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Involvement of pro- and antinociceptive factors in minocycline analgesia in rat neuropathic pain model

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ABSTRACT

In neuropathic pain the repeated minocycline treatment inhibited the mRNA and protein expression of the microglial markers and metalloproteinase-9 (MMP-9). The minocycline diminished the pronociceptive (IL-6, IL-18), but not antinociceptive (IL-1alpha, IL-4, IL-10) cytokines at the spinal cord level. *In vitro* primary cell culture studies have shown that MMP-9, TIMP-1, IL-1beta, IL-1alpha, IL-6, IL-10, and IL-18 are of microglial origin. Minocycline reduces the production of pronociceptive factors, resulting in a more potent antinociceptive effect. This change in the ratio between pro- and antinociceptive factors, in favour of the latter may be the mechanism of minocycline analgesia in neuropathy.

1. Introduction

Studies confirm the complexity of endogenous factors that initiate and regulate neuropathic pain conditions (Austin and Moalem-Taylor, 2010). Substances with diverse mechanisms of action that modulate the neuroimmune system have been shown to relieve neuropathic pain. One of these substances is minocycline, an antibiotic that belongs to the group of semisynthetic tetracyclines and has been used for 30 years (Yong et al., 2004). Minocycline has antiinflammatory, antiapoptotic, and immunomodulatory properties (Golub et al., 1991, 1992; Greenwald and Golub, 1993; Zemke and Majid, 2004; Sapadin and Fleischmajer, 2006; Kielian et al., 2007). Minocycline also has neuroprotective activity (Kim and Suh, 2009), as demonstrated in numerous neurodegenerative disease models in mice and rats (Du et al., 2001; Brundula et al., 2002; Nessler et al., 2002; Thomas and Le, 2004). Many reports revealed that minocycline administration diminished the development of neuropathic pain (Raghavendra et al., 2003; Ledeboer et al., 2005a,b; Mika et al., 2007, 2009).

In 2006, Piao et al. reported that the molecular mechanism of minocycline action consists of inhibiting p38 mitogen-activated protein kinases (p38MAPKs) in microglial cells, while recent publications suggest that minocycline also inhibits the activity of metalloproteinase (MMP-9) (Niimi et al., 2013). These two molecular minocycline targets could be targeted for the development of new treatments for neuropathic pain. p38MAPK has a significant contribution to neuropathic pain, and high levels of p38MAPK are observed in microglia in neuropathic pain models (Jin et al., 2003; Tsuda et al., 2004; Hains and Waxman, 2006; Clark et al., 2007; Hains and Wen et al., 2007). The activation of p38MAPK occurs as the result of the activity of cytokines (IL-1beta, TNFalpha), triggering the production of pronociceptive agents, such as nitrogen oxide, IL-1beta, IL-6, and IL-18 (DeLeo and Yezierski, 2001; Miyoshi et al., 2008). Increased p38 MAPK activity leads to the activation of a number of transcription factors that regulate the expression of genes involved in nociception (Potucek et al., 2006). During neuropathy, neurons and glia exchange signals through chemokines, cytokines and the complement system (Mika, 2008; Austin and Moalem-Taylor, 2010). Antiinflammatory cytokines such as IL-1alpha, IL-4, or IL-10 play an important role in nociception, thereby inhibiting the development of neuropathic pain. Pain develops due to a disturbance in the equilibrium between algesic and analgesic factors. In 2008, Kawasaki et al. demonstrated the crucial role of MMP-9 in the development of neuropathic pain, and the intrathecal administration of MMP-9 leads to the development of allodynia, activation of microglial cells, and p38MAPK upregulation. Administration of the tissue inhibitor of metalloproteinase 1 (TIMP-1) blocked the development of neuropathy. The authors suggest that MMP-9 enhances nociception by transforming inactive proIL-1beta to the active 1beta form.

The use of substances inhibiting microglial activation and formation of proinflammatory cytokines, namely minocycline, pentoxifylline, or propentofylline, inhibits the development of neuropathic pain (Neuner et al., 1994; Lundblad et al., 1995; Sweitzer et al., 2001;

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Raghavendra et al., 2003; Mika et al., 2007, 2008, 2009). Minocycline appears to be a very interesting substance that, on the one hand, reduces microglial activation by inhibiting the activity of p38MAPK in microglial cells (Piao et al., 2006) and, on the other hand, suppresses MMP-9 activity that was recently shown in experimental autoimmune encephalomyelitis models (Niimi et al., 2013).

The objective of our studies was to assess whether and how minocycline, which inhibits the development of neuropathic pain by inhibiting microglia activation (Mika et al., 2010), affects the activation of CD40-positive cells in the spinal cord and DRG during neuropathic pain. Simultaneously, we studied the effect of minocycline on the biosynthesis of proinflammatory cytokines (IL-1beta, IL-6, IL-18) and antiinflammatory cytokines (IL-1alpha, IL-10, IL-4) as well as MMP-9 and MMP-2 and TIMP-1 and TIMP-2. The objective of the *in vitro* studies was to determine whether the above factors involved in the development of neuropathic pain changed in LPS-treated primary microglia cell cultures. In addition, the efficacy of intrathecal MMP-9 inhibitor (MMP-9 INH.I) and p38MAPK inhibitor (SB203580) was compared.

2. Experimental procedures

2.1. Animals

Male Wistar rats (300–350 g) from Charles River (Hamburg, Germany) were housed in cages lined with sawdust under a standard 12/12 h light/dark cycle (lights on at 08:00 h) with *ad libitum* access to food and water. All experiments were performed according to the recommendations of the International Association for the Study of Pain (IASP) (Zimmermann, 1983) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and these experiments were approved by the local Bioethics Committee (Krakow, Poland).

2.2. Surgical preparations

Intrathecal (*i.t.*) catheters were chronically implanted in rats according to the methods of Yaksh and Rudy (1976) under pentobarbital anaesthesia (60 mg/kg; *i.p.*). The catheter (PE 10, INTRAMEDIC, Clay Adams, Becton Dickinson and Company, Rutherford, NJ, USA) was sterilised by immersion in 70% ethanol and fully flushed with sterile water prior to insertion. The catheter was carefully introduced through the atlanto-occipital membrane to the subarachnoid space at the rostral level of the spinal cord lumbar enlargement (L4–L6). Water for injection or respective drugs were delivered slowly (1–2 min) in a volume of 5 μl through the *i.t.* catheter, followed by 10 μl of water, which flushed the catheter. The injury to the sciatic nerve was performed five days after catheter implantation.

Chronic constriction injury (CCI) was performed according to Bennett and Xie (1988). The right sciatic nerve was exposed under sodium pentobarbital anaesthesia (60 mg/kg; *i.p.*). Four ligatures (4/0 silk) were made around the nerve, distal to the sciatic notch with 1 mm spacing, until a brief twitch in the respective hind limb was observed. After surgery, all rats developed symptoms of long-lasting neuropathic pain, such as allodynia and hyperalgesia.

