



Delta-Opioid Receptor Analgesia Is Independent of Microglial Activation in a Rat Model of Neuropathic Pain

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Abstract

The analgesic effect of delta-opioid receptor (DOR) ligands in neuropathic pain is not diminished in contrast to other opioid receptor ligands, which lose their effectiveness as analgesics. In this study, we examine whether this effect is related to nerve injury-induced microglial activation. We therefore investigated the influence of minocycline-induced inhibition of microglial activation on the analgesic effects of opioid receptor agonists: morphine, DAMGO, U50,488H, DPDPE, Deltorphin II and SNC80 after chronic constriction injury (CCI) to the sciatic nerve in rats. Pre-emptive and repeated administration of minocycline (30 mg/kg, i.p.) over 7 days significantly reduced allodynia and hyperalgesia as measured on day 7 after CCI. The antiallodynic and antihyperalgesic effects of intrathecally (i.t.) administered morphine (10–20 μg), DAMGO (1–2 μg) and U50,488H (25–50 μg) were significantly potentiated in rats after minocycline, but no such changes were observed after DPDPE (10–20 μg), deltorphin II (1.5–15 μg) and SNC80 (10–20 μg) administration. Additionally, nerve injury-induced down-regulation of all types of opioid receptors in the spinal cord and dorsal root ganglia was not influenced by minocycline, which indicates that the effects of opioid ligands are dependent on other changes, presumably neuroimmune interactions. Our study of rat primary microglial cell culture using qRT-PCR, Western blotting and immunocytochemistry confirmed the presence of mu-opioid receptors (MOR) and kappa-opioid receptors (KOR), further we provide the first evidence for the lack of DOR on microglial cells. In summary, DOR analgesia is different from analgesia induced by MOR and KOR receptors because it does not depend on injury-induced microglial activation. DOR agonists appear to be the best candidates for new drugs to treat neuropathic pain.

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Introduction

Neuropathic pain has been shown in clinical and animal studies to be resistant to alleviation by morphine [1,2,3,4,5], but the mechanism of this effect is unclear. The classical opioid system modulates nociception through three G-protein-coupled receptors: delta-opioid receptors (DOR) [6,7] kappa-opioid receptors (KOR) [8,9] and mu-opioid receptors (MOR) [8]. Opioid receptors do not necessarily function independently and can exist as heterodimers that modulate their pharmacology [10,11,12]. Several experimental studies have emphasised that the effects of DOR agonists are distinctively more potent than those of MOR and KOR receptors [13,14,15] in neuropathic pain. The field of DOR analgesia has been widely studied [16,17,18] and the DOR agonists seems to be a good drugs that would be effective in neuropathic pain, but still some of the aspects of DOR ligands interactions have to be clarified.

Reduction of morphine antinociceptive potency has been postulated to be a consequence of changes in the activity of opioid systems or opioid-specific signalling [3,19]. Although a reduction in the number of receptors may be a major factor in the

reduced efficacy of opioids, it has become clear that many other factors affect the efficacy of morphine. Such factors include heterologous desensitisation between opioid and proinflammatory chemokine receptors via shared G-protein-coupled systems [20], down-regulation of glutamate transporters in glial cells [21] or release of such substances as NO, ATP, excitatory amino acids, prostaglandins, and proinflammatory cytokines from activated glia [22,23,24,25,26,27]. In a previous study, we used minocycline, which is a tetracycline derivative with pleiotropic biological effects, to clarify whether the analgesic opioid effect is associated with the activation of microglia. Minocycline is a potent inhibitor of the microglial activation [22,28,29,30] that impairs microglial viability and migration [31,32]. Minocycline also up-regulates a tissue inhibitors of matrix metalloproteinases (TIMPs) [33], inhibits MMP-9 [34] and has been shown in many studies to have neuroprotective effects [35,36]. Some authors have suggested that minocycline may also reduce pain by inhibiting Ca^{2+} and Na^{+} currents in sensory neurons in DRG [35,37]. It was shown that pain after nerve injury depended on activation of the p38 MAPK signalling pathway in the spinal cord and p38 MAPK was co-

localized with activated microglia [38,39]. It was shown [40,41,42] that the level of phospho-p38 MAPK in microglia was reduced after minocycline treatment, which suggests that this kinase is a minocycline target. Little is known about its influence on neurons, but some authors suggest its neuroprotective effects in dopaminergic and glutamatergic neurons [43,44]. We demonstrated in our previous study that chronic administration of minocycline attenuated the development of neuropathic pain and enhanced morphine analgesia [45,46,47]. Filipovic and Zecevic [48] demonstrated *in vitro* studies that minocycline protects neurons from LPS-induced inflammation by inhibiting the activation of microglia. Activated microglia may weaken morphine analgesia by releasing pronociceptive factors, and the administration of minocycline antagonises this weakening [23,46,47].

The aim of the present study was to determine the extent to which the activation of microglia induced by injury to the sciatic nerve (by chronic constriction injury, CCI) in rats changes the analgesic effect of opioid receptors agonists. Minocycline, a well characterized drug for inhibiting microglial activity [49], was intraperitoneally (i.p.) administered pre-emptively and repeatedly to CCI-exposed rats, subsequently its influence on allodynia and hyperalgesia was measured. On day 7 after CCI, vehicle-treated and minocycline-treated rats received the opioid receptor agonists morphine, DAMGO, U50,488H, DPDPE, deltorphin II and SNC80 intrathecally. We conducted behavioural experiments and simultaneously analysed molecular changes in the transcription of *MOR*, *KOR*, and *DOR* mRNA in the dorsal horn of the lumbar spinal cord and in DRG (L4–L6) at day 7 after injury in CCI-exposed rats after minocycline or vehicle administration. We also analysed the presence of MOR, KOR and DOR in rat primary microglial cell cultures by using qRT-PCR, Western blotting and immunocytochemistry.

Methods

Animals

Male Wistar rats (200–350 g) were housed in cages that were lined with sawdust under a standard 12/12 h light/dark cycle (lights on at 08:00 h), with food and water available *ad lib*. Care was taken to reduce the number of animals used, and all experiments were performed according to the recommendations of IASP [50] and the NIH Guide for the Care and Use of Laboratory Animals and were approved by the II Local Bioethics Committee branch of the National Ethics Committee for Experiments on Animals based at the Institute of Pharmacology, Polish Academy of Sciences (Cracow, Poland).

Surgical preparations

Chronic constriction injury (CCI) was produced in rats by tying four ligatures around the sciatic nerve under sodium pentobarbital anaesthesia (60 mg/kg; i.p.). The *biceps femoris* and the *gluteus superficialis* were separated, and the right sciatic nerve was exposed. The 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. The procedure has been described in detail by Bennett and Xie, [51]. After the surgery, all 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 naïve and sham rats [52], we used naïve animals for the behavioural experiments in the present study.

Intrathecal (i.t.) injection

Rats were prepared for intrathecal (i.t.) injection by implanting catheters under pentobarbital anaesthesia (60 mg/kg i.p.). The intrathecal catheter consisted of polyethylene tubing that was 12 cm long (PE 10, Intramedic; Clay Adams, Parsippany, NJ) with an outside diameter of 0.4 mm and a dead space of 10 µl that had been sterilised by immersion in 70% (v/v) ethanol and fully flushed with sterile water before insertion. Rats were placed on a stereotaxic table (David Kopf Instruments, Tujunga, CA), and an incision was made in the atlanto-occipital membrane. The catheter (7.8 cm of its length) was carefully introduced into the subarachnoid space at the rostral level of the spinal cord lumbar enlargement (L4–L5) according to the method of Yaksh and Rudy [53]. After the implantation, the first injection of 10 µl of water was performed slowly and the catheter was tightened. One day after catheter implantation, the rats were monitored for physical impairments. Those showing motor deficits were excluded from further study. Animals were allowed a minimum 1 week of recovery after the surgery before the experiment began. Water for injection or respective drugs were delivered slowly (1–2 min) in a volume of 5 µl through the i.t. catheter and were followed by 10 µl of water for injection, which flushed the catheter.

Behavioural tests

Tactile allodynia (von Frey test). Allodynia was measured in rats subjected to CCI by the use of an automatic von Frey apparatus (Dynamic Plantar Aesthesiometer Cat. No. 37400, Ugo Basile Italy). Rats were placed in plastic cages with a wire net floor 5 min before the experiment. The von Frey filament was applied to the midplantar surface of the hind foot, and measurements were taken automatically [23]. The strength of the von Frey stimuli ranged from 0.5 to 26 g in rats.

