Cerebrospinal Fluid Oxaliplatin Contributes to the Acute Pain Induced by Systemic Administration of Oxaliplatin

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ABSTRACT

Background: Systemic administration of oxaliplatin has no effect on the tumors in the central nervous system (CNS) due to the limited concentration of oxaliplatin in the cerebrospinal fluid (CSF), while it was clinically reported that oxaliplatin can induce acute encephalopathy. Currently, the impairment of neuronal functions in the CNS after systemic administration of oxaliplatin remains uninvestigated.

Methods: The von Frey test and the plantar test were performed to evaluate neuropathic pain behavior after a single intraperitoneal administration of oxaliplatin (4 mg/kg) in rats. Inductively coupled plasma–mass spectrometry, electrophysiologic recording, real-time quantitative reverse transcription polymerase chain reaction, chromatin immunoprecipitation, Western blot, immunohistochemistry, and small interfering RNA were applied to understand the mechanisms.

Results: Concentration of oxaliplatin in CSF showed a time-dependent increase after a single administration of oxaliplatin. Spinal application of oxaliplatin at the detected concentration (6.6 nM) significantly increased the field potentials in the dorsal horn, induced acute mechanical allodynia (n = 12 each) and thermal hyperalgesia (n = 12 each), and enhanced the evoked excitatory postsynaptic currents and spontaneous excitatory postsynaptic currents in the projection neurokinin 1 receptor–expressing lamina I to II neurons. The authors further found that oxaliplatin significantly increased the nuclear factor-κB p65 binding and histone H4 acetylation in *cx3cl1* promoter region. Thus, the upregulated spinal CX3CL1 markedly mediated the induction of central sensitization and acute pain behavior after oxaliplatin administration.

Conclusions: The findings of this study suggested that oxaliplatin in CSF may directly impair the normal function of central neurons and contribute to the rapid development of CNS-related side effects during chemotherapy. This provides novel targets to prevent oxaliplatin-induced acute painful neuropathy and encephalopathy. **(Anesthesiology 2016; 124:1109-21)**

PAINFUL neuropathy manifested as allodynia and tingling frequently occurs in the cancer patients treated with chemotherapeutic drugs such as oxaliplatin, paclitaxel, and bortezomib.[1,2] Previous studies demonstrated that such painful neuropathy was largely mediated by axonopathy and/or myelinopathy, especially intraepidermal nerve fiber degeneration, a delayed neurotoxic effect of chemotherapeutic drugs.[3,4] However, clinical evidences also showed that chemotherapeutic drugs, in spite of the limited permeability across the blood–brain barrier (BBB), can induce acute encephalopathy manifested as seizures and progressive gait and balance difficulties.[5,6]

Oxaliplatin is a specific first-line antitumor agent.[7] Application of oxaliplatin induces severe painful neuropathy, characterized by two types of neurologic symptoms including acute and chronic peripheral neuropathy. Although cold hypersensitivity is the most prominent symptom in the patients receiving oxaliplatin, oxaliplatin evoked allodynia and hyperalgesia in the rodents.[8,9] However, chemotherapy-induced neuropathic pain is a dose-limiting side effect, which may persist beyond

What We Already Know about This Topic

- Oxaliplatin is a commonly used chemotherapeutic agent for non–central nervous system tumors
- The low concentrations of oxaliplatin reaching the central nervous system after systemic administration might contribute to the drug's side effects including pain and encephalopathy

What This Article Tells Us That Is New

- The administration of oxaliplatin to rats leads to nociceptive sensitization and the accumulation of the drug in cerebrospinal fluid
- Oxaliplatin may support sensitization of spinal cord neurons through an epigenetic mechanism resulting in the up-regulation of CX3CL1

Corresponding article on page 992. Drs. Huang, Li, and Ou-Yang contributed equally to this work.

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termination of treatment and lead to a sustained condition as painful or more painful than the original cancer.[4] For chronic peripheral neuropathy, the accumulation of platinum in the dorsal root ganglia (DRG) induces the axonopathy of peripheral nerves, thus leading to the persistent sensory abnormality.[3] Meanwhile, oxaliplatin-induced acute neuropathy is characterized by a rapid onset and emergence of symptoms immediately after infusion,[10] while axonopathy of peripheral nerves can unlikely occur in such a short time. Notably, low concentration of chemotherapeutic drugs can be detected in the central nervous system (CNS) after a single systemic administration,,[11–13] which implies that the chemotherapeutic drug entering the CNS might directly disturb the normal function of central neurons to mediate acute neuropathy. In this study, oxaliplatin-induced, acute pain–related behavior is adopted as a model to elucidate the mechanism of acute CNS disorder caused by the chemotherapeutic drugs.

A large body of evidence has shown that central sensitization, referring to the augmented response of central signaling neurons, plays a key role in the induction and maintenance of neuropathic pain.[14] Long-term potentiation at the synapses between afferent fibers and spinal dorsal horn neurons is an attractive cellular model of central sensitization.[15,16] Studies also showed that neurokinin 1 receptor (NK1R)–expressing neurons in the spinal dorsal horn played an important role in the processing of acute or sustained nociceptive information. For example, selective elimination of NK1R-expressing neurons by intrathecal injection of saporin-conjugated substance P remarkably attenuated the development of thermal hyperalgesia and mechanical allodynia.[17,18] It was also well known that large proportion of spinothalamic and spinal brachial neurons located in lamina I expressed NK1R, suggesting that these NK1R-expressing neurons participated in the ascending conduction of nociceptive information.[19]

Therefore, in this study, we aimed to determine the critical involvement of low concentration of cerebrospinal fluid (CSF) oxaliplatin, and the underlying mechanism, in the central sensitization and painful behavior induced by systemic administration of the chemotherapeutic agent. We hypothesized that the CSF oxaliplatin crossing the brain–blood barrier may directly activate transcriptional factor nuclear factor (NF)-κB signaling and epigenetic up-regulation of CX3CL1 in the dorsal horn neurons, thus contributing to central sensitization and acute neuropathy induced by systemic administration of oxaliplatin.

Materials and Methods

Animals

Male Sprague-Dawley rats (220 to 250 g) were housed in a temperature-controlled room (22° ± 1°C) with a 12-h light–dark cycle. Rats were randomly divided into different experimental groups. All animal experimental procedures were approved by the Sun Yat-Sen University Animal Care and Use Committee (Guangzhou, Guangdong, China) and carried out in accordance with the National Institutes of Health (Bethesda, Maryland) guideline on animal care.

Drug Administration and Behavioral Test

Oxaliplatin was purchased from Sigma Aldrich (USA) and freshly dissolved in 5% glucose/H_2O as a stock solution of 1 mg/ml. Rats received a single intraperitoneal administration of oxaliplatin (4 mg/kg) to induce acute mechanical allodynia and thermal hyperalgesia. The control animals received an equivalent volume of 5% glucose/H_2O. Intrathecal injection of neutralizing antibody against CX3CL1 (10 μg in 10 μl, Torrey Pines Bio Labs, USA), isotype IgG (10 μg in 10 μl, R&D system, USA), ammonium pyrrolidinedithiocarbamate (PDTC; 200 ng in 10 μl, Sigma, USA), NF-κB p65 small interfering RNA (siRNA; 50 μg in 15 μl, Ribobio, China), or scramble siRNA (50 μg in 15 μl, Ribobio) was performed 30 min before oxaliplatin application.

Intrathecal injection was preformed according to our previously described method.[20] In brief, laminectomy of the L5 vertebra was performed during anesthesia using sodium pentobarbital (50 mg/kg, intraperitoneally). After the dura was probed with an 8G needle, a polyethylene-10 catheter was inserted into the subarachnoid space of the rat through the L5/L6 intervertebral space, and the tip of the catheter was placed at the L5 spinal segmental level. After intrathecal implantation, the rats were allowed to recover from surgery for at least 5 days before subsequent drug injection. Any rats exhibiting hind limb paralysis or paresis after surgery were excluded from the study.

