Attenuation of rodent neuropathic pain by an orally active peptide, RAP-103, which potently blocks CCR2- and CCR5-mediated monocyte chemotaxis and inflammation

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Chemo...
the clinic may be due to this type of receptor cooperativity or redundancy, providing some explanation as to why small-molecule chemokine antagonists, specific for a single receptor, did not provide the expected therapeutic benefits [24].

In view of the close structural homology between CCR2 and CCR5 [20], we suspected that both of these receptors may contribute to pain states, and that treatments with dual targeting of CCR2 and CCR5 may have better therapeutic potential than so far evidenced by selective chemokine antagonists [32]. DAPTA (D-alapeptide T-amide), a HIV gp120-derived CCR5 entry inhibitor [19], had been shown to block neuroinflammation in Alzheimer’s disease models [25], and inhibition of CCR5-mediated monocyte migration [21] might attenuate neuropathic pain. We now show that RAP-103, a stabilized analog of DAPTA, was able to block CCR2/CCR5-mediated human monocyte chemotaxis. Oral administration of RAP-103 not only prevented the development of neuropathic pain, but also attenuated established mechanical and thermal hypersensitivity after a partial ligation on the sciatic nerve. The mechanism of RAP-103 action may include effects at either or both CCR2/CCR5 to reduce spinal microglial activation and monocyte infiltration and to inhibit inflammatory responses evoked by peripheral nerve injury that cause chronic pain; this mechanism suggests new compounds for treating chronic pain by blocking CCR2/CCR5.

2. Materials and methods

2.1. Animals

Animals were acclimatized to standard laboratory conditions (14-h light, 10-h dark cycle) and given free access to rat chow and water. Adult male Sprague Dawley rats (Charles River, Quebec, Canada) were used and weighed 250–275 g at the time of surgery. Green fluorescent protein (GFP)-positive chimeric mice were obtained from the CHUL Research Center, Laval University (Dr. S. Rivest). Adult male CCR5 knockout (KO) mice (B6.129p2-CCR5tm1kuz/J) were purchased from Jackson Labs (Bar Harbor, ME). All protocols were performed in accordance with guidelines from the Canadian Council on Animal Care and were approved by the McGill University and Laval University animal care committees.

2.2. Peptides and chemotaxis assay

RAP-103 (synthesized by RAPID Laboratories, C Pert@rapidpharma.com) was purified to >95% homogeneity and verified by high-performance liquid chromatography isolation, amino acid analysis, and mass spectroscopy. Peptides were dissolved in sterile water and stored as frozen (−20 °C) aliquots at 0.1 mM until use. Chemotaxis was assayed in 96-well plates (NeuroProbes, Cabin John, MD) with 5 μM pore size, PVP-free membranes. Purified human monocytes (>95%) prepared from healthy adult human donors by centrifugal elutriation (>95% pure, gift of L. Wahl, NIDR, NIH) were resuspended in chemotaxis assay buffer (Dulbecco modified Eagle medium supplemented with 0.1% bovine serum albumin) at a density of 2 × 10^6 cells/mL. Cells were labeled with 1.0 μM Calcein AM (Invitrogen, Carlsbad, CA) for 30 min at 37 °C, 5% CO2. After incubation, cells were washed once and resuspended in chemotaxis assay buffer (Dulbecco modified Eagle medium, 1 mg/mL bovine serum albumin, 25 mM HEPES) at a density of 2 × 10^6 cells/mL. Cells were then further treated with the indicated concentrations of RAP-103 for 30 min at 37 °C. Lower wells were filled with either buffer, MCP-1 (also known as CCL2) or MIP-1α (PeproTech) as test chemotacticants. The filter plate was snap-on, and monocytes that had been treated with RAP-103 or buffer only (30 min, 37 °C) were loaded onto the upper filter surface (50,000 cells in 25 μL). Chambers were then incubated at 37 °C for 90 min. At the conclusion of the test period, nonmigrating were wiped off the upper filter surface and relative fluorescence units of the migrating cells from the lower surface determined by bottom reading in a spectrometer (M5 SpectraMax) at 485/530 nm (Ex/Em). Triplicate determinations were made and results expressed as the mean chemotactic index, the ratio of cell migration of the indicated chemokine compared to cells that had been treated with the indicated dose of RAP-103, of 2 independent determinations.

2.3. Animal model of neuropathic pain

The current project uses the well-established rat neuropathic pain model described by Seltzer et al. [27]. With the animal under isoflurane anesthesia, the left common sciatic nerve was exposed via blunt dissection through the biceps femoris muscle. The dorsum of the nerve was carefully freed from surrounding connective tissues at a site near the trochanter. A 6-0 suture was inserted into the nerve with a 3/8 curved, reversed cutting mininneedle (Tyco Health Care, Ontario, Canada) and tightly ligated so that the dorsal one-third to one-half of the nerve thickness was trapped in the ligature. The muscle and skin layers were closed with 2 muscle sutures (4-0) and 3 to 4 skin sutures (4-0). Sham-operated rats underwent the same surgical procedure but the nerve was exposed and left intact. Survival times were 7 and 12 days after surgery. A group of naive rats was included in the protocol to obtain basal levels of certain gene and protein expressions.

