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The excitatory effects of the chemokine CCL2 on DRG somata are greater after an injury of the ganglion than after an injury of the spinal or peripheral nerve

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ABSTRACT

We compared the expression of chemokine receptor CCR2 protein in the dorsal root ganglia (DRG) injured by the chronic constriction injury (CCI), the spinal nerve ligation (SNL) and a chronic compression of DRG (CCD). Each of these injuries produced the same significant increase in CCR2 protein in the DRG, as assessed by Western blot analyses. Whole-cell patch-clamp recordings revealed that CCL2, a ligand for CCR2 receptor, depolarized nociceptive DRG neurons from rats of the all three models. A greater percentage of these neurons was depolarized by CCL2 after CCD than after either of the other injuries. Furthermore, CCL2 significantly lowered current threshold only in CCD neurons but not in CCI or SNL neurons. CCL2 significantly lowered the net whole-cell potassium currents in neurons after CCD but not after CCI or SNL. Thus, the injury-induced effects of CCL2 in increasing the excitability of the cell bodies of DRG neurons depend on the site of the injury – with greater effects occurring after an injury of the ganglion than after an injury of the spinal or peripheral nerve.

Chemokines are small secreted proteins. In addition to acting as chemotactic mediators for the recruitment of immune cells to sites of injury under neuropathic conditions, it is now recognized that chemokines also play pivotal roles in the peripheral mechanisms of neuropathic pain [1,19]. For example, intradermal injection of chemokine CCL2 (MCP-1) produces hyperalgesia and allodynia in rats [6,14]. CCL2 was up-regulated exclusively in dorsal root ganglion (DRG) neurons after an injury either of the peripheral nerve or the ganglion [9,16,20,21]. CCL2 binds to its cognate receptor CCR2. DRG neurons up-regulate the synthesis of the CCR2 in addition to CCL2 following a chronic compression of the DRG (CCD) or after a demyelination of axons in the sciatic nerve [4,20]. Mice deficient in CCR2 did not show mechanical allodynia after a partial ligation of the nerve [2], suggesting that up-regulation of CCR2 is a crucial step in the generation and/or maintenance of neuropathic pain. The contribution of CCL2/CCR2 to chronic pain is associated with an increase in neuronal excitability because CCL2 excites the nociceptive DRG neurons through the activation of CCR2 after CCD injury [15,20]. However, little is known of the expression level and the excitatory role of CCR2 in DRG neurons with the injuries to their peripheral branches following chronic constriction injury (CCI) or spinal nerve ligation (SNL) injuries. In this study, we compared the effects of CCI, SNL, and CCD on the level of expression of CCR2 in the DRG and the excitatory effects of CCL2 on nociceptive DRG neurons.

Rats (180–200 g) were anesthetized with pentobarbital sodium (40 mg/kg, administered intraperitoneally) for all surgical procedures. The surgical procedure for CCI model was similar to that described by Bennett and Xie [3]. Briefly, the right common sciatic nerve was exposed at the level of mid-thigh and four chromic catgut (4-0) ligations were tied loosely with about 1 mm spacing, proximal to the sciatic nerve's trifurcation. The spinal nerve ligation (SNL) model was performed as described [11]. Briefly, an incision was made into the left of the spine at the L4–S2 level. After the left L6 vertebral transverse process was removed, and the left L5 spinal nerve exposed and then tightly ligated with 6-0 silk thread. The CCD model was produced as described [15]. Briefly, the ipsilateral, right transverse process and intervertebral foramina of L4 and L5 were exposed and a stainless steel L-shaped rod (0.63 mm in diameter and 4 mm in length) inserted into each foramen, one at L4 and the other at the L5 ganglion. Un-operated rats were used as controls for all experiments. Sham operations for CCI, SNL and CCD treatment were not considered necessary because of the absence of the effects of such surgeries on the electrophysiological properties of DRG neurons [20,22]. Seven days after surgery, the animals were sacrificed and the DRGs harvested for either protein analysis or electrophysiological recording because the injured animals in three groups exhibited mechanical hyperalgesia at this time point according to our behavioral tests and previous reports.