The 50% withdrawal threshold was assessed using von Frey hairs as described previously.[21,22] Briefly, each animal was loosely restrained in a plastic box on a metal mesh and allowed to acclimate for at least 15 min per day for 3 consecutive days. On the first testing day, the animals were reintroduced to the testing environment and allowed to accommodate, and then von Frey filaments (bending force: 0.8*g*, 1.12*g*, 1.68*g*, 3.2*g*, 4.89*g*, 6.3*g*, 8.6*g*, 12.6*g*, and 20.2*g*) were presented alternately from the underneath to the midplantar surface of each hind paw. Each von Frey hair was applied 10 times. A nociceptive response was defined as a brisk paw withdrawal or flinching of the paw after von Frey filament application. The responses to all filaments for both paws were tabulated as a single value, and 50% withdrawal threshold was defined as the lowest bending force that produced 10 or more responses.

Thermal hyperalgesia was tested using a plantar test (7370, Ugo Basile Plantar Test Apparatus, Italy) according to the described method.[23] Briefly, a radiant heat source beneath a glass floor was aimed at the plantar surface of the hind paw. Three measurements of hind paw withdrawal latency were taken for each hind paw in each test session. The hind paw was tested alternately with more than 5-min intervals between the consecutive tests. Three measurements of latency per side were averaged as the result of each test. A 20-s cutoff was set to prevent the tissue damage.

The experimenter who conducted the behavioral tests was blinded to all treatments.

Inductively Coupled Plasma–Mass Spectrometry

CSF was drawn from a temporary foramen magnum, and bilateral plantar subcutaneous tissues (including skins and superficial muscle) were excised. CSF samples (0.1 ml) and plantar subcutaneous tissues (about 0.1 g/total, 0.05 g/per side) were obtained at different time points from different animals (*i.e.*, only one group of sample including CSF and plantar subcutaneous tissue was collected from one rat). The total platinum level in CSF samples or plantar subcutaneous tissues was assessed by inductively coupled plasma–mass spectrometry (ICP-MS; iCAP Qc; Thermo Fisher Scientific, USA) according to the method previously described.[24] Briefly, CSF samples (0.1 ml) or plantar subcutaneous tissues (0.1 g) were digested in a water bath at 90° to 100°C in a Teflon bomb with optimal-grade HNO_3 (2 ml) and then diluted in 10 ml distilled water. The resulting solutions were injected into the ICP-MS device to quantify the platinum accumulated in CSF samples or plantar subcutaneous tissues. Iridium (0.005 μg/ml in 1% nitric acid) was added as an internal standard to correct instrument drift during analysis. Blank samples were also digested and analyzed to provide correction. Each measurement used the average platinum value of the regent blanks as blank correction. CSF samples or plantar subcutaneous tissues from naive animals were used as control. Each sample was measured in triplicate.

In Vivo Field Potentials Recordings and Nerve Stimulations

Recording of C-fiber–evoked potentials in spinal dorsal horn was performed according to our previously described method.[25,26] Briefly, the rats were anesthetized with urethane (1.5 g/kg, intraperitoneally) and successfully maintained normal vital signs. The left sciatic nerve or sural nerve was dissected free for bipolar stimulation with a silver hook electrode. A laminectomy was performed to expose lumbar segments 4 and 5. A- or C-fiber–evoked field potentials were recorded at a depth of 50 to 400 μm from the dorsal surface of the spinal cord in ipsilateral lumbar enlargement with glass electrodes (impedance, 1 to 2 MΩ) in response to stimulation of sciatic nerve fibers or sural nerve fibers. An A/D converter card (DT2821-F-16SE, Data Translation, USA) was used to digitize and collect data at a sampling rate of 10 kHz. Single square pulses (0.5 ms duration in 1-min intervals) were delivered to the sciatic nerve or sural nerve as test stimuli. The strength of stimulation was adjusted to 1.5 to 2 times of the threshold for C-fiber response. The amplitudes of C-fiber–evoked field potentials were determined by long-term potentiation program.[16,25] In each experiment, responses to five consecutive test stimuli were averaged. The mean amplitudes of C-fiber responses before drug or vehicle application served as baseline. Only one recording was conducted on each animal.

Patch Clamp Recording in Spinal Slices

Spinal cord slices from 22 rats were prepared as described previously.[27] Briefly, the spinal cord was quickly removed and immersed in oxygenated (95% O_2^- and 5% CO_2) cold artificial cerebrospinal fluid (ACSF; in millimolar): 127 NaCl, 3.1 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose, pH 7.3, osmolarity 300 to 310 mOsm/l. Then, 500-μm thick sagittal spinal L4 to L6 cord slices with an attached root were cut with a vibratome (DTK-1000, Dosaka, Japan). Slices were incubated in gassed ACSF for at least 1 h at 32°C. After incubation, an individual slice with attached dorsal root was transferred to a recording chamber and continually perfused with oxygenated ACSF solution at room temperature. The dorsal root was gently led into the suction electrode. Lamina I to II neurons were visualized using a 60× water-immersion objective on an upright infrared Nikon microscope (Nikon, Japan). EPC-10 amplifier and the PULSE program (HEKA Electronics, Germany) were used with pipette (4 to 6 MΩ) containing the internal solution as following (in millimolar): K-gluconate 135, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, biocytin 5, and Mg-ATP 5. A seal resistance of more than or equal to 2 GΩ and an access resistance of 20 to 35 MΩ were considered acceptable. The series resistance was optimally compensated by more than or equal to 70% and constantly monitored throughout the experiments. Spontaneous EPSC and dorsal root stimulation–evoked EPSCs were recorded at the holding potential of −70 mV. Three neurons per slice were sampled for electrophysiologic recording.

Immunohistochemistry to Identify the NK1R Neurons in Lamina I

During the whole cell recording experiment, biocytin was included in the pipette solution to identify whether the recorded neurons were projection neurons. After completion of the recording, the spinal slice was fixed by immersion in 4% paraformaldehyde/phosphate buffer overnight. The sections were then blocked with 10% normal goat serum in Tris-Triton buffer and incubated for 72 h with rabbit anti-NK1R (1: 500, Novus, USA). After washing in Tris-buffered saline, sections were incubated for 24 h with a cocktail of Alexa Fluor 488-coupled goat anti-rabbit IgG (1:800; Jackson Immuno Reseach, USA) and Cy3-streptavidin (Invitrogen, USA). The stained sections were then examined with a Zeiss confocal microscope (Zeiss, Germany) equipped with a Zeiss digital camera (Zeiss).

RNA Extraction and Real-time Quantitative PCR

Total RNA was extracted from the rat dorsal horn tissues with Trizol reagent (Invitrogen). Reverse transcription was performed using oligo-dT primer and M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol. Specific primers sequences of the examined mRNA and β-actin for polymerase chain reaction (PCR) reactions were listed in table 1. Real-time quantitative PCR was

Table 1. Specific Primer Sequences

Gene	Primer	Sequence
CCL1	Forward	5′-AGAAAGCTGCGCCTTAA-3′
	Reverse	5′-CTCTGGTGCTGGGATGG-3′
TNF-α	Forward	5′-GGCCACCACGCTCTTCTGTC-3′
	Reverse	5′-GGGCTACGGGCTTGTCACTC-3′
IL-1β	Forward	5′-GGATGATGACGACCTGCTA-3′
	Reverse	5′-CACTTGTTGGCTTATGTTCTG-3′
IL-6	Forward	5′-CCACTGCCTTCCCTACTT-3′
	Reverse	5′TTGCCATTGCACAACTCT-3′
CX3CL1	Forward	5′-CTCCAGCCATCCAGCCATG-3′
	Reverse	5′-CATTTCGTCATGCCGAGGTG-3′
MCP-1	Forward	5′-CATTGTGGCCAAGGAGATCTG-3′
	Reverse	5′-CTTCGGAGTTTGGGTTTGCTT-3′
IL-10	Forward	5′-TGGACAACATACTGCTGACAG-3′
	Reverse	5′-GGTAAAACTTGATCATTTCTGACAAG-3′
IFN-γ	Forward	5′-CATCGCCAAGTTCGAGGTGA-3′
	Reverse	5′-CACCGACTCCTTTTCCGCTT-3′
β-Actin	Forward	5′-AGGGAAATCGTGCGTGACAT-3′
	Reverse	5′-GAACCGCTCATTGCCGATAG-3′

IFN = interferon; IL = interleukin; MCP = monocyte chemotactic protein; TNF = tumor necrosis factor.

performed using SYBR Green qPCR SuperMix (Invitrogen) and the ABI PRISM 7500 Sequence Detection System (USA). The reactions were set up based on the manufacture's protocol. PCR reaction conditions were incubation at 95°C for 3 min followed by 40 cycles of thermal cycling (10 s at 95°C, 20 s at 58°C, and 10 s at 72°C). The relative expression ratio of mRNA in the spinal tissues was quantified by the 2-ΔΔCT method.