The partial ligation was also performed on the left sciatic nerve of GFP-positive chimeric mice (10 weeks after irradiation and bone marrow transplantation) and CCR5 KO mice, according to the method described by Malmberg and Basbaum [15]. All mice were kept for 14 days.

2.4. Treatment paradigms

2.4.1. Physiological nociceptive response

A set of naive rats and sham-operated rats were treated with RAP-103 (1 mg/kg, p.o.) daily, for 4 consecutive days (n = 4 rats/group).

2.4.2. Prevention

To investigate whether blockade of both CCR2 and CCR5 can prevent the development of behavioral hypersensitivity after nerve injury, a set of rats was treated with H2O or RAP-103 (0.05, 0.1 or 1 mg/kg b.w., p.o.) administered immediately after surgery and continued once daily for 7 days after nerve injury (n = 4 for H2O and n = 4 for each RAP-103 dose).

2.4.3. Reversal

To further ascertain whether blockade of both CCR2 and CCR5 could also reverse already established neuropathic sensitivities, H2O or RAP-103 (0.1, 0.2 or 1 mg/kg b.w., p.o.) was administered to separate groups of rats starting from day 8 after injury, when both mechanical allodynia and thermal hyperalgesia had reached their lowest level. The treatment lasted for 5 days (days 8–12) (n = 5 for H2O and n = 4–8 for each RAP-103 dose).

A group of GFP-positive chimeric mice received RAP-103 or saline treatment (days 0–14, 1 mg/kg b.w., p.o.) to examine the effects of RAP-103 in monocyte trafficking into the spinal cord (n = 4/group).

2.5. Nociceptive behavioral testing

Both rats and mice subject for behavioral testing were habituated to the testing environment daily for at least 2 days before baseline testing. All animals were assessed for mechanical allodynia and thermal hyperalgesia of both hind paws before surgery and at
specified time points after injury until they were sacrificed for histological studies. The behavioral tests started 3–4 h after the drug administration. The investigator was blinded to the treatments the rats received. Mechanical sensitivity was assessed by using calibrated von Frey hairs as described by Chaplan et al. [6]. Animals were placed in boxes on an elevated metal mesh floor and allowed 40 to 60 min for habituation before testing. A series of von Frey filaments with logarithmically increasing stiffness (Stoelting) was applied perpendicular to the midplantar region of the hind paw. The 50% paw withdrawal threshold was determined by Dixon’s up–down method as previously described [9]. Thermal hyperalgesia was measured with the paw withdrawal test. Animals were placed on a glass floor within Plexiglas cubicles. After habituation, a focused high-intensity projector lamp was directed below onto the midplantar surface of the hind paw, and the reaction time (withdrawal latency of the hind paw) of the rats/mice was recorded automatically [11]. The commercial device (ITC Model 336) was calibrated so that the presurgical baseline paw withdrawal latencies were approximately 10–12 s. Twenty seconds was used as a cutoff time to avoid damage to the animal’s skin. The measurements were repeated 4 times for rats and 8 times for mice, at 3-min intervals on each paw. The initial pair of measurements was not used. The average of the 3 or 7 remaining pairs of measurements was taken as data. Efficacy of RAP-103 was determined according to the following formula: [(mean RAP-103 – mean control)/mean naive baseline – mean control] × 100%.

2.6. Tissue preparation

For histological studies, rats and mice were deeply anaesthetized with ketamine/xylazine and then perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). The ipsilateral and contralateral L4–L6 dorsal root ganglia (DRGs) from rats, as well as the corresponding levels of the spinal cords from both rats and mice, were removed and placed in the same fixative overnight, then transferred to 30% sucrose for cryoprotection. Frozen spinal cords were cut transversely into 30-μm-thick sections on a sliding microtome, collected in an antifreeze solution, and stored at −20 °C until use. The DRG were embedded in OCT compound (Tissue Tek, Miles Laboratories, Elkhart, IN), cut longitudinally at 14-μm thickness in a cryostat (Microm, Heidelberg, Germany), mounted onto Superfrost Plus slides, and stored at −80 °C until use.

For real-time polymerase chain reaction (PCR) experiments, 7 days after the nerve ligation, rats were deeply anaesthetized with ketamine/xylazine and decapitated. Lumbar (L4–L6) spinal cords and sciatic nerves were quickly removed and then snap frozen in liquid nitrogen, then stored at −80 °C until use.

