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Chemokines CCL2 and CCL7, but not CCL12, play a significant role in the development of pain-related behavior and opioid-induced analgesia

Klaudia Kwiatkowski, Katarzyna Popiolek-Barczyk, Anna Piotrowska, Ewelina Rojewska, Katarzyna Ciapała, Wioletta Makuch, Joanna Mika*

Institute of Pharmacology, Polish Academy of Sciences, Department of Pain Pharmacology, Krakow, Poland

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ABSTRACT

The complex neuroimmunological interactions mediated by chemokines are suggested to be responsible for the development of neuropathic pain. The lack of knowledge regarding the detailed pathomechanism of neuropathy is one reason for the lack of optimally efficient therapies. Recently, several lines of evidence indicated that expression of CCR2 is increased in spinal cord neurons and microglial cells after peripheral nerve injury. It was previously shown that administration of CCR2 antagonists induces analgesic effects; however, the role of CCR2 ligands in neuropathic pain still needs to be explained. Thus, the goal of our studies was to investigate the roles of CCL2, CCL7, and CCL12 in neuropathic pain development and opioid effectiveness. The experiments were conducted on primary glial cell cultures and two groups of mice: naive and neuropathic. We used chronic constriction injury (CCI) of the sciatic nerve as a neuropathic pain model. Mice intrathecally received chemokines (CCL2, CCL7, CCL12) at a dose of 10, 100 or 500 ng, neutralizing antibodies (anti-CCL2, anti-CCL7) at a dose of 1, 4 or 8 μg, and opioids (morphine, buprenorphine) at a dose of 1 μg. The pain-related behaviors were assessed using the von Frey and cold plate tests. The biochemical analysis of mRNA expression of glial markers, CCL2, CCL7 and CCL12 was performed using quantitative reverse transcriptase real-time PCR. We demonstrated that CCI of the sciatic nerve elevated spinal expression of *CCL2*, *CCL7* and *CCL12* in mice, in parallel with microglia and astroglial activation markers. Moreover, intrathecal injection of CCL2 and CCL7 induced pain-related behavior in naive mice in a dose-dependent manner. Surprisingly, intrathecal injection of CCL12 did not influence nociceptive transmission in naive or neuropathic mice. Additionally, we showed for the first time that intrathecal injection of CCL2 and CCL7 neutralizing antibodies not only attenuated CCI-induced pain-related behaviors in mice but also augmented the analgesia induced by morphine and buprenorphine. *In vitro* studies suggest that both microglia and astrocytes are an important cellular sources of the examined chemokines. Our results revealed the crucial roles of CCL2 and CCL7, but not CCL12, in neuropathic pain development and indicated that pharmacological modulation of these factors may serve as a potential therapeutic target for new (co)analgesics.

1. Introduction

Neuropathic pain is a chronic state resulting from sensory neurons damage in the peripheral and/or central nervous system (CNS). Various conditions may induce nerve injury, including tumors, mechanical injuries (e.g., postsurgical injury), demyelinating diseases (e.g., multiple sclerosis), metabolic disorders (e.g., diabetes mellitus), and viral infections (e.g., HIV-associated peripheral neuropathy, postherpetic neuralgia) and lead to the neuropathic pain [1,2]. The first-line drugs for mild neuropathic pain include antidepressants and anticonvulsants. With persisting and increasing pain, second- and third-line drugs are used, mainly opioids, which, despite their efficacy, may cause serious adverse effects and lead to abuse, diversion, or addiction. Thus, the use of polytherapy, using substances acting through different targets, is currently increasing [3]. As pharmacotherapy for neuropathic pain remains challenging, and given the complex and insufficiently understood mechanisms caused by different conditions underlying this phenomenon, there remains a lack of effective treatment for many patients. The identification of new molecular targets for potential analgesics or co-analgesics is needed, since such discoveries may contribute to the creation of effective and long-term therapies for neuropathic pain that are free of adverse effects, and improvement of patient quality of life is

* Corresponding author at: Institute of Pharmacology, Polish Academy of Sciences, Department of Pain Pharmacology, 12 Smetna Str., 31-343 Krakow, Poland.
E-mail address: joamika@if-pan.krakow.pl (J. Mika).

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now a healthcare priority.

Undoubtedly, the amplified neuronal response is critical for neuropathic pain development; however, extensive preclinical studies have also indicated that glial cells are extremely important [1,4,5]. It is now suggested that neuronal-glial interactions mediated by cytokines are crucial in the mechanism underlying different neuropathic pain states [6–10]. Among cytokines, chemokines from the CC subfamily play interesting roles [11]. It has been previously shown that CCR2, a specific G-protein-coupled receptor that binds several chemokines, including CCL2, CCL7 and CCL12 [11,12], is strongly upregulated in spinal neurons and microglia after peripheral nerve injury [13]. Recently, we have demonstrated that indirect modulation of the CCL2/CCR2 pathway by a microglial cell inhibitor (minocycline) as well as direct modulation by a CCR2 antagonist (RS504393) is an effective target for the treatment of neuropathic pain [14,15]. One of the most discussed CCR2 endogenous ligands in the context of neuropathic pain is CCL2. It was previously reported that CCL2 may promote spinal microglial activation leading to neuropathic pain development in different animal models [14,16]. Another interesting CC chemokine is CCL7, which is also increased within the spinal cord after nerve injury [17,18]; however, little is known about its role in nociception. CCL12 is a potent monocyte chemoattractant involved in allergic inflammation [19], but the role of CCL12 in neuropathic pain development is still unclear. The exact roles of endogenous ligands of CCR2 in the generation and maintenance of neuropathic pain require further explanation.

Therefore, the first goal of our studies was to investigate time course changes in the levels of *C1q*, *GFAP*, *CCL2*, *CCL7* and *CCL12* in the spinal cord in mice following chronic constriction injury (CCI) of the sciatic nerve. The next aim was to examine the role of CCL2, CCL7 and CCL12 in the generation of pain-related behaviors in naive mice. Then, we investigated the effects of a single intrathecal injection of CCL12, as well as the effects of CCL2 and CCL7 neutralizing antibodies, on neuropathic pain in CCI-exposed mice. Subsequently, we examined the effects of CCL2 and CCL7 neutralizing antibodies on the analgesic potency of morphine and buprenorphine in neuropathic mice. Additionally, using primary microglial and astroglial cell cultures, we analyzed the cellular source of the examined chemokines.

2. Materials and methods

2.1. Animals and ethical statement

Adult male Albino Swiss mice (20–25 g) were obtained from Charles River Laboratories International, Inc., Germany (total number of mice = 320). All animals were kept in cages lined with sawdust, in room with 12-h light cycles, and food and water available *ad libitum*. The experimental procedures were conducted according to recommendations of the National Institutes of Health (NIH) and the International Association for the Study of Pain (IASP) [20] and were approved by Ethical Committee of the Institute of Pharmacology of the Polish Academy of Sciences (permission numbers: 1277/2015, 1055/2013 and 75/2017). According to the 3R policy the number of animals was reduced to essential minimum.

2.2. Neuropathic pain model

We used chronic constriction injury (CCI) of the sciatic nerve as a neuropathic pain model. The procedure was performed according to Bennett and Xie [21] under isoflurane anesthesia, as was described in our previous papers [22,23]. All operated mice developed long-lasting neuropathic pain-related behaviors.

2.3. Drug administration

Recombinant mouse CCL2/CCL7/CCL12 protein, normal rabbit IgG and mouse CCL2/CCL7 antibody were purchased from R&D Systems (Minneapolis, USA); morphine hydrochloride from Fagron (Krakow, Poland), and buprenorphine from Polfa S.A. (Warsaw, Poland). All substances were dissolved in water for injection and administered intrathecally. The control group received water for injection. Intrathecal administrations were performed using the lumbar puncture technique according to Hylden and Wilcox [24], as reported in our previous papers [25–27].

Reconstituted CCL2, CCL7 and CCL12 in naive mice were administered once at the following doses: 10, 100, and 500 ng/5 µl. The concentrations of the examined chemokines were selected based on our previous experiments investigating other chemokines [27,26,28]. Reconstituted CCL12 was injected once at the following doses: 500 and 1000 ng/5 µl, and neutralizing antibodies against CCL2 or CCL7 were administered once at the following doses: 1, 4 and 8 µg/5 µl based on our previous studies [26,27]. Behavioral assessments were performed 1, 4 and/or 24 h after chemokine or neutralizing antibody administration. In order to eliminate the possibility that high concentrations of proteins non-specifically modulate the nociceptive transmission, a control antibody at a dose of 8 µg/5 µl (normal rabbit IgG) was administered intrathecally, and behavioral tests were conducted on the same experimental schedule.