2.3. Drug administration and experimental scheme

Minocycline hydrochloride (30 mg/kg; Sigma, Schnelldorf, Germany) was dissolved in water for injections and administered preemptively by intraperitoneal (*i.p.*) injections 16 and 1 h before CCI and then twice daily for seven days, as we previously described (Mika et al., 2007, 2009). This method of minocycline administration was used throughout the study and is referred to in the text as "repeated administration". This administration schedule was used because systemic microglia inhibitors attenuate the activation of microglia more efficiently when the inhibitor is injected before injury (Raghavendra et al., 2003; Ledeboer et al., 2005a, b; Mika et al., 2009) and repeatedly. For pharmacological studies, MMP-9 INH.I (5 μg/5 μl; Merc Darmstadt, Germany) and 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580; 5 μg/5 μl; Sigma, Schnelldorf, Germany) were *i.t.* administered preemptively 16 and 1 h before CCI, then once daily for seven days. The inhibitors of MMP-9 and p38MAPK were dissolved in DMSO according to the previously published data (Tsuda et al., 2004). The control groups received vehicle according to the same schedule. Naïve animals were processed (behavioural tests and tissue collection) in parallel to the V-CCI and MC-CCI groups with the exception of CCI surgery and anaesthesia.

2.4. Behavioural tests

2.4.1. Tactile allodynia (von Frey test)

Allodynia was measured 60 min after the last administration of minocycline (30 mg/kg) according to our previous study (Mika et al., 2007). Other drugs were examined in the same time point (Fig. 5A). Allodynia 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 wire net floors 5 min before the experiment. The strengths of the von Frey stimuli used in our experiments ranged up to 26 g. The filament was applied to the midplantar surface of the ipsilateral hind paw, and measurements were taken automatically as described by us previously (Mika et al., 2007; Makuch et al., 2013). There was almost no response to the highest strength (26 g) in the naïve animals. No significantly different paw reactions were observed between the contralateral hind paw of CCI-exposed and naïve rats. Therefore, a line was drawn at this value to represent the naïve animals in the figures.

2.4.2. Hyperalgesia (cold plate test)

The cold plate test was conducted 65 min after the last injection of MMP-9 INH.I and SB203580 (Fig. 5A). Hyperalgesia was assessed using the cold plate test (Cold/Hot Plate Analgesia Meter No. 05044, Columbus Instruments, USA) as previously described (Mika et al., 2007; Makuch et al., 2013). The temperature of the cold plate was kept at 5 °C, and the cut-off latency was 30 s. The animals were placed on the cold plate, and the time until the hind paw was lifted was recorded. In the naïve rat group, the reaction of the first hind paw lift was measured. In the rats subjected to nerve injury, the ipsilateral paw reacted first.

2.5. Biochemical tests

2.5.1. Microglial cell cultures and treatments

Primary cultures of microglial cells were prepared from 1-day-old Wistar rat pups as previously described (Zawadzka and Kaminska, 2005). Briefly, cells were isolated from the rats' cerebral cortices and were plated at a density of 3×10^5 cells/cm^2 in a culture medium that consisted of DMEM/Glutamax/high glucose (Gibco, USA) supplemented with heat-inactivated 10% foetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, USA) on poly-L-lysine coated 75 cm^2 culture flasks and were maintained at 37 °C and 5% CO_2. The culture medium was changed after 4 days. The loosely adherent microglial cells were recovered after 9 days by mild shaking and centrifugation. The microglial cells were suspended in a culture medium and plated at a final density of 2×10^5 cells onto 24-well plates for mRNA analysis. Adherent cells were incubated for 48 h in a culture medium before being analysed. Primary microglial cell cultures were treated with LPS [100 ng/ml] (Sigma-Aldrich) for 6 h. Cell specificity was determined in cultures of primary microglia by Western blot assay using an antibody to OX-42 (microglial marker) and by qRT-PCR using primers for *C1q* (a microglial marker) and *GFAP* (an astroglial marker). The homogeneity of the microglial population was high (more than 95% positive for OX-42 and C1q), and our homogeneity was similar to that obtained by Zawadzka and Kaminska (2005).

2.5.2. Tissue collection and RNA isolation

The ipsilateral sides of the dorsal lumbar (L4–L6) spinal cord and DRG (L4–L6) were collected immediately after decapitation on day 7 after CCI (4 h after the last drug administration). The tissue samples were placed in individual tubes containing the tissue storage reagent RNAlater (Qiagen Inc.) and were stored at −70 °C until RNA isolation. Total RNA was extracted using the TRIzol reagent (Invitrogen) as previously described (Chomczynski and Sacchi, 1987). The RNA concentration was measured using a NanoDrop ND-1000 Spectrometer (NanoDrop Technologies), and the RNA quality was determined by chip-based capillary electrophoresis using an RNA 6000 Nano LabChip Kit and an Agilent Bioanalyzer 2100 (Agilent) according to the manufacturer's instructions.

2.5.3. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Reverse transcription was performed on 2 μg of total RNA (for tissue analysis) and 500 ng (for cell culture analysis) using Omniscript reverse transcriptase (Qiagen Inc.) at 37 °C for 60 min. RT reactions were carried out in the presence of an RNAse inhibitor (rRNAsin, Promega) and an oligo(dT16) primer (Qiagen Inc.). The cDNA was diluted 1:10 with H_2O, and for each reaction ~50 ng of cDNA synthesised from the total RNA of an individual animal was used for the 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 the reactions were performed on an iCycler device (BioRad, Hercules). The following TaqMan primers and probes were used: Rn01527838_g1 (*Hprt*, hypoxanthine guanine rat hypoxanthine guanine phosphoribosyl transferase); Rn01423590_m1 (*CD40*); Rn00579162_m1 (*MMP-9*, metalloproteinase 9); Rn01538170_m1 (*MMP-2*, metalloproteinase 2); Rn00587558_m1 (*TIMP-1*, metallopeptidase inhibitor 1); Rn00587558_m1 (*TIMP-2*, metallopeptidase inhibitor 2); Rn00580432_m1 (*IL-1beta*, interleukin 1 beta); Rn00561420_m1 (*IL-6*, interleukin 6); Rn01422083_m1 (*IL-18*, interleukin 18); Rn00566700_m1 (*IL-1 alpha*, interleukin 1 alpha); Rn01456866_m1 (*IL-4*, interleukin 4); Rn00563409_m1 (*IL-10*, interleukin 10).

The amplification efficiency for each assay (between 1.7 and 2) was determined by running a standard dilution curve. The cycle threshold values were calculated automatically using the iCycler IQ 3.0 software with the default parameters. RNA abundance was calculated as $2^{-(\text{threshold cycle})}$. Because the HPRT transcript levels do not significantly change in rats exposed to CCI (Korostynski et al., 2006; Mika et al., 2008), they served as an adequate housekeeping gene.

2.5.4. Western blot analysis

Ipsilateral, dorsal rat spinal cords (L4–L6) were collected 6 h after the last minocycline treatment for protein analyses. Tissue lysates were collected in RIPA buffer with a protease inhibitor cocktail and cleared by centrifugation (14,000 ×*g* for 30 min, 4 °C). Samples containing 20 μg of protein were heated in a loading buffer (50 mM Tris–HCl, 2% SDS, 2% β-mercaptoethanol, 4% glycerol and 0.1% bromophenol blue) for 8 min at 98 °C and were resolved on 10–20% or 4–20% Criterion™ TGX™ pre-cast polyacrylamide gels. Following gel electrophoresis, the proteins were transferred to Immune-Blot PVDF membranes (Bio-Rad) 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-IBA1, 1:500, ProteinTech; rabbit polyclonal anti-MMP-9, 1:500, Millipore; mouse polyclonal anti-GAPDH, 1:5000 as loading control, Millipore), and incubated for 1 h at RT with a secondary goat polyclonal antibody conjugated to horseradish peroxidase (goat anti-rabbit IgG, 1:5000, BioRad). Both primary and secondary antibodies were diluted in solutions from SignalBoost Immunoreaction Enhancer Kit (Merck Millipore). The membranes were washed for 2 × 2 min and 3 × 5 min with TBST. Immunocomplexes were detected using an Immun-Star HRP Chemiluminescent Substrate Kit (BioRad) and visualised using a Fujifilm LAS-4000 FluorImager system. The relative levels of immunoreactivity were quantified using the Fujifilm Multi Gauge software.

