

Maraviroc reduces neuropathic pain through polarization of microglia and astroglia – Evidence from *in vivo* and *in vitro* studies



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ABSTRACT

Recent studies suggest that CCR5 and its ligands are important regulators for the development of neuropathic pain and that their modulation can have some beneficial properties. Therefore, the aim of our study was to investigate the influence of maraviroc (MVC, a CCR5 antagonist) on glial polarization markers and intracellular signaling pathways in the spinal cord 7 days after chronic constriction injury (CCI) to the sciatic nerve and in primary glial cultures after LPS stimulation. Our results demonstrated that chronic intrathecal administration of MVC diminished neuropathic pain symptoms and nociceptive threshold ~60 min after drug administration on days 3 and 7 post-CCI. MVC downregulated the levels of phosphorylated p38 MAPK, ERK1/2 and NF- κ B proteins in the spinal cord and upregulated STAT3 in the dorsal root ganglia (DRG). Additionally, using Western blot analysis, we demonstrated that MVC effectively diminished “classical” activation markers: IL-1 β , IL-18, IL-6 and NOS2 in the spinal cord. In contrast, MVC upregulated “alternative” antinociceptive activation markers: IL-1RA, IL-18BP and IL-10 in the spinal cord. In parallel, MVC downregulated the levels of phosphorylated p38 MAPK, ERK1/2 and NF- κ B proteins and upregulated STAT3 in microglial and astroglial cell cultures. Similarly, MVC reduced pronociceptive (IL-1 β , IL-18, IL-6, NOS2) and enhanced the antinociceptive (IL-1RA, IL-18BP, IL-10) factors after LPS stimulation. Our studies provide new evidence that MVC attenuates neuropathy symptoms, promotes spinal glial “alternative” polarization and restores the balance between pro- and antinociceptive factors. Our results suggest the modulation of CCR5 by MVC as a novel therapeutic approach for neuropathy.

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1. Introduction

Many research studies have confirmed that the treatment of neuropathic pain is a complex and difficult issue (Baron, 2006; Woolf and Mannion, 1999). The development of neuropathic pain involves not only neuronal pathways but also glial cells, which interact with neurons and thereby modulate pain transmission under pathophysiological conditions (Scholz and Woolf, 2007; White et al., 2007; Gao and Ji, 2010; Zhuo et al., 2011; Mika et al., 2013). First, the active microglia/macrophages proliferate, polarize and begin to produce nociceptive factors, including cytokines, capable of modulating nociception by exerting an effect on both neuronal and non-neuronal cells (Milligan and Watkins, 2009,

Gosselin et al., 2010). The process of microglia/macrophage polarization leads to two phenotypes: the potentially neurotoxic M1 phenotype from “classical activation” and the neuroprotective M2 phenotype from “alternative activation” (Kigerl et al., 2009; Crain et al., 2013; Nakagawa and Chiba, 2014). The M1 phenotype is characterized by pro-inflammatory factors with pronociceptive properties (IL-1 β , IL-6, IL-18, NOS2) (Crain et al., 2013; Burke et al., 2014); in contrast, the M2 phenotype is characterized by anti-inflammatory factors with antinociceptive properties (IL-10) (Burke et al., 2014; Popiolek-Barczyk et al., 2015). Interestingly, has recently been found that astrocytes also exhibit functional polarization, which plays a crucial role in neuroinflammation (Peng and Carbonetto, 2012; Jang et al., 2013). The precise regulation of neuropathic polarization processes is still unclear (Gemechu et al., 2011).

It is known that CCR5, which is up-regulated in the spinal cord after nerve injury, plays a crucial role in the development of

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neuropathic pain in rodents (Kiguchi et al., 2012; Matsushita et al., 2014; Kwiatkowski et al., 2016). We hypothesize that CCR5 is important for glial activation, their polarization into the phenotype from “classical activation”, and consequences responsible for neuropathic pain development. Recently, we reported that maraviroc (MVC, a selective antagonist of CCR5) effectively reduced the symptoms of neuropathic pain and intensified opioid analgesia (Kwiatkowski et al., 2016). In addition, MVC significantly decreased spinal microglia/astroglia activation and reduced the expression of some CCR5 ligands (Kwiatkowski et al., 2016). MVC was approved by the Food and Drug Administration for clinical use in the treatment of HIV-infected patients who are infected by the R5-tropic virus (Lieberman-Blum et al., 2008). Therefore, because it is used clinically, we believe that the study of its mechanism of action in neuropathic pain is very important.

The objective of our studies was to assess whether and how MVC, which inhibits the development of neuropathic pain (Kwiatkowski et al., 2016), affects the activation of some intracellular pathways and nociceptive factors of neuropathic pain. We used Western blotting to examine how chronic intrathecal (*i.t.*) administration of MVC influenced the nerve injury-induced increase of phosphorylated p38 MAPK, NF- κ B, ERK1/2, and STAT3 in the spinal cord and DRG. Simultaneously, we assessed influence of MVC on the increased levels of pro- (IL-1 β , IL-18, IL-6, NOS2) and antinociceptive factors (IL-1RA, IL-18BP, IL-10) that occur in neuropathy. Moreover, the objective of our *in vitro* studies was to determine the effect of MVC on the above factors in primary microglia and astroglia cultures.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–350 g) were housed in cages lined with sawdust under a standard 12/12-h light/dark cycle (lights on at 08:00) with food and water *ad libitum*. The animals were allowed to acclimate to the environment for approximately 5 min prior to the behavioral tests. All experiments were performed in accordance with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983) and the NIH Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the local Bioethics Committee (Krakow, 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 by receiving catheter implants while under sodium pentobarbital anesthesia (60 mg/kg *i.p.*), according to the method described by Yaksh and Rudy (1976). The *i.t.* catheter consisted of 13-cm long polyethylene tubing (PE 10,

Intramedic; Clay Adams, Parsippany, NJ, USA) with a dead space of 10 μ l 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 μ l), 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 was measured in the rats subjected to CCI using an automated von Frey apparatus (Dynamic Plantar Anesthesiometer, Cat. No. 37400, Ugo Basile, Italy) exactly as previously published (Makuch et al., 2013; Rojewska et al., 2014). The rats were placed in plastic cages with a wire-net floor 5 min before the experiment. The rats were moving freely on the surface of the metal mesh in the enclosed area. The machine's touch stimulator was moved under the animal and, using an angled and adjustable mirror, the filament was placed below the surface of the paw. The von Frey filament was applied to the midplantar surface of the hind foot, and measurements were taken automatically. The animal's response, which was a paw-withdrawal reflex, was recorded automatically using the force at which the paw was withdrawn, in grams. The strength of the von Frey stimuli was up to 26 g.

2.4.2. Thermal hyperalgesia (cold plate test)

Hyperalgesia was assessed using a Cold/Hot Plate Analgesia Meter (No. 05044, Columbus Instruments, USA). The temperature of the cold plate was maintained at 5 °C. The animals were placed on the cold plate, and the time until the hind paw was lifted was recorded; because the ipsilateral paw of the rats subjected to CCI always reacted first to a cold stimulus, this response reflected the reactivity of the ipsilateral hind paw. The cut-off latency was 30 s (Mika et al., 2007; Makuch et al., 2013).

2.4.3. Nociceptive threshold (tail flick test)

The tail-flick test was performed to evaluate the pain threshold to a thermal stimulus using a Tail-Flick Analgesic Meter (Analgesia Meter; Ugo Basile, Comerio, Italy), which is a standard method used in our study (Mika et al., 2009). During the procedure, an animal is placed on the apparatus surface and gently held by an experimenter. In this test, a beam of light is focused on the dorsal tail surface, at 2 cm from the tail tip. When the animal flicks its tail, the timer stops automatically, and the recorded time (latency) is measured. The animals were tested only once by this test, and the cut-off time was 9 s.

