chinese hamster ovary cells was reported (Chen et al., 2004). The authors proposed that MOR-CCR5 heterodimers may contribute to the observed cross-desensitization.

Therefore, the observed MVC-induced reversal of the CCR5 up-regulation caused by nerve injury can influence opioid agonist effectiveness. MVC may function by directly affecting the interaction between opioid receptors and CCR5. On the other hand, the drug diminishes microglial and astroglial cell activation. In 2003, Raghavendra et al. (2003) indicated that the activation of microglia and subsequent increased level of pronociceptive cytokines decreased opioid effectiveness and the development of morphine tolerance. In the current study, we showed that MVC diminishes glial activity and thus decreases the levels of pronociceptive CCL3, CCL4 and CCL5. We hypothesize that these mechanisms can enhance the analgesic effects of morphine and buprenorphine in neuropathy. These results are in line with those obtained in our department previously with the glial inhibitors minocycline and pentoxifylline (Mika et al., 2007, 2009). In fact, our published data show that similar chronic administration of those inhibitors not only attenuated the development of allodynia and hyperalgesia but also potentiated the analgesic activity of morphine under neuropathic pain in rats and mice (Mika et al., 2007, 2009). Pentoxifylline has been used in the clinic since 2000, and its influence on the effectiveness of morphine in the postoperative period has been clinically confirmed (Wrodliczek et al., 2000). Thus, our results suggest that the pharmacological modulation of CCR5 by MVC is a potential novel therapeutical approach for the co-treatment of patients receiving opioid therapy for neuropathic pain.

5. Conclusion

In summary, MVC diminished neuropathic pain and in parallel the protein level of Iba-1 and GFAP. Moreover, in the spinal cord and DRG, CCI-induced up-regulation of CCR5 was diminished after MVC administration. Furthermore, the restoration of the analgesic activity of morphine and buprenorphine by MVC suggests that increased glial activity and consequent expression of CCR5 ligands (CCL3, CCL4 and CCL5) may account for the diminished analgesic efficacy of opioids observed in the neuropathy. However, the primary culture experiments revealed that CCR5 ligands, namely CCL3, CCL4, CCL5, CCL7 (but not CCL11), may originate from both microglia and astroglia, and that their expression is potentiated by inflammatory status. Our results implicate the modulation of glia cells and the activation of neuroimmune factors as potential therapeutic targets for the neuropathic pain treatment.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

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Fig. 5. The influence of maraviroc (MVC) on the mRNA levels of selected chemokines (CCL3, CCL4, CCL5, CCL7, CCL11) in the spinal cord (A, C, E, G, I) and DRG (B, D, F, H, J) during neuropathic pain. Maraviroc (20 μg/s/k; i.c.; 16 h and 1 h before CCI and then once daily for 7 days); diminished the mRNA expression of CCL3 (A), CCL4 (C) and CCL5 (E), but did not influence the mRNA expression of CCL7 (G) in the dorsal part of the lumbar spinal cord 7 days after CCI. Addition of MVC decreased the level of CCL3 mRNA (F) and increased the level of CCL5 mRNA (H) in the DRG, but did not influence the mRNA expression of CCL4 (B), CCL4 (D) and CCL11 (J) in the DRG. In the spinal cord, the mRNA level of CCL11 was undetectable. The data are presented as the mean ± SEM. The intergroup differences were analyzed using ANOVA with Bonferroni’s multiple comparisons test. *p < 0.05, ***p < 0.01 and ****p < 0.001 indicate differences between naïve rats and V-treated CCI-exposed rats; *p < 0.05, **p < 0.01 and ***p < 0.001 indicate differences between V-treated CCI-exposed rats and MVC-treated CCI-exposed rats. N, naive; V, vehicle; MVC, maraviroc.