2.5.5. Immunocytochemical analysis

The commercially available, specific anti-Iba-1 antibody was used. The cells were vehicle- or LPS-stimulated for 6 h and then were fixed for 20 min in 4% paraformaldehyde in a 0.1 M phosphate buffer (PB, pH 7.4) and incubated with primary antibody (rabbit anti-Iba-1, 1:500, ProteinTech) for 2 days at 4 °C. After three washes in PB, double immunofluorescence was revealed by incubation for 2 h in the fluorochrome-conjugated secondary antibody, Alexa Fluor 555 (Molecular Probes, USA) donkey anti-mouse, diluted 1:500 in 5% NDS. Sections were then washed with PB and coverslipped with an Aquatex mounting medium (Merck, Darmstadt, Germany). Sections without primary antibodies were used as negative controls.

2.5.6. Statistical analysis

The behavioural data in Fig. 1 are presented in grams and seconds (mean ± SEM), and the groups contained the number of rats as follows: naïve = 12; V-CCI = 9; MC-CCI = 11. The behavioural data in Fig. 5 are presented also in grams and seconds (mean ± SEM), and the groups contained the number of rats as follows: naïve = 12; V-CCI = 20; DMSO-CCI = 7; MMP-9INH.I-CCI = 7; SB203580-CCI = 14. The results of the experiments were evaluated using a one-way analysis of variance (ANOVA). The data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate a significant difference compared with the control group (naïve rats); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate a significant difference compared with the vehicle-treated CCI-exposed rats; $^{\&}p < 0.05$ and $^{\&\&\&}p < 0.001$ indicate differences compared with the DMSO-treated CCI-exposed group.

The results of the qRT-PCR and Western blot analyses are presented as the fold change compared with the control group (naïve rats) and were calculated for the ipsilateral sides of the spinal cords and DRGs of the CCI-exposed rats. For the Western blot analysis the groups contained the number of rats as follows: naïve = 6; V-CCI = 7; MC-CCI = 7. The quantitative qRT-PCR data are presented as the mean ± SEM and represent the normalised averages derived from the threshold cycle in the qRT-PCR analysis of 10 to 12 samples for each group. For the qRT-PCR analysis the groups contained the number of rats as follows: naïve = 10; V-CCI = 12; MC-CCI = 12. The inter-group differences were analysed using an ANOVA, followed by Bonferroni's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate a significant difference compared with the control group (naïve rats); $^{\#}p < 0.05$ $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate a significant difference compared with the vehicle-treated CCI-exposed rats.

The results of the primary microglia cultures are presented as the fold change compared with the control group (naïve rats). The data are presented as the mean ± SEM. Inter-group differences were analysed with a t test. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate differences compared with the LPS-treated cells.

All graphs were prepared using the GraphPad Prism software (version 5.0).

3. Results

3.1. The influence of repeated administration of minocycline on the development of allodynia and on CD40, C1q mRNA in the spinal cord and DRG 7 days after CCI

All CCI-exposed rats developed allodynia (Fig. 1A, B). In the von Frey test, strong tactile allodynia on the paw ipsilateral to the injury was observed on day seven after CCI. At this time, the ipsilateral paw responded to a stimulation of 14.8 ± 0.7 g (Fig. 1B), and naïve rats

responded to 26.0 g. Minocycline significantly attenuated the mechanical allodynia from 14.8 ± 0.7 g to 21.2 ± 0.8 g (Fig. 1B).

In the spinal cord, *CD40* mRNA (1.0 ± 0.05 *vs.* 3.4 ± 0.2) was upregulated compared with that of naïve rats (Fig. 1C). Minocycline diminished the level of *CD40* mRNA in the spinal cord from 3.4 ± 0.2 to 3.0 ± 0.2. Additionally, in the DRG, *CD40* mRNA was significantly upregulated from 1.0 ± 0.04 to 1.4 ± 0.04 (Fig. 1D) in CCI-exposed rats compared with naïve animals. Minocycline significantly diminished the level of *CD40* in the DRG from 1.4 ± 0.04 to 1.17 ± 0.05 (Fig. 1D).

In the spinal cord (Fig. 1E), upregulation of C1q mRNA (1 ± 0.1 *vs.* 8.6 ± 0.5) was observed compared to naïve rats. Repeated treatment with minocycline significantly diminished (from 8.6 ± 0.5 to 4.8 ± 1.1) the level of *C1q* mRNA in the spinal cord. In the DRG (Fig. 1F) upregulation of *C1q* mRNA (1 ± 0.3 *vs.* 6.0 ± 0.4) was observed compared to naïve rats. Repeated treatment with minocycline significantly diminished (from 6.0 ± 0.4 to 3.0 ± 0.1) the level of *C1q* in the DRG.

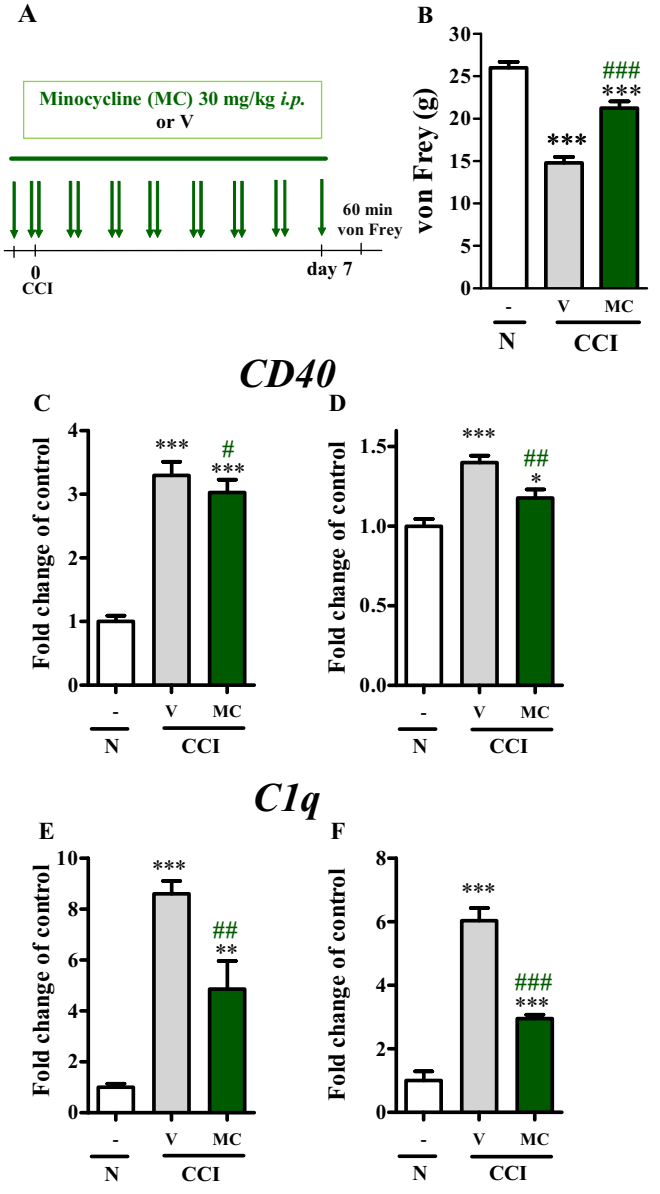


Fig. 1. Effect of minocycline (30 mg/kg; *i.p.*; 16 h and 1 h before CCI and then twice daily for 7 days) on the development of mechanical allodynia (A; von Frey test) and *CD40* mRNA expression in the spinal cord (B) and DRG (C) on day 7 after CCI. Allodynia was assessed 60 min after the last drug administration, and the data are presented as the mean ± SEM (9–12 rats per group). The data for the biochemical studies were analysed as fold changes compared with controls and represent the normalised averages derived from the qRT-PCR threshold cycles of 10–12 samples from each group. The inter-group differences were analysed using an ANOVA with Bonferroni's multiple comparison test. $^{*}p < 0.05$ and $^{***}p < 0.001$ indicate a significant difference compared with the control (naïve rats); and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ indicate a significant difference compared with the V-CCI rats (ANOVA, Bonferroni's test). N, naïve; V, vehicle (water for injection); MC, minocycline.