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 1-day-old Wistar rats according to the procedure described by Zawadzka and Kaminska (2005). Cells were isolated from the cerebral cortex and were then plated in poly-L-lysine-coated, 75-cm² culture bottles at a density of 3×10^5 cells/cm² in high-glucose DMEM with GlutaMAX (Gibco, New York, USA), heat-inactivated 10% fetal bovine serum, 0.1 mg/ml streptomycin and 100 U/ml penicillin (Gibco, New York, USA). The cultures were maintained at 37 °C and 5% CO₂. On the fourth day of culture, the culture medium was changed. On the ninth day, the cultures were gently shaken and centrifuged to recover the loosely adherent microglia. Then, the medium was changed, and on the twelfth day, the microglia were

recovered again. Once more, the culture medium was replaced, and the cultures were allowed to grow on a rotary shaker at 37 °C for 24 h (200 rpm) to remove the remaining non-adherent cells. The medium was removed, and astrocytes were plated on culture plates and were cultured for 3 days. Then, the astrocytes were trypsinized (0.005% trypsin EDTA solution, Sigma-Aldrich, St. Louis, USA). Microglia/astrocytes were seeded at a final density of 1.2×10^6 cells per 6-well plate for analysis of the protein in the culture medium and were incubated for 48 h. Primary microglial and astrocyte cell cultures were treated with MVC [100 nM] 30 min before LPS administration [100 ng/ml] (Sigma-Aldrich, St. Louis, USA) and incubated for 1 h and 24 h. To identify microglia and astrocytes in the cell cultures, we used IBA1 (a microglial marker, SC-327 225, Santa Cruz Biotechnology Inc., Santa Cruz, USA) and GFAP (an astrocyte marker, SC-166 458, Santa Cruz Biotechnology Inc., Santa Cruz, USA) staining. We obtained highly homogeneous microglial and astroglial populations (more than 95% positive for IBA1 and GFAP, respectively) (Zawadzka and Kaminska, 2005).

2.6. Drug administration

Maraviroc (MVC, Tocris, Warsaw, Poland; (20 µg/5 µl)) was dissolved in 12% DMSO and was administered preemptively *i.t.* injection 16 h and 1 h before CCI; subsequently, it was administered once daily for 7 days. The control groups received vehicle (V, 12% DMSO) according to the same schedule. The respective substances were delivered slowly in a volume of 5 µl through the *i.t.* catheter, followed by an injection of 10 µl of water, which flushed the catheter. The behavioral tests were performed 60 min (von Frey), 65 min (cold plate) and 70 min (tail flick) after MVC administration on day 7 post-CCI.

2.7. Biochemical tests

2.7.1. Western blot

Tissue for Western blot analysis were 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 ($14,000 \times g$ for 30 min at 4 °C). Samples containing 20 µg of protein were heated in a loading buffer (4x Laemmli Buffer, Bio-Rad, Warsaw, Poland) for 5 min at 98 °C. Then, 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). The membranes were then washed in TBST and incubated overnight at 4 °C with the following primary antibodies: rabbit polyclonal (IL-1β (Abcam) 1:1000; IL-18 (R&D Systems) 1:1000; IL-6 (Invitrogen) 1:500; NOS2 (Santa Cruz) 1:500; IL-1RA (Abcam) 1:1000; IL-18BP (Novus Biologicals) 1:1000; IL-10 (Invitrogen) 1:500; p38 MAPK (Cell Signaling) 1:1000; p-p38 MAPK (Cell Signaling) 1:1000; ERK1/2 (Cell Signaling) 1:1000; p-ERK1/2 (Cell Signaling) 1:1000; NF-κB (Santa Cruz) 1:500; NF-κB (Santa Cruz) 1:500; STAT3 (Cell Signaling) 1:1000; and p-STAT3 (Cell Signaling) 1:500) and mouse polyclonal (GAPDH (Millipore) 1:5000). The membranes were then incubated for 1 h in horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies at a dilution of 1:5000. 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 (Fig. 1) are presented in grams and seconds as the mean ± SEM. One-way analysis of variance (ANOVA) with Bonferroni's post-hoc test were used to evaluate the experimental results and analyze differences between groups. ***p* < 0.01 and ****p* < 0.001 indicate differences between naive (control) 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.

The results of the Western blot (Figs. 2 and 3) analyses are presented as the fold change compared with the results from naive rats on the 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. In the Western blot analysis, each groups contained from 5 to 7 samples. 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* < 0.05, ##*p* < 0.01 and ###*p* < 0.001 indicate differences between V-treated CCI-exposed and MVC-treated CCI-exposed rats.

For the glial cell cultures, the results of the Western blot (Figs. 4 and 5) analyses are presented as the fold change compared with vehicle-treated, non-stimulated cells. The results were obtained from four groups: vehicle-treated non-stimulated cells (K/–, control group); MVC-treated non-stimulated cells (MVC/–); vehicle-treated LPS-stimulated cells (K/+); and MVC-treated LPS-stimulated cells (MVC/+). For the Western blot analysis, each group contained from 3 to 5 samples. 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 compared with the K/– group. #*p* < 0.05, ##*p* < 0.01 and ###*p* < 0.001 indicate differences between K/+ and MVC/+. \$\$*p* < 0.01 and \$\$\$*p* < 0.001 indicate differences compared with the MVC/– group.

All graphs and analyses were prepared using GraphPad Prism5.

3. Results

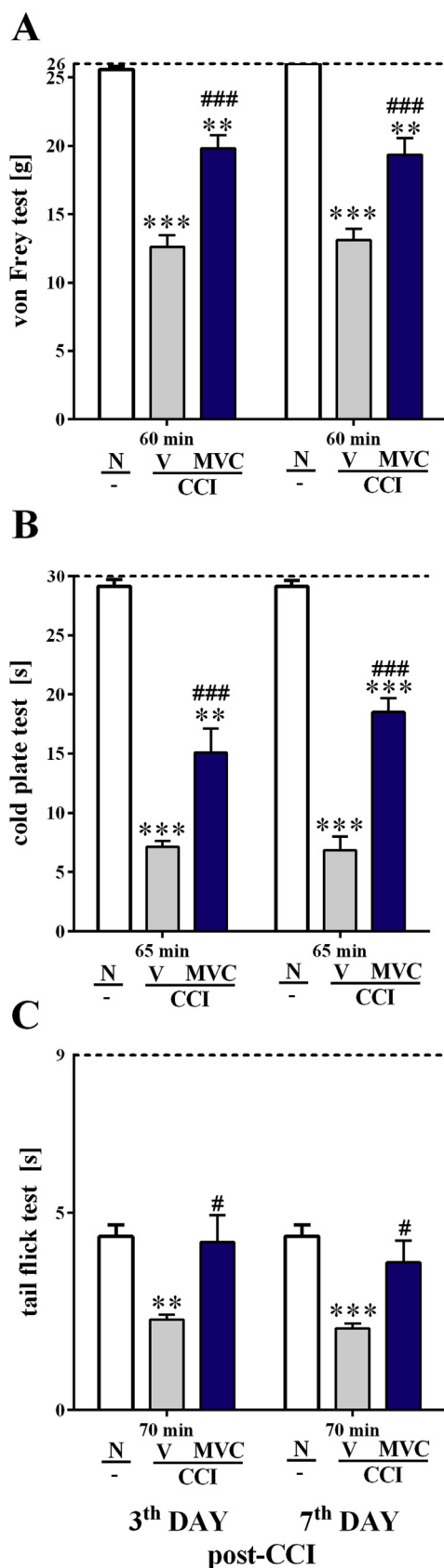
3.1. In vivo studies – rat model of neuropathic pain

3.1.1. The influence of repeated administration of MVC on neuropathic pain symptoms and nociceptive threshold measured on days 3 and 7 after CCI

In the von Frey test, strong mechanical allodynia of the paw ipsilateral to the injury was observed on days 3 and 7 after CCI. The ipsilateral paw responded to a stimulation of 12.6 ± 0.9 g and 13.1 ± 0.8 g on days 3 and 7, respectively (Fig. 1A). Naive rats responded to 25.6 ± 0.2 g and 26.0 ± 0.0 g. MVC significantly attenuated the mechanical allodynia 60 min after injection from 12.6 ± 0.9 g to 19.8 ± 1.0 g and 13.1 ± 0.8 g to 19.3 ± 1.2 g on days 3 and 7, respectively (Fig. 1A).

In the cold plate test, strong thermal hyperalgesia of the paw ipsilateral to the injury was observed on days 3 and 7 after CCI. The ipsilateral paw responded to a stimulation of 7.1 ± 0.5 s and 6.8 ± 1.1 s on days 3 and 7, respectively (Fig. 1B). Naive rats responded to 29.2 ± 0.6 s and 29.1 ± 0.5 s. MVC significantly attenuated the thermal hyperalgesia 60 min after injection from 7.1 ± 0.5 s to 15.1 ± 2.0 s and 6.8 ± 1.1 s to 18.5 ± 1.2 s on days 3 and 7, respectively (Fig. 1B).