Western Blot

Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) at different time points. The spinal cord was removed and sectioned in a cryostat. The spinal dorsal horn punch was taken with a 15-gauge cannula and frozen at −80°C until used. Samples (about 40 mg) were homogenized on ice in 15 mmol/l Tris buffer containing a cocktail of proteinase inhibitors and phosphatase inhibitors. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The blots were placed in the block buffer for 1 h at room temperature and incubated with primary antibody against CX3CL1 (1:1000, Torrey Pines Bio Labs), NF-κB p65 (1:1000, Abcam, USA), phosphorylated NF-κB p65 (Ser311; 1:1000, CST, USA), acetylated histone H4 (1:1000, Millipore, USA), histone H4 (1:1000, Millipore), acetylated histone H3 (K9; 1:500, Abcam), or histone H3 (1:500, Abcam) overnight at 4°C. The blots were then incubated with horseradish peroxidase–conjugated secondary antibodies. Electrochemiluminescence (Pierce, USA) was used to detect the immune complex. The band was quantified with computer-assisted imaging analysis system (ImageJ; NIH, USA).

siRNA Preparation, Transfection, and Screening

Specific siRNAs were applied to targetedly knockdown the expression of NF-κB p65 as previously described.[28] Three siRNAs targeting rat *Rela* (NF-κB p65) gene were designed and synthesized by Ribobio (China). The nucleotide sequences were as follows: siRNA1: 5′-GCAUCCAGACCAACAAUAAdTdT-3′ (sense) and 3′-dTdTCGUAGGUCUGGUUGUUAUU-5′ (antisense); siRNA2: 5′-C UCAAGAUCUGCCGAGUAAdTdT-3′ (sense) and 3′-dTdTGAGUUCUAGACGG CUCAUU-5′ (antisense); and siRNA3: 5′-GCAGUUCGAUGCUGAUGAAdTdT-3′ (sense) and 3′-dTdTCGUCAAGCUACGACUACUU-5′ (antisense). According to our previous screening test,[28] the siRNA with sequence 1 demonstrated remarkable efficacy to suppress the expression of NF-κB p65 subunit in the HBZY-1 cell line and spinal cord *in vivo*. Hence, the synthesized siRNA1 was chosen for the subsequent experiments *in vivo*.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP Assay Kit (Thermo, USA).[28] The rat L4 to L5 spinal dorsal horns were removed quickly and placed in 1% formaldehyde for 2 min. The DNA was fragmented by sonication and digested with micrococcal nuclease. After the addition of ChIP dilution buffer, 100 μl samples were saved as input. Eight microliters of NF-κB p65 antibody (Abcam) or acetylated histone H4 antibody (Millipore) was added to 500 μl precleared chromatin solution, and the sample was incubated overnight. Immunoprecipitation with control rabbit IgG (Cell Signaling Technology, USA) was performed as a negative control. Antibody/DNA complexes were captured, washed, and eluted, and the cross-link was reversed. The DNA was purified from the complexes and input fractions. The precipitated DNA was resuspended in 60 μl of nuclease-free water, and quantitative real-time PCR or semiquantitative PCR was performed on 5 μl of samples as described above. ChIP/input ration was calculated. Primers (5′-GCTGCCCTGACCATAAAT-3′ and 5′-AGCTGTACGGCACTCACC-3′) were designed to amplify a −1941/−1931 region relative to the transcription start site of rat *cx3cl1* promoter, which contains an NF-κB–binding site.[29]

Statistical Analysis

All data were expressed as means ± SD and analyzed using SPSS 13.0 (SPSS, USA). Western blot, reverse transcription PCR, and electrophysiologic data were analyzed by two-way ANOVA followed by Tukey *post hoc* test. For behavioral test, one-way or two-way ANOVA with repeated measures followed by Tukey *post hoc* test was carried out. The criterion for statistical significance was $P < 0.05$. While no power analysis was performed, the sample size was determined according to our and peers' previous publications in painful behavior and pertinent molecular studies.

Results

CSF Oxaliplatin Increased Field Potentials in the Spinal Dorsal Horn and Induced Acute Pain-related Behavior in Rats

This study showed that a single intraperitoneal administration of oxaliplatin (4 mg/kg) elicited the rapid development of pain-related behavior. As shown in figure 1, A and B, significant decreases in the 50% paw withdrawal threshold ($F = 38.56$, $P < 0.01$) and paw withdrawal latency ($F = 62.89$, $P < 0.01$) were detected in 60 min and persisted for at least 10 h after injection of oxaliplatin. We also found that oxaliplatin with a single intraperitoneal injection significantly enhanced C-fiber–evoked field potentials in dorsal horn in 60 min with a peak in about 120 min (fig. 1C). As the thermal hyperalgesia and mechanical allodynia were mediated by different nerve fibers, we further examined the responses of sciatic-evoked C- or A-fiber potentials in the spinal dorsal horn. The results showed that the amplitude of C- and A-fiber field potentials significantly increased at 60 min after single oxaliplatin treatment compared with vehicle group (fig. 1, D and E). The rapid onset of pain behaviors and electrophysiologic adaptation suggested that the behavioral hypersensitivity might result from the central sensitization directly induced by oxaliplatin in spinal dorsal horn.

To determine whether the oxaliplatin entering the CNS directly induced central sensitization and nociceptive transmission in the spinal dorsal horn, we detected the amount of oxaliplatin in both CSF and plantar subcutaneous tissue by ICP-MS after a single injection of oxaliplatin (4 mg/kg, intraperitoneally). The amount of oxaliplatin in CSF was 6.6 ± 0.53 nM in 1 h (8.7 ± 0.66 nM in 2 h and 18.4 ± 1.53 nM in 6 h) and that in plantar subcutaneous tissue was 5.5 ± 0.42 ng/100 mg tissue in 1 h (6.9 ± 0.94 ng in 2 h and 19.1 ± 2.08 ng in 6 h) after a single intraperitoneal administration of oxaliplatin (fig. 1, F and G). In view of a total approximate 580 μl volume of CSF in rats,[30] we directly applied 38 nM oxaliplatin (the final concentration after dilution in CSF was similar with the detected concentration in CSF in 1 h after systemic administration) onto the