3.2. The influence of repeated minocycline administration on MMP-9, MMP-2, TIMP-1 and TIMP-2 mRNA in the spinal cord and DRG 7 days after CCI

MMP-9 mRNA was upregulated in the spinal cord from 1 ± 0.1 to 2.7 ± 0.4 (Fig. 2A) and in the DRG from 1 ± 0.1 to 5.5 ± 1.1 (Fig. 2B) compared with naïve rats. Minocycline diminished the levels of *MMP-9* mRNA in the spinal cord from 2.7 ± 0.1 to 1.1 ± 0.1 and in the DRG from 5.5 ± 1.1 to 2.9 ± 0.5 (Fig. 2A and B, respectively).

MMP-2 mRNA was upregulated in the spinal cord from 1.0 ± 0.6 to 1.5 ± 0 (Fig. 2C) and in the DRG from 1 ± 0.06 to 1.35 ± 1.1 (Fig. 2D) compared with naïve rats. Minocycline did not influence the level of *MMP-2* mRNA in the spinal cord (Fig. 2C) but decreased the level of *MMP-2* from 1.35 ± 1.1 to 1.0 ± 0.07 in the DRG (Fig. 2D).

TIMP-1 mRNA was upregulated in the spinal cord from 1.0 ± 0.3 to 2.6 ± 0.2 (Fig. 2E) and in the DRG from 1 ± 0.03 to 1.9 ± 0.1 (Fig. 2F) compared with naïve rats. Minocycline did not influence the level of *TIMP-1* mRNA in the spinal cord (Fig. 2E) but decreased the level of *TIMP-1* from 1.9 ± 0.1 to 1.6 ± 0.04 in the DRG (Fig. 2F).

In the spinal cord and DRG, no changes were observed in *TIMP-2* mRNA compared with naïve rats (Fig. 2G, H). Minocycline did not influence the level of *TIMP-2* mRNA in either structure.

3.3. The influence of repeated minocycline administration on Iba-1 and MMP-9 protein levels in the spinal cord level 7 days after CCI

In the spinal cord, Iba-1 protein was upregulated from 1.0 ± 0.07 to 3.2 ± 0.06 compared with naïve animals (Fig. 3A). Minocycline decreased the level of microglial activation marker from 3.2 ± 0.06 to 2.3 ± 0.1 (Fig. 3A).

Similar to Iba-1 protein regulation, MMP-9 protein was upregulated from 1.0 ± 0.006 to 1.3 ± 0.01 in the spinal cord. Minocycline decreased the level of MMP-9 protein from 1.3 ± 0.01 to 1.2 ± 0.01 (Fig. 3B).

3.4. The influence of repeated minocycline administration on the transcription levels of proinflammatory cytokines (IL-1beta, IL-6, IL-18) in the spinal cord and DRG 7 days after CCI

IL-1beta mRNA was upregulated from 1.0 ± 0.07 to 3.4 ± 0.3 in the spinal cord (Fig. 4A) and from 1.0 ± 0.1 to 2.2 ± 0.1 in the DRG (Fig. 4B) compared with naïve rats. Minocycline did not influence the observed levels of *IL-1beta* mRNA in the spinal cord or DRG.

IL-6 mRNA was upregulated from 1.0 ± 0.07 to 9.1 ± 2.0 in the spinal cord (Fig. 4C) and from 1 ± 0.16 to 79.1 ± 4.2 in the DRG (Fig. 4D) compared with naïve rats. Minocycline diminished the levels of *IL-6* mRNA from 9.1 ± 2.0 to 4.6 ± 1.0 in the spinal cord and from 79.1 ± 4.2 to 47.5 ± 5.2 in the DRG.

IL-18 mRNA was upregulated from 1.0 ± 0.6 to 1.2 ± 0.05 in the spinal cord (Fig. 4E) and from 1 ± 0.1 to 2.2 ± 0.1 in the DRG (Fig. 4F) compared with naïve rats. Minocycline diminished the levels of *IL-18* mRNA in the spinal cord from 1.2 ± 0.05 to 1 ± 0.1 and in the DRG from 2.2 ± 0.1 to 1.8 ± 0.1.

3.5. The influence of repeated minocycline administration on mRNA levels of antiinflammatory cytokines (IL-1alpha, IL-4, IL-10) in the spinal cord 7 days after CCI

In the spinal cord, no changes in *IL-1alpha* mRNA (Fig. 4G) were observed, but in the DRG, *IL-1alpha* mRNA was upregulated from 1 ±