Cold hyperalgesia (cold plate test). Hyperalgesia was assessed using the cold plate test (Cold/Hot Plate Analgesia Meter No. 05044 Columbus Instruments, USA) as has been described previously [23,45]. The temperature of the cold plate was maintained at 5°C, and the cut-off latency was 30 s. The rats were placed on the cold plate, and the time until lifting of the hind foot was recorded. The injured foot was the first to react in every case.

Drug administration

The chemicals used in this study and their sources were as follows: morphine hydrochloride – Polfa (Kutno, Poland); DAMGO (Sigma-Aldrich, USA), Deltorphan II (Sigma-Aldrich, USA), DPDPE (Sigma-Aldrich, USA), U50,488H (Tocris, UK), SNC80 (Sigma-Aldrich, USA) and minocycline hydrochloride (Sigma-Aldrich, USA). Minocycline (MC; 30 mg/kg; i.p.) was dissolved in sterile water and pre-emptively administered intraperitoneally 16 h and 1 h before CCI and then twice daily for 7 days. This method of minocycline administration was used throughout the work and is referred to in the text as “repeated administration”. The control groups received a vehicle (water for injection) according to the same schedule. One hour after the last morning of minocycline or vehicle administration on day 7 after CCI, morphine (M; 20; 40 µg; 62.3 nM; 124.6 nM), DAMGO (MOR selective ligand; 1; 2 µg; 1.94 nM; 3.9 nM), U50,488H (KOR selective ligand; 25; 50 µg; 61.6 nM; 123.2 nM), DPDPE (DOR I selective ligand; 10; 20 µg; 15.48 nM; 30.96 nM), deltorphan II (del II; DOR II selective ligand; 1.5; 15 µg; 1.9 nM; 19 nM), SNC80 (10; 20 µg; 22.24 nM; 44.48 nM) or a vehicle was intrathecally injected. The von Frey test (25 and 45 min later) and cold plate tests (30 and 50 min later) were

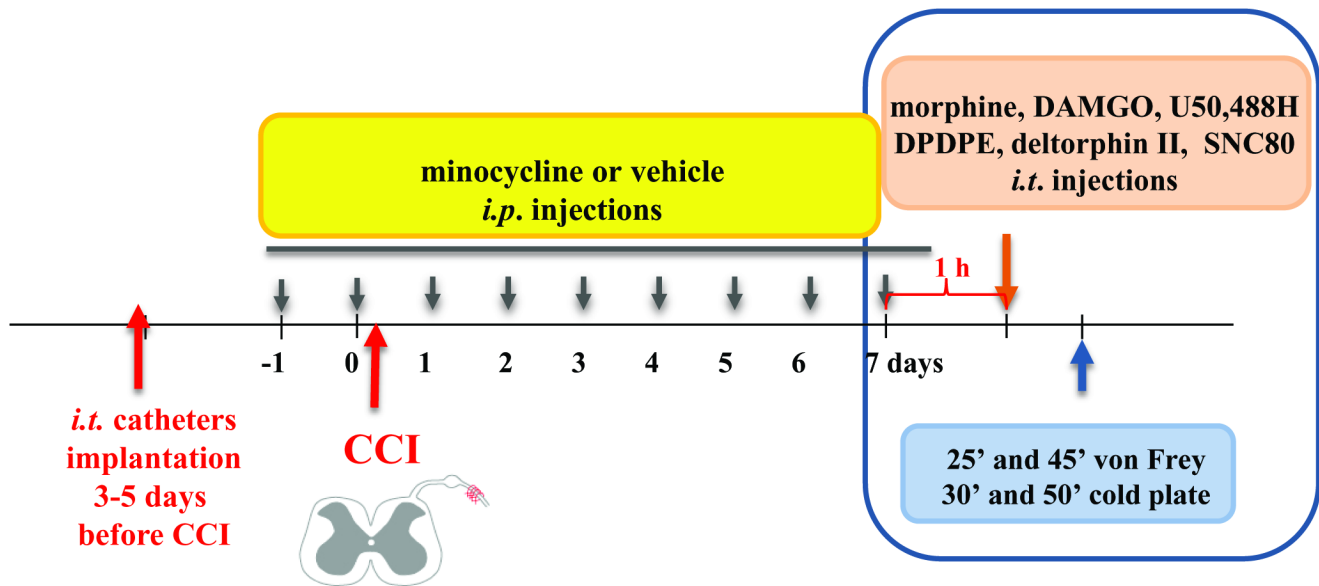


Figure 1. Drug administration.
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carried out after vehicle or opioid agonist administration (Figure 1).

Microglial cell cultures and treatments

Primary cultures of microglial cells were prepared from 1-day-old Wistar rat pups as has been previously described [54]. Briefly, cells were isolated from the rats' cerebral cortices and were plated at a density of 3×10^5 cells/cm² 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 cm² culture flasks and were maintained at 37°C and 5% CO₂. The culture medium was changed after 4 days. The loosely adherent microglial cells were recovered after 9 days by mild shaking and centrifugation. Microglial cells were suspended in a culture medium and plated at a final density of 2×10^5 cells onto 24 well plates and 1.2×10^6 cells onto 6 well plates. Adherent cells were incubated for 48 h in a culture medium before being used for the analyses. Cell specificity was determined using an antibody to OX-42 (microglial marker) in cultures of primary microglia. Levels of mRNA for *Clq* (a microglial marker) and *GFAP* (an astroglial marker) were also investigated. Cultured primary microglia were more than 95% positive for OX-42 and *Clq*.

We have also analyzed the level of MOR, DOR and KOR mRNA level after LPS-stimulation (data not shown). Cells were prepared as described above and then incubated for 6 h with LPS [100 ng/ml] [55] or vehicle (PBS). We have not observed any significant changes in expression of MOR and KOR transcripts in comparison with vehicle-treated cells. In the LPS-stimulated microglial cells the presence of *DOR* mRNA also haven't been detected. Lipopolysaccharides from *Escherichia coli* 0111:B4 (Sigma-Aldrich, USA).

qRT-PCR analysis of gene expression

Total RNA was extracted according to the method Chomczynski and Sacchi [56] using the TRIzol reagent (Invitrogen) as previously described [57]. The RNA concentration was measured

using a NanoDrop ND-1000 Spectrometer (NanoDrop Technologies). Reverse transcription was performed on 500 ng (from cell cultures) or 1000 ng (from tissue) of total RNA using Omniscript reverse transcriptase (Qiagen Inc.) at 37°C for 60 min. cDNA was diluted 1:10 with H₂O. qRT-PCR was performed using Assay-On-Demand TaqMan probes according to the manufacturer's protocol (Applied Biosystems) and run on a Real-Time PCR iCycler device (BioRad, Hercules, CA, USA). Rn00561699_m1 (*Oprd1*), Rn01430371_m1 (*Oprm1*), and Rn00567737_m1 (*Oprk1*) were used as TaqMan primers and probes. The expression of *HPRT* (a housekeeping gene) was quantified to control for variation in cDNA amounts. Cycle threshold values were calculated automatically by iCycler IQ 3.0 software with default parameters. Abundance of RNA was calculated as $2^{-(\text{threshold cycle})}$.

Western blot analysis

Cell lysates were collected in a RIPA buffer with a protease inhibitor cocktail and cleared by centrifugation (14000×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 5 min at 70°C and resolved by SDS-PAGE on 12% polyacrylamide gels. Following gel electrophoresis, the proteins were electrophoretically transferred to Immune-Blot PVDF membranes (Bio-Rad). The blots were blocked for 30 min using 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline with 0.1% Tween 20 (TBST). The blots were incubated with primary antibodies (rabbit polyclonal anti-MOR, 1:500 [58]; rabbit polyclonal anti-DOR, 1:500 [59]; rabbit polyclonal anti-KOR 1:500, Abcam [58] that had been diluted in a SignalBoost Immunoreaction Enhancer Kit (Merck Millipore) for 24 h at 4°C and then incubated with a goat polyclonal antibody that had been conjugated to horseradish peroxidase (goat anti-rabbit IgG, BioRad) at a dilution of 1:1000 for 1 h at room temperature. After four 5-minute washes in TBST, immunocomplexes were detected using a Lumi-Light Western Blotting Kit and visualised using a Fujifilm LAS-4000 fluorimager system. The blots were washed 4 times for 5 minutes each in TBST and reprobbed with a mouse antibody against GAPDH (1:5000, Millipore) as a

loading control. The relative levels of immunoreactivity were quantified using Fujifilm Image Gauge software.