2.7. In situ hybridization (ISH)

Detection of mRNAs encoding CCR5 was performed on lumbar spinal cord and DRG sections with 35S-labeled riboprobes. Radio-labeled CCR5 probe was synthesized with a 702 bp-cDNA cloned into expression vector pCR–Blunt II–TOPO. The sequence chosen was verified by BLAST (Basic Local Alignment Search Tool) analysis in GenBank. Hybridization were performed according to a previously described protocol [30]. Briefly, plasmids were linearized, and sense and antisense cRNA probes were synthesized with appropriate RNA polymerase. Sections were postfixed in 4% PFA and digested by proteinase K (10 μg/mL), after which spinal cord sections were rinsed in water and a solution of 0.1 M triethanolamine (TEA, pH 8.0), acetylated in 0.25% acetic anhydride in 0.1 M TEA, and then dehydrated. Hybridization of the sections by riboprobe involved 90 μl hybridization mixture containing 10⁶ cpm/mL radioactivity and incubation at 55 °C overnight in a slide warmer. Slides were rinsed in standard saline citrate (1 × SSC: 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0) and digested by RNase A at 37 °C (20 μg/mL), rinsed in descending concentrations of SSC, and dehydrated through graded concentrations of ethanol. Sections were exposed to X-ray film (BioMax, Kodak, Rochester, NY) for 2–3 days and dipped in NTB2 nuclear emulsion (Kodak). Slides were kept at 4 °C for 3–5 weeks safe from light, developed in D19 developer (Kodak), and counterstained with thionine.

2.8. Combination of immunohistochemistry with ISH

Immunohistochemistry was combined with ISH to determine whether CCR5 is expressed on microglia. Spinal cord sections were processed by the avidin–biotin method with peroxidase used as a substrate. Rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1) polyclonal antibody was used as a marker for microglia.

Briefly, sections were incubated with rabbit Iba-1 polyclonal antibody (1:1000; Wako Chemicals, Richmond, VA) at room temperature (21–23 °C) for 2 h, followed by a 2-h incubation with a biotinylated secondary antibody (Vector Laboratories, Burlington, CA), before final incubation with an avidin–biotin–peroxidase complex (Vectastain ABC Elite Kit; Vector Laboratories). The stainings were visualized by reacting in 0.05% diaminobenzidine and 0.003% hydrogen peroxide. Thereafter, sections were mounted, desiccated, fixed in 4% PFA, and digested by protease K. Prehybridization, hybridization, and posthybridization steps were performed according to the above protocol, with shorter dehydration times in ascending alcohol to prevent decoloration of immunoreactive cells. The slides were dried, exposed, and developed as described above.

2.9. Immunohistochemistry

Regular immunofluorescent staining was performed to characterize the spinal glial cell reaction to peripheral nerve injury and to RAP-103 treatment. Free-floating sections were incubated overnight at 4 °C with rabbit anti-Iba-1 polyclonal antibody (for microglia, 1:1000; Wako Chemicals) and rabbit anti-β-gal fibrillary acidic protein (GFAP) polyclonal antibody (for astrocytes, 1:1000; DakoCytomation, Carpenteria, CA), followed by 60-min incubation at room temperature in fluorochrome-conjugated goat secondary antibody.

2.10. RNA extraction and real-time quantitative PCR

Total RNA was extracted from spinal cords and sciatic nerves with RNeasy lipid tissue mini kit (Qiagen, Toronto, ON, Canada or Valencia, CA). Synthesis of cDNA from total RNA was performed with SuperScript VILO cDNA synthesis kit (Invitrogen). Primers were produced by Qiagen QuantiTect (IL-1β-QT00181657, IL6-QT00182896, GAPDH-QT00199633). Spinal cords and sciatic nerves collected from the following groups were analyzed: 3 naive animals; 3 nerve injured H2O-treated animals, and 4 nerve injured RAP-103-treated animals. Experiments were performed in triplicate with the SYBR Green I Dye technology. Levels of target mRNAs were normalized to the housekeeping gene GAPDH. Fold changes versus naive animals in their respective ipsilateral and contralateral sides were analyzed by using the comparative Ct (dCt) method [14].

2.11. Image processing and analysis

Images were acquired with an Olympus BX51 (Tokyo, Japan) microscope equipped with a color digital camera (Olympus DP71) and Olympus confocal laser-scanning biological microscope (Fluoview 1000). Quantitative analysis of both Iba-1 and GFAP staining was performed on images digitized with a constant set of parameters (exposure time, gain, and postimage processing).
with special attention to avoid signal saturation. Four areas of interest (AOI), defined as 2 rectangles (192 × 397 pixels) on the dorsal horn (DH) (lamina I–III) and 2 rectangles (287 × 343 pixels) on the ventral horn (VH) (lamina IX), on both sides relative to the side of injury, were selected. Two thresholds of fluorescence intensity for Iba-1 and GFAP, respectively, were established according to the signals on naive animals. All objects within the AOI having fluorescence intensity above the chosen threshold were considered as parts of Iba-1- or GFAP-positive cells and were subject to the quantitative analysis. We measured the area occupied by Iba-1-positive or GFAP-positive cells within the AOI by Image Pro Plus 6.2 for Windows (Media Cybernetics, Bethesda, MD) in different groups of animals: naive (mice and rats), CCR5 KO mice (sham and nerve injured), and nerve-injured rats treated with H2O or RAP-103. Samples from 5–6 sections per animal and 3–4 animals per group were included for each quantitative analysis.