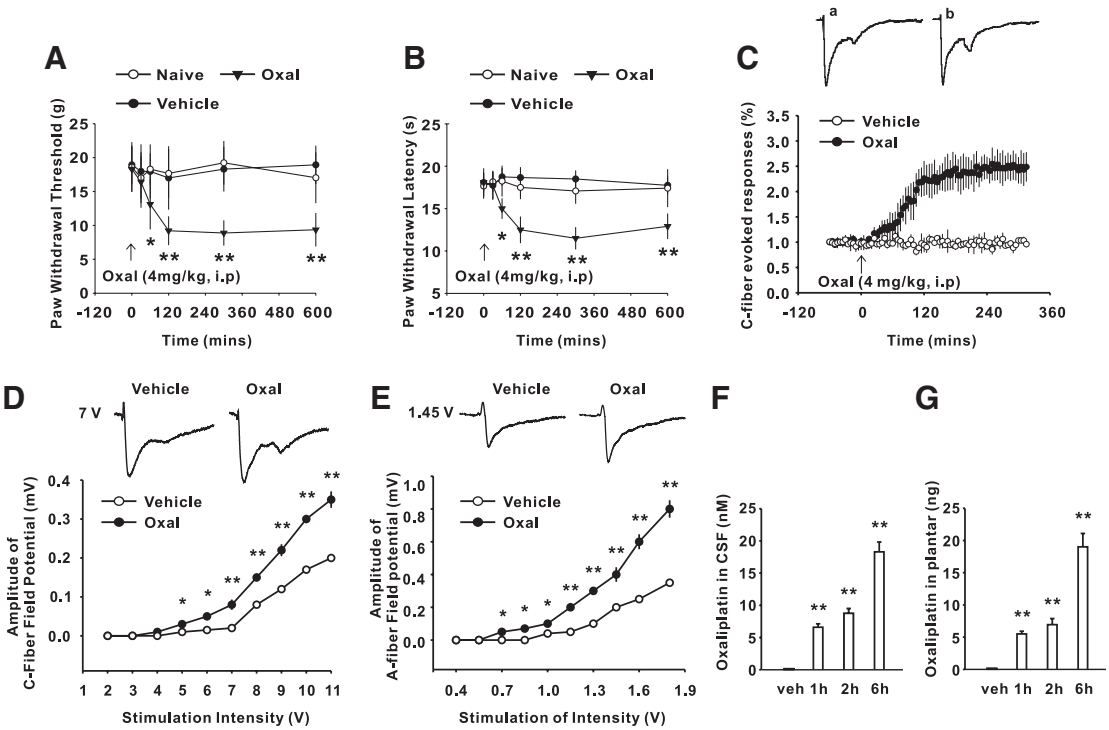

Fig. 1. A single intraperitoneal administration of oxaliplatin (oxal) induced the pain-related behavior and the increases of sciatic-evoked C- or A-fiber responses in the spinal dorsal horn. A single intraperitoneal injection of oxal (4 mg/kg) produced significant mechanical allodynia (*A*) and thermal hyperalgesia (*B*), which started at 1 h and lasted for at least 10 h. n = 12 in each group; **P* < 0.05, ***P* < 0.01 *versus* the vehicle (veh) group. (*C*) Long-term potentiation of C-fiber–evoked field potentials in the spinal cord was induced after oxal treatment (4 mg/kg, intraperitoneally [i.p.]). n = 12 in each group. Traces at *top* were recorded before (*a*) and 120 min after (*b*) oxal application. (*D*) The stimulus-response curves of the C-fiber–evoked field potentials. Traces at *top* were the representative original recordings of spinal C-fiber–evoked field potentials on stimulus intensity of 7 V after veh or oxal treatment. n = 12 in each group; **P* < 0.05, ***P* < 0.01 *versus* the veh group. (*E*) Single oxal treatment significantly increased the amplitude of A-fiber–evoked field potential. Traces at *top* were the representative original recordings of spinal A-fiber–evoked field potentials on stimulus intensity of 1.45 V after veh or oxal treatment. n = 12 in each group; **P* < 0.05, ***P* < 0.01 *versus* the veh group. The amount of oxal in cerebrospinal fluid (CSF) (*F*) and plantar subcutaneous tissue (*G*) at 1, 2, and 6 h after injection of oxal (4 mg/kg, intraperitoneally). n = 6 in each group; ***P* < 0.01 *versus* the veh group.

spinal dorsal surface, and the alterations of field potentials and pain behavior were examined. The results showed that the application of oxaliplatin directly onto the spinal dorsal surface at the detected amount in CSF (38 nM, 100 μl) significantly enhanced the field potentials in dorsal horn. At 60 min after oxaliplatin application, the potentiation of C-fiber–evoked field potentials was 145.36 ± 17.35% compared to the baseline. The potentiation reached maximum in 120 min and remained at high level until the end of experiments (fig. 2A). Behavioral analysis also showed that oxaliplatin by intrathecal injection (380 nM in 10 μl) decreased the 50% withdrawal threshold (fig. 2B) and withdrawal latency (fig. 2C). Recent study showed that intraplantar administration of higher dose of oxaliplatin (1 or 40 μg) can induce hyperalgesia.[31,32] Our results showed that intraplantar injection of oxaliplatin at the detected concentration in plantar subcutaneous tissue (20 ng in 10 μl) did not show obvious effect on the 50% withdrawal threshold compared with the vehicle group (fig. 2D). Consistent with the behavioral finding, intraplantar injection of oxaliplatin (20 ng in 10μl) did not change the field potentials in dorsal horn (fig. 2E). Furthermore, to rule out the disturbance of the tibial nerve and the gastrocnemius-soleus nerve,[33] we recorded the C-fiber–evoked field potential by electrical stimulation of the sural nerve. The results showed that C-fiber–evoked field potentials by electrical stimulation of the sural nerve did not change in the rats with intraplantar injection of oxaliplatin (20 ng in 10 μl; fig. 2F). These results implied that the oxaliplatin entering the CNS, but not that distributed in the plantar subcutaneous tissue, played an important role in the induction of rapid onset of central sensitization and pain-related behavior.

Up-regulation of CX3CL1 Contributes to Pain-related Behavior and Central Sensitization Induced by Systemic Administration of Oxaliplatin

In view of the pivotal role of neuroinflammation in the induction of central sensitization and hyperalgesia,[34] we here determined whether oxaliplatin-induced central sensitization and behavioral hypersensitivity were mediated by regulating the expression of cytokine and chemokines. The results showed that a single intraperitoneal treatment of oxaliplatin induced significant increase in the mRNA levels of several surveyed cytokines including monocyte chemotactic protein 1, interleukin-6, CX3CL1, interferon-γ, tumor necrosis factor-α, and interleukin-1β at different time points, respectively (fig. 3A). Among these cytokine/chemokines, CX3CL1 underwent a significant dynamic change, which was coincident with behavioral hypersensitivity induced by oxaliplatin. Considering its critical role in the central sensitization and neuropathic pain,[35] including paclitaxel-induced neuropathic pain,[28] subsequent investigation was carried out to study the