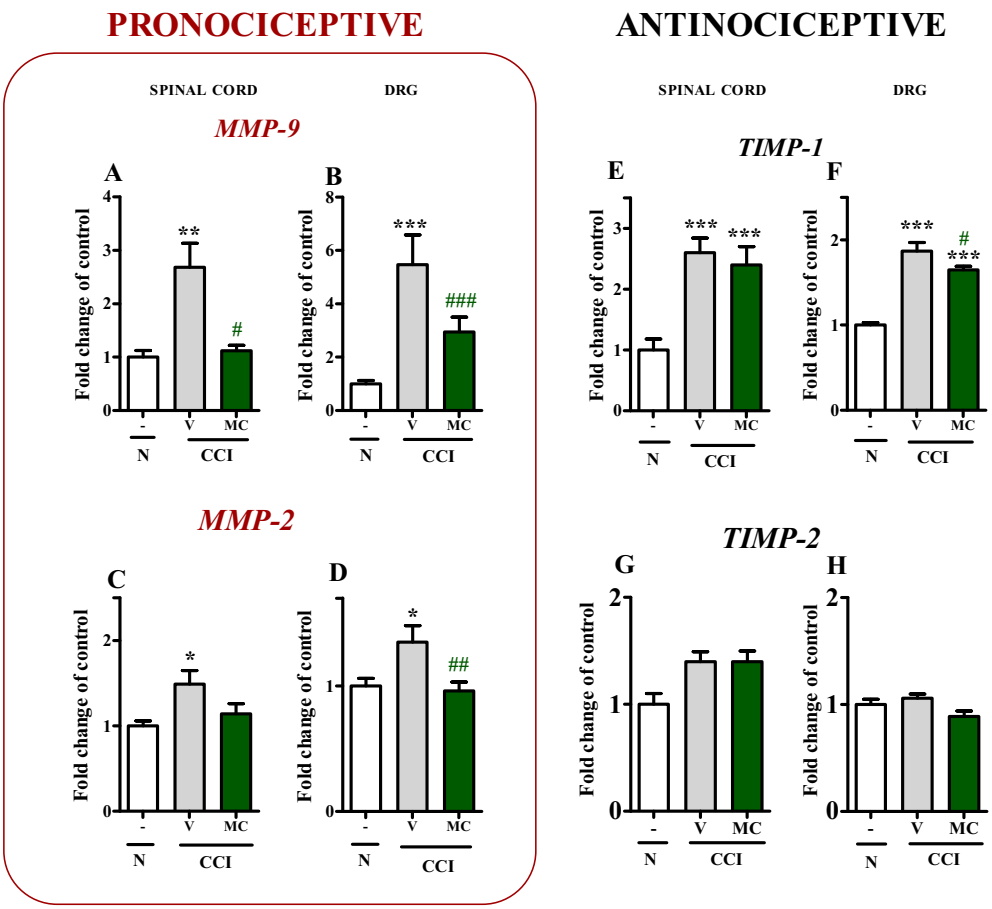


Fig. 2. Effects of minocycline (MC) on mRNA levels of pro- (*MMP-9*, *MMP-2*) and antiinflammatory factors (*TIMP-1*, *TIMP-2*) in the spinal cord and DRG during neuropathic pain. Minocycline (30 mg/kg; *i.p.*; 16 h and 1 h before CCI and then twice daily for 7 days) diminished the mRNA expression of *MMP-9* (A, B) in the dorsal part of the lumbar spinal cord and in the DRG 7 days after CCI. Minocycline also influenced the levels of *TIMP-1* (F) and *MMP-2* (D) mRNA in the DRG. The data were analysed as fold changes compared with controls and represent the normalised averages derived from the qRT-PCR threshold cycles of 10–12 samples from each group. The data are presented as the mean ± SEM. Inter-group differences were analysed with Bonferroni's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate differences compared with the naïve rats. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate differences compared with the CCI-exposed group. N, naïve; V, vehicle (water for injection); MC, minocycline.

0.1 to 29.9 ± 2.8 (Fig. 4H) compared with naïve rats. Minocycline did not influence these changes in *IL-1alpha* mRNA in the spinal cord or DRG.

In the spinal cord and DRG, no changes in *IL-4* mRNA were observed compared with naïve rats (Fig. 4I, J). Minocycline did not influence the levels of *IL-4* mRNA in either the spinal cord or DRG.

IL-10 mRNA was upregulated from 1 ± 0.2 to 4.4 ± 0.3 in the spinal cord (Fig. 4K) and from 1 ± 0.2 to 7.3 ± 0.9 in the DRG (Fig. 4L) compared with naïve rats. Minocycline did not influence these changes in *IL-10* mRNA in the spinal cord but decreased these changes in the DRG from 7.3 ± 0.9 to 4.5 ± 0.5.

3.6. The influence of LPS stimulation on mRNA levels of pro- and antiinflammatory factors in microglial cells — in vitro studies

Our immunocytochemical analyses showed changed morphology of microglial cell from a ramified (non-stimulated) to an amoeboid after 6 h LPS treatment. The mRNA levels for *MMP-9*, *IL-1beta*, *IL-6*, *IL-18*, *TIMP-1*, *TIMP-2*, *IL-1alpha* and *IL-10* were significantly upregulated 6 h after LPS treatment (Table 1). The mRNA for *IL-4* was undetectable.

3.7. Comparison of analgesic effects of MMP-9 INH.I and SB203580 on CCI-induced development of allodynia and hyperalgesia 7 days after CCI in rats

V- and DMSO-treated CCI-exposed rats developed similarly strong allodynia and hyperalgesia compared to naïve rats as demonstrated in the von Frey and cold plate tests (Fig. 5A, B, C).

MMP-9 INH.I administration at a dose of 5 μg/5 μl attenuated the development of allodynia from 12.2 ± 0.7 g to 16.2 ± 2.4 g (Fig. 5B) and of hyperalgesia from 6.3 ± 0.5 s to 15.4 ± 2.3 s (Fig. 5C) on day 7 after CCI. We used the same dose to directly compare the effectiveness of the MMP-9 INH.I with that of the p38MAPK inhibitor (SB203580). The results obtained after SB203580 administration were weaker than those of MMP-9 INH.I SB203580 attenuated the development of allodynia from 11.8 ± 0.4 g to 13 ± 0.8 g (Fig. 5B) and of hyperalgesia from 6.3 ± 0.5 s to 10.7 ± 0.9 s (Fig. 5C).

4. Discussion

Studies in recent years, including those conducted in our group, suggest that repeated administrations of minocycline inhibit microglial activation, as demonstrated using markers, such as CD40, C1q, Iba-1, and OX42 (Chatzigeorgiou et al., 2009; Mika et al., 2009). This effect contributes to the reduction of neuropathy development (Raghavendra et al., 2003; Ledeboer et al., 2005a,b; Mika et al., 2007, 2010). Minocycline is an interesting, biologically active substance that has potential therapeutic use. However, the exact mechanism of minocycline action has not been fully elucidated. Many reports, including those published by our group, revealed that preemptive systemic minocycline administration diminished the development of neuropathic pain states (Raghavendra et al., 2003; Ledeboer et al., 2005a,b; Mika et al., 2007, 2009), but did not reduce pain that was already established (Raghavendra et al., 2003). Ledeboer et al. (2005a,b) suggested that microglia play a crucial role in initiating,

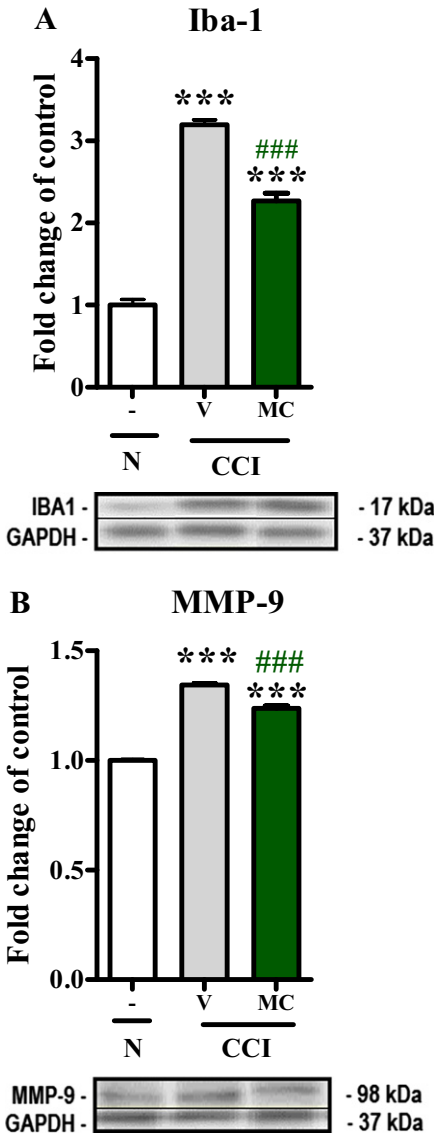


Fig. 3. Effects of minocycline (MC) on the protein levels of Iba-1 and proinflammatory MMP-9 in the spinal cord during neuropathic pain. Minocycline (30 mg/kg; *i.p.*; 16 h and 1 h before CCI and then twice daily for 7 days) diminished the protein levels of Iba-1 (A) and MMP-9 (B) in the dorsal part of the lumbar spinal cord and in the DRG 7 days after CCI. The data are presented as the mean ± SEM of 6–7 samples from each group. Inter-group differences were analysed using Bonferroni's multiple comparison test. $^{***}p < 0.001$ indicates differences compared with the naïve rats. $^{###}p < 0.001$ indicates differences compared with the CCI-exposed group. N, naïve; V, vehicle (water for injection); MC, minocycline.