Behavioural Tests



In the tail flick test, a lower pain threshold to a thermal stimulus was observed on days 3 and 7 after CCI. The tail responded to a stimulation of 2.3 ± 0.1 s and 2.1 ± 0.1 s on days 3 and 7, respectively (Fig. 1C), and naive rats responded to a stimulation of 4.4 ± 0.3 s. MVC significantly attenuated the pain threshold 60 min after injection from 2.3 ± 0.1 s to 4.3 ± 0.7 s and 2.1 ± 0.1 s to 3.7 ± 0.5 s on days 3 and 7, respectively (Fig. 1C).

3.1.2. The influence of repeated administration of MVC on p-p38, p-ERK1/2, p-NF- κ B and p-STAT3 in the spinal cord and DRG 7 days after CCI

The p-p38 protein level was upregulated in the spinal cord from 1.0 ± 0.1 to 1.5 ± 0.1 (Fig. 2A) and in the DRG from 1.0 ± 0.1 to 1.4 ± 0.1 (Fig. 2B) in CCI-exposed rats compared with that of naive rats. MVC diminished the protein level of p-p38 from 1.5 ± 0.1 to 1.1 ± 0.1 in the spinal cord (Fig. 2A) and from 1.4 ± 0.1 to 1.0 ± 0.1 in the DRG (Fig. 2B).

The p-ERK1/2 protein level was upregulated in the spinal cord from 1.0 ± 0.1 to 1.5 ± 0.1 (Fig. 2C) and in the DRG from 1.0 ± 0.05 to 1.3 ± 0.03 (Fig. 2D) in CCI-exposed rats compared with that of naive rats. MVC diminished the protein level of p-ERK1/2 from 1.5 ± 0.1 to 0.88 ± 0.1 in the spinal cord (Fig. 2C) and from 1.3 ± 0.03 to 0.95 ± 0.04 in the DRG (Fig. 2D).

The p-NF- κ B protein level was upregulated in the spinal cord from 1.0 ± 0.05 to 1.2 ± 0.03 (Fig. 2E) and in the DRG from 1.0 ± 0.04 to 1.2 ± 0.1 (Fig. 2F) in CCI-exposed rats compared with that of naive rats. MVC diminished the protein level of p-NF- κ B from 1.2 ± 0.03 to 1.0 ± 0.1 in the spinal cord (Fig. 2E) and increased from 1.2 ± 0.1 to 1.5 ± 0.02 in the DRG (Fig. 2F).

The p-STAT3 protein level was up-regulated in the spinal cord from 1.0 ± 0.03 to 1.3 ± 0.04 (Fig. 2G), but no changes were observed in the p-STAT3 protein level in the DRG (Fig. 2H) in CCI-exposed rats compared with that of naive rats. MVC diminished the protein level of p-STAT3 from 1.3 ± 0.04 to 1.0 ± 0.1 in the spinal cord (Fig. 2G) and increased it from 1.5 ± 0.1 to 2.3 ± 0.2 in the DRG (Fig. 2H).

3.1.3. The influence of repeated administration of MVC on nociceptive factors in the spinal cord and DRG 7 days after CCI

3.1.3.1. Pronociceptive factors (IL-1 β , IL-18, IL-6, NOS2). The IL-1 β protein level was upregulated in the spinal cord from 1.0 ± 0.1 to 1.4 ± 0.1 (Fig. 3A), but no changes were observed in the IL-1 β protein level in the DRG (Fig. 3B) in CCI-exposed rats compared with that of naive rats. MVC diminished the protein level of IL-1 β from 1.4 ± 0.1 to 0.5 ± 0.1 in the spinal cord (Fig. 3A) and from 1.15 ± 0.01 to 0.81 ± 0.1 in the DRG (Fig. 3B).

The IL-18 protein level was upregulated in the spinal cord from 1.0 ± 0.1 to 1.9 ± 0.1 (Fig. 3C) and in the DRG from 1.0 ± 0.1 to 2.2 ± 0.1 (Fig. 3D) in CCI-exposed rats compared with that of naive rats. MVC diminished the protein level of IL-18 from 1.9 ± 0.1 to 1.5 ± 0.1 in the spinal cord (Fig. 3C) and from 2.2 ± 0.1 to 1.8 ± 0.1 in the DRG (Fig. 3D).

The IL-6 protein level was upregulated in the spinal cord from

Fig. 1. The effects of repeated maraviroc administration (MVC; 20 μ g/5 μ l 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), thermal hyperalgesia (B; cold plate test) and threshold pain (C; tail flick test) on days 3 and 7 after CCI. Allodynia, hyperalgesia and nociceptive thresholds were assessed 60, 65 and 70 min after the last MVC injection. The behavioral results are presented as the mean \pm SEM (10–20 rats per group), and the horizontal dotted line shows the cut-off value. The intergroup differences were analyzed using ANOVA with Bonferroni's multiple comparisons test. ** $p < 0.01$ and *** $p < 0.001$ indicate differences compared with the naive group; # $p < 0.05$ and ### $p < 0.001$ indicate differences between V-treated and MVC-treated CCI-exposed rats. **Abbreviations:** CCI, chronic constriction injury; MVC, maraviroc; V, vehicle.

Protein kinases and transcription factors

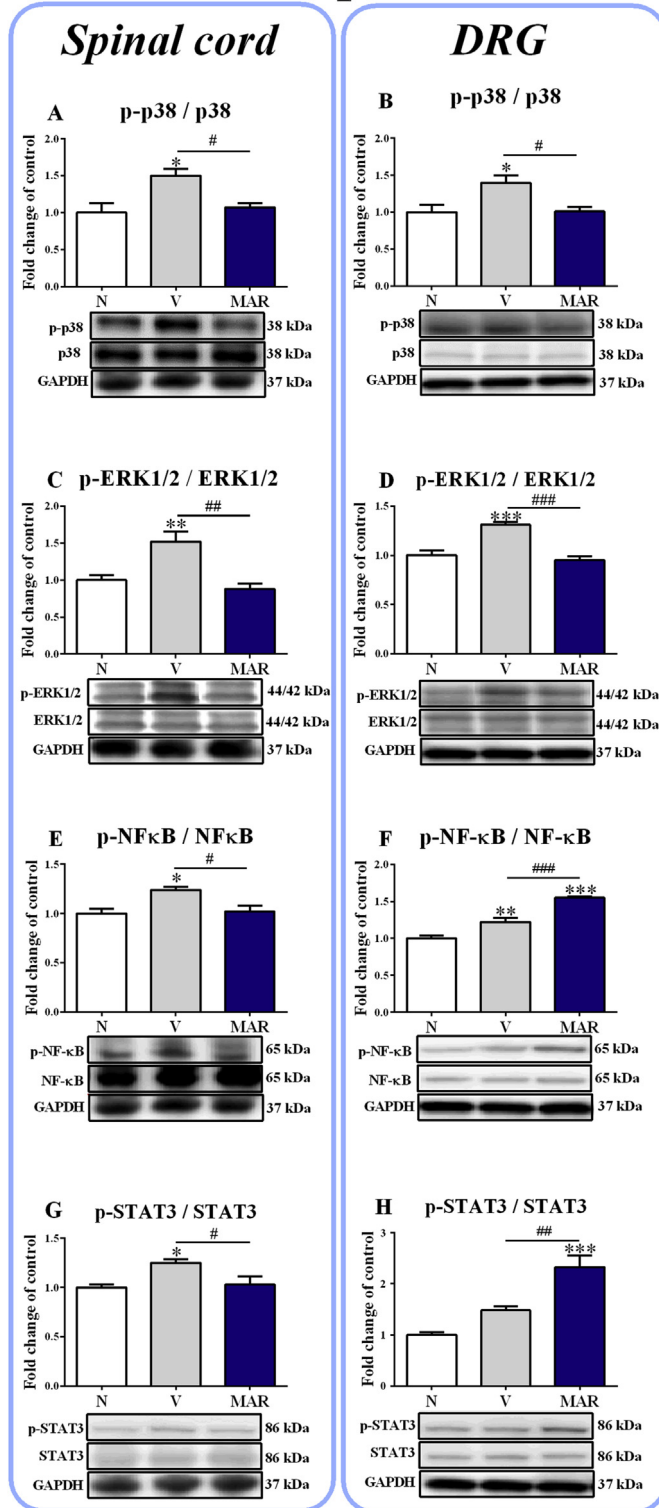


Fig. 2. The effects of repeated maraviroc administration (MVC; 20 µg/5 µl i.t.; 16 h and 1 h before CCI and then once daily for 7 days) on the protein levels of protein kinases (p-p38: A, B and p-ERK1/2: C, D) and transcription factors (p-NF-κB: E, F and p-STAT3: G, H) in the spinal cord (A, C, E, G) and the DRG (B, D, F, H) on day 7 after CCI. The data are shown as the mean fold changes in expression relative to the control (naive group) levels ± SEM (4–8 samples for each group). The intergroup differences were analyzed using ANOVA with Bonferroni's multiple comparisons test. *p < 0.05, **p < 0.01 and

1.0 ± 0.1 to 1.3 ± 0.1 (Fig. 3E) and in the DRG from 1.0 ± 0.1 to 1.4 ± 0.1 (Fig. 3F) in CCI-exposed rats compared with that of naive rats. MVC diminished the protein level of IL-6 from 1.3 ± 0.1 to 1.0 ± 0.03 in the spinal cord (Fig. 3E) and from 1.4 ± 0.1 to 0.9 ± 0.1 in the DRG (Fig. 3F).