Immunocytochemical analysis

We used commercially available specific anti-MOR, anti-KOR and anti-OX/42 antibodies. Cells were fixed for 20 minutes in 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) and incubated with primary antibodies (rabbit anti-MOR, 1:400 [60], rabbit anti-KOR, 1:400 [61], rabbit anti-DOR, 1:400, [62] Neuromics; mouse anti-OX/42, 1:500, Serotec) for 2 days at 4°C. After three washes in PB, double immunofluorescence was revealed by incubation for 2 h in the appropriate fluorochrome-conjugated secondary antibody, Alexa Fluor546 donkey anti-rabbit and Alexa Fluor488 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.

Data analysis

The behavioural data are presented as the mean \pm S.E.M of 8–16 rats per group. The results of the experiments were statistically evaluated using one-way analysis of variance (ANOVA). All of the differences between the treatment groups were further analysed with Bonferroni's post-hoc tests. Significant differences in comparisons with vehicle-treated CCI-exposed rats are indicated by **P* (<0.05), ***P* (<0.01) and ****P* (<0.001). Significant differences between vehicle-treated CCI-exposed rats that had received a single dose of opioid receptor ligands and minocycline-treated CCI-exposed rats that had received a single dose of opioid receptor ligands are indicated by #*P* (<0.05), ##*P* (<0.01) and ###*P* (<0.001).

The qRT-PCR analyses were performed in three groups: naïve, CCI-exposed and minocycline-treated CCI-exposed rats. The results are presented as fold changes compared with the naïve rats in the ipsilateral dorsal lumbar spinal cord and DRG. The qRT-PCR data are presented as the mean \pm S.E.M and represent the normalised averages that were derived from the threshold qRT-PCR cycles from four to eight samples for each group. Intergroup differences were analysed using ANOVAs followed by Bonferroni's multiple comparison tests. Significant differences resulting from comparison with naïve rats are indicated by **P* (<0.05), ***P* (<0.01) and ****P* (<0.001). The data from 3 to 10 cell cultures are presented.

Results

Repeated administration of minocycline significantly diminished allodynia and hyperalgesia in neuropathic pain in rats

All vehicle-treated CCI rats exhibited neuropathic pain symptoms in the behavioral tests. The rats exhibited strong allodynia on the seventh day after ligation as measured by the von Frey test (11.6 $g \pm 0.6$ vs. 25.8 $g \pm 0.2$ for naïve rats) (Fig. 2A,C,E; 3A,C,E) and potent hyperalgesia as measured by the cold plate test (7.6 $s \pm 0.9$ vs. 29.7 $s \pm 0.3$ for naïve rats) (Fig. 2B,D,F; 3B,D,F). Repeated administration of minocycline (MC; 30 mg/kg; i.p.) attenuated allodynia (17.4 $g \pm 0.5$ vs. 11.6 $g \pm 0.6$ for the vehicle-treated CCI rats) (Fig. 2A,C,E; 3A,C,E) and also hyperalgesia (12.1 $s \pm 0.9$ vs. 7.6 $s \pm 0.9$ for the vehicle-treated CCI rats) (Fig. 2B,D,F; 3B,D,F) to a similar extent at both time points.

Repeated administration of minocycline significantly influenced the effects of morphine in neuropathic pain in rats

The doses of morphine effective in neuropathic pain (M; 20 and 40 μg ; i.t.) were used. Morphine was injected one hour after the last morning dose of minocycline (30 mg/kg; i.p. repeatedly) or vehicle, and allodynia and hyperalgesia were measured (see Figure 1). Morphine at a dose of 20 μg significantly diminished allodynia 25 and 45 minutes after injection compared with vehicle-treated animals (18.60 $g \pm 0.4$ vs. 11.6 $g \pm 0.6$ and 17.57 $g \pm 0.39$ vs. 11.9 $g \pm 0.5$, respectively; Fig. 2A). In minocycline-treated animals, the antiallodynic effect of morphine at a dose of 20 μg was potentiated by minocycline at both times as shown in the von Frey test (25 minutes: 22.04 $g \pm 0.52$ vs. 18.60 $g \pm 0.4$ for morphine-treated rats, 45 minutes: 22.3 $g \pm 0.34$ vs. 17.57 $g \pm 0.39$ for morphine-treated rats; Fig. 2A). Morphine at a dose of 20 μg did not influence thermal hyperalgesia but in the group which was injected repeatedly with minocycline, the antihyperalgesic effect of morphine was demonstrated (30 minutes: 18.30 $s \pm 1.6$ vs. 11.3 $g \pm 0.8$ for morphine-treated rats, 50 minutes: 22.5 $s \pm 1.7$ vs. 13.5 $s \pm 1.5$ for morphine-treated rats; Fig. 2B). A higher dose of morphine (40 μg) was effective in reducing mechanical allodynia 25 and 45 minutes after injection compared with vehicle-treated animals (25 minutes: 21.1 $g \pm 0.48$ vs. 11.6 $g \pm 0.6$, 45 minutes: 23.9 $g \pm 0.6$ vs. 11.9 $g \pm 0.5$; Fig. 2A). In minocycline-treated rats, the antiallodynic effect of morphine at a dose of 40 μg was potentiated at both times (25 minutes: 24.84 $g \pm 0.27$ vs. 21.1 $g \pm 0.48$, 45 minutes: 23.98 $g \pm 0.51$ vs. 20.25 $g \pm 0.6$; Fig. 2A). In opposite to the lower dose, morphine at a dose of 40 μg showed antihyperalgesic effect at both time points (30 minutes: 16.5 $s \pm 1.9$ vs. 7.6 $s \pm 0.9$, 50 minutes: 19.1 $s \pm 2.2$ vs. 8.2 $s \pm 0.8$; Fig. 2B). The increased effect of morphine at a dose of 40 μg in minocycline-treated group was observed at both time points, however, this effect was more significant after 50 minutes (25.8 $s \pm 1.0$ vs. 19.1 $s \pm 2.2$; Fig. 2B).

Repeated administration of minocycline significantly influenced the effects of MOR (DAMGO) and KOR (U50,488H) agonists in neuropathic pain in rats

DAMGO at a dose of 1 μg significantly diminished allodynia 25 and 45 minutes after injection compared with vehicle-treated animals (25 minutes: 20.4 $g \pm 0.5$ vs. 11.6 $g \pm 0.6$, 45 minutes: 18.7 $g \pm 0.4$ vs. 11.9 $g \pm 0.5$; Fig. 2C). In minocycline-treated animals, the antiallodynic effect of DAMGO (1 μg) was potentiated at both times as shown in the von Frey test (25 min: 22.6 $g \pm 0.7$ vs. 20.4 $g \pm 0.5$ for DAMGO-treated rats, 45 min: 21.4 $g \pm 1.0$ vs. 18.7 $g \pm 0.4$ for DAMGO-treated rats; Fig. 2C). DAMGO at a dose of 1 μg did not influence thermal hyperalgesia but in minocycline- and DAMGO-treated group the antihyperalgesic effect occurred at both time points but the better effect we observed 50 min after injection (21.2 $s \pm 1.8$ vs. 16.4 $s \pm 1.7$ for DAMGO-treated rats; Fig. 2D). The higher dose of DAMGO (2 μg) was effective in reducing mechanical allodynia 25 and 45 minutes after injection compared with vehicle-treated animals (25 min: 21.3 $g \pm 0.9$ vs. 11.6 $g \pm 0.6$, 45 min: 20.6 $g \pm 1.1$ vs. 11.9 $g \pm 0.5$; Fig. 2C). As shown in the von Frey test, repeated minocycline administration potentiated effect of DAMGO at a dose of 2 μg at both times, but a better effect was present after 45 minutes (24.9 $g \pm 0.5$ vs. 20.6 $g \pm 1.1$; Fig. 2C). In opposite to the lower dose, DAMGO at a dose of 2 μg showed antihyperalgesic effect at both time points (30 min: 18.0 $s \pm 2.9$ vs. 7.6 $s \pm 0.9$, 50 min: 16.9 $s \pm 1.5$ vs. 8.2 $s \pm 0.8$; Fig. 2D). We also observed increased effect of DAMGO in minocycline-treated