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For Western blot analysis, rats were deeply anesthetized with diethyl ether and killed by decapitation. The L4/L5 DRGs were quickly removed and homogenized in a SDS sample buffer containing a mixture of proteinase and phosphatase inhibitors. Equal amounts of the samples (50 μg) were loaded per lane, separated by 12% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 2% BSA in Tris-buffered saline containing 0.1% Tween20 (TBST) at room temperature for 1 h and then incubated overnight at 4 °C with antibody against CCR2 (antirabbit, 1:50; Santa Cruz Biotechnology, USA) in 2% BSA in TBST. After washing with TBST, horseradish peroxidase-conjugated antirabbit serum (Santa Cruz Biotechnology) was used as a secondary antibody and the antigen–antibody complexes were visualized using an enhanced chemiluminescence detection reagent (Amersham). Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories), and quantification was performed using Multi-Analyst 1.0.2 software (Bio-Rad).

For cell dissociation, isolated L4/L5 DRGs were dissected and placed in complete saline solution (CSS) containing (in mM): 137 NaCl, 5.3 KCl, 1 $MgCl_2$, 3 $CaCl_2$, 25 sorbitol, and 10 HEPES, adjusted to pH 7.2 with NaOH. The DRGs were then digested for 25 min with collagenase A (1 mg/ml; Boehringer Mannheim, Germany) and, for another 25 min, with collagenase D (1 mg/ml; Boehringer) and papain (30 units/ml, Worthington Biochemical, Lakewood, NJ) in CSS containing 0.5 mM EDTA and 0.2 mg/ml cysteine at 37 °C. The cells were dissociated by trituration in culture medium containing 1 mg/ml bovine serum albumin and 1 mg/ml trypsin inhibitor (Boehringer) and plated on glass coverslips coated with 0.1 mg/ml polyornithine and 1 mg/ml laminin (Boehringer). The culture medium contained equal amounts of Dulbecco's Modified Eagle Medium and F12 (Gibco, Grand Island, NY) with 10% fetal calf serum (HyClone Laboratories, Logan, UT) and 1% penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Life Technologies, Rockville, MD). The cells were incubated at 37 °C (5% CO_2 balanced air) for 1 h after which culture medium without the trypsin inhibitor was added. Twelve to eighteen hours after cell dissociation, the coverslip with adherent DRG neurons was placed in a recording chamber that was mounted on the stage of an upright microscope (BX50-WI, Olympus Optical, Tokyo, Japan).

Whole-cell electrophysiological recordings were obtained at room temperature by means of an Axonclamp 200B amplifier and pClamp 9 software (Axon Instruments). Electrodes were fabricated from borosilicate glass and pulled on a PC-10 puller (Narishige, Japan). The pipette solution contained (in mM): 120 K-gluconate, 10 KCl, 5 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES, 2 Mg-ATP, and 1 Li-GTP. The pH (7.2) of the solution was adjusted with Tris-base and the osmolarity (310 mOsm) adjusted with sucrose. The impedance of a typical patch pipette was 2–4 MΩ. The electrophysiological recordings were filtered at 3 kHz and digitized by A/D converter (Digidata 1322A, Axon Instruments) at 5 kHz. The recording chamber was perfused continuously at a rate of 2–3 ml/min with a bath solution containing (in mM) 145 NaCl, 3 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, and 10 glucose and adjusted to a pH of 7.4 and osmolarity of 300–310 mOsm. All command voltages were corrected for junction potentials between the internal and the external solutions. For K^+ current recordings, the bath solution contained (in mM): 145 N-methyl-D-glucamine, 3 KCl, 2.5 $CdCl_2$, 0.6 $MgCl_2$, 10 Hepes, and 10 glucose, adjusted to pH 7.4 using HCl.

All chemicals were purchased from Sigma–Aldrich (Shanghai, China) unless otherwise stated. Chemokine CCL2 was purchased from R&D Systems (Minneapolis, MN). A concentration of 100 nM of CCL2 in BSA was prepared just before each day's experiment. Chemicals and other bath solutions were each delivered directly to the soma of each recorded neuron through a pipette via a 100 μm-diameter tip perfusion pipette controlled by a DAD-12 superfusion system and computer interface (ALA Scientific Instruments Inc., USA). The data were expressed as means ± S.E.Ms. Differences between two groups were determined using *t*-tests. Comparisons of proportions were made using the chi-square test or Fisher's exact test. A one-way ANOVA or two-way repeated-measures ANOVA was used for multiple comparisons. Statistical significance was established at $p < 0.05$.