For co-administration with opioids the CCL2 or CCL7 neutralizing antibodies were administered once intrathecally at the dose 8 µg/5 µl based on previously obtained results. Then 1 h (CCL2) or 4 h (CCL7) after the neutralizing antibody injection a single injection of morphine or buprenorphine (1 µg/5 µl) was performed, and behavioral tests were repeated 30 min after opioid administration. The time point at which the opioid was administered was chosen based on the neutralizing antibody effectiveness determined in the previous experiment, and thus the time point was different for each chemokine.

2.4. Behavioral tests

2.4.1. Von Frey test

Hypersensitivity to mechanical stimuli was measured using calibrated nylon monofilaments (0.6–6 g; Stoelting, USA). The animals were placed in plastic cages with wire net floors. Then, von Frey filaments of increasing strength were applied to the midplantar surface of the hind paw, as described previously [22,23,28]. In naive mice, both hindpaws were tested, and in CCI-exposed mice, the ipsilateral paw was tested two times. The behavioral tests were always conducted in the same order: first von Frey and then the cold plate test.

2.4.2. Cold plate test

Hypersensitivity to thermal stimuli was measured using a cold plate apparatus (Cold/Hot Plate Analgesia Meter No. 05044, Columbus Instruments, USA) as described previously [22,23,28]. The temperature was kept at 2 °C, and the cut-off latency was 30 s. Animals were placed on the cold plate, and the time to lift the hind paw was recorded. In naive mice, both hindpaws were observed, and in CCI-exposed mice, only the injured paw was observed, as it reacts earlier.

2.5. Primary microglial and astroglial cell cultures

In vitro studies was conducted using primary microglial and astroglial cell cultures prepared from the cerebral cortex obtained from 1-day-old Wistar rats according to Zawadzka and Kaminska [29], and as described in our previous papers [30,31]. The cells were plated at a seeding density of 3×10^5 cells/cm^2 (in DMEM, high glucose, GlutaMAX™ with 10% FBS, heat inactivated, 0.1 mg/ml streptomycin, and 100 U/ml penicillin; Gibco, New York, USA) in poly-L-lysine-coated, 75-cm^2 culture flasks and grown in a 37 °C incubator with a humidified atmosphere of 5% CO_2 in air. After 4 days, the culture medium was changed and cultures were grown for the next 5 days. On day 9, the loosely attached microglial cells to the monolayer were harvested by gentle shaking and cell viability was determined (TC20-automated cell

counter; Bio-Rad, Poland). After the medium was changed, the procedure was repeated on day 12. Astroglia cells were prepared by shaking the flasks, after 12 days in culture, at 37 °C for 24 h (200 rpm). After 3 days, the adherent cells were trypsinized (0.005% trypsin-EDTA solution, Sigma-Aldrich) and seeded into fresh flasks with a medium change 24 h after plating. The microglia/astroglia cells were seeded into plates (2×10^5 cells/well in a 24-well plates) and incubated for 48 h. Both cell cultures were treated with minocycline (20 μM) or fluorocitrate (1 nM) 30 min before administration of lipopolysaccharide from Escherichia coli 0111:B4 (100 ng/ml) and incubated for 24 h. To identify the microglia and astrocytes the IBA1 (anti-IBA1, 1:500, Santa Cruz) and GFAP (anti-GFAP, 1:500, Santa Cruz, CA, USA) were used, respectively. We obtained homogeneities of our cultures similar to those reported by Zawadzka and Kaminska [29].

2.6. Analysis of gene expression by RT-qPCR

For *in vivo* studies, tissue derived from the ipsilateral fragments of the lumbar (L4-L6) spinal cord for quantitative real-time PCR (RT-qPCR) analysis was collected immediately after decapitation from naive and CCI-induced mice on days 2, 7, and 14 after operation. Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, USA) based on a protocol described by Chomczynski and Sacchi [32] and as described in our previous papers [15,17,33]. RNA quality and concentration were measured by a DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, USA). The Omniscript RT Kit (Qiagen Inc., Hilden, Germany), oligo (dT16) primer (Qiagen Inc., Hilden, Germany) and RNAse inhibitor (rRNasin, Promega, Mannheim, Germany) were used for reverse transcription performed using 1 μg of total RNA for tissue and 500 ng of total RNA for cultured cells. The obtained cDNA was diluted 1:10 with RNase-/DNase-free H_2O. RT-qPCR reaction was conducted with ~50 ng of cDNA from each sample using Assay-On-Demand TaqMan probes (Applied Biosystems, Foster City, USA), and run in an iCycler device (Bio-Rad, Hercules, Warsaw, Poland). The following TaqMan primers and probes were used: Mm03024075_m1 (*Hprt*, hypoxanthine-guanine phosphoribosyltransferase); Mm00432142_m1 (*C1q*); Mm01253033_m1 (*GFAP*); Mm00441242_m1 (*Ccl2*); Mm00443113_m1 (*Ccl7*); Mm01617100_m1 (*Ccl12*); Rn01527840_m1 (*Hprt*, hypoxanthine-guanine phosphoribosyltransferase); Rn00580555_m1 (*Ccl2*); Rn01467286_m1 (*Ccl7*); and Rn01464638_m1 (*Ccl12*). The *Hprt* gene served as an endogenous control, as it did not reveal any significant changes across groups.

2.7. Statistical analysis

Behavioral data (Figs. 1 and 4–8) are presented as the mean ± SEM (n = 5–9 animals per group). The results were evaluated using one-way ANOVA followed by Bonferroni's multiple comparisons *post hoc* test of selected pairs measured separately at each time point. Additionally, if applicable, the results were evaluated using two-way ANOVA to determine the time × drug interaction, and the description is presented in the Sections 3.3 and 3.5. **RT-qPCR analysis data** (Fig. 2 n = 9 and Fig. 3 n = 6–8 samples per group) are presented as the fold change relative to the controls (naive mice or vehicle-treated non-stimulated cells) ± SEM. Differences between groups were evaluated using a *t*-test (Fig. 3) or one-way ANOVA followed by Bonferroni's multiple comparisons *post hoc* test (Figs. 2 and 3). All graphs and statistical analyses were performed using Prism (GraphPad Software, Inc., USA). Significant differences between groups are indicated when $P < 0.05$.

3. Results

3.1. Behavioral assessment and time-dependent changes in spinal CCL2, CCL7, CCL12 and glial markers levels in CCI-exposed mice

All examined mice exhibited strong tactile (Fig. 1A) and thermal (Fig. 1B) hypersensitivity, relative to control (naive) group, 2, 7 and 14 days after CCI.

We demonstrated that *C1q* mRNA levels were already increased 2 days after CCI and then gradually decreased on day 7 and 14 (Fig. 2A). Similarly, the *GFAP* mRNA levels were also upregulated 2 days after CCI, then decreased with time (Fig. 2B).

Parallelly, we demonstrated that *CCL2* mRNA levels were already increased 2 days after CCI and then gradually decreased on day 7; however, *CCL2* levels were still significantly higher relative to naive mice until day 14 (Fig. 2C). The strongest changes were observed for *CCL7*; mRNA levels strongly increased 2 days after CCI and then, similar to *CCL2*, gradually decreased from the 7th to the 14th day after sciatic nerve injury relative to naive mice (Fig. 2D). The highest *CCL12* mRNA levels were observed on the 2nd day after CCI then gradually decreased. However, a significant increase in *CCL12* levels relative to naive mice was also observed 7 days after CCI, in contrast to levels on day 14 post-CCI (Fig. 2E).

3.2. The influence of minocycline and fluorocitrate on CCL2, CCL7 and CCL12 in vehicle- and LPS-treated microglial and astroglial cell cultures

We detected the expression of *CCL2* mRNA in nonstimulated microglial (Fig. 3A) and astroglial (Fig. 3B) cell cultures. Minocycline reduced the mRNA levels of *CCL2* in nonstimulated microglia (Fig. 3A) but not astroglia (Fig. 3B) relative to vehicle-treated, nonstimulated cells. We did not observe any significant changes in *CCL2* levels in fluorocitrate-treated, nonstimulated microglia and astroglia relative to controls (Fig. 3A and B). Moreover, *CCL2* mRNA levels were strongly increased after LPS treatment relative to controls in both studied cell cultures (Fig. 3A and B). Minocycline decreased *CCL2* levels in LPS-stimulated cells relative to vehicle-treated LPS-stimulated cells in microglia (Fig. 3A) and astroglia (Fig. 3B). Fluorocitrate diminished the mRNA levels of *CCL2* only in LPS-stimulated astroglia relative to vehicle-treated LPS-stimulated cells (Fig. 3B).