2.12. Statistical analysis

Data are presented as means ± SEM. Statistic significance was determined by using the following: (1) for behavioral analysis, 1-way ANOVA followed by Dunnet’s test for the changes of all time points vs presurgery baseline; unpaired t test for the difference between groups (RAP-103 treated vs saline treated at each time point); (2) unpaired t test for the difference between groups in glial responses and in cytokine expression (CCR5 KO vs wild type, RAP-103 treated vs H2O treated, injured H2O treated vs naive) and for monocyte chemotaxis (RAP-103 treated vs H2O treated); and (3) paired t test for the difference between 2 sides (ipsilateral and contralateral) in cytokine expression. The criterion for statistical significance was P < 0.05.

3. Results

3.1. RAP-103 blocked CCR2- and CCR5-mediated human monocyte chemotaxis

CCR2 and CCR5 are 2 CC chemokine receptors that control trafficking of monocyte/macrophages and mediate the inflammatory response during infectious disease and tissue injury. MCP-1 is a specific ligand for CCR2, while MIP-1β is specific for CCR5. In order to identify the receptor targets of RAP-103, we tested the ability of RAP-103 to antagonize the function of chemokine receptors by blocking the chemotactic migration of human monocytes in the presence of specific ligand. Monocytes were treated for 30 min at 37 °C with RAP-103 before testing. The results showed that RAP-103 was a potent antagonist of both CCR2 (half maximal inhibitory concentration [IC50] 4.2 pM) and CCR5 (IC50 0.18 pM) (Fig. 1). RAP-103 was apparently 20-fold more potent at blocking MIP-1β than MCP-1 chemotaxis.

3.2. Oral administration of RAP-103 potently attenuated nerve injury-induced mechanical and thermal hypersensitivity

3.2.1. Systemic administration of RAP-103 did not affect either mechanical or thermal sensitivities in naive and sham-operated animals

Consecutive daily administration of RAP-103 (1 mg/kg) for 4 days in naive rats did not affect their paw withdrawal threshold in response to von Frey hair stimulation and their paw withdrawal latency to heat stimuli. A similar response was observed in the groups of sham-operated rats treated with RAP-103 (10 mg/kg) (Fig. 2).

3.2.2. RAP-103. prevented the development of neuropathic pain

To test the hypothesis that RAP-103 alleviates behavioral signs of neuropathic pain, we first evaluated the effects of RAP-103 on the development of mechanical allodynia and thermal hyperalgesia in rats after nerve injury. Rats started to receive RAP-103 or H2O on the day of the surgery; the drug was delivered orally, once per day, for 7 days. Shortly after partial ligation of the left sciatic nerve, rats receiving H2O showed an exaggerated bilateral decrease of paw withdrawal threshold in response to von Frey hair stimulation (Fig. 3A and B) and a sharp unilateral response to heat stimuli (Fig. 3C and D) at the plantar surface, which confirmed a similar observation reported originally by Seltzer et al. [27]. Oral administration of RAP-103 for 7 days almost completely prevented the development of mechanical allodynia induced by nerve injury, eg, oral daily RAP-103 (1 mg/kg b.w.) reached 106 ± 33% and 88 ± 32% attenuation of mechanical allodynia at 3 days and at 7 days after injury, respectively. Paw withdrawal threshold in response to von Frey hair stimulation in rats treated with RAP-103 was 11.23 ± 2.26 g (day 3) and 9.89 ± 2.39 g (day 7) vs 3.84 ± 0.45 and 2.91 ± 0.37 g in animals receiving H2O treatment (P < 0.001 vs H2O treated), whereas presurgery paw withdrawal latency was 10.83 ± 0.71 g (Fig. 3A). RAP-103 provided systemically also improved the mechanical sensitivity of the contralateral side (Fig. 3B). The efficacy of RAP-103 on nerve injury-induced thermal hyperalgesia was assessed in the same group of animals at day 4 and day 6 after injury. All 3 tested doses (0.05, 0.1, 1 mg/kg b.w.) resulted in a sustained attenuation of thermal hyperalgesia (Fig. 3C). The effects were less potent than that observed in mechanical sensitivity testing, ranging from 43 ± 11% to 67 ± 22% reduction at different time points with 1 mg/kg b.w.