rather than maintaining, enhanced pain responses. Our *in vitro* studies revealed that the mRNA levels of *MMP-9*, *TIMP-1*, *IL-1beta*, *IL-1alpha*, *IL-6*, *IL-10*, and *IL-18* are elevated in LPS-treated primary microglia rat cultures. These results suggest the involvement of factors of microglial origin in the mechanism of minocycline pain inhibition. The analgesic effect of minocycline was already thought to be due to the inhibition of the spinally activated proinflammatory cytokines IL-1beta and TNFalpha (Ledeboer et al., 2005a,b). In 2006, Zanjani et al. observed that minocycline reduced the serum concentrations of IL-6 following nerve injury. Our present comprehensive studies on the involvement of neuroimmune factors showed that the preemptive and repeated *i.p.* administration of minocycline reduced the *MMP-9*, *IL-6* and *IL-18* mRNA levels within the spinal cord and DRG in CCI-exposed rats. Moreover, the *TIMP-1*, *MMP-2* and *IL-10* mRNA levels were also diminished by minocycline in the DRG.

Piao et al. (2006) reported that the molecular mechanism of minocycline consisted of p38MAPK inhibition within microglia, while other authors demonstrated that minocycline also inhibited MMP-9 in ischemia models (Machado et al., 2006; Murata et al., 2008). In 2008, MMP-9 upregulation in the DRG after spinal nerve ligation was demonstrated (Kawasaki et al., 2008). We were the first to demonstrate the significant upregulation of *MMP-9* and *TIMP-1* mRNA in the spinal cord and DRG of CCI-exposed rats. These results confirm the hypothesis proposed by Kawasaki et al. (2008), who demonstrated that the intrathecal administration of MMP-9 caused pain associated with microglial activation and increased IL-1beta levels. As early as 1998, Schönbeck et al. suggested that MMP-9 is an enzyme that converts proIL1beta into its active form, *i.e.*, IL-1beta (Schönbeck et al., 1998). The effect was not observed in MMP-9 knock-out mice. Moreover, the administration of MMP-9 siRNA reduced the development of neuropathy (Wang et al., 2000), and the intrathecal administration of anti-IL-1beta antibodies reduced MMP-9-induced allodynia (Kawasaki et al., 2008). A hypothesis was formulated that the nerve damage caused burst discharges within sensory neurons, releasing MMP-9 and IL-1beta, which, upon being transformed into the active form, enhanced the excitability of neurons. This suggestion has been confirmed in our studies (the mRNA levels of *MMP-9* and *IL-1beta* simultaneously increased). Minocycline reduced the level of the active IL-1beta form by reducing the level of MMP-9 biosynthesis. In 2001, Rosenberg et al. demonstrated that MMP-2 is produced by activated astrocytes and satellite cells (Rosenberg et al., 2001). In 2009, we described the activation of astrocytes, which begins slowly around day 7. This result indicates that the activation of astroglia follows the earlier activation of microglia in CCI-exposed rats (Mika et al., 2009). Kawasaki et al. (2008) demonstrated increases in MMP-2 levels within the DRG as late as day 10 following spinal nerve ligation. As shown by our studies, on day 7 following sciatic nerve ligation, a slight increase in the *MMP-2* mRNA levels is observed within the spinal cord and DRG. However, we were unable to observe upregulation in animals receiving repeated minocycline administration, suggesting that at the initial stages of neuropathic pain, MMP-2 may originate from the microglia. This hypothesis is supported by the fact that MMP-2 is produced by primary microglial cell cultures. The intrathecal administration of endogenous inhibitors of TIMP-1 and TIMP-2 (inhibitors of MMP-9 and MMP-2, respectively) strongly inhibited the development of neuropathic pain. Our results suggest that the administration of minocycline prevents the spinal upregulation of *MMP*-9 and *MMP-2* mRNA levels in CCI-subjected rats, thereby reducing the levels of proinflammatory factors. The beneficial effects of minocycline are also due to the CCI-induced upregulation of antinociceptive factors. TIMP-1 and TIMP-2 are not downregulated after injection, suggesting that the drug restores the equilibrium. Considering the role of *MMP-9* in the development of neuropathy, the activity of minocycline may result from its beneficial effects, which are both analgesic and neuroprotective. Therefore, the MMP-9 INH.I was also used in our studies. Following the intrathecal administration of MMP-9 INH.I, the development of allodynia and hyperalgesia was impaired in our neuropathic pain model. The intrathecal administration of MMP-9 leads to a strong activation of p38MAPK in microglial cells (Kawasaki et al. 2008) and these changes are similar to those observed in the development of neuropathic pain (Jin et al., 2003). MMP-9-induced allodynia can also be reduced by a p38MAPK inhibitor (FR167653) (Kawasaki et al., 2008). In our studies, the MMP-9 INH.I had better effectiveness under neuropathic pain then SB203580, a p38MAPK inhibitor, which is in agreement with others, who have shown that SB203580 is very effective in the early phase (1–5 days after ligation of the L5 spinal nerve) of neuropathic pain development (Jin et al., 2003). Our data suggests that the analgesic effect of minocycline is related to the inhibition of both MMP-9 and p38MAPK.

In our studies, we have focused on the proinflammatory interleukins IL-6, IL-18 and IL-1beta, which are important for nociception. The latter is known to be strongly activated upon neural damage (Hopkins and Rothwell, 1995; Mika et al., 2008). The IL-1beta or IL-6 intrathecal administration induces allodynia and hyperalgesia in rats (Oka et al.,