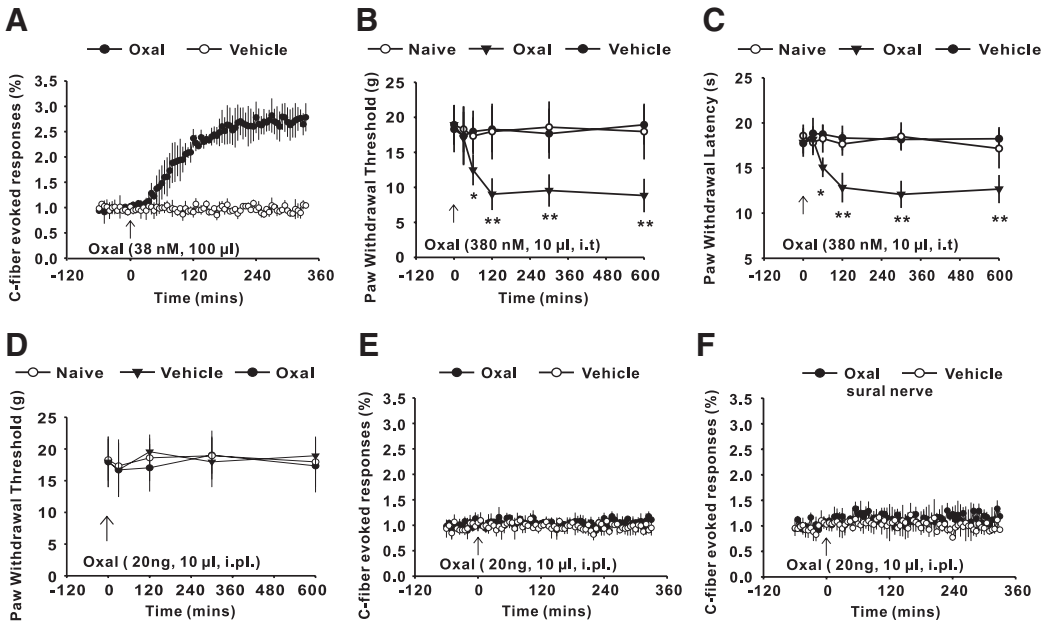

Fig. 2. Local application of oxaliplatin (oxal) induced the long-term potentiation in dorsal horn and pain-related behavior. (*A*) After basal threshold was determined, local bath application of oxal (38 nM in 100 μl) to the spinal cord surface was carried out, and C-fiber–evoked field potentials began to increase at 60 min and lasted for 6 h. n = 12 in each group. (*B*) Intrathecal (i.t.) injection of oxal (380 nM in 10 μl) induced significant mechanical allodynia. n = 12 in each group; **P* < 0.01, ***P* < 0.01 *versus* the vehicle group. (*C*) Paw withdrawal latency was significantly decreased after i.t. injection of oxal (380 nM in 10 μl). n = 12 in each group; **P* < 0.01, ***P* < 0.01 *versus* the vehicle group. (*D*) Intraplantar injection of oxal (20 ng in 10 μl) had no effect on mechanical withdrawal threshold. n = 12 in each group. (*E*) Intraplantar injection of oxal (20 ng in 10 μl) had no effect on the C-fiber–evoked field potentials in the dorsal horn. n = 12 in each group. (*F*) Intraplantar injection of oxal (20 ng in 10 μl) had no effect on the C-fiber–evoked field potentials in the spinal cord by electrical stimulation of the sural nerve. n = 12 in each group. i.pl. = intraperitoneally.

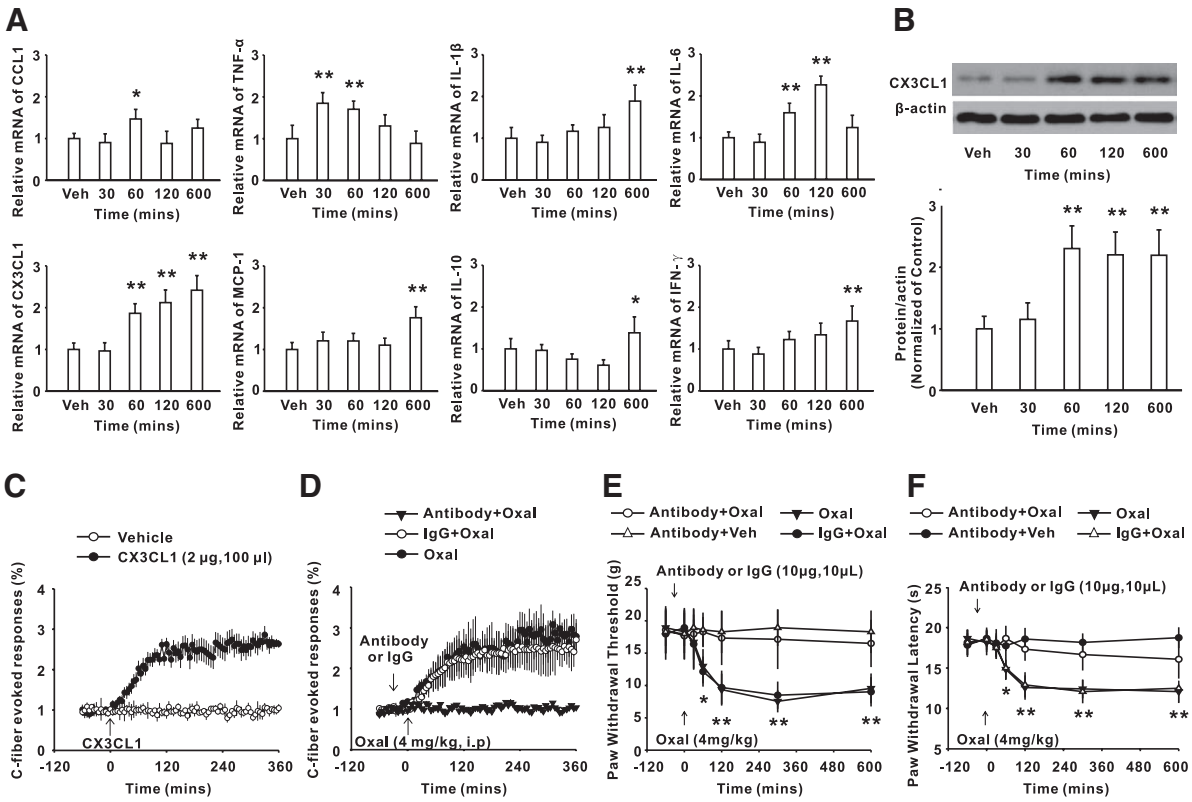

Fig. 3. Increased CX3CL1 in the dorsal horn mediated oxaliplatin (oxal)-induced long-term potentiation (LTP) and pain-related behavior. (*A*) mRNA levels of cytokines/chemokines in the spinal dorsal horn after single oxal treatment (4 mg/kg, intraperitoneally). n = 6 in each group; **P* < 0.05, ***P* < 0.01 *versus* vehicle (veh) group. (*B*) Expression of CX3CL1 protein began to increase at 60 min after oxal treatment (4 mg/kg, intraperitoneally) and lasted for 600 min. n = 6 in each group; ***P* < 0.01 *versus* veh group. (*C*) Local application of CX3CL1 (2 μg in 100 μl) induced LTP in the spinal dorsal horn. n = 12 in each group. (*D*) Local application of CX3CL1 neutralizing antibody (10 μg in 100 μl) on the spinal cord blocked LTP induced by the intraperitoneal injection of oxal. n = 12 in each group. (*E*, *F*) Intrathecal injection of CX3CL1 neutralizing antibody (10 μg in 10 μl) blocked oxal-induced mechanical allodynia (*E*) and thermal hyperalgesia (*F*). n = 12 in each group; **P* < 0.05, ***P* < 0.01 *versus* veh group. IFN = interferon; IL = interleukin; i.pl. = intraperitoneally; MCP = monocyte chemotactic protein; TNF = tumor necrosis factor.

role of CX3CL1 in the oxaliplatin-induced central sensitization and pain-related behavior. Consistent with the change of CX3CL1 mRNA, oxaliplatin also induced an increase in the CX3CL1 protein expression in the spinal dorsal horn at 60 min, which maintained till the end of the experiment (10 h) when compared with the vehicle group (fig. 3B).

To define the role of CX3CL1 in oxaliplatin-induced central sensitization and pain-related behavior, we first observed whether the application of CX3CL1 onto the spinal dorsal surface can induce the potentiation of nociceptive transmission. The results showed that markedly increased field potentials in the dorsal horn were evoked by CX3CL1 (2 μg, fig. 3C), mimicking the spinal plasticity induced by oxaliplatin. Local spinal application with neutralizing antibody against CX3CL1 (10 μg/100 μl), but not IgG (10 μg/100 μl), significantly inhibited the oxaliplatin (4 mg/kg, intraperitoneally)-induced increase of field potentials in dorsal horn (fig. 3D). Consistently, intrathecal injection of CX3CL1 neutralizing antibody, but not IgG, also attenuated acute mechanical allodynia (fig. 3E) and thermal hyperalgesia (fig. 3F) induced by oxaliplatin (4 mg/kg, intraperitoneally). Intrathecal injection of neutralizing antibody alone had no effect on the pain-related behavior in the naive rats (fig. 3, E and F). These findings suggested that increased expression of CX3CL1 substantially contributed to the spinal sensitization and painful behavior induced by oxaliplatin in the rodent model.