3.2.3. RAP-103. attenuated already established neuropathic pain

We also examined the effects of RAP-103 on already established hypersensitivity after nerve lesion. RAP-103 was provided orally on day 8 after injury, where both paw withdrawal threshold and latency in response to mechanical and thermal stimuli, respectively, had already reached their lowest level (Fig. 4). The treatment continued afterward for 5 days. As revealed in Fig. 4A and B, the dose of 0.1 mg/kg b.w. of RAP-103 was not able to reverse the mechanical allodynia at both ipsilateral and contralateral sides, but partially rescued the low withdrawal latency in response to heat stimuli at day 8 (Fig. 4C). However, the dose of 0.2 mg/kg b.w. started to exhibit more consistent effect in relieving both mechanical and thermal hypersensitivity, eg, the paw withdrawal threshold reached...
5.45 ± 1.11 g at day 9 and 6.72 ± 1.24 g at day 11 in RAP-103-treated rats vs 2.06 ± 0.13 g and 2.11 ± 0.44 g in rats treated with H2O at their respective time points; the paw withdrawal latency reached 9.28 ± 2.04 s at day 8 and 10.82 ± 1.48 s at day 10 in RAP-103-treated rats vs 5.23 ± 0.32 s and 5.71 ± 0.76 s in rats treated with H2O at their respective time points (Fig. 4C). The dose of 1 mg/kg b.w. also effectively attenuated the nerve injury-evoked hypersensitivity to heat stimuli (Fig. 4C).

3.3. RAP-103-inhibited nerve injury-induced spinal microglial activation

To understand the potential underlying mechanisms in relieving pain behavior, we examined the effects of RAP-103 on spinal microglia. In nerve-injured rats receiving H2O treatment, there was a strong increase in Iba-1 immunoreactivity (ir) condensed at the ipsilateral side of spinal DH and VH at 7 days after surgery. This pattern of Iba-1 expression in microglial cells was no longer observed in rats treated with RAP-103 (Fig. 5A). High-magnification images revealed that stereotypical microglial morphological changes during activation were also prevented by RAP-103. Seven days after nerve injury, without RAP-103 treatment, microglia in the spinal cord ipsilateral side to the injury displayed large cell bodies, shortened and thickened branches, and intense Iba-1 labeling, whereas Iba-1-positive cells in the spinal cord of RAP-103-treated rats had small cell bodies with long and fine ramifications and much less intense Iba-1 staining (Fig. 5A). To compare with naive rats, nerve injury dramatically increased the total surface occupied by Iba-1-positive cells on the ipsilateral side spinal cord DH and VH, which reflects a significant increase of cell number and an enlargement of cell size, two major characteristics of microglial cell activation (Fig. 5B). This nerve injury-induced changes in Iba-1-positive cells was reduced by 7-day preventive RAP-103 treatment and also attenuated by a reversal RAP-103.
treatment paradigm (Fig. 5B). Moreover, by blocking both CCR2 and CCR5, RAP-103 successfully prevented the infiltration of bone marrow-derived monocytes/macrophages from circulation into the spinal cord parenchyma (Fig. 5C). Fourteen days after nerve injury, a cluster of ramified GFP-positive cells found in the spinal parenchyma DH (14.09 ± 2.27 cells/region) and VH (27.03 ± 2.58 cells/region) was reduced to 4.22 ± 0.98 cells/region and 7.69 ± 1.89 cells/region, respectively, in the presence of RAP-103 (Fig. 5D).

3.4. Effect of RAP-103 on nerve injury-induced spinal astrocyte activation

As a result of nerve injury, microglia and astrocytes activation usually occur together, although they may not occur at the same time course. We also examined the effects of RAP-103 on astrocyte activation in the spinal cord after nerve injury. Spinal astrocytes were labeled with an antibody against GFAP. Changes in GFAP ir after peripheral nerve injury with and without RAP-103 treatment are depicted in Fig. 6A. Hypertrophic GFAP-positive cells on the ipsilateral side DH were visualized with confocal microscope. RAP-103 treatment (days 0–7) did not alter significantly nerve injury-induced astrocyte hypertrophy (Fig. 6B). The cumulative surface occupied by GFAP-positive cells within defined AOI was significantly higher than that of naive animals; however, no significant changes were observed with the RAP-103 preventive treatment (Fig. 6C). The reversal paradigm had a slight effect in reducing nerve injury-induced spinal astrocytes activation (Fig. 6C).

3.5. RAP-103 reduced the increase of cytokines in the spinal cord after the lesion on the sciatic nerve

To explore whether RAP-103, a potent dual CCR2/CCR5 antagonist, could alter inflammatory response and thereby explain its potential effect on hypersensitivity, we measured levels of proinflammatory cytokines interleukin (IL)-1β and IL-6 transcripts in the spinal cords and in sciatic nerves with quantitative real-time PCR. IL-1β and IL-6 mRNAs were significantly upregulated at the ipsilateral spinal cord and in damaged sciatic nerve after nerve injury. The increase of these proinflammatory mediators within the spinal cords, as well as in the peripheral nerves, was significantly reduced with the treatment of RAP-103 (Fig. 7).