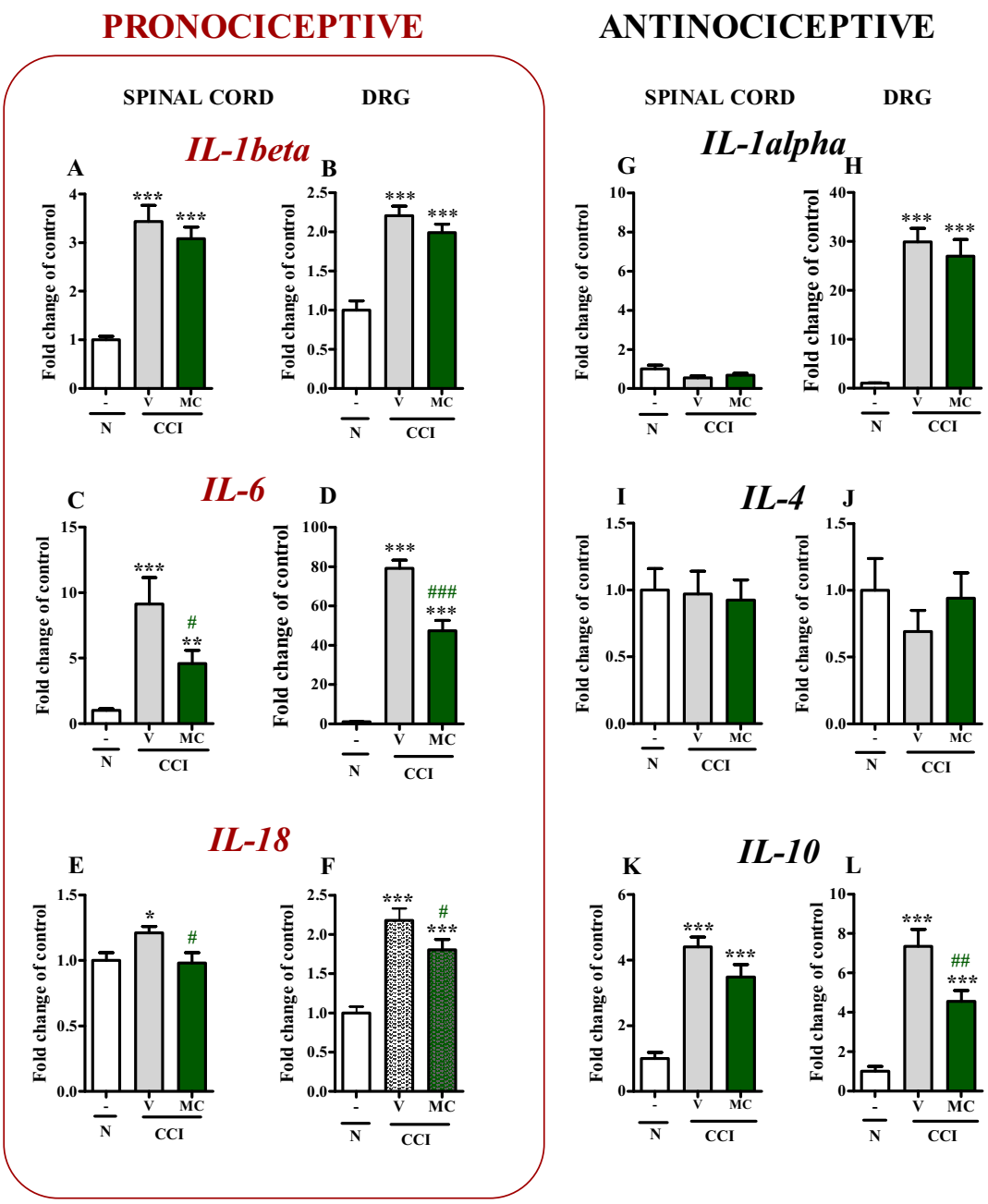
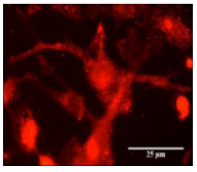
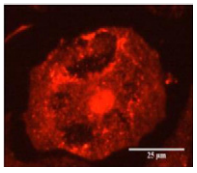


Fig. 4. Effects of minocycline (MC) on the mRNA levels of proinflammatory (*IL-1beta*, *IL-6*, *IL-18*) and antiinflammatory cytokines (*IL-1alpha*, *IL-4*, *IL-10*) in the spinal cord and DRG during neuropathic pain. Minocycline (30 mg/kg; *i.p.*; 16 h and 1 h before CCI and then twice daily for 7 days) diminished the mRNA expression levels of *IL-6* (C, D) and *IL-18* (E, F) in the dorsal part of the lumbar spinal cord and in the DRG 7 days after CCI. Minocycline also influenced the levels of *IL-10* (L) mRNA in the DRG. The data were analysed as fold changes compared with controls and represent the normalised averages derived from the qRT-PCR threshold cycles of 10–12 samples from each group. The data are presented as the mean ± SEM. Inter-group differences were analysed using Bonferroni's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate differences compared with the naïve rats. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ indicate differences compared with the CCI-exposed group. N, naïve; V, vehicle (water for injection); MC, minocycline.

1993; Oprée and Kress, 2000; Obreja et al., 2002; Mika et al., 2008). As shown by our studies, sciatic nerve injury caused a strong increase in the mRNA levels of *IL-1beta* at the spinal cord and DRG level, while minocycline had no effect on these changes. IL-6 is secreted mainly not only by monocytes and macrophages but also by microglial cells (Kreutzberg, 1996; Milligan et al., 2003) and has been implicated in neuropathic pain (De Jongh et al., 2003, Mika et al., 2008). In the aetiology of numerous neurodegenerative and neuroimmune disorders associated with pain symptoms, allodynia was correlated with the IL-6 immunoreactivity or mRNA levels within the sciatic nerve and DRG (DeLeo et al., 1996; Gruol and Nelson, 1997; Osamura et al., 2005). The importance of IL-6 in nociception has been further demonstrated in IL-6 knock-out mice, which do not develop hypersensitivity in response to nerve injury (Ramer et al., 1998; Murphy et al., 1999). Studies revealed that the intrathecal administration of IL-6 to naïve animals caused allodynia (DeLeo et al., 1996). Our studies showed that sciatic nerve ligation led to a significant increase in the *IL-6* mRNA levels within the spinal cord and the dorsal root ganglia on day 7 following CCI. Minocycline administration prevented these changes, which is in agreement with previous studies (Zanjani et al., 2006; Lee et al., 2010). In 2008, the first report suggesting an important role of IL-18 in neuropathic pain was published. IL-18 is a proinflammatory cytokine that is observed in numerous autoimmune diseases (Boraschi and Dinarello, 2006). Some published data showed that administration of IL-18 leads to increased

Table 1
Proinflammatory and antiinflammatory factor expression in primary microglial cell cultures. The mRNA levels of proinflammatory (*MMP-9*, *MMP-2*, *IL-1beta*, *IL-6*, *IL-18*) and antiinflammatory (*TIMP-1*, *TIMP-2*, *IL-1alpha*, *IL-4*, *IL-10*) factors in non-stimulated and LPS-treated microglial primary cell cultures. The reverse transcriptase-polymerase chain reaction shows that mRNA for *MMP-9*, *MMP-2*, *IL-1beta*, *IL-6*, *IL-18*, *TIMP-1*, *TIMP-2*, *IL-1alpha*, *and IL-10*, but not *IL-4*, is expressed in rat primary microglial cell cultures. After 6 h, LPS-treatment upregulated the mRNA for *MMP-9*, *IL-1beta*, *IL-6*, *IL-18*, *TIMP-1*, *IL-1alpha*, *and IL-10*, but not for *MMP-2* and *TIMP-2*. The mRNA level for IL-4 was undetectable. The data are presented as the mean ± SEM. Inter-group differences were analysed using a t test. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate differences compared with the LPS-treated cells. K/− (not stimulated), K/+ (LPS-stimulated). Microphotographs – an immunocytochemistry analysis of microglial cells – *in vitro* studies. Cell specificity was determined using commercially available Iba-1 (a microglial marker) antibody in immunocytochemistry. The scale bar for all microphotographs is 25 μm. The data from 3 cell cultures are presented.

Primary microglia cell culture	K/−	K/+
		
	mRNA level (fold change of control)	
IL-1beta	1 ± 0.1	55.1 ± 5.4***
IL-6	1 ± 0.2	17,822.0 ± 4365.0***
IL-18	1 ± 0.3	5.4 ± 0.5**
IL-1alpha	1 ± 0.3	321.1 ± 35.8***
IL-4	Not detected	Not detected
IL-10	1 ± 0.4	20.5 ± 1.4**
MMP-9	1 ± 0.3	5.1 ± 0.8**
TIMP-1	1 ± 0.1	3.1 ± 0.4***
MMP-2	1 ± 0.2	1.1 ± 0.2
TIMP-2	1 ± 0.1	0.8 ± 0.1

microglial activation (Prinz and Hanisch, 1999). Studies suggest that IL-18 released by microglial cells is responsible for the development of allodynia (Miyoshi et al., 2008; Chu et al., 2012; Daigo et al. 2012), which is consistent with our results. On day 7 following CCI, the *IL-18* mRNA levels in the spinal cord and DRG increase and are reduced by minocycline administration. Because the activation of the proinflammatory cytokine IL-18 occurs *via* p38MAPK, the administration of minocycline, which is an inhibitor of that kinase, may reduce neuropathic pain.