The NOS2 protein level was upregulated in the spinal cord from 1.0 ± 0.04 to 1.3 ± 0.1 (Fig. 3G), but no changes were observed in the NOS2 protein level in the DRG (Fig. 3H) in CCI-exposed rats compared with that of naive rats. MVC diminished the protein level of NOS2 from 1.3 ± 0.1 to 0.8 ± 0.04 in the spinal cord (Fig. 3G) but did not influence the protein level of NOS2 in the DRG (Fig. 3H).

3.1.3.2. Antinociceptive factors (IL-1RA, IL-18BP, IL-10). The IL-1RA protein level did not change in the spinal cord (Fig. 3I) or in the DRG (Fig. 3J) in CCI-exposed rats compared with that of naive rats. MVC increased the protein level of IL-1RA from 0.82 ± 0.1 to 1.3 ± 0.04 in the spinal cord (Fig. 3I) and from 0.7 ± 0.1 to 1.1 ± 0.1 in the DRG (Fig. 3J).

The IL-18BP protein level was downregulated in the spinal cord from 1.0 ± 0.1 to 0.6 ± 0.1 (Fig. 3K) and in the DRG from 1.0 ± 0.1 to 0.5 ± 0.1 (Fig. 3L) in CCI-exposed rats compared with that of naive rats. MVC increased the protein level of IL-18BP from 0.6 ± 0.1 to 1.0 ± 0.1 in the spinal cord (Fig. 3K) and from 0.5 ± 0.1 to 1.2 ± 0.1 in the DRG (Fig. 3L).

The IL-10 protein level was downregulated in the spinal cord from 1.0 ± 0.1 to 0.7 ± 0.04 (Fig. 3M) and in the DRG from 1.0 ± 0.04 to 0.7 ± 0.1 (Fig. 3N) in CCI-exposed rats compared with that of naive rats. MVC increased the protein level of IL-10 from 0.7 ± 0.04 to 1.2 ± 0.1 in the spinal cord (Fig. 3M) and from 0.73 ± 0.1 to 1.1 ± 0.04 in the DRG (Fig. 3N).

3.2. In vitro studies- rat primary glial cell cultures

3.2.1. The influence of MVC on p-p38, p-ERK1/2, p-NF-κB and p-STAT3 in the microglia and astroglia 1 h after LPS stimulation

The p-p38 protein level was upregulated in the microglia from 1.0 ± 0.03 to 1.3 ± 0.03 (Fig. 4A) and in the astroglia from 1.0 ± 0.1 to 1.8 ± 0.2 (Fig. 4B) in LPS-stimulated cells compared with that of non-stimulated cells. MVC diminished the protein level of p-p38 from 1.3 ± 0.03 to 1.0 ± 0.0 in the microglia (Fig. 4A) and from 1.8 ± 0.2 to 0.9 ± 0.1 in the astroglia (Fig. 4B) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

The p-ERK1/2 protein level was upregulated in the microglia from 1.0 ± 0.04 to 1.3 ± 0.03 (Fig. 4C) and in the astroglia from 1.0 ± 0.1 to 1.6 ± 0.1 (Fig. 4D) in LPS-stimulated cells compared with that of non-stimulated cells. MVC diminished the protein level of p-ERK1/2 from 1.3 ± 0.03 to 0.9 ± 0.1 in the microglia (Fig. 4C) and from 1.6 ± 0.1 to 1.2 ± 0.04 in the astroglia (Fig. 4D) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

The p-NF-κB protein level was upregulated in the microglia from 1.0 ± 0.1 to 2.8 ± 0.3 (Fig. 4E) and in the astroglia from 1.0 ± 0.1 to 3.0 ± 0.3 (Fig. 4F) in LPS-stimulated cells compared with that of non-stimulated cells. MVC diminished the protein level of p-NF-κB from 2.8 ± 0.3 to 1.1 ± 0.1 in the microglia (Fig. 4E) and from 3.0 ± 0.3 to 1.3 ± 0.2 in the astroglia (Fig. 4F) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

***p < 0.001 indicate differences compared with the naive group. #p < 0.05, ##p < 0.01 and ###p < 0.001 indicate differences between V-treated and MVC-treated CCI-exposed rats. Abbreviations: CCI, chronic constriction injury; MVC, maraviroc; N, naive; V, vehicle.