3.6. Nerve injury induced CCR5 expression in activated spinal microglia

Because the expression of CCR2 in spinal microglia has been reported previously [2], and because whether CCR5 can be induced within the spinal cord by nerve injury is unclear, to gain insight into the site of action of RAP-103 in this specific neuropathic pain condition, we examined the expression and the cellular localization of CCR5 in rat spinal cord. Although no positive ISH signal was detected in the spinal cords of nerve-injured rats by using sense probe (Fig. 8A) and in the spinal cords of naive rats with an antisense probe (Fig. 8B), CCR5 mRNA was found to be induced on the ipsilateral side of rat spinal cords at days 3–12 after partial ligation of the sciatic nerve. An example of CCR5 mRNA expression at day 7 after injury is depicted in Fig. 8C. Positive signals (silver grains) for CCR5 mRNAs were observed on some glial cells in both DH (Fig. 8D) and VH, which were heavily stained with thionine. Double labeling combining immunohistochemistry for Iba-1 and ISH for CCR5 mRNA confirmed that these CCR5 expressing cells are Iba-1-positive-activated microglia (Fig. 8E–J). Although CCR5 mRNA expression in DRG neurons has been reported previously in a chemical-induced sciatic nerve demyelination model [4], we did not detect any significant positive signals for CCR5 mRNAs in...
Fig. 5. Effect of RAP-103 on spinal microglial reaction. (A) Photomicrographs depicting Iba-1 labeling in lumbar spinal cord sections 7 days after nerve injury. Partial sciatic nerve ligation induced a marked increase of Iba-1 immunoreactivity at the ipsilateral DH and VH of spinal cord compared to that of naive animals. After administration of RAP-103 (1 mg/kg, p.o., for 1 week) started immediately after nerve injury reduced Iba-1 ir in spinal microglial cells. High-magnification images demonstrated multidimensional changes of activated microglia (ipsilateral), including cell density, cell shape and size, and Iba-1 ir intensity, which were prevented by RAP-103 treatment. Scale bar = 500 μm (top) and 20 μm (bottom). (B) Quantitative analysis using the same method described in Fig. 9B showing a significant increase of area occupied Iba-1-positive cells within a specific AOI after nerve injury, which was strikingly reduced after RAP-103 treatment in both prevention and reversal paradigms. Data are presented as mean ± SEM. **P < 0.01, ***P < 0.001, RAP-103 vs saline in their respective regions; ##P < 0.01, ###P < 0.001, naive vs injured in their respective regions; n = 3–4 animals per group and 5–6 sections per region. (C) Fourteen days after nerve injury, bone marrow-derived GFP-positive monocytes/macrophages accumulated in the ipsilateral side DH and VH, which was completely abolished at the presence of RAP-103 (1 mg/kg b.w., p.o. days 0–14). Scale bar = 500 μm. (D) Number of ramified GFP-positive cells within the spinal cord parenchyma was significantly lower in RAP-103-treated animals than those that received saline treatment. Data are presented as mean ± SEM. **P < 0.01, ***P < 0.001, RAP-103 vs H2O in their respective regions.
DRG sensory neurons from days 3 to 12 after partial ligation of the sciatic nerve (data not shown).

### 3.7. CCR5 is required for the development of neuropathic pain and spinal microglial activation after nerve injury

The critical role of CCR2 in the pathogenesis of neuropathic pain has been well established [2,31]. To further confirm that CCR5 is also necessary for the development of neuropathic pain, we made use of CCR5 KO mice. As depicted in Fig. 9, CCR5 gene deletion did not affect mouse withdrawal threshold and latency to mechanical heat stimuli without injury (baseline before surgery and contralateral paw). However, although wild-type mice showed a robust decrease in withdrawal threshold from 0.54 ± 0.06 g before surgery to 0.16 ± 0.03 g (P < 0.001) at day 3 after surgery, and maintained this hypersensitivity (approximately 0.1 g) to the end of the testing period (day 14), mechanical allodynia was completely abolished in CCR5 KO mice. Similar responses were obtained to heat stimulation. CCR5 KO mice did not develop thermal hyperalgesia after nerve injury, and in wild-type mice, the withdrawal latency decreased from 11.36 ± 1.09 s before surgery to 6.73 ± 0.27 s at day 14 after injury. In addition, spinal microglial reaction to the peripheral nerve injury observed in wild-type mice was significantly impaired in CCR5 KO mice (Fig. 10A), which was confirmed by a quantitative analysis based on the cumulative area occupied by Iba-1-positive cells within a defined AOI (Fig. 10B and C).

### 4. Discussion

To our knowledge, this is the first report showing a benefit of RAP-103 in a model of neuropathic pain. Although CCR2 has a
well-established role in the inflammation underlying chronic pain, some evidence for the involvement of CCR5 in development of neuropathic pain after injury is suggested by studies showing that injection of MIP-1α and RANTES into peripheral nerve elicited pain behaviors [13,18]. Our results support such a view; the use of genetically deficient animals demonstrated that CCR5 is required for the development of neuropathic pain. Mice lacking CCR5 develop neither mechanical nor thermal hypersensitivity after injury on the nerve.