CSF Oxaliplatin Increased the CX3CL1 Expression and Excitability of the NK1R+ Project Neurons of Spinal Lamina I

Spinal lamina I projection neurons play a major role in the processing and integration of peripheral nociceptive information that is relayed to supraspinal brain regions. To further define the mechanism underlying the increased efficiency of synaptic transmission induced by local application of oxaliplatin, we examined the potential involvement of CX3CL1 signaling in the spinal plasticity and painful behavior induced by local application of oxaliplatin. First, the results showed a significantly increased expression of CX3CL1 in the spinal cord slices incubated with oxaliplatin (6.6 nM) for 1 and 2 h (fig. 4A). Next, we performed whole cell recording on the

lamina I projection neurons in spinal cord slices with attached dorsal root to examine the involvement of CX3CL1 signaling in the central sensitization induced by oxaliplatin. The results showed that amplitude of C-response elicited by dorsal roots C-fiber stimulation in the lamina I NK1R+ projection neurons was significantly increased after oxaliplatin (6.6 nM) incubation for 1 h (fig. 4B). Moreover, amplitude, but not the frequency, of spontaneous excitatory postsynaptic currents (sEPSCs) was significantly increased in the surveyed NK1R+ neurons in the spinal cord slices incubated with oxaliplatin (fig. 4C). Furthermore, our results also showed that incubation of spinal cord slices with CX3CL1 (2 μg) for 1 h significantly increased the amplitude of C-response of NK1R+ projection neurons elicited by dorsal roots C-fiber stimulation (fig. 3B). We also found that incubation of the slices with CX3CL1 neutralizing antibody (10 μg) significantly attenuated the enhancement of dorsal root stimulation–elicited evoked excitatory postsynaptic currents (eEPSC) induced by oxaliplatin (fig. 4B). Meanwhile, CX3CL1 incubation significantly increased the amplitude of sEPSCs, and inhibition of CX3CL1 with neutralizing antibody significantly attenuated the increases of sEPSCs amplitude induced by oxaliplatin (fig. 4C). Note that NK1R+ projection neurons of spinal lamina I were afterward confirmed by immunohistochemistry results that the recorded biocytin-positive cells were colocalized with the NK1R-positive cells (fig. 4D). These results further suggested the critical involvement of CX3CL1 signaling in the enhanced excitatory synaptic transmission in dorsal horn induced by oxaliplatin.

NF-κB p65 Mediated Pain-related Behavior and Increase of Field Potentials via Up-regulation of CX3CL1 after Oxaliplatin Treatment

NF-κB, an important transcriptional factor, regulates the expression of many cytokines and chemokines.[36] Next, we determined whether NF-κB signaling participated in the oxaliplatin-induced pain-related behavior and central

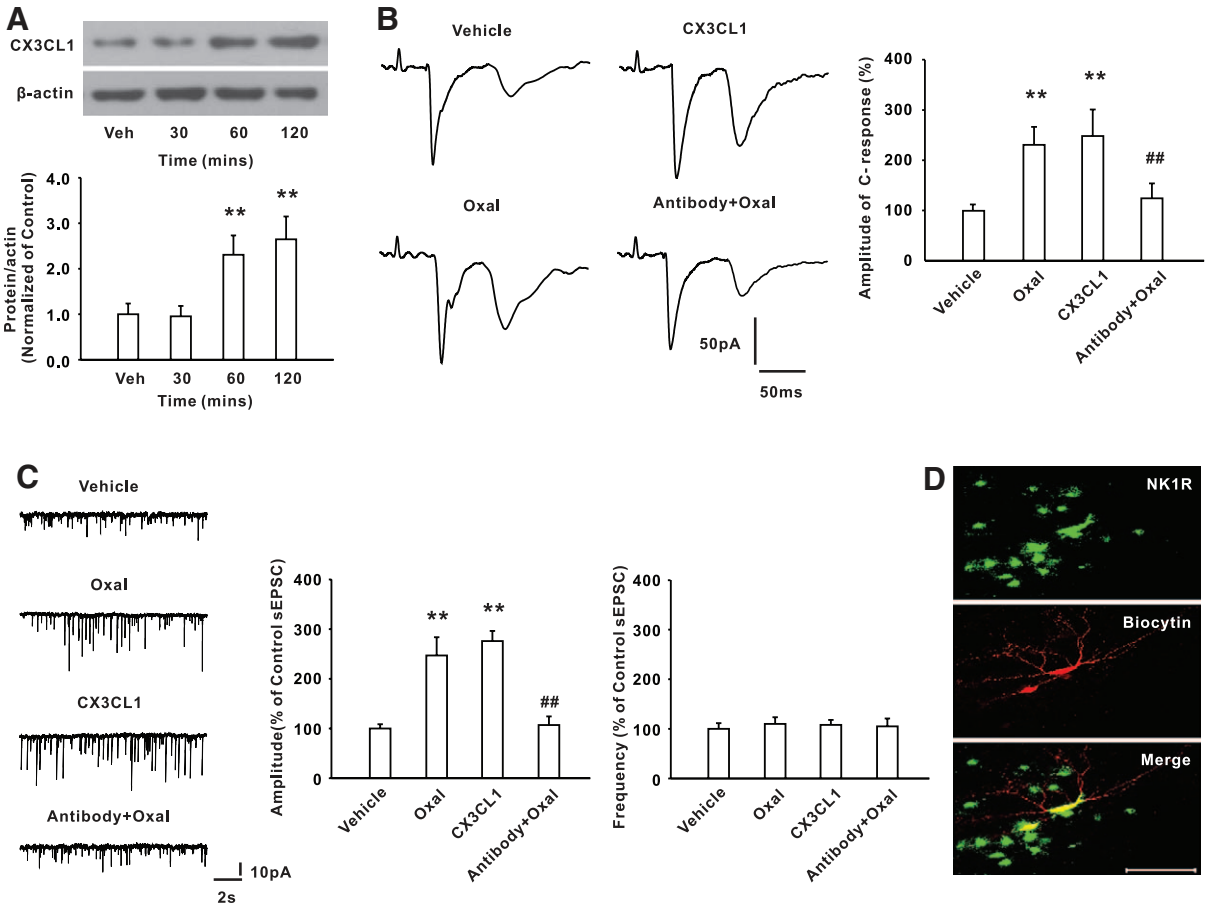

Fig. 4. CX3CL1 mediated the enhanced synaptic transmission. (*A*) Up-regulation of CX3CL1 in spinal cord slice was induced after incubation of oxaliplatin (oxal) (6.6 nM) for 1 and 2 h. n = 8 in each group; $^{**}P < 0.01$ *versus* vehicle (veh) group. (*B*) Inhibition of CX3CL1 with neutralizing antibody attenuated the increase of evoked excitatory postsynaptic currents amplitude in the spinal slices after oxal incubation (6.6 nM). n = 31 in each group; $^{**}P < 0.01$ *versus* veh group, $\#\#P < 0.01$ *versus* oxal group. (*C*) CX3CL1 neutralizing antibody inhibited the increase of amplitude, but not frequency, of spontaneous excitatory postsynaptic currents (sEPSCs) in the spinal slices after oxal incubation (6.6 nM). n = 28 in each group; $^{**}P < 0.01$ *versus* veh group; $\#\#P < 0.01$ *versus* oxal group. (*D*) Projection neurons in the spinal lamina I were identified by intracellular biocytin labeling and immunohistochemistry for neurokinin 1 receptor (NK1R) after electrophysiologic study.

sensitization *via* upregulating CX3CL1 expression. First, the expression of NF-κB p65 phosphorylated at Ser311 was significantly increased at 30 min and persisted to the end point of the experiment (10 h) after a single intraperitoneal oxaliplatin injection (fig. 5A). Electrophysiologic results showed that pretreatment of NF-κB p65 inhibitor PDTC (200 ng in 100 μl), but not vehicle, onto the spinal dorsal surface significantly prevented the oxaliplatin-induced increase of field potentials (fig. 5B). Furthermore, pretreatment of siRNA targeting NF-κB p65 (50 μg in 100 μl), but not scramble RNA, by direct application onto the spinal dorsal surface also prevented the increase of field potentials induced by single intraperitoneal treatment of oxaliplatin (fig. 5C). Consistently, intrathecal administration of PDTC (200 ng in 10 μl) or specific p65 siRNA (50 μg in 10 μl) significantly inhibited oxaliplatin-induced acute mechanical allodynia (fig. 5D) and thermal hyperalgesia (fig. 5E). Meanwhile, intrathecal injection of PDTC (200 ng in 10 μl) or specific p65 siRNA (50 μg in 10 μl) significantly reduced the up-regulation of CX3CL1 protein (fig. 5F) and mRNA (fig. 5G) in the spinal dorsal horn at 10 h after the treatment with oxaliplatin. These results suggested that up-regulation of CX3CL1 induced by oxaliplatin was dependent on NF-κB p65 signaling pathway.