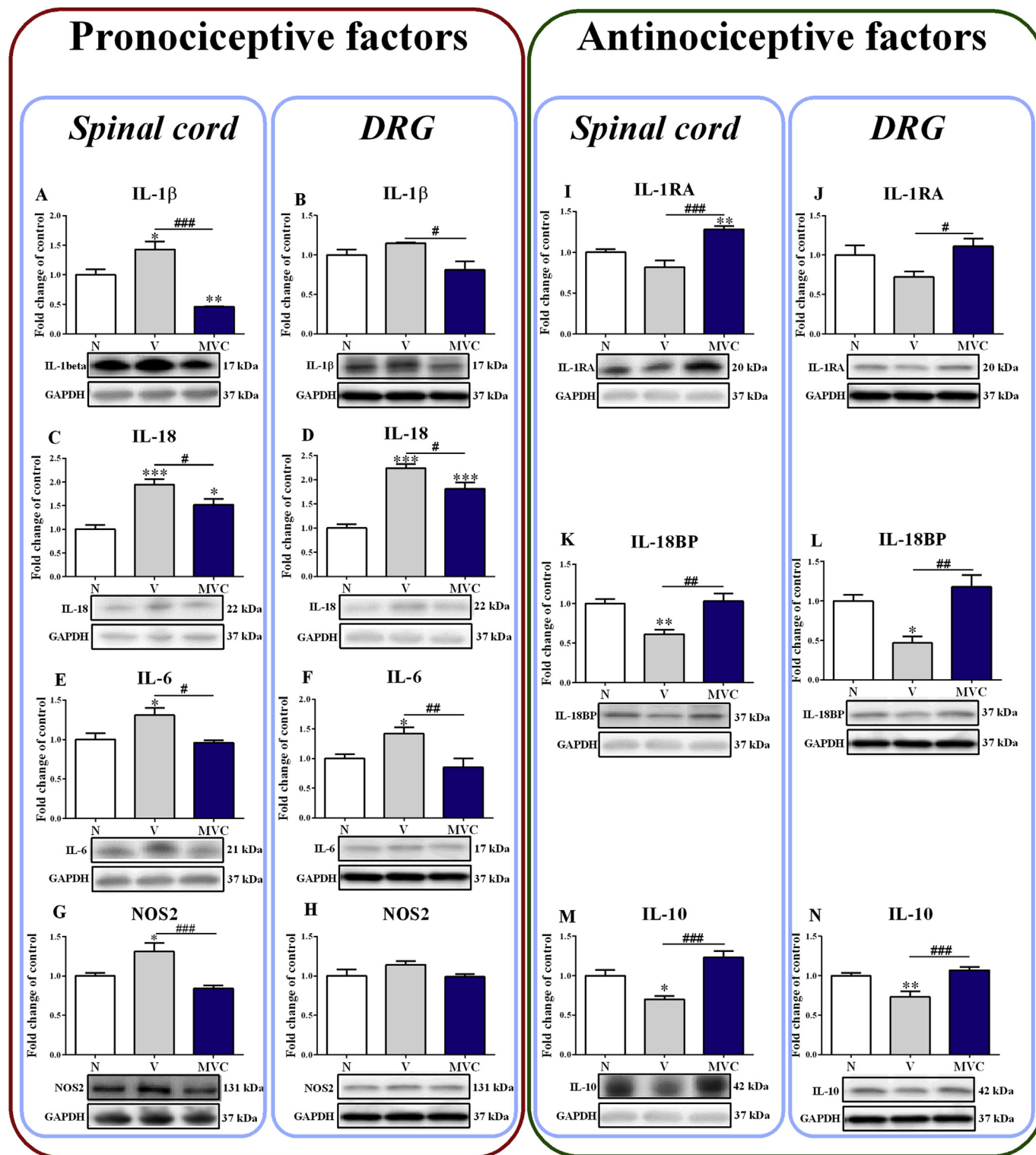


Fig. 3. The effects of repeated maraviroc administration (MVC; 20 μ g/5 μ l i.t.; 16 h and 1 h before CCI and then once daily for 7 days) on the protein levels of pronociceptive (IL-1 β : **A**, **B**; IL-18: **C**, **D**; IL-6: **E**, **F**; NOS2: **G**, **H**) and antinociceptive factors (IL-1RA: **I**, **J**; IL-18BP: **K**, **L**; IL-10: **M**, **N**) in the spinal cord (**A**, **C**, **E**, **G**, **I**, **K**, **M**) and the DRG (**B**, **D**, **F**, **H**, **J**, **L**, **N**) on day 7 after CCI. The data are shown as the mean fold changes in expression relative to the control (naive group) levels \pm SEM (4–8 samples for each group). 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 compared with the naive group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ indicate differences between V-treated and MVC-treated CCI-exposed rats. **Abbreviations:** CCI, chronic constriction injury; MVC, maraviroc; N, naive; V, vehicle.

The p-STAT3 protein level was upregulated in the microglia from 1.0 ± 0.1 to 3.3 ± 0.1 (Fig. 4G) and in the astroglia from 1.0 ± 0.1 to

1.8 ± 0.2 (Fig. 4H) in LPS-stimulated cells compared with that of non-stimulated cells. MVC increased the protein level of p-STAT3

Protein kinases and transcription factors

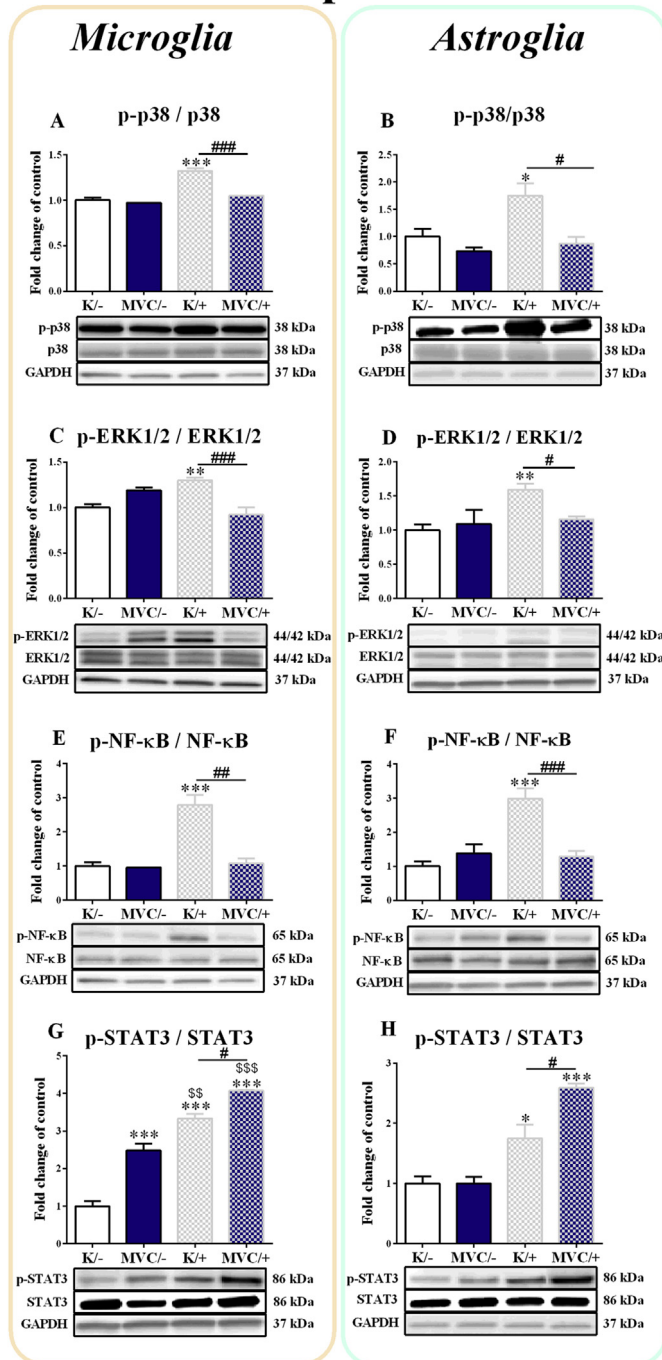


Fig. 4. The effects of maraviroc administration (MVC; 100 nM, 30 min before LPS stimulation; 100 ng/ml) on the protein levels of protein kinases (p-p38; **A, B** and p-ERK1/2; **C, D**) and transcription factors (p-NF-κB; **E, F** and p-STAT3; **G, H**) in unstimulated and LPS-stimulated microglial (**A, C, E, G**) and astroglial cell (**B, D, F, H**) cultures 1 h after the LPS stimulation. The data are shown as the mean fold changes in expression relative to the control (non-stimulated cells) levels \pm SEM (3–6 samples for each group). 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 compared with the K/- group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ indicate differences between K/+ and MVC/+ groups. \$ $p < 0.01$ and \$\$\$ $p < 0.001$ indicate differences compared with the MVC/- group. **Abbreviations:** (K/-) vehicle-treated non-stimulated cells (control group); (MVC/-) MVC-treated non-stimulated cells; (K/+) vehicle-treated LPS-stimulated cells; and (MVC/+) MVC-treated LPS-stimulated cells.

from 1.0 ± 0.1 to 2.5 ± 0.2 in the microglia (**Fig. 4G**) in MVC-treated non-stimulated cells compared with that of vehicle-treated non-stimulated cells. MVC increased the protein level of p-STAT3 from 2.5 ± 0.2 to 4.1 ± 0.02 in the microglia (**Fig. 4G**) in MVC-treated LPS-stimulated cells compared with that of MVC-treated non-stimulated cells. MVC increased the protein level of p-STAT3 from 3.3 ± 0.1 to 4.1 ± 0.02 in the microglia (**Fig. 4G**) and from 1.8 ± 0.2 to 2.6 ± 0.1 in the astroglia (**Fig. 4H**) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

3.2.2. The influence of MVC on nociceptive factors in microglia and astroglia 24 h after stimulation

3.2.2.1. Pronociceptive factors (IL-1 β , IL-18, IL-6, NOS2). The IL-1 β protein level was upregulated in the microglia from 1.0 ± 0.3 to 37.3 ± 1.9 (**Fig. 5A**) and in the astroglia from 1.0 ± 0.1 to 490.5 ± 70.3 (**Fig. 5B**) in LPS-stimulated cells compared with that of non-stimulated cells. MVC diminished the protein level of IL-1 β from 37.3 ± 1.9 to 20.5 ± 3.9 in the microglia (**Fig. 5A**) and from 490.5 ± 70.3 to 296.4 ± 2.5 in the astroglia (**Fig. 5B**) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

The IL-18 protein level was upregulated in the microglia from 1.0 ± 0.05 to 1.9 ± 0.1 (**Fig. 5C**) and in the astroglia from 1.0 ± 0.04 to 2.5 ± 0.2 (**Fig. 5D**) in LPS-stimulated cells compared with that of non-stimulated cells. MVC diminished the protein level of IL-18 from 1.9 ± 0.1 to 1.2 ± 0.1 in the microglia (**Fig. 5C**) and from 2.5 ± 0.2 to 1.9 ± 0.03 in the astroglia (**Fig. 5D**) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

The IL-6 protein level was upregulated in the microglia from 1.0 ± 0.1 to 2.2 ± 0.2 (**Fig. 5E**) and in the astroglia from 1.0 ± 0.01 to 2.7 ± 0.1 (**Fig. 5F**) in LPS-stimulated cells compared with that of non-stimulated cells. MVC diminished the protein level of IL-6 from 2.2 ± 0.2 to 1.5 ± 0.1 in the microglia (**Fig. 5E**) and from 2.7 ± 0.1 to 1.7 ± 0.2 in the astroglia (**Fig. 5F**) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