Additionally, the observed mRNA levels of *CCL7* significantly decreased after minocycline treatment in nonstimulated microglial but not astroglial cell cultures (Fig. 3C and D). Fluorocitrate treatment did not influence *CCL7* levels in any nonstimulated cell cultures (Fig. 3C and D). After LPS stimulation, we observed strong upregulation of *CCL7* relative to control cells in both microglia and astroglia. Minocycline treatment significantly reduced *CCL7* levels in LPS-stimulated microglia and astroglia (Fig. 3C and D). Fluorocitrate treatment did not influence *CCL7* levels in both LPS-stimulated microglial and astroglial cell cultures (Fig. 3C and D).

In nonstimulated microglia and astroglia, we also detected the expression of *CCL12* (Fig. 3E and F). Minocycline significantly decreased *CCL12* levels in nonstimulated microglia (Fig. 3E) but not in astroglia (Fig. 3F) relative to control cells. Interestingly, fluorocitrate treatment potentiated the expression levels of *CCL12* in astroglia relative to vehicle-treated, nonstimulated cells, whereas it did not influence *CCL12* levels in microglial cell cultures (Fig. 3E and F). The expression of *CCL12* was strongly increased after LPS stimulation; however, none of tested drugs affected *CCL12* levels in both types of cell cultures (Fig. 3E and F).

3.3. Effects of a single intrathecal injection of CCL2, CCL7 or CCL12 on pain-related behaviors in naive mice

Single intrathecal injection of CCL2 induced hypersensitivity to mechanical (Fig. 4A) and thermal (Fig. 4B) stimuli in a dose-dependent

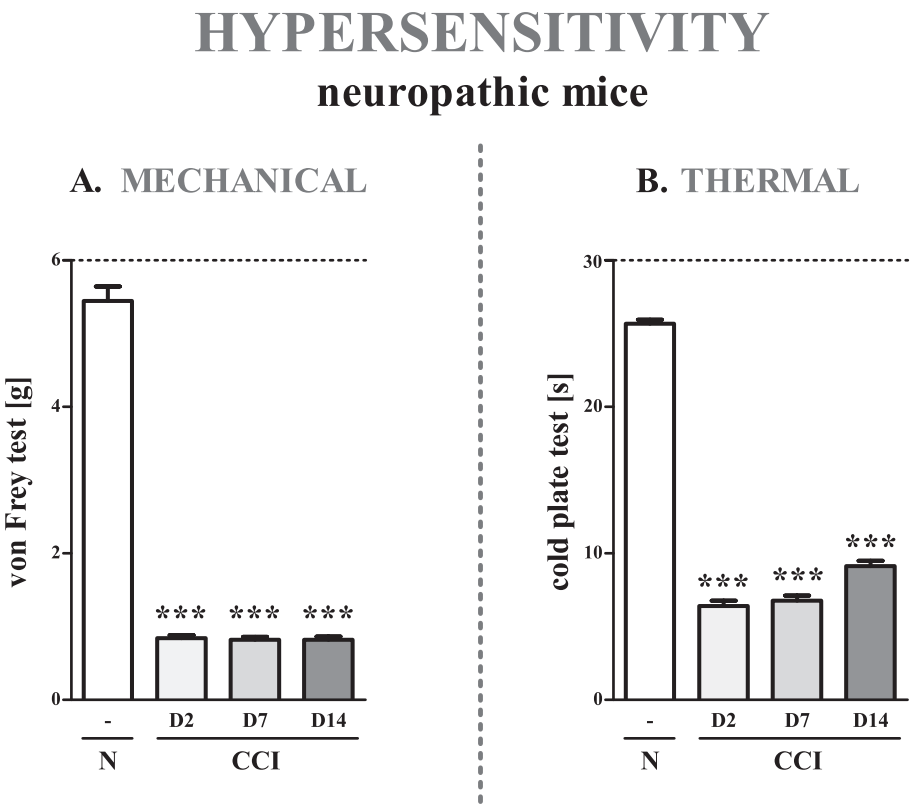

Fig. 1. Time-dependent changes in development of mechanical (A) and thermal (B) hypersensitivity 2, 7 and 14 days after CCI. Data are presented as the mean ± SEM (Naive n = 9; CCI-exposed n = 9). The horizontal dotted line represents the cut-off value. The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs; $^{***}P < 0.001$ comparing naive mice vs. each group at each time point. *Abbreviations: CCI, chronic constriction injury; D, day; N, naive.*

manner. Reactions to non-noxious stimuli were observed 1 h after the injection of 10-ng, 100-ng and 500-ng of CCL2. After 4 h, CCL2-induced pain decreased but was still observed for the two higher doses (Fig. 4A). In the cold plate test, hypersensitivity to thermal stimuli was detected 1 h after the injection of the two higher doses but not with the 10-ng dose (Fig. 4B). After 4 h, thermal hypersensitivity was even stronger for the group receiving the 500-ng dose and slightly lower for the group receiving the 100-ng dose (Fig. 4B). The pronociceptive effects of CCL2 completely disappeared after 24 h (Fig. 4A and B). Moreover, our study provided the first evidence that a single intrathecal injection of CCL7 induced the development of hypersensitivity in a dose-dependent manner, as measured by the von Frey (Fig. 4C) and cold plate (Fig. 4D) tests. Painful reactions to mechanical and thermal stimuli were observed 1 h after the injection of the 100-ng and 500-ng doses. We did not observe these effects for the lowest tested dose of CCL7 (Fig. 4C and D). In contrast to CCL2, the pronociceptive effects of CCL7 were completely abolished after 4 h in both tests (Fig. 4C and D). Surprisingly, we did not observe any pain-related behaviors in the groups of mice receiving a single intrathecal injection of CCL12 at any dose used for the previous chemokines (Fig. 4E and F) nor at a much higher dose, 8 μg/5 μl (data not shown on the graph), in both tests.

Two-way ANOVA confirmed a significant interaction between CCL2 or CCL7 treatment and the time points investigated in both the von Frey (for CCL2: $F_{9,124} = 5.516$; $P < 0.0001$, for CCL7: $F_{9,88} = 12.28$; $P < 0.0001$) and cold plate (for CCL2: $F_{9,103} = 12.24$; $P < 0.0001$, and for CCL7: $F_{9,73} = 5.97$; $P < 0.0001$) tests. CCL2 significantly enhanced mechanical ($F_{3,124} = 50.21$; $P < 0.0001$) and thermal ($F_{3,103} = 47.64$; $P < 0.0001$) hypersensitivity, showing its pronociceptive dose-dependent effect in the von Frey and cold plate test. Additionally, CCL7 significantly enhanced mechanical ($F_{3,88} = 37.55$; $P < 0.0001$) and thermal ($F_{3,73} = 12.78$; $P < 0.0001$) hypersensitivity, showing its pronociceptive dose-dependent effect in both tests.

3.4. The effects of a single intrathecal injection of CCL12 on neuropathic pain in CCI-exposed mice

To determine if CCL12 has some antinociceptive properties, we performed single intrathecal injections of CCL12 in neuropathic mice. We did not observe any significant changes in neuropathic-pain related behaviors in mice receiving CCL12 at a dose of 500 ng/5 μl or in the group receiving CCL12 at a dose of 1000 ng/5 μl relative to vehicle-treated animals in both tests (Fig. 5A and B). Additionally, we injected CCL12 at a dose of 8 μg/5 μl; however, it also did not reveal any beneficial effects in CCI-exposed mice (data not shown on the graph).

3.5. The effects of a single intrathecal injection of a CCL2 or CCL7 neutralizing antibody on neuropathic pain in CCI-exposed mice

Single intrathecal injections of CCL2 neutralizing antibody at doses of 1 and 4 μg did not influence the development of CCI-induced hypersensitivity at any time point during the experiment in both tests (Fig. 6A and B). Significant antinociceptive effects were only observed after the administration of the highest dose (8 μg/5 μl) of CCL2 neutralizing antibody (Fig. 6A and B). The strongest analgesic effect was observed 1 h after neutralizing antibody injection, then gradually decreased; however, the antinociceptive effect was significant until 4 h after injection. Two-way ANOVA confirmed a significant interaction between the investigated treatment and time points investigated in the von Frey ($F_{9,108} = 14.86$; $P < 0.0001$) and cold plate ($F_{9,105} = 6.04$; $P < 0.0001$) tests. The CCL2 neutralizing antibody significantly reduced mechanical ($F_{3,108} = 19.16$; $P < 0.0001$) and thermal ($F_{3,105} = 11.38$; $P < 0.0001$) hypersensitivity, showing its analgesic dose-dependent effect in both tests.