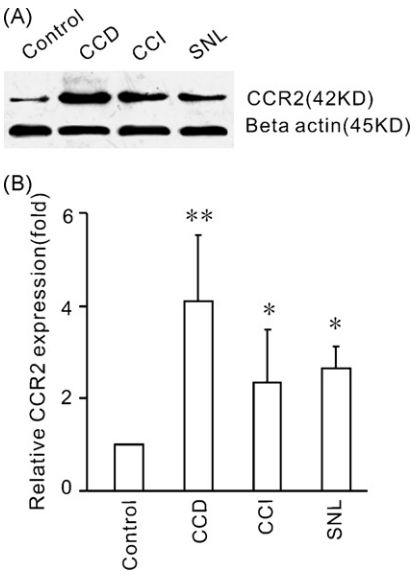

Fig. 1. Up-regulation of CCR2 receptor protein in the injured DRG. (A) Western blots of DRG protein extracts probed for CCR2. β-Actin serves as the loading control. (B) CCR2 levels of signal significantly increased in CCD-injured L4/5 DRGs, CCI-injured L4/5 DRGs and SNL-injured L5 DRGs, respectively, compared with the naïve control (**$p < 0.01$, *$p < 0.05$, one-way ANOVA with a Tukey test). The relative quantification of CCR2 protein was described as fold change from control.

We first evaluated the differential changes in the expression of CCR2 in the intact DRGs in rats that received CCI, SNL and CCD surgery, respectively. CCR2 protein level in the ipsilateral L4/5 DRG from naïve control rats ($n = 8$ rats, 16 L4/5 DRGs in 4 groups) and injured rats was determined by Western blotting ($n = 8$ rats, 16 L4/5 DRGs in 4 groups for CCD or CCI model, respectively; $n = 16$ rats, 16 L5 DRGs in 4 groups for SNL model). As illustrated in Fig. 1A, Western blotting analysis with a CCR2 antibody showed the 42 kDa band in the DRG tissues of naïve rats but greater CCR2 band densities in the injured DRGs. As illustrated in Fig. 1B, densimetric analysis of immunoblot band intensities demonstrated 4.1 ± 1.4, 2.6 ± 0.47 and 2.4 ± 1.1 fold increases in band intensities of DRG after CCD, SNL and CCI injury, respectively, compared with the naïve control ($p < 0.01$, $F = 4.80$, Tukey test). However, the degree of increase in CCR2 expression in DRG was not significantly different among CCD, SNL and CCI groups ($p = 0.405$, $F = 0.956$).

We then examined the effects of CCL2 in altering the excitability of nociceptive DRG neurons. The acutely dissociated small DRG neurons were recorded under the whole-cell current clamp configuration CCI neurons had a capacitance of 40.2 ± 2.2 pF ($n = 25$) and a resting membrane potential of −56.5 ± 0.9 mV ($n = 25$). Of 25 neurons tested, 11 were depolarized (≥2 mV) by CCL2 (100 nM, 1 min) [15] for (11 of 25, 44%). The mean magnitude of depolarization of the responsive CCI neurons was 19.9 ± 8.2 mV ($n = 11$). Five responsive CCI neurons (5/11, 45.5%) exhibited spontaneous action potentials. SNL neurons had a capacitance of 40.3 ± 1.9 pF ($n = 21$) and a resting membrane potential of −55.3 ± 0.9 mV ($n = 21$). Of 21 neurons tested, 7 were depolarized (≥2 mV) after a local, puff application of CCL2 (7 of 21, 30.4%). The mean magnitude of depolarization of the responsive SNL neurons was 18.4 ± 5.2 mV ($n = 7$). One responsive SNL neuron (1/7, 14.3%) exhibited spontaneous action potentials. CCD neurons had a capacitance of 36.9 ± 1.3 pF