Oxaliplatin Increased NF-κB p65-mediated Histone H4 Acetylation in the cx3cl1 Promoter Region

Upon activation of NF-κB pathway, NF-κB p65 can bind to the promoter regions and modify the acetylation of histones,[37,38] thereby facilitating the expression of target genes. Therefore, we determined whether treatment with oxaliplatin promoted the binding of NF-κB p65 to the *cx3cl1* promoter in the dorsal horn using ChIP-PCR assay. A potent binding site administration of NF-κB p65 in the

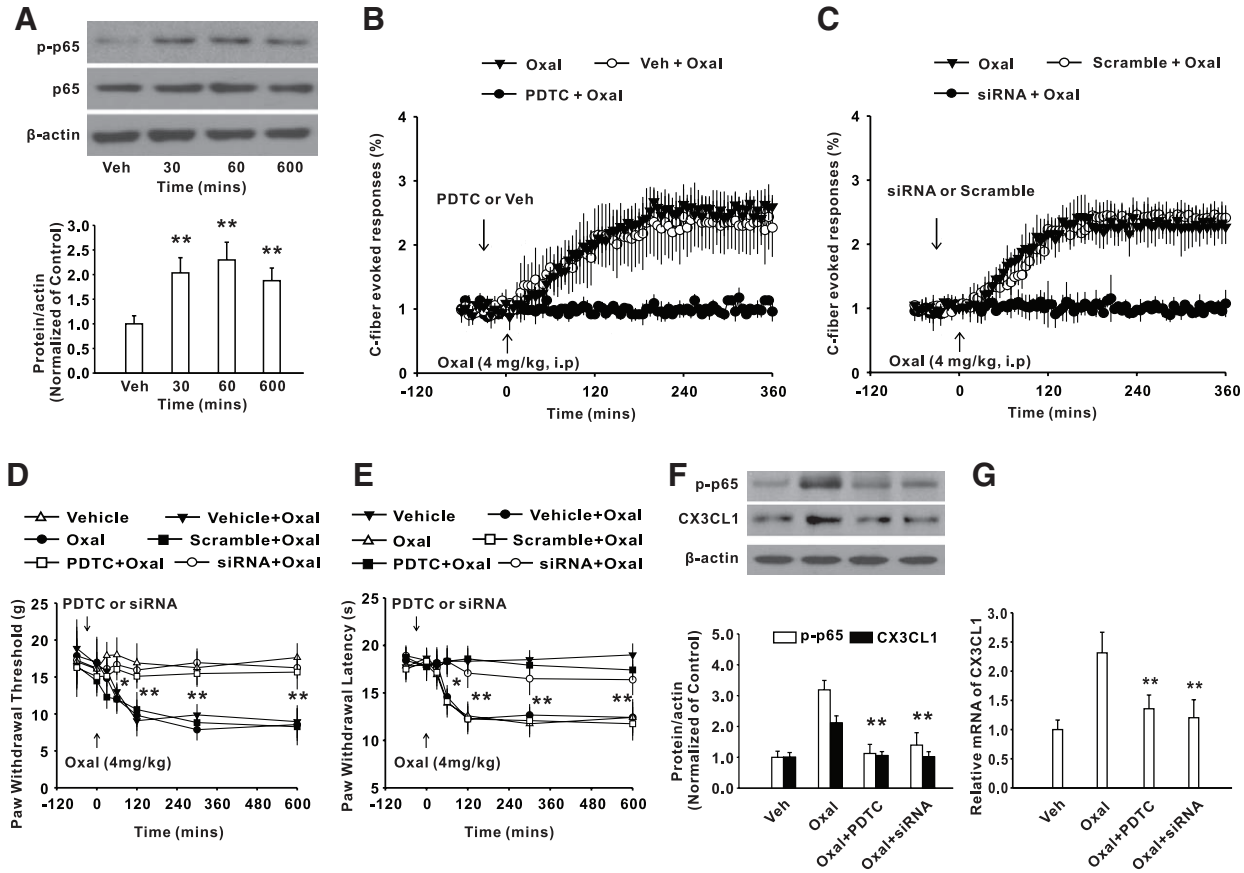

Fig. 5. Activation of transcriptional factors such as nuclear factor (NF)-κB p65 was involved in oxaliplatin (oxal)-induced long-term potentiation (LTP) and pain-related behavior through modulating CX3CL1 signaling in the dorsal horn. (*A*) Phosphorylation of NF-κB p65 was increased after single oxal treatment. n = 6 in each group; $^{**}P < 0.01$ *versus* vehicle (veh) group. Pretreatment with intrathecal ammonium pyrrolidinedithiocarbamate (PDTC) (*B*) or p65 siRNA (*C*) 30 min before oxal administration blocked the induction of spinal LTP compared with oxal-treated group. n = 12 in each group. (*D*) Inhibition of NF-κB signaling pathway by PDTC or siRNA attenuated oxal-induced acute mechanical allodynia. n = 12 in each group; $^{*}P < 0.01$, $^{**}P < 0.01$ *versus* oxal group. (*E*) Inhibition of NF-κB signaling pathway by PDTC or siRNA attenuated oxal-induced acute thermal hyperalgesia. n = 12 in each group; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* oxal group. Pretreatment with intrathecal PDTC (*F*) or p65 siRNA (*G*) reduced the up-regulation of CX3CL1 protein and mRNA induced by oxal. n = 12 in each group; $^{**}P < 0.01$ *versus* oxal group. i.p. = intraperitoneally.

cx3cl1 gene at position −1941/−1931 was firstly identified using TFSEARCH and JASPAR database. Then the DNA precipitated by the NF-κB p65 antibody was subjected to PCR with the primers designed to amplify a 162-bp fragment (−2029/−1867) of the *cx3cl1* promoter flanking the NF-κB–binding site. The results showed that the recruitment of p65 to the *cx3cl1* promoter in the dorsal horn was significantly increased after oxaliplatin treatment at 60 and 600 min compared with vehicle group by semiquantitative (fig. 6A) and quantitative real-time PCR analysis (fig. 6B). Next, we examined whether treatment with oxaliplatin may modify histone acetylation in the *cx3cl1* promoter region. The western blotting results showed that global acetylation of H4 in the dorsal horn was significantly increased at 60 and 600 min after application of oxaliplatin (fig. 6C). No alteration of acetylation of H3 (K9) was detected (fig. 6D). For ChIP assay, the DNA precipitated by the acetylated H4 antibody was used for PCR analysis, and the *cx3cl1* promoter region flanking the NF-κB–binding site was amplified. The results showed that the level of H4 acetylation on the *cx3cl1* gene promoter was enhanced at 60 min after treatment with oxaliplatin (fig. 6E). Furthermore, the effect of oxaliplatin on H4 acetylation in *cx3cl1* promoter region was reduced by intrathecal administration of PDTC or p65 siRNA (fig. 6E). Taken together, these results indicated that treatment with oxaliplatin remarkably increased the binding of NF-κB and H4 acetylation in *cx3cl1* promoter region, thus potentially contributing to the CX3CL1 up-regulation induced by oxaliplatin.