Our results further suggest that pharmacological blockade by RAP-103 of either or both CCR2 and CCR5 have therapeutic potential in injury-associated neuropathic pain. RAP-103 acts as a potent antagonist for both CCR5- and CCR2-mediated human monocyte chemotaxis. In vivo experiments revealed that this dual antagonist is orally active and exerts potent antiinflammatory and antihyperalgesic effects in both preventive and reversal treatment paradigms in rats after peripheral nerve injury. RAP-103 relieves neuropathic pain through reducing CCR2/CCR5-mediated inflammatory reaction because in parallel with the behavioral outcomes, we observed first that pharmacological blockade of both CCR2/CCR5 by RAP-103 reduced spinal microglial activation triggered by nerve injury, including the abolishment of blood born monocyte/macrophage recruitment into the spinal parenchyma, and second that RAP-103 was able to prevent the increase of proinflammatory cytokines along the pain-signaling pathway.

DAPTA, the parent compound from which RAP-103 was derived, had previously been shown to have anti-inflammatory effects, including inhibition of CCR5/MIP-1α chemotaxis on human monocytes [21], attenuation of neuroinflammation in an Alzheimer's disease model [25], and reduction of the inflammatory cytokines tumor necrosis factor-α, IL-1, and IL-6 in patients with human immunodeficiency virus [26]. In the current study, our in vitro experiments clearly demonstrated that RAP-103 potently blocks MIP-1α- and MCP-1-elicited monocyte chemotaxis with IC50s of 0.18 pM and 4.2 pM, respectively. The remarkably potent inhibitory effect of RAP-103 on both CCR5 and CCR2 mediated monocyte trafficking suggests that dual antagonists of CCR2 and CCR5 may provide new tools for the study of chemokine signaling in different pathological conditions, and potentially therefore better treatment outcomes.

The roles of MCP-1/CCR2 signaling in chronic pain have been well documented. Overexpression of MCP-1 showed enhanced pain sensitivity [17]. Neutralizing MCP-1 prevented the development of nerve injury-evoked hypersensitivity [10]. Drugs that block CCR2 receptors can reduce hypersensitivity in human immunodeficiency virus [5] and focal nerve demyelination [4] associated peripheral neuropathy. Lack of CCR2 in mice impaired the development of mechanical allodynia after nerve injury [2,31]. Relative to CCR2, the function of CCR5 in chronic pain is less well defined. However, some recent evidence suggested the potential involvement of CCR5 in different aspects of pain modulation. Microinjection of RANTES, a natural ligand for CCR5, into the periaqueductal gray matter, a brain region critical to the processing of pain signals, induced hyperalgesia, which was prevented by pretreatment with antibodies against RANTES [3]. Partial sciatic nerve ligation induced expression of MIP-1α, another natural ligand for CCR5, on macrophages and Schwann cells in injured nerve. Tactile allodynia and thermal hyperalgesia developed after the nerve lesion was prevented by perineural injection of neutralizing anti-MIP-1α and CCR5 siRNA [13]. The results of our current investigation that used CCR5 KO mice clearly demonstrated that similar to CCR2, CCR5 is required for the development of neuropathic pain after nerve injury. Pharmacological intervention with RAP-103, a dual antagonist of both CCR2 and CCR5, delivered orally not only prevented the initiation of mechanical and thermal hypersensitivity, but also attenuated both mechanical and thermal hypersensitivity already established in rats having ligation on the sciatic nerve.

Together with its effect in relieving nerve injury-induced behavioral hypersensitivity, we also observed that RAP-103 efficiently inhibited spinal microglial activation, including changes in cell density, cell size, and cell shape. In addition, RAP-103 successfully blocked the entrance of bloodborne monocytes/macrophages into the spinal cord parenchyma. All these most likely occurred through the interaction of RAP-103, with both CCR2 and CCR5 receptors present on circulating monocytes, macrophages, and activated spinal microglia. Both CCR2 [31] and CCR5 KO (current study) mice exhibited an impaired microglial response after an injury on the nerve. In coincidence with the inhibition of microglial activation, the levels of some proinflammatory cytokines, such as IL-1β and IL-6, most likely released by activated spinal glial cells and peripheral immune cells, were also reduced with the treatment of RAP-103. The roles of these proinflammatory mediators in the pathophysiology of neuropathic pain has been extensively studied [23]. They contribute significantly to enhance excitability of sensory neurons and to maintain pathological pain states. Therefore, we assume that RAP-103 attenuate mechanical and thermal hypersensitive response, at least partially, through modulation of nerve injury-induced glial activation and subsequent inflammatory reaction.

Because of their key roles in inflammation-related diseases, CCR2 and CCR5 constitute attractive therapeutic targets. However,
it should be noted that the chemokine network is notorious for its redundancy and receptor promiscuity. Apparent redundancy in the chemokine system, such as CCR2 and CCR5, might exist to confer robustness to the control of inflammation [7,8,12]. Moreover, the fact that chemokine receptors form hetero-oligomeric complexes composed of at least 3 chemokine receptors, CCR2, CCR5, and CXCR4, add an additional layer of complexity to this system [29].