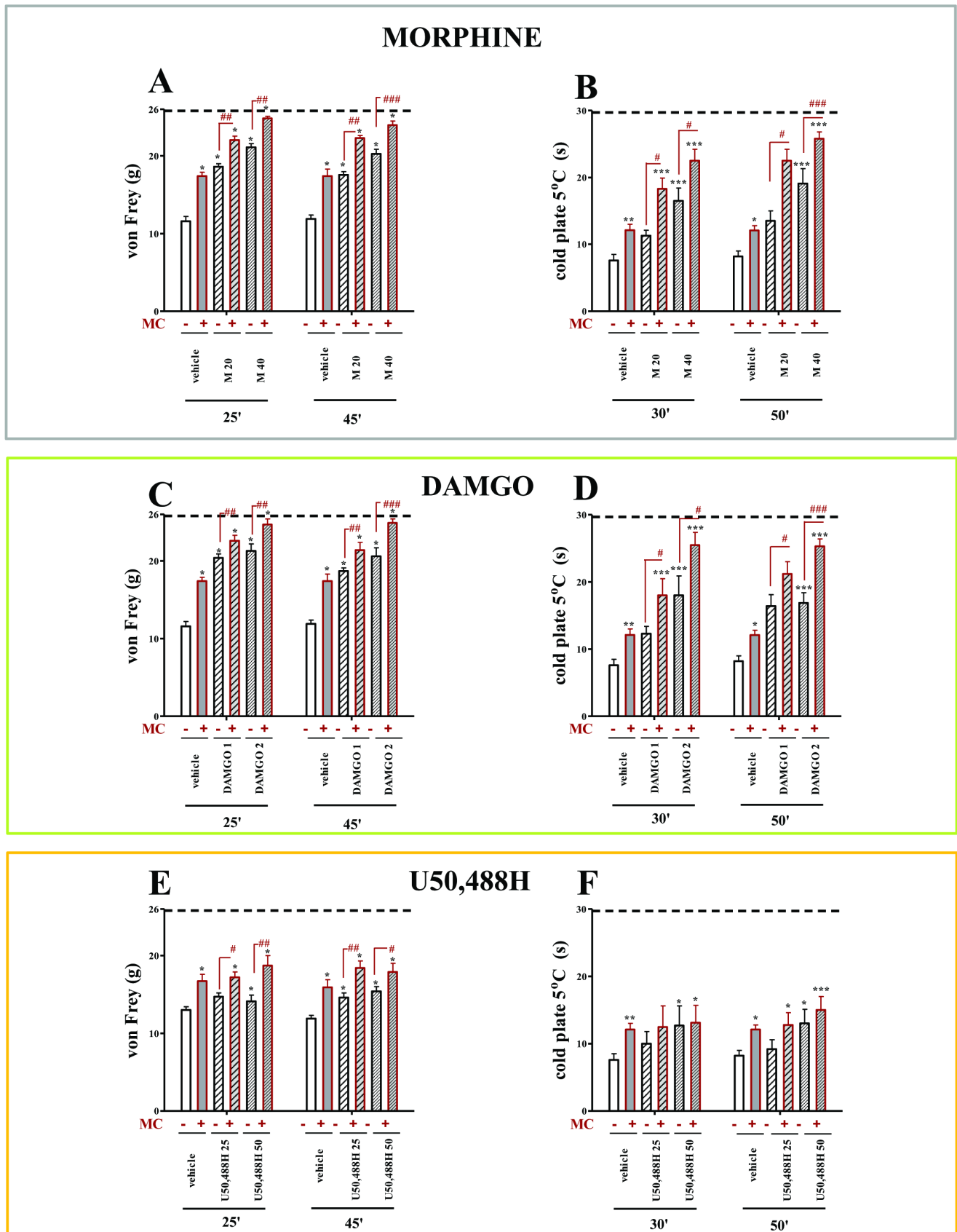


Figure 2. The effects of morphine, DAMGO and U50,488H on vehicle- and minocycline-treated CCI-exposed rats. The response to morphine, DAMGO and U50,488H was measured 25 and 45 minutes after administration by the von Frey test (A, C, E) and 30 and 50 minutes after

administration by the cold plate test (B, D, F). Minocycline (MC; 30 mg/kg; i.p.) was administered intraperitoneally pre-emptively 16 h and 1 h before CCI, and then repeatedly twice daily for 7 days. Vehicle-treated and minocycline-treated rats received intrathecal morphine (M; 20; 40 $\mu\text{g}/5 \mu\text{l}$), DAMGO (1; 2 $\mu\text{g}/5 \mu\text{l}$) or U50,488H (25; 50 $\mu\text{g}/5 \mu\text{l}$) one hour after the last morning administration on day 7 after CCI. The data are presented as the mean response \pm S.E.M. (8–16 rats per group). The results of the experiments were statistically evaluated using One-way Analyses of Variance (ANOVA). The differences between the treatment groups throughout the study were further analysed with Bonferroni's *post-hoc* tests. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate significant differences compared with vehicle-treated CCI-exposed rats; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ indicate significant differences between vehicle-treated CCI-exposed rats that received a single dose of morphine and minocycline-treated CCI-exposed rats that received a single dose of morphine, DAMGO or U50,488H. Dotted line is a value for naïve animals (for von Frey test 25.8 g; for cold plate test 29.7 s).

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group 30 minutes after opioid injection, however, this effect was more significant after 50 minutes (25.3 \pm 1.1 vs. 16.9 \pm 1.5; Fig. 2D).

During neuropathy, the antiallodynic effect of U50,488H appeared after doses of 25 μg i.t. only 45 minutes after injection (14.6 \pm 0.6 vs. 11.9 \pm 0.5; Fig. 2E). The effect of this dose of U50,488H was potentiated in minocycline-treated group as shown in the von Frey test at both times, but at 45 minutes after injection the effect was more pronounced (18.4 \pm 0.9 vs. 14.6 \pm 0.6; Fig. 2E). U50,488H at a dose of 25 μg did not influence hyperalgesia and minocycline did not change this action (Fig. 2F). The higher dose (50 μg ; i.t.) diminished allodynia at both times (25 min: 14.1 \pm 0.8 vs. 11.6 \pm 0.6, 45 min: 15.4 \pm 0.6 vs. 11.9 \pm 0.5; Fig. 2E). In minocycline-treated rats, the antiallodynic effect of U50,488H at a dose of 50 μg was potentiated at both times, but its potentiation was higher after 25 minutes (18.7 \pm 1.3 vs. 14.1 \pm 0.8; Fig. 2E). This dose of U50,488H slightly diminished hyperalgesia at 30 (12.7 \pm 2.9 vs. 7.6 \pm 0.9; Fig. 2F) and 50 (13.0 \pm 2.1 vs. 8.2 \pm 0.8; Fig. 2F) minutes after injection. Repeated minocycline administration did not change the action of U50,488H at a dose of 50 μg (Fig. 2F).

Repeated administration of minocycline did not influence the effects of DOR agonists (DPDPE, Deltorphin II and SNC80) in neuropathic pain in rats

DPDPE at a dose of 10 μg diminished allodynia 25 and 45 minutes after injection compared with vehicle-treated animals (25 min: 19.8 \pm 2.1 vs. 11.6 \pm 0.6, 45 min: 16.6 \pm 2.4 vs. 11.9 \pm 0.5; Fig. 3A) and minocycline treatment did not change this effect (Fig. 3A). Higher dose of DPDPE (20 μg) was effective in reducing mechanical allodynia only 25 minutes after injection compared with vehicle-treated animals (20.3 \pm 1.9 vs. 11.6 \pm 0.6, Fig. 3A) and this antiallodynic effect was not changed by minocycline (Fig. 3A). As shown in the cold plate test, DPDPE significantly diminished thermal hyperalgesia after both doses of 10 and 20 μg (30 min: 16.1 \pm 2.3 vs. 7.6 \pm 0.9; 50 min: 17.50 \pm 1.9 vs. 8.2 \pm 0.8 and 30 min: 20.3 \pm 1.9 vs. 7.6 \pm 0.9; 50 min: 23.6 \pm 1.7 vs. 8.2 \pm 0.8; respectively; Fig. 3B) and minocycline did not modulate this action (Fig. 3B).