A single intrathecal injection of CCL7 neutralizing antibody at a dose of 1 μg did not influence the development of CCI-induced hypersensitivity at any time during both tests (Fig. 6C and D). Strong antinociceptive effects were observed after injection of the two highest doses; however, the effects in the group receiving the dose of 4 μg was

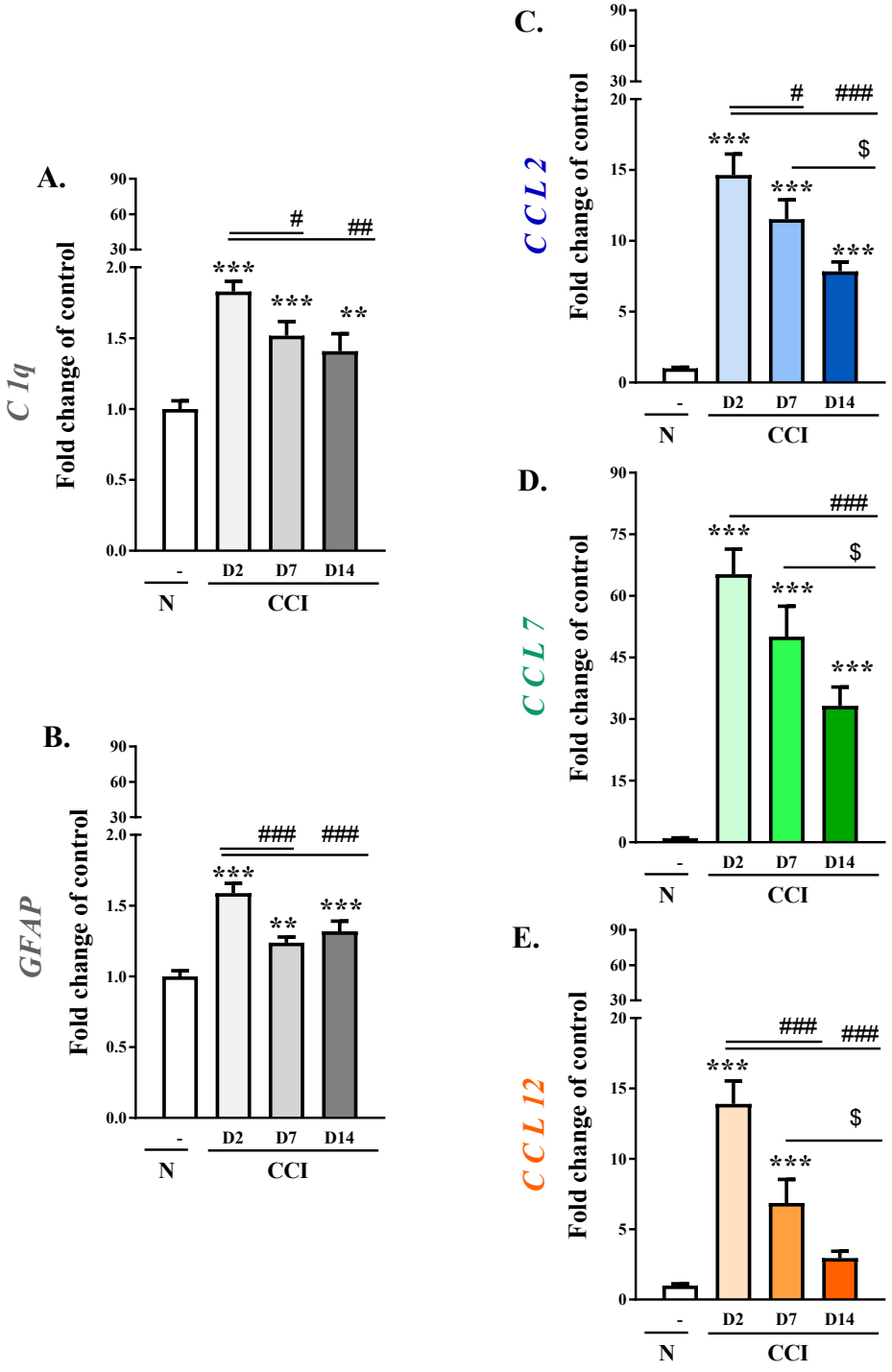

Fig. 2. Time-dependent changes in the spinal mRNA levels of *C1q* (A), *GFAP* (B), *CCL2* (C), *CCL7* (D) and *CCL12* (E) associated with neuropathic pain development (CCI model). Data are presented as the fold change relative to control mice (naive) ± SEM (Naive n = 9; CCI-exposed n = 9). The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with naive mice; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with the D2 group; $^{\$}P < 0.05$ compared with the D7 group. *Abbreviations: CCI, chronic constriction injury; D, day; N, naive.*

nearly two times weaker than that observed in the group treated with 8 µg (Fig. 6C and D). The strongest effect was observed 4 h after neutralizing antibody injection. Two-way ANOVA confirmed a significant interaction between the investigated treatment and the time points investigated in both the von Frey ($F_{9,112} = 3.82$; $P < 0.0001$) and cold plate ($F_{9,111} = 7.162$; $P < 0.0001$) tests. The CCL7 neutralizing antibody significantly reduced mechanical ($F_{3,112} = 47.79$; $P < 0.0001$) and thermal ($F_{3,111} = 13.45$; $P < 0.0001$) hypersensitivity, showing its analgesic dose-dependent effect in both tests.

The injection of a control antibody did not influence the development of CCI-induced mechanical and thermal hypersensitivity at any time point in both tests (Fig. 6A–D).

3.6. The effects of single intrathecal injection of CCL2 or CCL7 neutralizing antibodies on morphine analgesic potency in CCI-exposed mice

The combined administration of CCL2 neutralizing antibody (8 µg/5 µl) and morphine (1 µg/5 µl) resulted in substantially more effective