Specific antagonism of one chemokine receptor can lead to functional cross-inhibition of the others [29]. It is perhaps more correct to consider that the functional and biologically relevant therapeutic targets are the naturally occurring mixed receptor complexes. Heterologous desensitization of CCR2-mediated responses may provide an explanation for the functional action of RAP-103, which is derived from the CCR5 antagonist DAPTA [19]. CCR5 expression

![Image](image-url)

**Fig. 8.** Expression of CCR5 mRNA in rat lumbar spinal cord ipsilateral to partial sciatic nerve ligation. The hybridization with the CCR5 sense probe did not show any positive signal in nerve-injured rat spinal cord sections (A). The expression of constitutive CCR5 mRNAs was not detected within the spinal cords of naive animals (B). After nerve injury, upregulation of CCR5 transcripts at both dorsal and ventral horns of spinal cord, ipsilateral to the nerve injury, was observed (C, highlighted areas). High-magnification microscopic analysis revealed that positive signals (silver grains) for CCR5 mRNAs were observed on thionine-stained small cell bodies (black arrowheads), presumably some glial cells within both DH (C) and VH. Double-labeling studies confirmed that the receptor transcripts expressing cells were Iba-1-positive activated microglia (E–J). Black arrows indicate Iba-1 ir and CCR5 mRNA double-labeled cells. Scale bar = 500 μm for A, B, C, E; 50 μm for D; and 100 μm for G, H, I, J.

![Image](image-url)

**Fig. 9.** Mechanical allodynia and thermal hyperalgesia after partial ligation on sciatic nerve in CCR5 KO mice. After partial ligation of the sciatic nerve, paw withdrawal thresholds and latencies remained at the same levels as before surgery in CCR5 KO mice, whereas in wild-type mice, injured paw withdrawal thresholds to von Frey stimulation decreased sharply from 0.54 ± 0.06 g before surgery to 0.09 ± 0.01 g 2 weeks after injury, and withdrawal latencies to heat stimulation decreased also from 11.36 ± 1.089 s before surgery to 6.73 ± 0.27 s at day 14 after injury. Data are shown as mean ± SEM. **P < 0.01, ***P < 0.001; n = 4–6 mice/group. Baseline data (day 0) were obtained by an average of 2 measurements at 1–2 days before surgery.
is low on resting cells, and its upregulation after injury in the context of likely heterodimer formation may serve to attenuate or further limit CCR2-driven inflammation. CCR2 may be more important for early migration responses into injured spinal cord because CCR5 is low. As CCR5 becomes expressed and R5 ligands are locally released, the CCR5 pathways also contribute to spinal microglial reactions.

The action of RAP-103 to limit CCR2 responses causing chronic pain may not occur directly via CCR2, but rather as a consequence of CCR5-mediated desensitization of CCR2 [29]. This model of joint CCR2/CCR5 interaction could help explain why selective or “pure” receptor-targeted therapeutic compounds that antagonize single chemokine receptors afford little efficacy in clinical use [32]. Useful antagonists might block multiple receptors or could target a functional receptor complex, rather than constituent single receptors. Receptor oligomerization represents a regulatory mechanism for a more nuanced control of CCR2- and CCR5-driven inflammatory activation that might be exploited clinically. Drugs that block more than one component of the chemokine system may overcome the functional redundancy and cross-regulation in the chemokine system that presumably limits effectiveness of CCR2 antagonists and might be a more efficacious strategy than targeting either receptor

**Fig. 10.** Spinal microglial activation in mice deficient in CCR5 receptor. (A) Peripheral nerve injury-induced spinal microglial activation observed in wild-type mice was significantly impaired in CCR5 KO mice. (B) Methodology for the quantitative evaluation on spinal microglial activation. A threshold of fluorescence intensity for Iba-1 (showed as a mask) was established according to the signals on naive animals. All objects within the AOI having fluorescence intensity above the chosen threshold were considered as parts of Iba-1-positive cells and were subjected to quantitative analysis. (C) Quantitative analysis based on the cumulative area occupied by Iba-1-positive cells showed an impairment of microglial activation in CCR5 KO mice. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01, injured vs sham. ***P < 0.01, wt vs KO mouse; n = 3–5 section/mouse, n = 4 mice/group. Scale bar = 500 μm.
alone. Our results support these hypotheses because a novel and potent, orally active dual CCR2/CCR5 antagonist, RAP-103, has multiple benefits in injury-induced neuropathic pain.

In conclusion, we provided evidence that in addition to chemokine receptor CCR2, CCR5 is equally necessary for the development of neuropathic pain. On the basis of the structural similarity and functional redundancy in controlling monocyte/macrophage trafficking and spinal microglial reaction, we suggest that targeting both CCR2 and CCR5 should provide greater efficacy than targeting CCR2 or CCR5 alone, and that RAP-103 has the potential for broad clinical use in neuropathic pain treatment.

Conflict of interest statement

CP and MR are inventors of RAP-103 and stakeholders in RAPID Pharmaceuticals, AG, the company that holds the patent rights.

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