Deltorphin II at a dose of 1.5 μg slightly diminished allodynia 25 and 45 minutes after injection compared with vehicle-treated animals (25 min: 17.7 \pm 1.7 vs. 11.6 \pm 0.6, 45 min: 17.4 \pm 1.4 vs. 11.9 \pm 0.5; Fig. 3C) and no changes in deltorphin II action in minocycline-treated group were observed (Fig. 3C). The higher dose of Deltorphin II (15 μg) was effective in reducing mechanical allodynia 25 (17.8 \pm 1.5 vs. 11.6 \pm 0.6; Fig. 3C) and 45 (17.2 \pm 2.1 vs. 11.9 \pm 0.5; Fig. 3C) minutes after injection compared with vehicle-treated animals. Repeated minocycline injection did not change the antiallodynic effect of deltorphin II (Fig. 3D). Deltorphin II at a dose of 1.5 μg did not influence hyperalgesia in CCI-exposed rats and minocycline also did not change this effect (Fig. 3D). As shown in the cold plate test, deltorphin II at a dose of 15 μg diminished thermal hyperalgesia

only 50 minutes after injection (11.3 \pm 2.8 vs. 8.2 \pm 0.8; Fig. 3D) and minocycline did not change the antihyperalgesic action of the higher dose of Deltorphin II (Fig. 3D).

SNC80 at a dose of 10 μg slightly diminished allodynia 25 and 45 minutes after injection compared with vehicle-treated animals (25 min: 16.4 \pm 0.8 vs. 11.6 \pm 0.6, 45 min: 17.9 \pm 1.7 vs. 11.9 \pm 0.5; Fig. 3E). The higher dose of SNC80 (20 μg) was effective in reducing mechanical allodynia 25 (19.9 \pm 0.6 vs. 11.6 \pm 0.6; Fig. 3E) and 45 (19.0 \pm 1.2 vs. 11.9 \pm 0.5; Fig. 3E) minutes after injection compared with vehicle-treated animals. Repeated minocycline injection did not change the antiallodynic effect of both doses of SNC80 (Fig. 3E). As shown in the cold plate test, SNC80 at a dose of 10 μg influenced hyperalgesia in CCI-exposed rats (30 min: 19.5 \pm 2.8 vs. 7.6 \pm 0.9; 50 min: 13.0 \pm 2.3 vs. 8.2 \pm 0.8; Fig. 3F) SNC80 at a dose of 20 μg diminished thermal hyperalgesia 30 (24.2 \pm 2 vs. 7.6 \pm 0.9; Fig. 3F) and 50 (20.8 \pm 4.0 vs. 8.2 \pm 0.8; Fig. 3F) minutes after injection. Minocycline did not change the antihyperalgesic action of both doses of SNC80 (Fig. 3F).

Repeated administration of minocycline did not influence MOR, DOR and KOR mRNAs during neuropathic pain in rats

In the spinal cord the downregulation of MOR mRNA from 1 \pm 0.05 to 0.7 \pm 0.07 (Fig. 4A) and in the DRG from 1 \pm 0.03 to 0.5 \pm 0.06 (Fig. 4B) was observed compared to the naïve rats. Minocycline not influence the level of MOR mRNA in the spinal cord and in the DRG (Fig. 4A and B, respectively).

In the spinal cord the downregulation of KOR mRNA from 1 \pm 0.06 to 0.8 \pm 0.05 (Fig. 4C) and in the DRG from 1 \pm 0.06 to 0.6 \pm 0.04 (Fig. 4D) was observed compared to the naïve rats. Minocycline not influence the level of KOR mRNA in the spinal cord and in the DRG (Fig. 4C and D, respectively).

In the spinal cord the downregulation of DOR mRNA from 1 \pm 0.1 to 0.67 \pm 0.05 (Fig. 4E) and in the DRG from 1 \pm 0.1 to 0.3 \pm 0.07 (Fig. 4F) was observed compared to the naïve rats. Minocycline not influence the level of DOR mRNA in the spinal cord and in the DRG (Fig. 4E and F, respectively).

Opioid receptor expression in primary microglial cell cultures

Using a reverse transcriptase-polymerase chain reaction, we found that mRNA for MOR and KOR, but not for DOR, is expressed in rat primary microglial cell cultures (Fig. 5A). In the Western blot analysis, we found that protein for MOR and KOR is present in microglia (Fig. 5B). The protein for DOR is undetectable (Fig. 5B). The expression of MOR and KOR, but not DOR, in microglial cells was confirmed by immunocytochemistry (Fig. 5C).

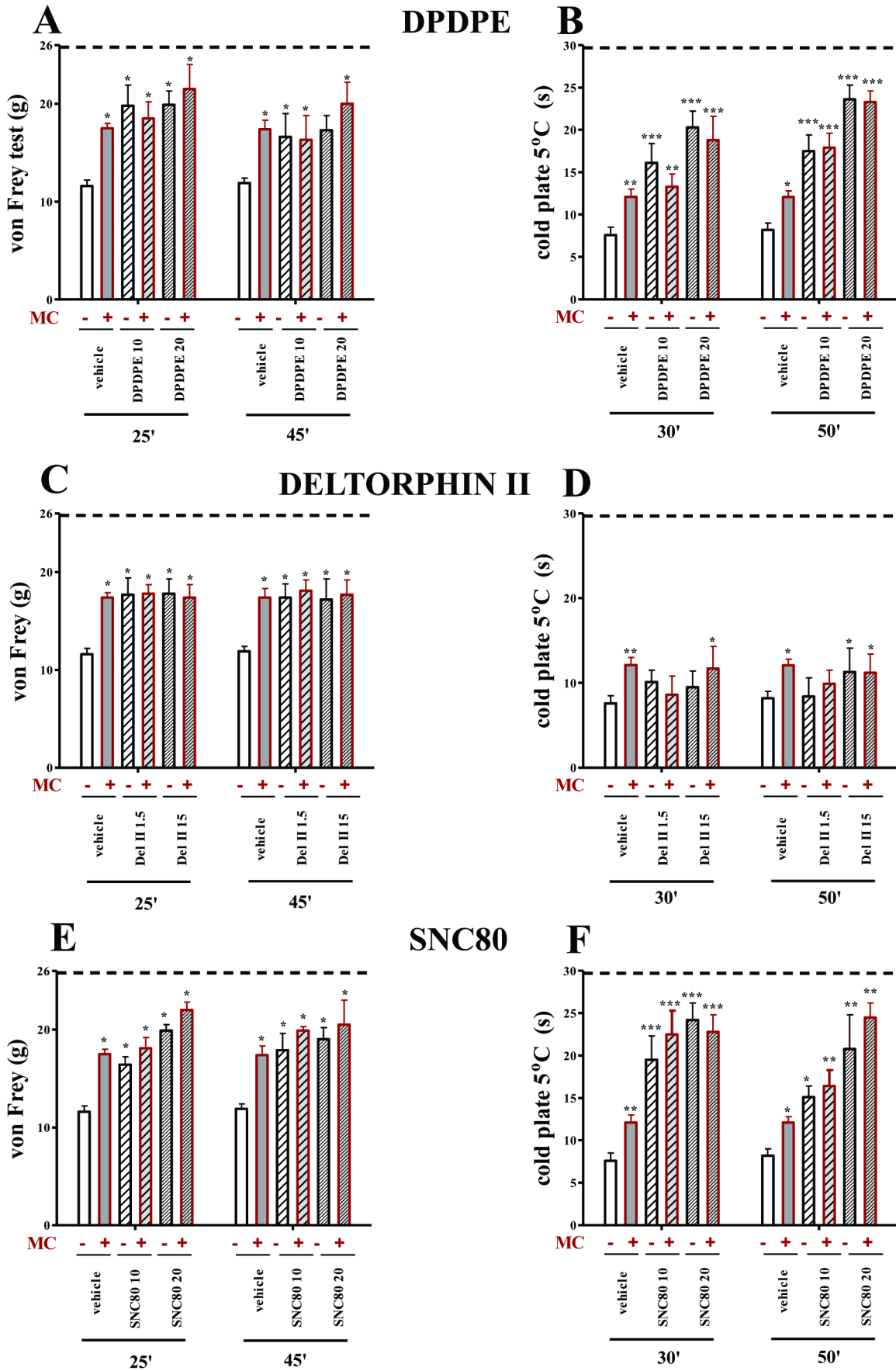


Figure 3. The effects of DPDPE, deltorphin II and SNC80 on vehicle- and minocycline-treated CCI-exposed rats. The response to DPDPE, deltorphin II and SNC80 was measured 25 and 45 minutes after administration by the von Frey test (A, C, E) and 30 and 50 minutes after administration by the cold plate test (B, D, F). Minocycline (MC; 30 mg/kg; i.p.) was administered intraperitoneally pre-emptively 16 h and 1 h before CCI, and then repeatedly twice daily for 7 days. Vehicle-treated and minocycline-treated rats received intrathecal DPDPE (10; 20 $\mu\text{g}/5 \mu\text{l}$), deltorphin II (del II; 1.5; 15 $\mu\text{g}/5 \mu\text{l}$) or SNC80 (10; 20 $\mu\text{g}/5 \mu\text{l}$) one hour after the last morning administration on day 7 after CCI. The data are presented as the mean response \pm S.E.M. (8–16 rats per group). The results of the experiments were statistically evaluated using ANOVAs. The differences between the treatment groups throughout the study were further analysed with Bonferroni's *post-hoc* tests. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate significant differences compared with vehicle-treated CCI-exposed rats. Dotted line is a value for naïve animals (for von Frey test 25.8 g; for cold plate test 29.7 s).