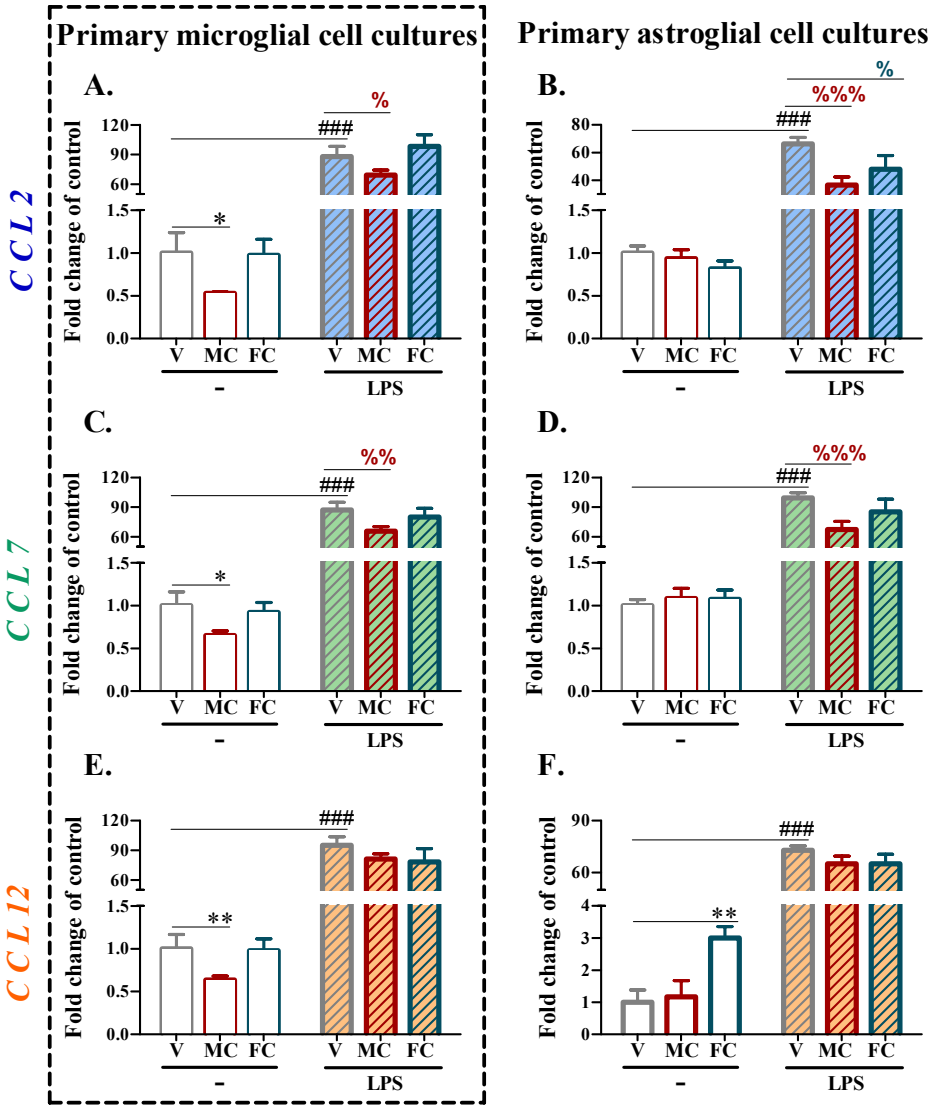

Fig. 3. The influence of minocycline (MC; 20 μM) or fluorocitrate (FC; 1 nM) on *CCL2* (A, B), *CCL7* (C, D), and *CCL12* (E, F) mRNA levels in non-stimulated and LPS-stimulated primary microglial (A, C, E) and astroglial (B, D, F) cell cultures. The data are presented as the fold change relative to the control group (V-treated nonstimulated cells), expressed as the mean ± SEM (n = 6–8 samples per group). The results were evaluated using t-tests for the comparison of V- vs. MC/FC-treated non-stimulated cells; $^{*}P < 0.05$ and $^{**}P < 0.01$. One-way ANOVA followed by Bonferroni's *post hoc* test was used to assess differences between V- and MC/FC-treated nonstimulated and LPS-stimulated cells; $^{###}P < 0.001$ V-treated nonstimulated vs. LPS-stimulated; $^{\%}P < 0.05$, $^{\%\%}P < 0.01$, $^{\%\%\%}P < 0.001$ V- vs. MC/FC-treated LPS-stimulated. *Abbreviations: FC, fluorocitrate; LPS, lipopolysaccharide; MC, minocycline; V, vehicle.*

analgesia to mechanical and thermal painful stimulation relative to groups of mice receiving only one of the examined drugs (Fig. 7B and C). Similar beneficial effects were observed in the group of mice receiving the CCL7 neutralizing antibody (8 μg/5 μl) with morphine (1 μg/5 μl). The combination of these two substances resulted in the strongest analgesic effects relative to mice treated with the CCL7 neutralizing antibody or morphine alone (Fig. 7D and E).

3.7. The effects of a single intrathecal injection of CCL2 or CCL7 neutralizing antibody on buprenorphine analgesic potency in CCI-exposed mice

Administration of the CCL2 neutralizing antibody (8 μg/5 μl) resulted in significant improvement in buprenorphine-induced analgesia to mechanical and thermal painful stimulation relative to groups of mice receiving only one of the examined drugs (Fig. 8B and C). In the group of mice receiving CCL7 neutralizing antibody (8 μg/5 μl) and then buprenorphine (1 μg/5 μl), we observed more effective analgesia only to mechanical stimuli relative to groups of animals receiving only one of the examined drugs. These effects were not observed in the cold plate test (Fig. 8D and E).

4. Discussion

Our studies showed that sciatic nerve injury performed in mice induced the upregulation of *CCL2*, *CCL7* and *CCL12* during the early phase of neuropathic pain development. The strongest changes were observed for CCL7. We observed that these changes were associated with the increase in the level of microglial (*C1q*) and astroglial (*GFAP*) activation markers. Additionally, our *in vitro* studies indicated that both microglial and astroglial cells may produce each of the examined chemokines. Thus, we suggest that activated glial cells are important source of CCL2, CCL7 and CCL12 in the CNS under neuroinflammation, however this still needs further investigation. Moreover, our data demonstrated that intrathecal injection of CCL2 and CCL7 induced pain-related behavior in naive mice in a dose-dependent manner. Surprisingly, the intrathecal injection of CCL12 did not modulate nociceptive transmission in naive, as well as neuropathic, mice. Additionally, our data provide the first evidence that the intrathecal injection of CCL2 or CCL7 neutralizing antibody not only attenuated CCI-induced neuropathic pain-related behaviors but also enhanced the analgesic potency of morphine and buprenorphine to mechanical and/or thermal stimulation.

The expression of CCR2 has been shown in spinal microglia, astrocytes and neurons [8,34,35], and several chemokines bind this receptor, including CCL2, CCL7 and CCL12 [8,36]. Recent studies

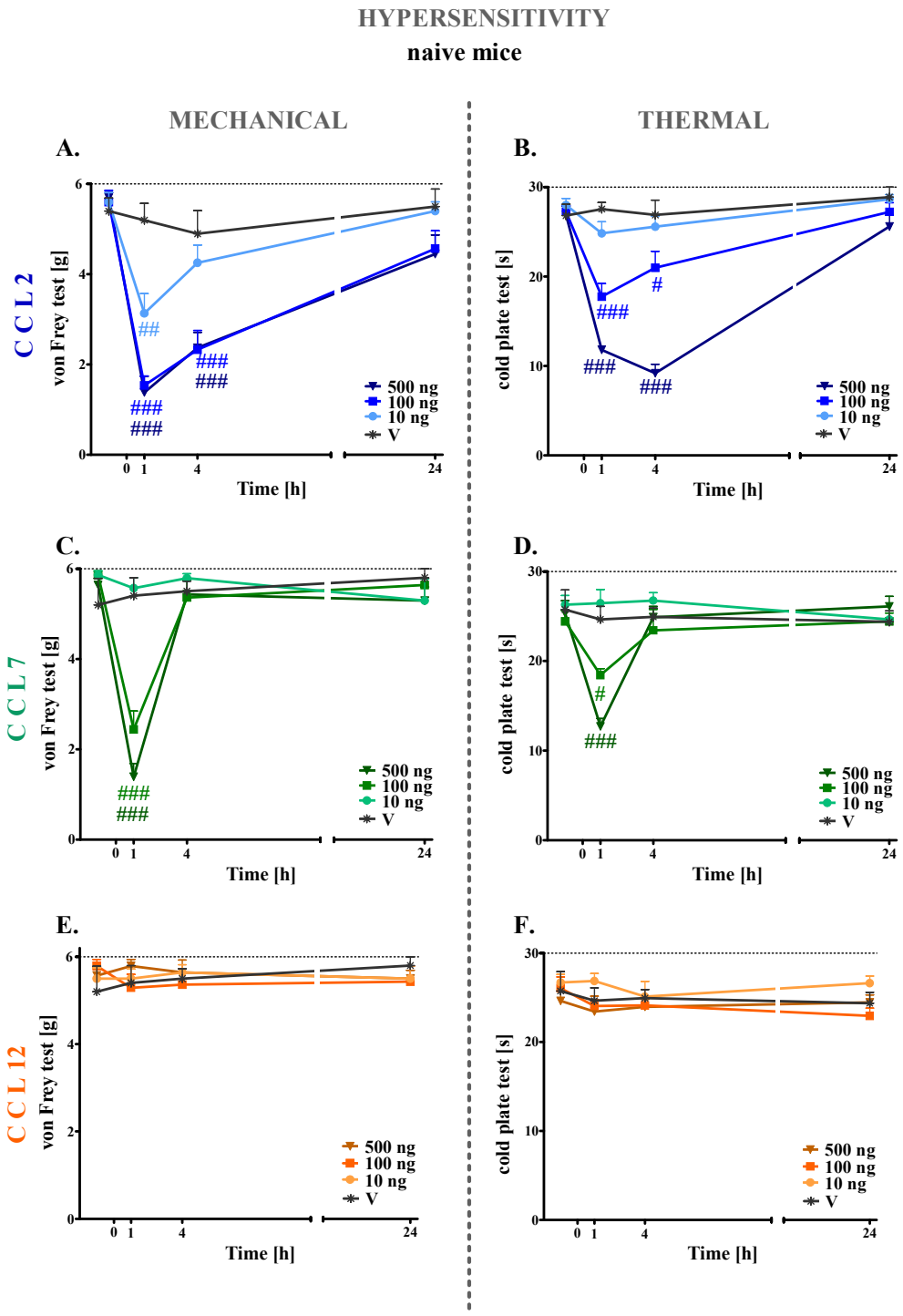

Fig. 4. The effects of CCL2 (A, B), CCL7 (C, D) and CCL12 (E, F) at a dose of 10, 100, or 500 ng/5 µl on mechanical (A, C, E), and thermal (B, D, F) hypersensitivity. Data are presented as the mean ± SEM (n = 5 V-treated; n = 7 CCL-treated mice per group). The horizontal dotted line shows the cut-off value. The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs measured separately at each time point; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ comparing V-treated mice vs. all groups at each time points. *Abbreviations: V, vehicle.*

revealed an increase in CCL2 levels after peripheral nerve injury in various structures of the nervous system, which was associated with the strong activation of glial cells [14,15,37] in animals with neuropathic pain. This is in agreement with our current results showing that the mRNA levels of spinal *CCL2* were strongly enhanced 2 and 7 days after CCI of the sciatic nerve and remained upregulated until day 14. Moreover, our current pharmacological studies give evidence that a single intrathecal injection of CCL2 induced pain-related behaviors in naive mice in a dose-dependent manner. These results agree with those of other studies showing that CCL2 injection induces long-lasting thermal hypersensitivity [35,38] and leads to microglia activation [13,39]. It was previously suggested that CCL2 released by neurons strongly induces spinal microglia activation, and phosphorylation of p38 MAPK leads to the production of pronociceptive cytokines [8,9]. Our recently published data showed that a CCR2 antagonist (RS504393) reduced microglial activation and protein levels of spinal CCL2, resulting in the diminished production of well-known pronociceptive factors, such as IL-1beta, IL-18, IL-6 and iNOS [14,15]. As a