Recently, an increasing number of studies have highlighted the crucial role of proinflammatory cytokines in pain processes. The intrathecal administration of IL-1alpha, in contrast to IL-1beta, is capable of suppressing nociception (Binshtok et al., 2008; Mika et al., 2008). A strong increase in the IL-1alpha levels was observed within the DRG (Mika et al., 2008). Such a strong elevation of the *IL-1alpha* mRNA levels is important for reducing neuropathy; importantly, minocycline does not inhibit this change. We also demonstrated a strong increase in *IL-10* mRNA in the spinal cord and DRG that is not affected by minocycline. As an antinociceptive cytokine, IL-10 affects microglial activation by inhibiting the activation of IL-1beta and IL-6 and reducing the activation of pronociceptive cytokine receptors (Moore et al., 1993; Sawada et al., 1999). The fact that minocycline does not reduce the levels of these antinociceptive factors is one of the reasons behind its analgesic effects. IL-4 is also considered to be an antinociceptive cytokine (Vale et al., 2003). However, no changes in *IL-4* mRNA levels were observed in our study, similar to the study conducted by Wen Tao et al. (2011).

Proinflammatory factors produced by activated glia and immune cells promote neuropathic pain conditions (Mika et al., 2013). At the spinal cord level, the microglia is highly activated, leading to the release of proinflammatory substances, such as IL-1beta, IL-6, and IL-18, and antiinflammatory substances, such as IL-10 and IL-1alpha. This hypothesis corresponds well with our *in vitro* studies, where we have shown that microglial cells produce IL-1beta, IL-6, IL-18, MMP-2 and MMP-9, TIMP-1 and TIMP-2, IL-10 and IL-1alpha, while IL-4 is undetectable. In 2013

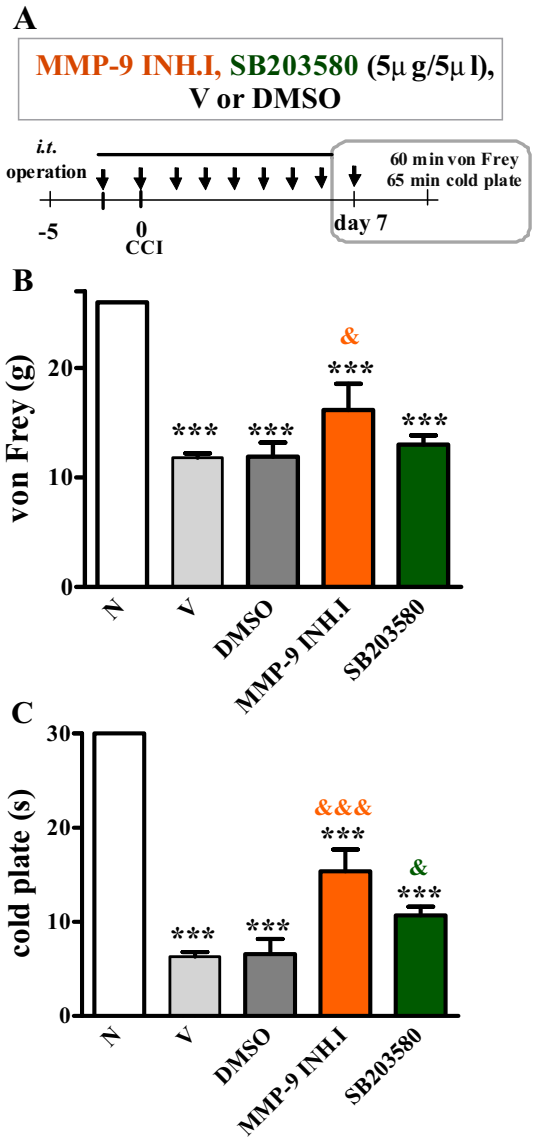


Fig. 5. Influence of preemptive and repeated administration of DMSO; a MMP-9 inhibitor (MMP-9 INH.I; 5 μg/5 μl) and a p38MAPK inhibitor (SB203580; 5 μg/5 μl) on the development of mechanical allodynia (A; von Frey test) and thermal hyperalgesia (B; cold plate test) 7 days after CCI in rats. The drugs were administered by intrathecal (*i.t.*) injection 16 and 1 h before CCI and then once daily for seven days. The control groups received water for injection (V) and DMSO according to the same schedule. Inter-group differences were analysed using ANOVA followed by Bonferroni's multiple comparison test. The data are presented as the mean ± SEM (7–20 rats per group). Pain symptoms were assessed 60 min after the last drug administration. $^{***}p < 0.001$ indicates differences compared with the naïve rats; $^{\&}p < 0.05$ and $^{\&\&\&}p < 0.001$ indicate differences compared with the DMSO-treated CCI-exposed group. N, naïve; V, water for injection.

Kobayashi et al. have shown the upregulation of IL-1beta mRNA induced by LPS in microglia cell culture, which is in agreement with our results (Kobayashi et al., 2013). The authors have also shown the parallel increase of IL-1beta protein level as measured by ELISA. In the DRG, the immune response is driven by macrophages, lymphocytes and satellite cells. The recruitment and activity of the immune cells in the DRG upregulate IL-1beta, IL-6, IL-18, and MMPs.

5. Conclusion

In summary, the development of neuropathic pain is the result of a disrupted equilibrium between algesic and analgesic factors. We were the first to demonstrate the reversal of these changes through minocycline as a result of reduced pronociceptive factor mRNA (*IL-6*

and *IL-18*) within the spinal cord and DRG in a CCI model. Minocycline had no impact on expression of antinociceptive factors, such as *IL-1alpha*, *IL-4* and *IL-10* in the spinal cord and DRG, with the exception of *IL-10* within the DRG. We observed that minocycline restores the neuroimmune balance that is biased towards pronociceptive factors in the development of neuropathic pain. In addition, our study significantly broadens the knowledge on the effects of minocycline in neuropathic pain and highlights the important role of MMP-9 as a factor involved in the development of neuropathic pain. We were the first to demonstrate that minocycline affects *MMP-9* mRNA within the spinal cord and *MMP-9*, *MMP-2* and *TIMP-1* mRNA within the DRG that are elevated following CCI. These results provide a rational basis for the further evaluation of minocycline in the treatment of neuropathic pain and perhaps other MMP-9-dependent pathologies.

Abbreviations

C1q	complement component C1q, marker of microglia
CCI	chronic constriction injury to the sciatic nerve
CD40	cluster of differentiation 40
DRG	dorsal root ganglia
Hprt	hypoxanthine guanine rat hypoxanthine guanine phosphoribosyl transferase
Iba-1	ionised calcium-binding adapter molecule 1
IL-1 alpha	interleukin 1 alpha
IL-10	interleukin 10
IL-18	interleukin 18
IL-1beta	interleukin 1 beta
IL-4	interleukin 4
IL-6	interleukin 6
LPS	lipopolysaccharide
MC	minocycline
MMP-2	metalloproteinase 2
MMP-9	metalloproteinase 9
MMP-9 INH.I	inhibitor of metalloproteinase 9
N	naïve
p38MAPK	p38 mitogen-activated protein kinase
TIMP-1	metallopeptidase inhibitor 1
TIMP-2	metallopeptidase inhibitor 2
V	vehicle

Competing interests

The authors declare no competing interests.

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