The NOS2 protein level was upregulated in the microglia from 1.0 ± 0.5 to 42.2 ± 3.3 (**Fig. 5G**) and in the astroglia from 1.0 ± 0.6 to 48.5 ± 5.2 (**Fig. 5H**) in LPS-stimulated cells compared with that of non-stimulated cells. MVC diminished the protein level of NOS2 from 42.2 ± 3.3 to 32.4 ± 0.8 in the microglia (**Fig. 5G**) and from 48.5 ± 5.2 to 30.1 ± 1.5 in the astroglia (**Fig. 5H**) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

3.2.2.2. Antinociceptive factors (IL-1RA, IL-18BP, IL-10). The IL-1RA protein level was downregulated in the microglia from 1.0 ± 0.03 to 0.6 ± 0.1 (**Fig. 5I**), but no differences were observed in the level of IL-1RA in the astroglia (**Fig. 5J**) between LPS-stimulated and non-stimulated cells. MVC increased the protein level of IL-1RA from 0.6 ± 0.1 to 1.2 ± 0.1 in the microglia (**Fig. 5I**) and from 0.8 ± 0.1 to 1.2 ± 0.1 in the astroglia (**Fig. 5J**) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

The IL-18BP protein level was downregulated in the microglia from 1.0 ± 0.03 to 0.7 ± 0.1 (**Fig. 5K**) and upregulated in the astroglia from 1.0 ± 0.05 to 1.4 ± 0.04 (**Fig. 5L**) in LPS-stimulated cells compared with that of non-stimulated cells. MVC increased the protein level of IL-18BP from 1.0 ± 0.03 to 1.3 ± 0.1 in the microglia (**Fig. 5K**) and from 1.0 ± 0.05 to 1.4 ± 0.1 in the astroglia (**Fig. 5L**) in MVC-treated non-stimulated cells compared with that of vehicle-treated non-stimulated cells. MVC increased the protein level of IL-18BP from 0.7 ± 0.1 to 1.1 ± 0.02 in the microglia (**Fig. 5K**) and from 1.4 ± 0.04 to 2.3 ± 0.1 in the astroglia (**Fig. 5L**) in MVC-treated LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

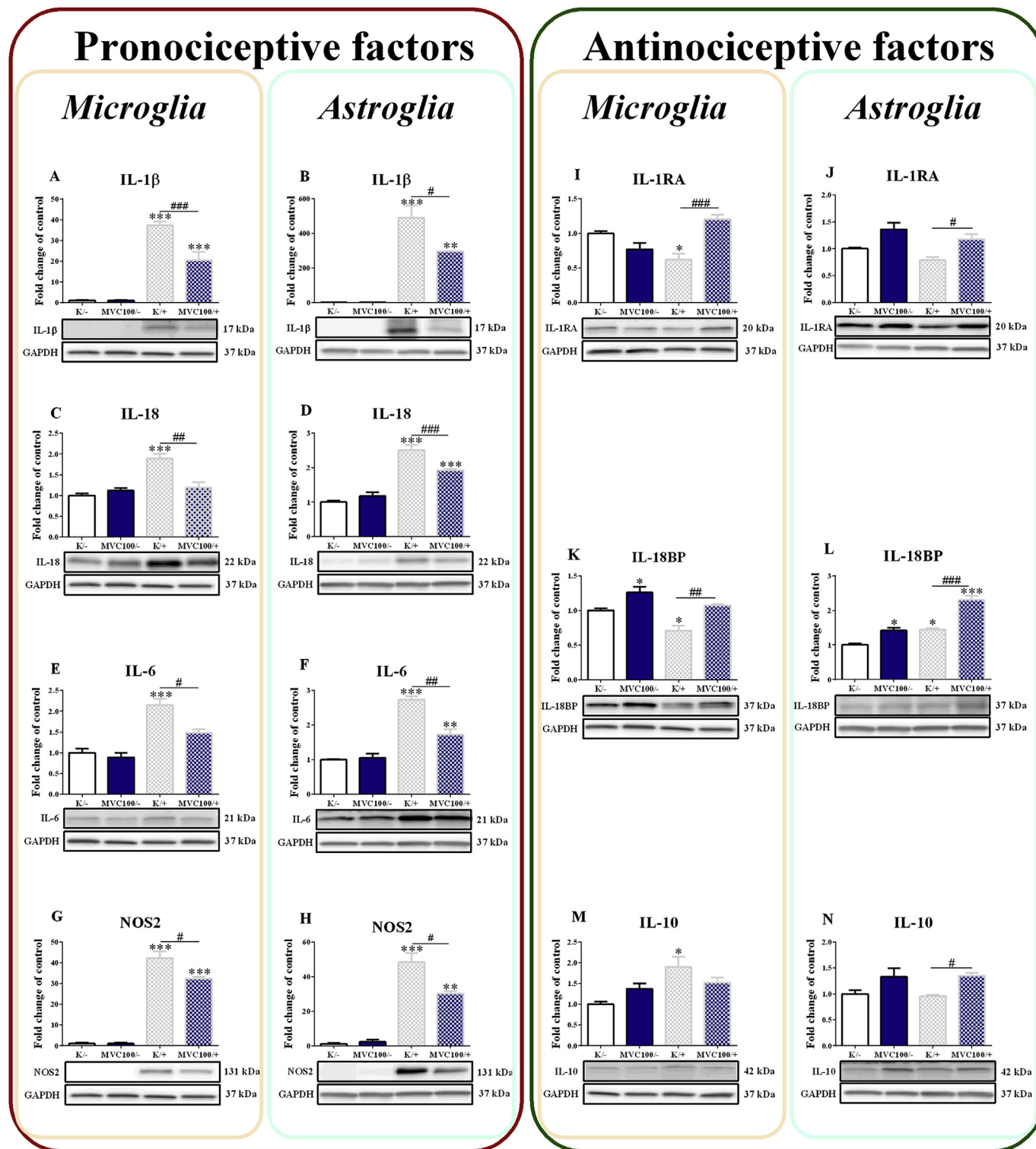


Fig. 5. The effects of maraviroc administration (MVC; 100 nM, 30 min before LPS stimulation; 100 ng/ml) on the protein levels of pronociceptive (IL-1 β : **A, B**; IL-18: **C, D**; IL-6: **E, F**; NOS2: **G, H**) and antinociceptive factors (IL-1RA: **I, J**; IL-18BP: **K, L**; IL-10: **M, N**) in unstimulated and LPS-stimulated microglial (**A, C, E, G, I, K, M**) and astroglial (**B, D, F, H, J, L, N**) cell cultures 24 h after the LPS treatment. The data are shown as the mean fold changes in expression relative to the control (non-stimulated cells) levels \pm SEM (3–6 samples for each group). 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 compared with the K/– group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ indicate differences between K/+ and MVC/+ groups. **Abbreviations:** (K/–) vehicle-treated non-stimulated cells (control group); (MVC/–) MVC-treated non-stimulated cells; (K/+) vehicle-treated LPS-stimulated cells; and (MVC/+) MVC-treated LPS-stimulated cells.

The IL-10 protein level was upregulated in the microglia from 1.0 ± 0.1 to 1.9 ± 0.2 (Fig. 5M), but no differences were observed in the level of IL-10 in the astroglia (Fig. 5N) between LPS-stimulated

and non-stimulated cells. MVC did not influence the protein level of IL-10 in the microglia (Fig. 5M) and increased the IL-10 protein level from 1.0 ± 0.02 to 1.4 ± 0.1 in the astroglia (Fig. 5N) in MVC-treated

LPS-stimulated cells compared with that of vehicle-treated LPS-stimulated cells.