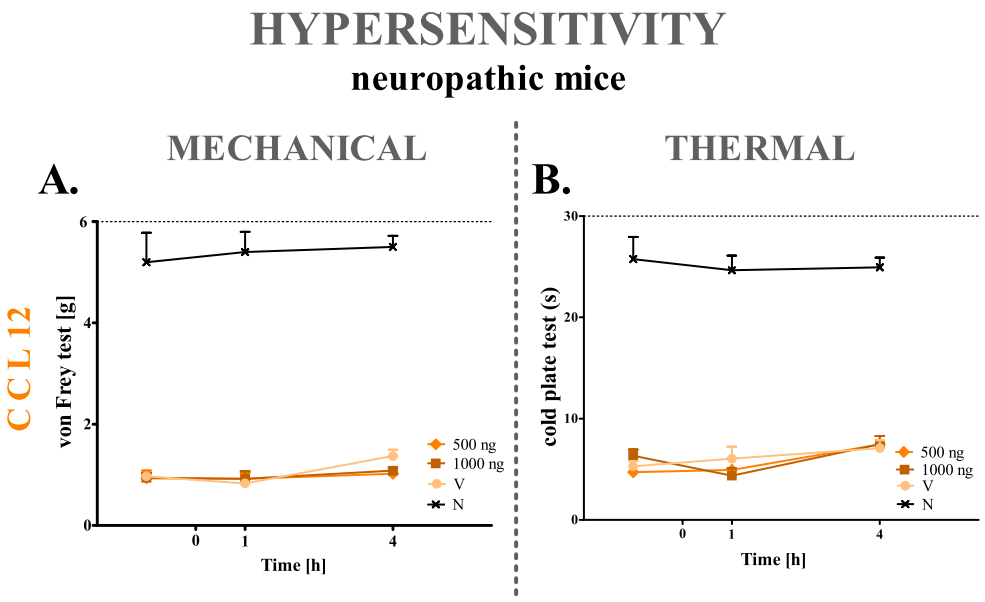

Fig. 5. The influence of CCL12 (500 or 1000 ng/5 µl) on mechanical (A), and thermal (B) hypersensitivity 5–6 days after CCI. Data are presented as the mean ± SEM (n = 5 V-treated; n = 7 CCL-treated mice per group). The horizontal dotted line shows the cut-off value. The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs measured separately at each time point. *Abbreviations: N, naive; V, vehicle.*

result, administration of RS504393, as well as other CCR2 antagonists (e.g., RS102895 and INCB3344), resulted in attenuation of neuropathic pain induced by peripheral nerve injury [14,15,40,41]. Astrocytes are another source of CCL2, which, after release, may activate CCR2 receptors localized on the spinal neurons and provoke central sensitization by NMDA receptors through ERK pathway activation [35]. Our *in vitro* studies revealed that not only astroglial but also microglial cells are able to produce CCL2. In these studies, we also treated primary cell cultures with glial modulators, i.e., minocycline and fluorocitrate. Minocycline inhibits p38 MAPK phosphorylation and thus may directly reduce both microglial and astroglial cell activation [42,31]. This effect is strongly associated with the attenuation of neuropathic pain symptoms in rodents [43,33], while fluorocitrate selectively affects astrocyte metabolism through the impairment of carbon flux through the Krebs cycle [44]. In the current study, we demonstrated that minocycline effectively diminished *CCL2* levels in both nonstimulated and LPS-stimulated cell cultures, which suggest that activated glial cells are an important source of CCL2 in the CNS under neuroinflammation.

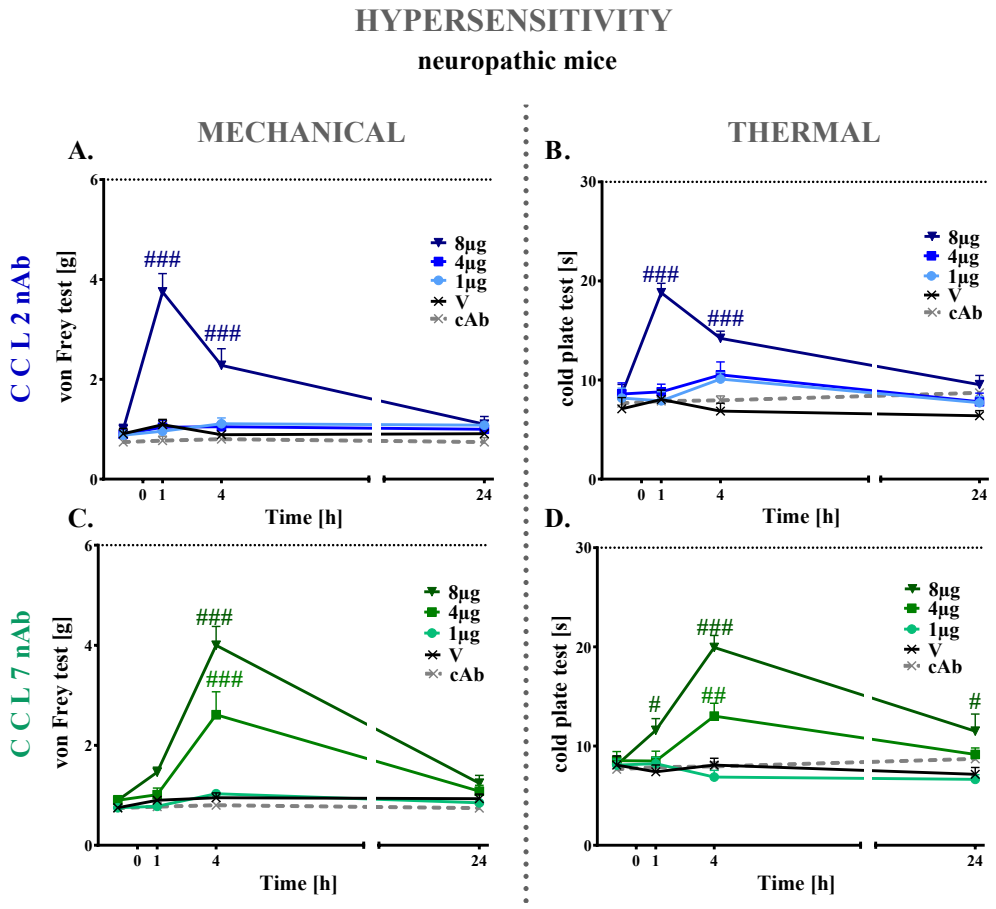

Fig. 6. The influence of CCL2 (A, B) and CCL7 (C, D) neutralizing antibodies at a dose of 1 µg, 4 µg, or 8 µg/5 µl, and control IgG at a dose of 8 µg/5 µl (A–D) on mechanical (A, C) and thermal (B, D) hypersensitivity 5–6 days after CCI. Data are presented as the mean ± SEM (n = 7 V-treated; n = 8 nAb CCL-treated mice per group). The horizontal dotted line shows the cut-off value. The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs measured separately at each time point; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ comparing V-treated vs. nAb-treated CCI-exposed mice at each time point. *Abbreviations: cAb, control IgG antibody; nAb, neutralizing antibody; V, vehicle.*