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Discussion

Opioids are fundamental to the treatment of pain, but their efficacy is limited by side effects, including tolerance and hyperalgesia [63]. Some authors suggest that reduced sensitivity to opioids and the increases in their dosages that are necessary to obtain adequate analgesia heighten the risk of side effects [4,5,64]. It remains unclear why morphine is a less potent analgesic in clinical [65,66] and experimental [15,46,67] studies of neuropathic pain in contrast to inflammatory pain [15,67]. In many studies it was shown that selective DOR agonists do not lose their effectiveness in the treatment of neuropathic pain [13,15,68]. It has been suggested that the lesser effects of morphine on neuropathic pain are due to the reduced number of presynaptic opioid receptors that results from nerve damage-induced degeneration of primary afferent neurons [64,69] besides other effects, like upregulation of the anti-opioid system CCK, and the NMDA receptor-dependent central sensitization, between others. Obara *et al.* [15] used ED₅₀ analysis to demonstrate in 2009 that much higher doses of MOR and KOR agonists injected intraplantarly are required to produce antinociception in neuropathic than in inflammatory pain. However, it remains unclear why the ED₅₀ of DOR agonists, but not those of MOR and KOR agonists, is comparable in both neuropathic and inflammatory pain [15]. Our study showed that the expression of all types of opioid receptor mRNA in the spinal cord and DRG was decreased in neuropathic pain. Also Stone *et al.* [70], using three animals models of peripheral nerve injury (CCI, SNL and SNT), observed decreases in spinal DOR expression at day 10th after operations at the side of injury. Herradon *et al.* [71] compared two strains of rats, Fischer 344 and Lewis rats, in neuropathic pain model and found significant down-regulation of DOR mRNA 28 days after CCI in the DRG of Lewis rats and the same trend was observed in F344 rats. Those data correspond well with our results obtained at day 7th after CCI in Wistar rats. In the study of Obara *et al.* [15] biochemical experiments were conducted at 3rd and 14th day (but not day 7th) after CCI and showed a non-significant, downward trend in the expression of mRNA for *MOR*, *DOR* and *KOR* at the spinal cord level. At the DRG level of neuropathic rats these authors observed down-regulation of mRNA level for *MOR* in L4–L5 DRGs (at day 3rd) and in L5 DRG (at day 14th), for *DOR* in L5 DRG (at both days) and for *KOR* in L4 and L5 DRGs (at day 3rd) and in L5 DRG (at day 14th) in comparison with naïve animals. Those data correspond well with our results obtained at day 7th after CCI, however, in our experiments we pooled L4–L6 DRGs [15]. In the paper of Kabli and Cahill [68] the up-regulation of DOR protein was observed 14 days after sciatic nerve injury at the DRG level. The authors suggest that the increase in DOR protein is correlated with DOR trafficking to the site of injury, which may explain the lower level of mRNA as shown by our results. Should be noted, however, that in some papers no decrease in opioid receptor expression was observed [71,72]. Those discrepant data may result from different animal

species used in experiments or time of the tissue collection after injury as it is shown in the mentioned works.

However, minocycline does not affect the reduced levels of all opioid receptor mRNAs, although it potentiates analgesia after such opioid agonists as morphine, DAMGO and U50,488H. Therefore, we should consider other mechanisms of weakened analgesia in neuropathic pain that can be restored by minocycline administration. These mechanisms may be important for sustained analgesia after administration of DOR ligands.

Several studies have suggested that the activation of glia and the enhancement of proinflammatory cytokine levels in the spinal cord and DRG are responsible for the development of both neuropathic pain and morphine tolerance [73,74,75]. We have previously observed an attenuation of the development of neuropathic pain when we administered minocycline, which is a substance that inhibits microglial activation in CCI-exposed rats [45,46]. Although minocycline can influence some neuronal functions, its ability to reduce microglial activation [76] as well as its selectivity of the action has been recently demonstrated [49]. We show in the present paper that the antiallodynic and antihyperalgesic effects of morphine, DAMGO and U50,488H, but not of DPDPE, deltorphin II or SNC80, were significantly potentiated with minocycline in CCI-subjected rats. In many studies we may find pharmacological evidence of distinct subtypes of DOR [77]. It is known that DOR₁ is activated by DPDPE, DOR₂ by deltorphin II and SNC80 is a highly selective agonist for DOR. We had demonstrated in our previous studies the occurrence of strong antiallodynic and antihyperalgesic effects of DOR₁ and DOR₂ agonists after their acute and chronic i.t. administration in a rat neuropathic pain model [13]. Analgesic effects of i.t. injected SNC80 was also shown in many pain models [77,78,79]. In the present paper we have shown that the effectiveness of DPDPE, deltorphin II and SNC80 is not enhanced by minocycline treatment. Our results suggest that DOR analgesia is not dependent on injury-induced microglial activation. DORs are therefore a promising target for the development of analgesics. Indeed as previously reported by Holdridge *et al.* [80,81] the prolonged morphine treatment-induced incensement in microglial cell size was not functionally relevant in DOR analgesia, which confirms our research concerning the presence of DOR on microglia.

In the *in vitro* study, we used qRT-PCR, Western blot and immunocytochemistry assays to confirm the presence of MOR and KOR in microglia, and we have shown for the first time that those microglial cells do not express DOR. Our results are in agreement with other studies that have shown that microglia express MOR and KOR [82,83,84]. Chao *et al.* [85] first reported in 1996 that KOR was present in human microglia using qRT-PCR and a ligand-binding assay. The expression of KOR in microglial cells was also confirmed by the membrane binding of selective ligand [³H]U69,593. Additionally, Chao *et al.* 1997 [86] have shown that morphine and DAMGO suppressed human microglia chemotaxis in a dose-dependent fashion and this effect is significantly attenuated by β -funaltrexamine (a MOR selective