4. Discussion

Because the involvement of glial cells in the generation and maintenance of neuropathic pain has been demonstrated by many studies (Scholz and Woolf, 2007; Mika et al., 2013; Tsuda et al., 2013; Zhuo et al., 2011) we searched for substances that modulate the activity of these cells. Our behavioral studies have shown that the chronic *i.t.* administration of MVC, a selective CCR5 antagonist, significantly reduced pain, according to measurements obtained 1 h after drug injection on days 3 and 7 after CCI. These findings complemented those of our previous studies, where we described the analgesic properties of MVC and its beneficial influence on CCI-induced spinal upregulation of the CCR5 and its pronociceptive ligands (CCL3, CCL4, CCL5) under neuropathic pain (Kwiatkowski et al., 2016). The data from the present study support our hypothesis that by the modulation of microglia/macrophage and astroglia activation, MVC promotes their alternative phase, strongly influencing on the intracellular signaling and release of nociceptive cytokines in a rat neuropathic pain model. Our Western blot studies have shown that MVC treatment prevented the CCI-elevated p-p38, p-ERK1/2 and p-NF- κ B protein levels in the spinal cord and DRG. Simultaneously, we demonstrated that MVC treatment diminished the CCI-elevated pronociceptive factors in the spinal cord and/or DRG (IL-1 β , IL-18, IL-6, NOS2). Additionally, we demonstrated that MVC administration increased the reduced/unchanged levels of antinociceptive IL-1RA, IL-18BP and IL-10 protein observed following injury in both examined structures. Most of our *in vivo* findings correspond with the results obtained after the LPS stimulation and MVC treatment of primary microglia and astroglia cultures. As shown by the *in vitro* studies, MVC downregulated the LPS-elevated p-p38, p-ERK1/2, p-NF- κ B and all analyzed pronociceptive factors. Similarly to the *in vivo* studies, we found that antinociceptive interleukins such as IL-1RA and IL-18BP increased after the MVC treatment in LPS-stimulated glial cells.

MVC is a potent and selective antagonist of CCR5 localized on the surface of a variety of cells (Levy, 2009). In the central nervous system, CCR5 is expressed by neurons, astrocytes and microglia (Rottman et al., 1997; Kaul et al., 2007). Glia, especially microglia, are known to dynamically modulate neuronal functions in pathological conditions; they are the first to become activated following nerve injury (Tanga et al., 2004; Milligan and Watkins, 2009; Zhuo et al., 2011), and they remain active for several weeks (Coyle, 1998; Clark et al., 2007). In our previously published paper (Kwiatkowski et al., 2016), we showed that MVC strongly diminished the CCI-induced activation of microglia and astroglia, cells which have been implicated in both causative and exacerbative roles in neuropathic pain. The propentofylline, pentoxifylline, and minocycline by reducing microglial activation, inhibit the secretion of numerous cytokines, thus inhibiting the development of neuropathic pain (Neuner et al., 1994; Lundblad et al., 1995; Sweitzer et al., 2001; Raghavendra et al., 2003; Mika et al., 2007; Rojewska et al., 2014).

The CCR5 is a seven-transmembrane, G protein-coupled receptor (Schlyer and Horuk, 2006; Allen et al., 2007). Interaction of CCR5 with its pronociceptive ligands (e.g., CCL3, CCL4, CCL5), which are enhanced by nerve injury, causes its activation and induces the complete internalization of the receptor bound to the G_i protein class (Ferain et al., 2011), contributing to further signal transduction through the activation of certain protein kinases (Proudfoot, 2002). The CCR5 stimulation induced some protein phosphorylation, which is an important molecular mechanism by which extracellular signals influence nociceptive transmission. The mitogen-

activated protein kinases (MAPKs) are a family of evolutionarily conserved molecules that play a critical role in cell signaling, gene expression and that have recently become of particular interest with respect to neuropathic pain (Ji et al., 2002; Milligan et al., 2003; Jin et al., 2003; Svensson et al., 2003; Kawasaki et al., 2006; Zhuang et al., 2006; Daulhac et al., 2006). It is well documented that p38 and ERK1/2 play a role in neuropathy and this effect is correlated with microglia and astroglia activation (Jin et al., 2003; Tsuda et al., 2004; Zhuang et al., 2005). We have shown for the first time under neuropathic pain conditions that chronic MVC treatment prevented the CCI-induced upregulation of both p-p38 and p-ERK1/2 protein levels as measured 7 days after nerve injury and by this mechanisms can decreased synthesis of pronociceptive factors (IL-1 β , IL-6, IL-18, NOS2). The only study about the impact of MVC on p38 and ERK was carried out in a hepatocellular carcinoma mouse model, where MVC inhibited hepatic stellate cell activation and increased mouse survival (Ochoa-Callejero et al., 2013). Several studies have also shown increased activity of NF- κ B, a key regulator of inflammatory processes in reactive glial cells, within the spinal cord and DRG in various animal models of neuropathy (Ma and Bisby, 1998; Meunier et al., 2007; Lee et al., 2011; Popiolek-Barczyk et al., 2015). The activation of the NF- κ B family of transcription factors induces many of the pronociceptive factor genes (Beg and Baltimore, 1996; Vanden Berghe et al., 1998; Niederberger and Geisslinger, 2008). In Fu et al. (2010) showed that the transgenic inhibition of glial NF- κ B reduces pain behavior. In the present study, we also observed that MVC treatment prevented the spinal CCI-elevated phosphorylation of NF- κ B 7 days after nerve injury and simultaneously prevented the upregulation of IL-1 β , IL-6, IL-18 and NOS2. Another important transcription factor involved in neuropathy is STAT3, which is a crucial regulator of the pronociceptive IL-6 and antinociceptive IL-10 (Dominguez et al., 2008). The upregulation of STAT3 signaling after nerve injury has been observed in animal models of neuropathic pain (Dominguez et al., 2008; Popiolek-Barczyk et al., 2015). In this study, we observed the increase of STAT3 in the spinal cord and a similar trend in the DRG 7 days after CCI. In Dominguez et al. (2008), showed that the inhibition of STAT3 in microglia attenuated neuropathic pain, which corresponds well with our results showing that MVC enhanced the level of CCI-elevated STAT3 and, in parallel, the level of antinociceptive IL-10 in the DRG. The effect of MVC on STAT3 in the spinal cord is the opposite to that in DRG, however the reason remains unclear and requires further investigation. These conflicting reports could be partly explained by the dualistic possibility of STAT3 activation (Koscsó et al., 2013; Przanowski et al., 2014; Dominguez et al., 2008; Popiolek-Barczyk et al., 2015). To better understand the mechanism of MVC action, we also studied intracellular pathways in primary cultures of rat microglia and astrocytes. Numerous studies, also ours, have indicated that LPS induced the activation of p38- and ERK1/2-mitogen-activated protein kinases and transcription factors (NF- κ B and STAT3) in primary microglia and astrocytes (Bhat et al., 1998; Schumann et al., 1998; Tanaka et al., 2008; Beurel and Jope, 2009). MVC treatment after LPS stimulation inhibits p38, ERK and NF- κ B activity, which is correlated with the production of pronociceptive factors (IL-1 β , IL-18, IL-6, NOS2). However, the increase in the level of STAT3 after LPS stimulation in the MVC-treated microglia and astrocytes was significantly higher, which, in turn, seems to be correlated with the production of IL-10 and IL-1RA. Our results supported a recently discovered mechanism, which indicates that IL-10-activated STAT3 modulates the expression of IL-1RA by promoting the recruitment of NF- κ Bp65 to the IL-1RA promoter in LPS-stimulated phagocytes (Tamassia et al., 2010). That interaction is an interesting target for further research.