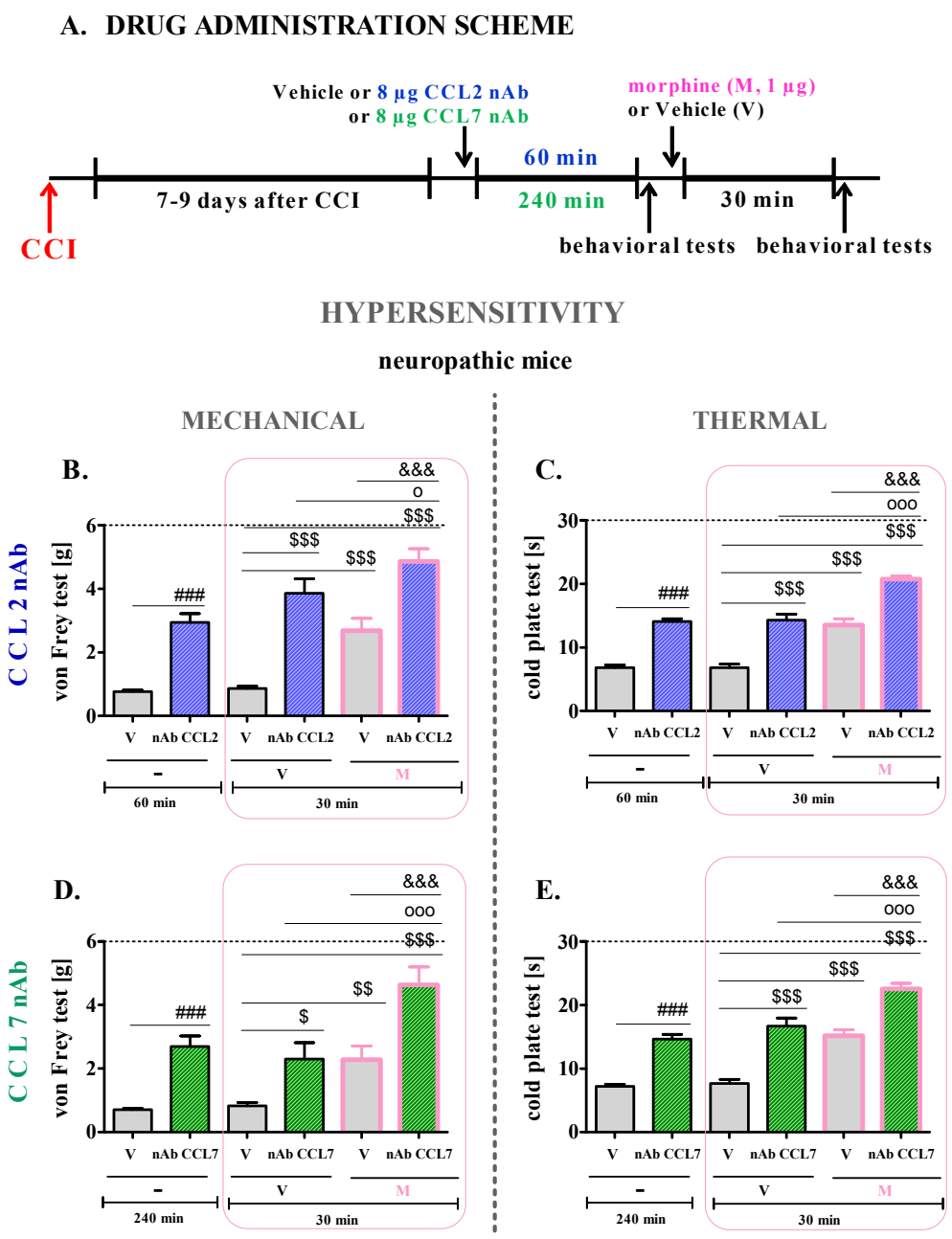

Fig. 7. The influence of a CCL2 (B, C) or CCL7 (D, E) neutralizing antibody on morphine (1 µg/5 µl) effectiveness 7–9 days after CCI. Data are presented as the mean ± SEM (n = 6 V-treated; n = 8 nAb CCL-treated mice per group). The horizontal dotted line shows the cut-off value. The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs; $^{###}P < 0.001$ comparing V-treated vs. nAb-treated CCI-exposed mice; $^{\$}P < 0.05$, $^{\$\$} < 0.01$ and $^{\$\$\$}P < 0.001$ comparing V + V-treated vs. nAb + V-treated/V + M-treated/nAb + M-treated CCI-exposed mice; $^{o}P < 0.05$ and $^{ooo}P < 0.001$ comparing nAb + V-treated vs. nAb + M-treated CCI-exposed mice; $^{\&\&\&}P < 0.001$ comparing V + M-treated vs. nAb + M-treated CCI-exposed mice. *Abbreviations: CCI, chronic constriction injury; M, morphine; nAb, neutralizing antibody; V, vehicle.*

Fluorocitrate reduced the mRNA levels of *CCL2* only in LPS-stimulated astrocytes. It seems that CCL2, produced by activated glia, directly influences spinal neurons and potentiates thermal and mechanical hypersensitivity. Furthermore, in our current study, we demonstrated that intrathecal injection of CCL2 neutralizing antibody effectively reversed neuropathic pain-related behavior in CCI-exposed mice. Others have shown that injection of CCL2 neutralizing antibody prevents microglia activation induced by peripheral nerve injury [13,39]. All of these data indicate the critical role of CCL2 in spinal glial activation and thus in the development of neuropathic pain.

CCL7 shares 60–71% homology with CCL2 and therefore also binds CCR2 [45]. Under physiological conditions, CCL7 stimulates immune cell trafficking to sites of inflammation [46]. Since CCL7 is mainly responsible for mediating inflammatory responses, it was suggested that this chemokine may majorly contribute to the development of chronic diseases [46]. It was previously shown that partial sciatic nerve ligation in mice induces strong upregulation of *CCL7* mRNA levels [47]. In our current study, we observed enhanced spinal expression of *CCL7* two days after nerve injury, and *CCL7* levels were significantly increased until the 14th day after CCI. Moreover, Imai et al. demonstrated that increased CCL7 immunoreactivity in the spinal dorsal horn was co-localized with an astrocyte marker (GFAP) but not with a microglial marker (IBA-1) or neuronal markers. Neutralization by a CCL7 antibody suppressed nerve injury-evoked microglial cell activation [47]. Thus, the authors suggested that spinal microglia might be activated through the CCR2 receptor by CCL7 released from astrocytes [47]. On the other hand, Ke et al. [18] demonstrated that neuron-derived CCL7 may promote astrocyte proliferation under neuropathic pain. Our *in vitro* studies revealed that both microglia and astrocytes may release CCL7, and minocycline significantly reduced *CCL7* mRNA levels in both LPS-stimulated microglia and astrocytes. In our opinion, the long-lasting upregulation of CCL7 induced by peripheral nerve injury is associated with enhanced multidirectional communication between neurons, microglia, and astrocytes in the spinal cord, which leads to sensory neuron sensitization. These interactions generally result in the generation and maintenance of neuropathic pain [17,47]. Here, we demonstrated that