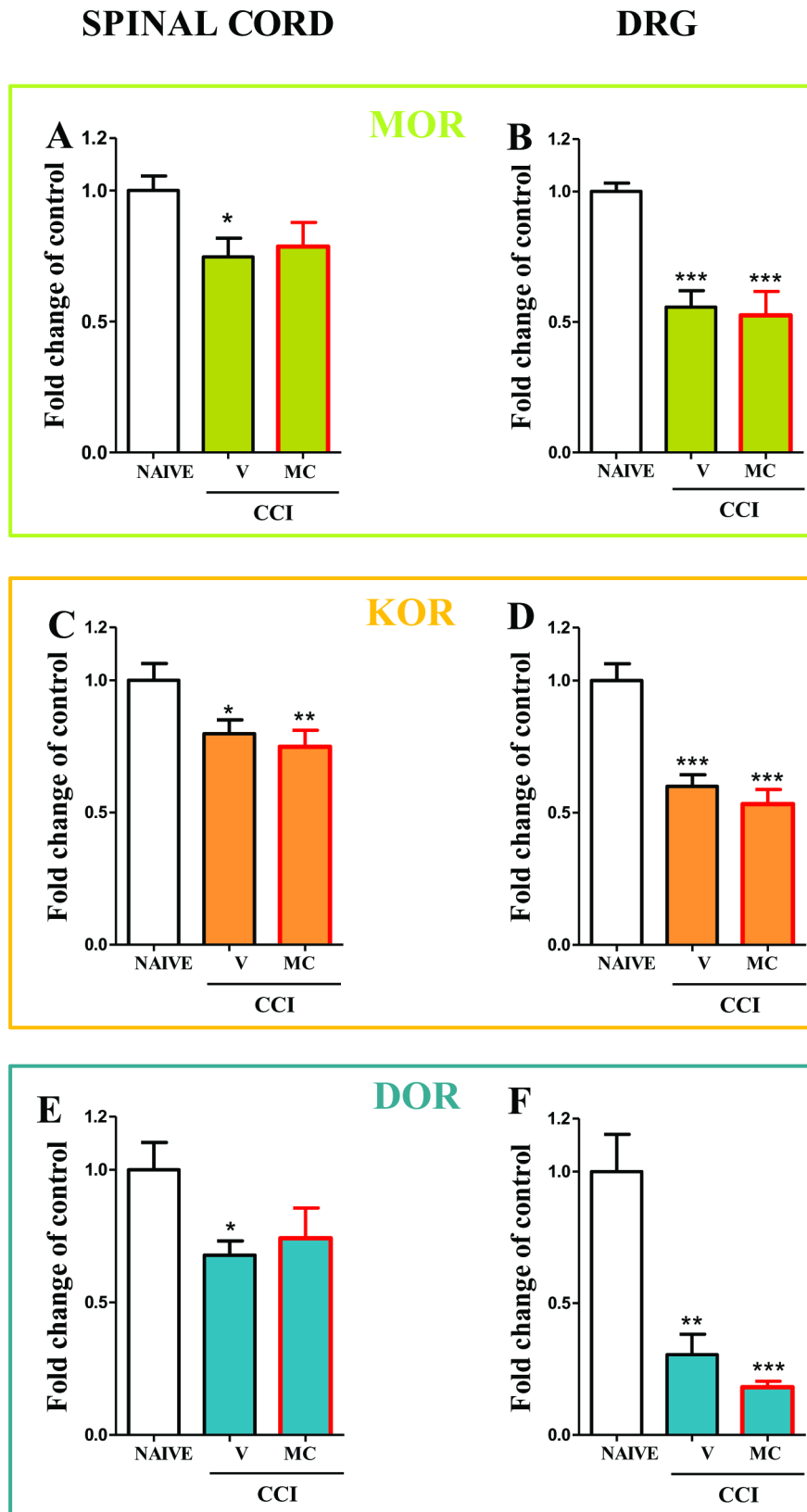


Figure 4. MOR, DOR, KOR mRNAs in spinal cord and DRGs in vehicle- and minocycline-treated CCI-exposed rats. Minocycline (MC; 30 mg/kg; i.p.) was administered intraperitoneally pre-emptively 16 h and 1 h before CCI, and then repeatedly twice daily for 7 days. On the seventh day, spinal cords (L4–L6) and DRG were collected for the qRT-PCR analysis of MOR (A, B), KOR (C, D) and DOR (E, F) gene expression. The data are presented as the means \pm SEM and represent the normalised averages derived from the threshold qRT-PCR cycles from four to eight samples for each group. Intergroup differences were analysed using ANOVAs followed by Bonferroni's multiple comparison tests. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ indicate significant differences compared with naïve rats. doi:10.1371/journal.pone.0104420.g004

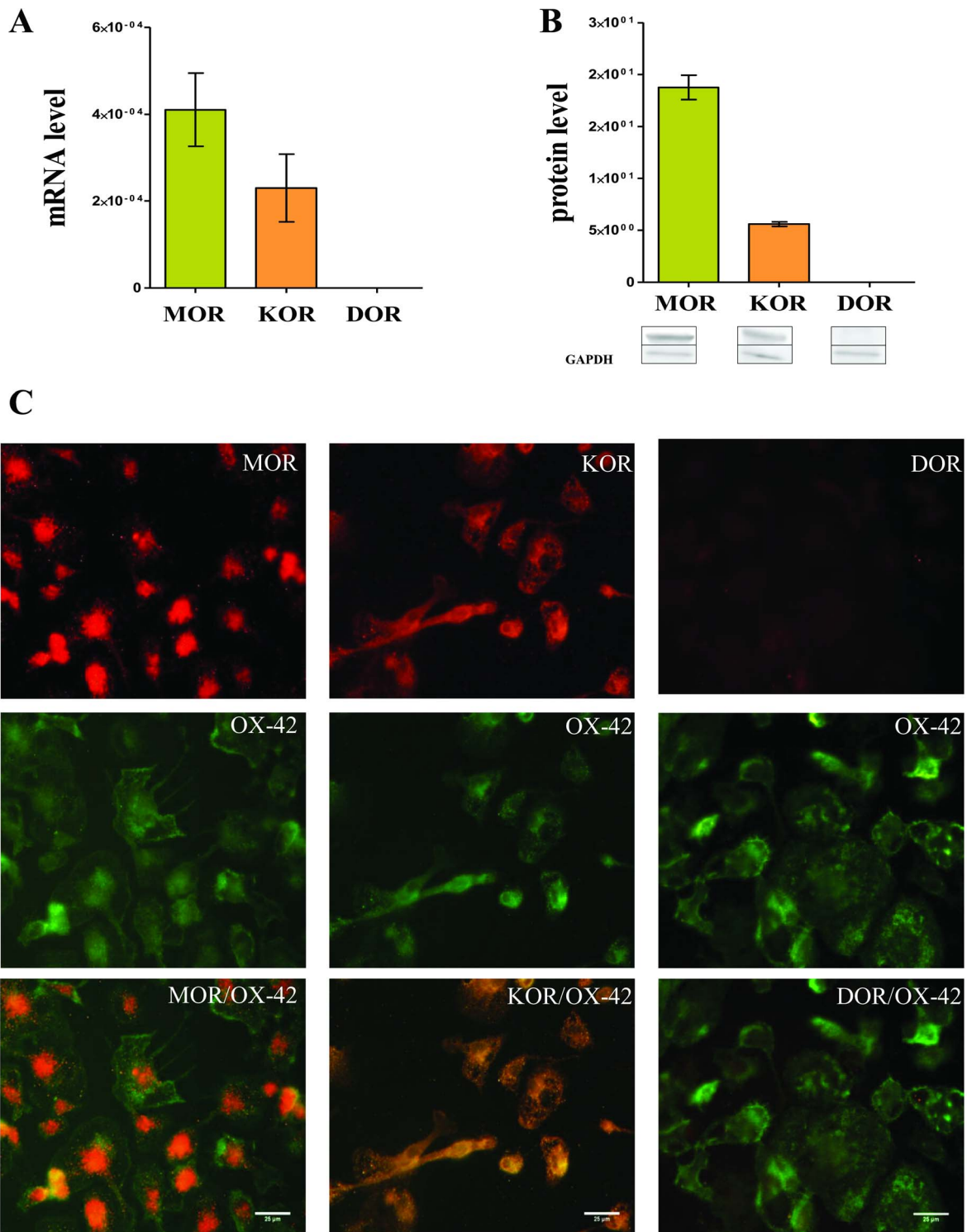


Figure 5. The presence of opioid receptors on microglia cells - *in vitro* studies. We analysed the presence of opioid receptors in primary microglial cell cultures. Using reverse transcriptase-polymerase chain reaction, we found that mRNA for MOR and KOR, but not DOR, is expressed in rat primary microglial cells cultures (A). Western blot analysis (B) detected proteins for MOR and KOR, but not DOR, in microglia. The presence of MOR and KOR, but not DOR, were confirmed by immunocytochemistry (C), and we show the colocalisation of MOR (left panel) and KOR (middle panel) antibodies (red) with OX/42 antibodies (green). The scale bar for all microphotographs is 25 μ m. The data from 3–10 cell cultures are presented. doi:10.1371/journal.pone.0104420.g005

antagonist), which is consistent with our confirmation of the presence of MOR in microglial cells. The occurrence of DOR in microglial cells is still controversial. Our results strongly suggest

that there is no DOR in rat primary microglial cells, although the studies of Turchan-Chlewo *et al.* [87] suggest their presence. However, Turchan-Chlewo *et al.* [87] used the PCR method (Sybr