According to the literature and our results, the p38- and ERK1/2-

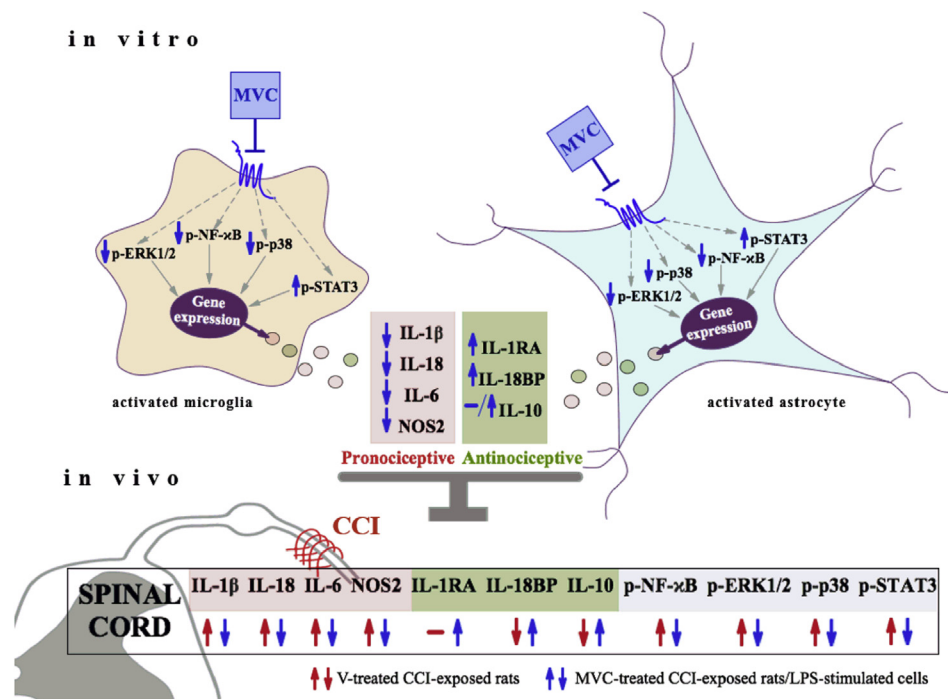


Fig. 6. Hypothetical participation of maraviroc in the process of glial cell polarization during neuropathic pain. It is well known that glial cells (microglia and astrocytes) become activated and initiate a series of signaling cascades that are believed to play an important role in nociceptive transmission (Mika et al., 2013; Tiwari et al., 2014). It seems that targeting glial signaling might lead to more effective pain treatment. It has been suggested that MVC blocks CCR5 signaling and regulates, in a direct or indirect manner, the intracellular pathways, which plays a key role in regulating the immune response (Ochoa-Callejero et al., 2013 and Lisi et al., 2012). Aforementioned hypothesis is supported by our *in vitro* studies, which indicated that the administration of MVC reduces p38, ERK1/2 and NF-κB activity and enhances STAT3 activity in primary cultures of microglia and astrocytes. In response to the modulation of intracellular pathways by MVC, the production of pronociceptive factors characteristic of the “classical” glial activation (IL-1β, IL-18, IL-6, NOS2) is reduced, and in contrast, the production of antinociceptive factors characteristic of the “alternative” activation markers (IL-1RA, IL-18BP and IL-10), which are “alternative” activation markers, is increased. Under neuropathic pain MVC diminishes CCR5 level in the spinal cord and DRG and in parallel microglial and astroglial activation (Lieberman-Blum et al., 2008; Kiguchi et al., 2012 and Kwiatkowski et al., 2016). Our present *in vivo* studies indicated that MVC downregulated the levels of phosphorylated p38 MAPK, ERK1/2 and NF-κB proteins in the spinal cord, and upregulated STAT3 in the DRG. Moreover, we demonstrated that MVC effectively diminished “classical” pronociceptive activation markers (IL-1β, IL-18, IL-6 and NOS2) in the spinal cord. Simultaneously, MVC spinally upregulated “alternative” antinociceptive activation markers (IL-1RA, IL-18BP and IL-10). To summarize, our studies provide new evidence that MVC has an essential influence on the polarization of both microglial and astroglial cells *in vitro* as well as *in vivo*. Our results identify novel targets for neuropathic pain therapies based on the pharmacological modulation of CCR5 by maraviroc. **Abbreviations:** CCI, chronic constriction injury; CCR5, C-C chemokine receptor type 5; DRG, dorsal root ganglion; ERK1/2, extracellular signal-regulated kinase 1/2; GFAP, glial fibrillary acidic protein; GPCRs, G protein-coupled receptors; IBA1, ionized calcium-binding adapter molecule 1; IL, interleukin; IL-18BP, interleukin 18 binding protein; MAPK, mitogen-activated protein kinases; MVC, maraviroc; NF-κB, nuclear factor-kappa B; NOS2, nitric oxide synthase 2; STAT, signal transducers and activators of transcription.

MAPK, NF-κB and STAT3 signaling pathways in neuropathic pain can induce the polarization of glial cells (Vanden Berghe et al., 1998; Zhuang et al., 2005; Niederberger and Geisslinger, 2008; Beurel and Jope, 2009; Popiolek-Barczyk et al., 2015; Rojewska et al., 2015). Precise regulation of the polarization process in neuropathic pain is still unclear; however, the data from the present study support the hypothesis that antagonists of CCR5 can shift the balance from M1 into M2.

iNOS is one of the most interesting M1 polarization markers (Popiolek-Barczyk et al., 2015). It is well known that the induction of NOS2 causes an overproduction of nitric oxide (NO) and contributes to pain processing (Simmons and Murphy, 1992; Moalem and Tracey, 2006; Hervera et al., 2010). NOS2 is spinally up-regulated after nerve injury in animal models of neuropathy (Martucci et al., 2008; Makuch et al., 2013; Rojewska et al., 2016) and in primary microglia and astrocytes upon the induction of inflammation (Simmons and Murphy, 1992; Hewett et al., 1993; Popiolek-Barczyk et al., 2015). In the present study, MVC significantly reduced the spinally CCI-upregulated and LPS-elevated NOS2 levels in microglia and astrocyte cultures. Those results elucidate the molecular mechanism for the anti-inflammatory action of MVC.

Cytokines belonging to the IL-1 superfamily, IL-1β and IL-18, as well as IL-6, are also characteristic markers of the M1 phase and

strong pronociceptive factors (Gottschall et al., 1994; Raivich et al., 1996; Milligan et al., 2003; Miyoshi et al., 2008; Kobayashi et al., 2013; Tam and Ma, 2014; Wang et al., 2014; Pilat et al., 2015). Overproduction of IL-1β, IL-18 and IL-6 is implicated in the pathophysiological changes that occur during different disease states, including neuropathic pain (Dinarello, 1996; Okamoto et al., 2001; Braddock and Quinn, 2004; Miyoshi et al., 2008; Ren and Torres, 2009; Pilat et al., 2015, 2016; Rojewska et al., 2016). Moreover, intrathecal administration of both IL-1β and IL-18 cause allodynia and hyperalgesia in rats (Malcangio et al., 1996; Mika et al., 2008; Miyoshi et al., 2008). In contrast, IL-1RA (Malcangio et al., 1996; Mika et al., 2008; Pilat et al., 2015) and IL-18BP promote anti-allodynic activity (Miyoshi et al., 2008; Pilat et al., 2016) under conditions of neuropathy. Our *in vitro* study indicates in both glial cell cultures the presence of IL-1β, IL-18 and IL-6 as well as their strong upregulation after LPS stimulation, as it has previously been shown in other studies (Gottschall et al., 1994; Kobayashi et al., 2013; Ledebøer et al., 2002; Nakamura et al., 1999; Pilat et al., 2016; Popiolek-Barczyk et al., 2015). Noticeably, the down-regulation of IL-18BP in microglia and its upregulation in astrocytes after LPS stimulation was observed; however, this cell-dependent, variable regulation requires further investigation. The results presented in this study indicated that MVC treatment prevented the CCI-elevated (*in vivo*) and LPS-elevated (*in vitro*) level of IL-1β, IL-18

and IL-6, and enhanced the level of antinociceptive IL-1RA and IL-18BP. Additionally, our study demonstrated that the application of MVC increased the level of antinociceptive IL-18BP, even in non-stimulated cells, in both primary cultures. The IL-10 is considered to be one of the strongest antinociceptive cytokines and it is able to induce the alternative activation of microglia and astroglia (Moore et al., 1993; Sawada et al., 1999; Jang et al., 2013; Tam and Ma, 2014). Our studies showed that IL-10 level in the spinal cord and DRG is reduced during neuropathic pain. Studies conducted by Milligan et al. (2006) and Ledeboer et al. (2007) have shown that the intrathecal administration of IL-10 suppressed neuropathic pain symptoms. Although the exact role of IL-10 is not known, existing evidence suggests that it may interact with microglial cells by inhibiting the release of pronociceptive factors such as IL-1 β and IL-6 (Sawada et al., 1999). Our results provide evidence that MVC enhanced the CCI-reduced IL-10 in the spinal cord and DRG. Moreover, MVC increased the level of IL-10 in astrocytes after LPS stimulation, which could be one of the reasons behind its beneficial effects for neuropathic pain.

5. Conclusion

The activation of glial cells leads to disturbances in the equilibrium between pro- and antinociceptive cytokines, favoring the former. We found that the intrathecal administration of MVC inhibited neuropathic pain by lowering the production of pronociceptive (M1 phase; IL-1 β , IL-18, IL-6 and NOS2) and increasing the production of antinociceptive (M2 phase; IL-1RA, IL-18BP) cytokines (Fig. 6). The data from our both *in vivo* and *in vitro* studies provide evidence that MVC promotes the spinal level of M2-phase microglia/macrophages and astroglia. This change in the proportion between pro- and antinociceptive factors in favor of the latter is emerging as a key mechanism underlying the genesis of MVC antiallodynic and antihyperalgesic properties. Moreover, we believe that the modulation of glia and, subsequently, neuro-immune factors, is a potential therapeutic target for the treatment of neuropathic pain. Our results suggest that the pharmacological modulation of CCR5 by MVC is promising for pain therapy; however, further investigation is required.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgments

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