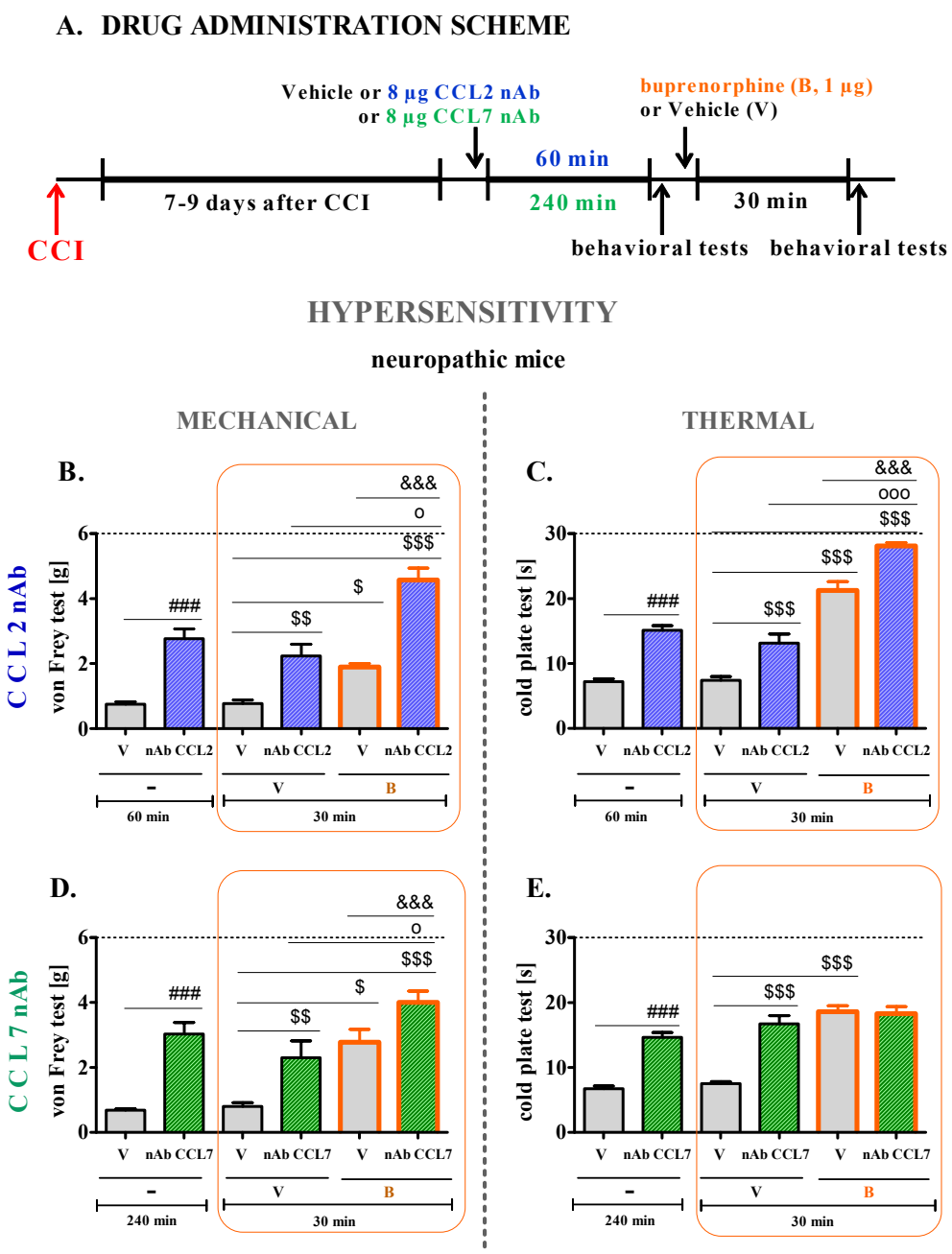

Fig. 8. The influence of a CCL2 (B, C) or CCL7 (D, E) neutralizing antibody on buprenorphine (1 µg/5 µl) effectiveness 7–9 days after CCI. Data are presented as the mean ± SEM (n = 6 V-treated; n = 8 nAb CCL-treated mice per group). The horizontal dotted line shows the cut-off value. The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs; $^{###}P < 0.001$ comparing V-treated vs. nAb-treated CCI-exposed mice; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ comparing V + V-treated vs. nAb + V-treated/V + B-treated/nAb + B-treated CCI-exposed mice; $^{o}P < 0.05$ and $^{ooo}P < 0.001$ comparing nAb + V-treated vs. nAb + B-treated CCI-exposed mice; $^{\&\&\&}P < 0.001$ comparing V + B-treated vs. nAb + B-treated CCI-exposed mice. *Abbreviations: B, buprenorphine; CCI, chronic constriction injury; nAb, neutralizing antibody; V, vehicle.*

intrathecal injection of CCL7 in naive mice induced pain-related behaviors. In 2016, Ke et al. showed that knockdown of CCL7 strongly reduced neuropathic pain development [18]. We have demonstrated that treatment with CCL7 neutralizing antibodies effectively attenuates neuropathic pain symptoms in mice after CCI. Interestingly, Li et al. [48] reported that decreasing CCL7 levels was more effective for pain relief than decreasing CCL2 levels in a spinal nerve ligation model. This finding corresponds well with our behavioral data, which demonstrated that intrathecal injection of a CCL7 neutralizing antibody effectively attenuated CCI-induced neuropathic pain in mice at lower doses than that required for a CCL2 neutralizing antibody.

CCL12 is constitutively expressed in lymph nodes. Under allergen exposure or inflammation, its release is increased mainly by activated macrophages to attract a broad range of immune cells [19]. Interestingly, it was previously demonstrated that monocyte levels in CCL12$^{-/-}$ mice were identical to those in wild type mice after infectious factor exposure; thus, in contrast to CCL2 and CCL7, CCL12 appears to have no effect on monocytosis [49]. Our *in vitro* studies demonstrated that *CCL12* can be expressed by microglia and astrocytes, although its cellular source at specific levels of the CNS must be confirmed in the future. The monocyte chemotactic protein family, including CCL12, was previously suggested to exert potent proinflammatory actions through the chemotaxis of inflammatory leukocytes to injured CNS [50,51]. Interestingly, in our *in vitro* studies, treatment with glial modulators changed the constitutive levels of *CCL12* but not its LPS-elevated expression. We observed that minocycline reduced *CCL12* levels in microglia, whereas fluorocitrate strongly elevated *CCL12* levels in non-stimulated astrocytes; however the mechanism underlying this action remains unclear and needs further investigation. Recently, the upregulation of CCL12 was demonstrated in the articular cartilage of osteoarthritic knees; thus, the involvement of CCL12 in chronic joint pain was suggested [52,53]. Our biochemical analysis revealed that *CCL12* was also strongly upregulated in the spinal cord in the CCI model two days after sciatic nerve injury and then gradually decreased until

14 days. This finding agrees with results provided by Denk et al., who demonstrated transient CCL12 transcriptional changes in a partial sciatic nerve ligation model in mice, with a similar peak on the 2nd day after nerve injury [54]. Considering the high homology between CCL2 and CCL12 and the CCI-induced elevation of *CCL12* levels, we expected that CCL12 also would have strong pronociceptive properties. However, our research provided evidence that, surprisingly, the intrathecal injection of CCL12 did not induce any pain-related behavior in naive mice. Thus, we thought that although CCL12 did not influence nociceptive transmission in naive mice, it may have some beneficial effects in mice with sciatic nerve injury. However, none of examined doses of CCL12 demonstrated analgesic properties in mice with neuropathic pain. In the light of these results, we suggest that spinal CCL12, in contrast to CCL2 and CCL7, is not critical for the development of pain-related behavior. However, there is still a lack of literature data explaining the actual role of CCL12 in the CNS, which is why further studies in different animal models are needed.

Opioids are often used for the treatment of chronic pain, but in neuropathic pain, opioid therapy exhibits lower effectiveness. The low benefits of opioid drugs in patients suffering from neuropathic pain are associated with significant molecular changes in the release of nociceptive factors. Recently, it was demonstrated that opioid potency is probably modulated not only by enzymes like iNOS [55] and interleukins such as IL-1beta or IL-18 [56–58] but also by several chemokines, e.g., CCL2, CCL3, CCL5, CCL9 and XCL1 [15,17,26,25]. It was suggested that some chemokines, among them CCL2, may interfere with opioid-induced analgesic effects due to heterologous desensitization [59,60]. We previously demonstrated that blocking CCR2 with an antagonist, RS504393, restores the analgesic potential of opioids [15]. RS504393 decreased the CCI-elevated level of pronociceptive IL-1beta, IL-18, IL-6, iNOS, and CCL2, due to inhibition of microglial cell activation [14,15]. In 2012, Zhao et al. showed that neutralization of spinal CCL2 diminished morphine-induced activation of microglial cells, and thus reduced development of morphine tolerance [61]. We provide the first evidence that spinal injection of a CCL2 or CCL7 neutralizing antibody enhanced the analgesic properties of morphine or buprenorphine in neuropathic mice. We hypothesize that the beneficial effects of CCL2 and CCL7 neutralization on opioid efficacy result from a reduction in spinal microglia activation. Our results point to critical roles for both CCL2 and CCL7 in spinal microglial activation and thus in the development of neuropathic pain. Currently, biologic therapies, including monoclonal antibodies, are being increasingly investigated for the treatment of various chronic pain conditions. This type of therapy is an interesting option, mainly because the high affinity and specificity of monoclonal antibodies, as well as the long elimination half-life, allowing for less frequent drug dosing. All of these properties may improve drug acceptability and reduce unwanted adverse effects. In clinical trials for chronic pain treatment, monoclonal antibodies directed against nerve growth factor, tumor necrosis factor alpha, epidermal growth factor receptor or calcitonin gene-related peptide have already been used [62]. However, our study gives the first evidence that neutralizing antibodies targeting chemokines, in particular CCL2 and CCL7, may also be attractive alternatives not only for new analgesics but also for co-analgesics, which would be used potentially with opioid drugs.

5. Conclusion

Neuropathic pain treatment remains problematic since long-term opioid therapy continues to be controversial. Our results demonstrated that the generation and maintenance of neuropathic pain, especially in the early phase, are accompanied by strong spinal upregulation of CCL2, CCL7, and CCL12. Based on *in vitro* and *in vivo* results we suggest that microglia and astroglia are the important cellular source of this chemokines in the CNS, however this still needs further studies. We observed that the CCL2 and CCL7, but not CCL12, induced pain-related behavior in naive mice in a dose-dependent manner. In addition, neutralization of CCL2 and CCL7 strongly attenuated CCI-induced neuropathic pain. Furthermore, we are the first to demonstrate that neutralization of these two chemokines beneficial impacted the analgesia induced by morphine and buprenorphine in neuropathic mice. Our results highlight the pivotal roles of CCL2 and CCL7 in neuropathic pain development and provide new evidence for their significant influence on opioid effectiveness. Thus, we suggest that CCL2 and CCL7 are attractive targets for novel pharmacotherapy of neuropathic pain.

Declarations of interest

None.

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