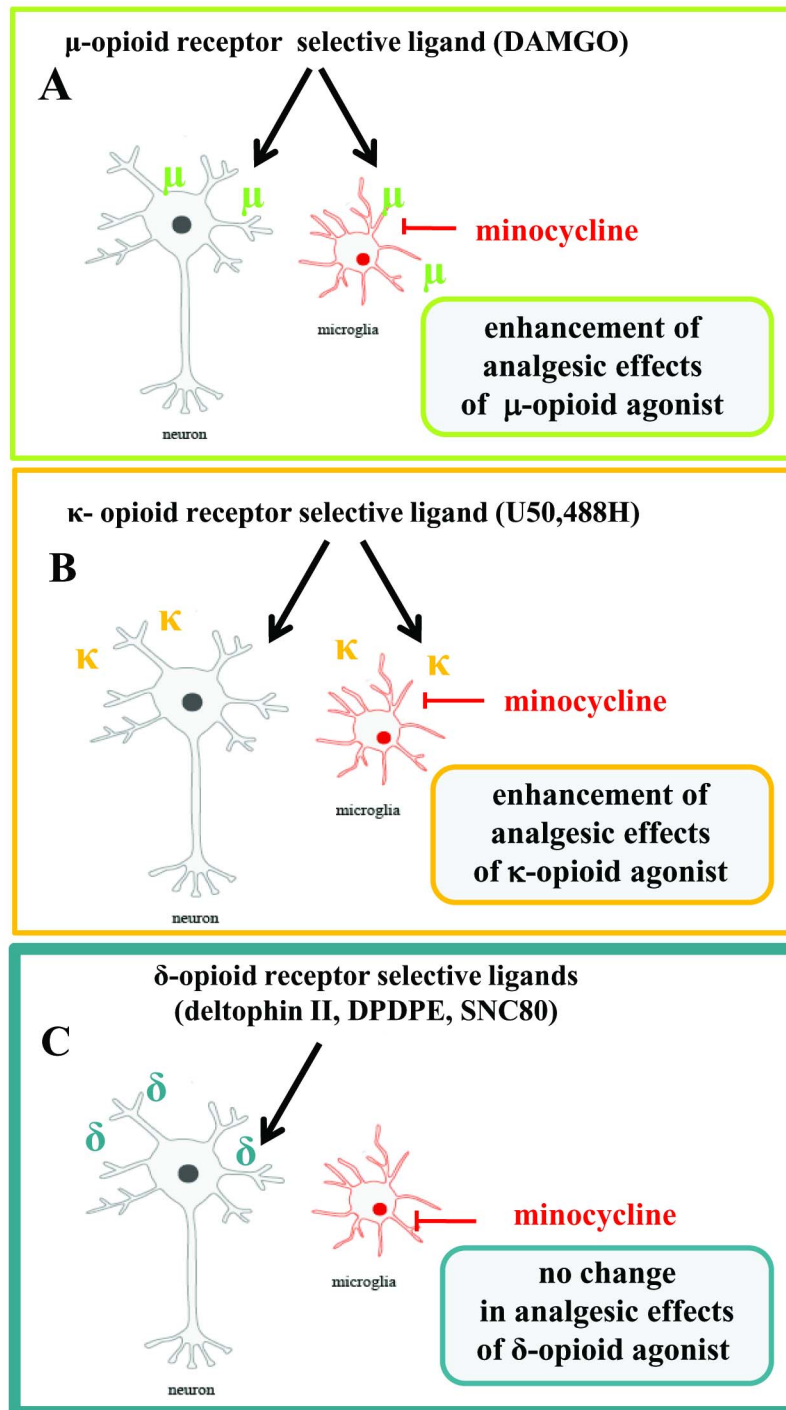


Figure 6. The possible influence of minocycline on analgesia after opioid receptors ligands. In our opinion, activated spinal microglia are key factors in the development of neuropathic pain and play a major role in the antagonizing of some opioids effectiveness. The results of our paper show for the first time that that DOR, in contrast to MOR and KOR, is not present in microglial cells. This phenomenon might be responsible for the different analgesic effects of MOR, KOR and DOR ligands (Fig. 2 and 3). We provide evidence that minocycline (a potent inhibitor of microglial activation and proliferation) enhances the effects of selective MOR (DAMGO; Fig. 2C,D) and selective KOR (U50,488H; Fig. 2E,F) agonists by inhibition of microglial cell activation. The effectiveness of DOR agonists (DPDPE, deltorphin II and SNC80) is not changed by minocycline (Fig. 3A–F). Our results indicate that an important element of the effectiveness of opioid drugs in neuropathic pain is the activation of microglia. The lack of DOR receptors in these cells causes that DOR receptor-mediated analgesia is not weaker under neuropathic pain, in which there is a strong activation of microglia. Earlier inhibition of microglial activation by minocycline administration therefore did not influence the effect of DOR selective agonists. The above results indicate not only that minocycline potentiates analgesia after MOR and KOR agonists but also that DOR is a potentially important target in the search for new drugs that would be effective against neuropathic pain.
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Green), which is not sufficiently selective and can give false positive results. The TaqMan assay that we used is standardised and highly specific, and we confirmed the lack of DOR in microglia cell cultures by using Western blot and immunocytochemistry, therefore, our results are not consistent with those that were obtained by immunostaining by Turchan-Cholewo *et al.* [87] and Thorlin *et al.* [88]. Moreover studies by Turchan-Cholewo *et al.* [87] used commercial antibodies against DOR, which are no longer recommended for such studies. Due to the lack of selective DOR antibody a unique genetic mouse model was developed in order to investigate the distribution of DOR in the nervous system [16,89]. Opioid receptors have high degrees of homology, and antibodies may recognise other subtypes within the same family. DOR expression has also been observed on glial-like cells in the dentate gyrus [90] and rat cervical spinal cord [91], but none of these authors distinguished between astrocytes and microglia. Our results concerning the absence of DOR in microglia are consistent with recently published results of pharmacological studies that were conducted by Merighi *et al.* [84]. These authors have shown in primary microglia cell cultures that DPDPE, a DOR agonist, does not change the level of protein for PKC after LPS stimulation, while morphine and DAMGO up-regulate this kinase. The ineffectiveness of DPDPE in this study may be explained by the lack of DOR receptors in microglial cells. Horvath and DeLeo, [92] showed that selective agonists of DOR₁ DPDPE had no effect on microglial migration, which confirms our results that DOR, in contrast to MOR and KOR, is not present in microglial cells. DOR shows a functional profile that is distinct from that of MOR or KOR [93] and plays an important role in chronic pain, for example DOR knockout mice showed augmented neuropathic pain [94,95]. DOR agonists are poor analgesics in acute pain [96], but are highly effective following inflammatory or neuropathic pain [13,96,97,98,99].

Happel *et al.* [100] suggested in 2008 that morphine and DAMGO influence the immune system. For example, opioids alter macrophage functions and they modulate cytokine production and chemokine and chemokine receptor expression. Activation of proinflammatory chemokine receptors is known to down-regulate the analgesic functions of opioid receptors, and this enhances the perception of pain [101]. Horvath *et al.* [102] showed that morphine increases microglial migration by means of an interaction between MOR and P2X₄ receptors. This interaction is dependent on PI3K/Akt pathway activation [102]. Under neuropathic pain, the phosphorylation of p38 MAPK in microglia results in increased synthesis of the proinflammatory cytokines IL-1 β , IL-6, and TNF- α . Spinal blockade of these cytokines is known to attenuate neuropathic pain and morphine tolerance [74,103]. Therefore, in cases where MOR and KOR agonists target

microglial signalling by inhibiting the actions of chemokines (fractalkine, CCL2), ATP receptors (P2X₄, P2X₇), MMP-9, p38 MAPK, or/and proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) improve their effectiveness. However, this is not the case with DOR agonists. This difference deserves future exploration.

MOR agonists, especially morphine, still remain the drugs of choice for the treatment of neuropathic pain, despite the side effects and limited efficacy. It was shown that DOR agonists were effective in persistent pain [13,68,81,104,105] and the mechanisms underlying this analgesic action was probably linked with trafficking to the cell membrane or better receptor coupling to signalling effectors [104]. Nadal *et al.* [95] demonstrated that neuropathic pain was enhanced in delta-opioid receptor knockout mice. Some authors suggest a delta opioid agonists as a promising alternative to mu analgesics in the treatment of chronic pain [13,95,104,106]. Currently, research is being conducted on analgesic effects of new DOR ligands [107] and some of these substances are being tested in clinical trials [108].

The results of the present study document for the first time that DOR, in contrast to MOR and KOR, are not present in microglial cells (Figure 6). In conclusion, we provide evidence that minocycline not only diminishes neuropathic pain-related behaviour but also enhances the effectiveness of morphine and selective MOR and KOR opioid ligands under neuropathic pain conditions. Our findings lend support to the view that neuroimmunological changes in the spinal cord and DRG are important for opioid effectiveness in neuropathic pain. In our opinion, activated spinal microglia are key factors in not only the development of neuropathic pain but also in the different efficacies of opioid analgesics. Our results also suggest that DOR analgesia is not dependent on injury-induced microglial activation. We therefore suggest that DOR is an interesting target for the development of new drugs that would be effective against neuropathic pain.

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Author Contributions

Conceived and designed the experiments: JM KP-B ER KS BP. Performed the experiments: JM KP-B ER WM KS BP. Analyzed the data: JM KP-B ER WM KS BP. Contributed reagents/materials/analysis tools: JM KP-B ER WM KS BP. Wrote the paper: JM KP-B ER KS BP.

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