Discussion

The painful neuropathy induced by chemotherapeutic drugs is generally thought to result from their indirect and chronic toxicities on the nervous system. Our studies, for the first time, demonstrated that chemotherapeutic drugs crossing the BBB in the CSF directly affected the normal function of central neurons in the model of oxaliplatin-induced acute neuropathy. The results showed that concentration of oxaliplatin in CSF gradually increased in a time-dependent manner after a single intraperitoneal administration, and intrathecal application of the mimicked amount of oxaliplatin in CSF (6.6 nM) to the lumbar spinal cord induced the sensitization of nociceptive synaptic transmission, mechanical allodynia, and thermal hyperalgesia. Incubation with oxaliplatin (6.6 nM) significantly increased the eEPSC and sEPSCs in the NK1R+ projection neurons of lamina I in the spinal cord slices. Meanwhile, injection of a low dose of oxaliplatin into the plantar subcutaneous tissue failed to alter the spinal field potentials after stimulation of sciatic nerve or sural nerve and pain-related behavior. This study also revealed that application of oxaliplatin substantially

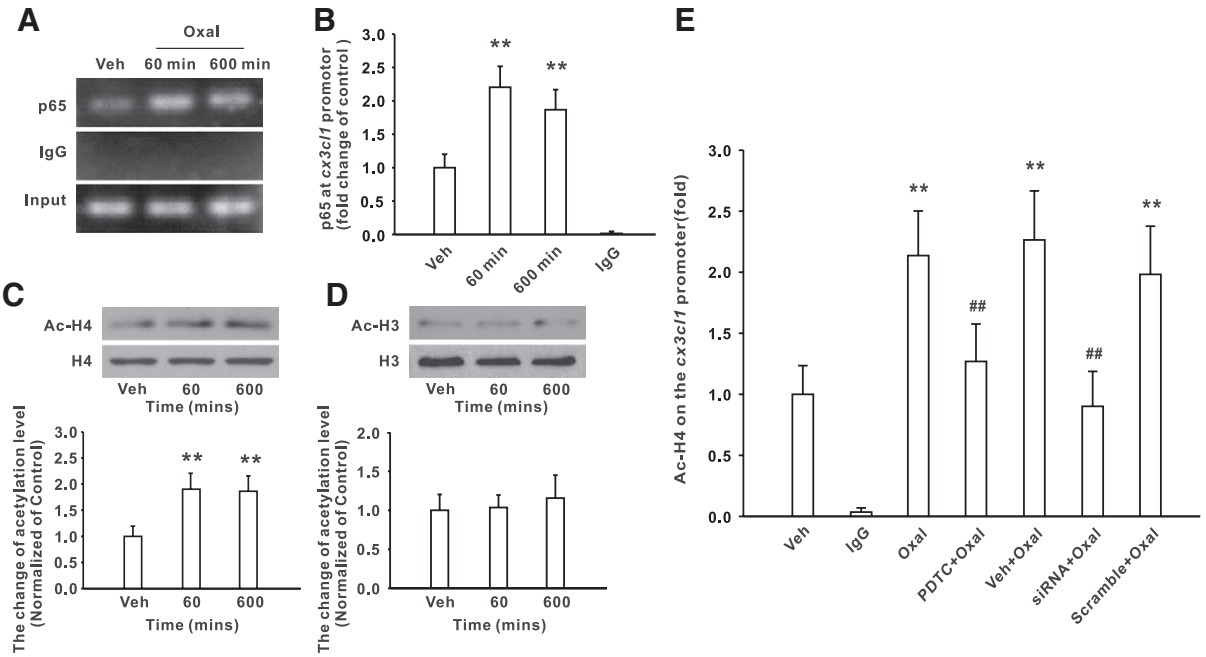

Fig. 6. Treatment with oxaliplatin enhanced nuclear factor (NF)-κB p65-dependent acetylation of histone H4 on the *cx3cl1* promoter region. Semiquantitative (*A*) and quantitative real-time polymerase chain reaction (*B*) showed the increased recruitment of NF-κB p65 to the *cx3cl1* gene promoter in oxaliplatin (oxal) group by chromatin immunoprecipitation (ChIP) assays. n = 8 in each group; ***P* < 0.01 *versus* vehicle (veh) group. Treatment with oxal significantly increased the global acetylation of histone H4 (*C*), but not H3 K9 (*D*), in the dorsal horn. n = 8 in each group; ***P* < 0.01 *versus* veh group. (*E*) ChIP assay was performed with acetylated H4 antibody or IgG. Increased acetylation of histone H4 on the *cx3cl1* promoter region flanking NF-κB p65 binding site was reduced by ammonium pyrrolidinedithiocarbamate (PDTC) or p65 siRNA in the rats with oxal treatment. n = 8 in each group; ***P* < 0.01 *versus* veh group, ##*P* < 0.01 *versus* oxal group.

upregulated the expression of CX3CL1 and NF-κB p-p65 in the dorsal horn of the intact animals or spinal slices, and inhibition of CX3CL1/p-p65 remarkably prevented the central sensitization *in vivo* and *in vitro* and acute pain-related behaviors induced by oxaliplatin. Further, ChIP assay demonstrated that inhibition of NF-κB with PDTC or siRNA significantly decreased the recruitment of NF-κB p65 and H4 acetylation in the *cx3cl1* promoter region and suppressed the upsurge of CX3CL1 expression induced by oxaliplatin. These findings suggested that the oxaliplatin entering CNS directly upregulated CX3CL1 expression by increasing the NF-κB p65 binding and H4 acetylation in the *cx3cl1* promoter region, thus contributing to the increased efficiency of spinal nociceptive synaptic transmission and the emergence of acute pain-related behavior.

While some chemotherapeutic drugs, such as oxaliplatin or paclitaxel, have limited BBB permeability,[11,12,39,40] clinical or animal studies show that these chemotherapeutic drugs can induce the acute or chronic encephalopathy.[5,6,41] Previous studies showed that oxaliplatin induced serious acute and chronic painful neuropathy.[42] It is generally accepted that axonopathy in peripheral nerves due to the damage of DRG plays an important role in oxaliplatin-induced chronic painful neuropathy.[43,44] However, oxaliplatin-induced acute painful behavior may appear immediately after the first injection,[45] while axonopathy unlikely occurs in such a short time. In this study, a low concentration of oxaliplatin in the CSF was detected at 1 h and peaked at 6 h after a single systemic administration (intraperitoneally). Actually previous studies also reported the detectable oxaliplatin in CSF after systemic administration of oxaliplatin (intravenously) in nonhuman primates,[11,12] while the time courses varied possibly due to the difference in the species and the delivery method of agent. Moreover, local spinal application of oxaliplatin (6.6 nM) rapidly enhanced field potentials in dorsal horn neurons and induced the pain-related behaviors, which was similar with those after the single systemic administration. Recent study showed that intraplantar administration of large dose of oxaliplatin (1 or 40 μg) significantly induced acute hyperalgesia in the rodents,[31,32] suggesting that peripheral mechanism might participate in the oxaliplatin-induced acute neuropathy. However, this study showed a remarkably lower dose of oxaliplatin (20 ng/100 mg tissue) in the plantar subcutaneous tissue after a single systemic administration of oxaliplatin with the routine dose (4 mg/kg). Furthermore, intraplantar administration of oxaliplatin at the detected dose (20 ng) did not change field potentials in dorsal horn neurons and pain-related behavior in the rodents. Therefore, our results provided the evidences that acute neuropathy induced by single systemic injection of oxaliplatin with routine dose might not be critically associated with the peripheral mechanism. Furthermore, incubation of oxaliplatin (6.6 nM) significantly increased the amplitude of eEPSC and sEPSCs of NK1R+ projection neurons in spinal cord slices, indicating that oxaliplatin entering the CNS can directly sensitize the projection neurons in the lamina I. A significant accumulation of oxaliplatin in dorsal root ganglions after systemic administration is well demonstrated.[46,47] While it is somehow unlikely that oxaliplatin in DRG may significantly induce sensitization of spinal neurons and painful behavior in a short time (1 h), we cannot exclude the possible involvement of oxaliplatin-induced DRG damage in the emergence of acute neuropathy induced by oxaliplatin. Besides, an acute thermal hyperalgesia was observed in the rats receiving oxaliplatin, implying that a potential sensitization of nociceptor, possibly the central terminal in dorsal horn, cannot be excluded. Although this study remained underdeveloped, these evidences indicated that the CSF oxaliplatin may directly affect the activity of dorsal horn neurons and mediate the emergence of acute painful behavior after the systemic administration of oxaliplatin.

Our study also showed that treatment with oxaliplatin significantly upregulated the expression of chemokines CX3CL1 and p-p65 in the dorsal horn, and inhibition of NF-κB p65/CX3CL1 signaling pathway markedly prevented the central sensitization and pain-related behavior induced by oxaliplatin. This study found that incubation of oxaliplatin with a mimicked dose (6.6 nM) significantly increased the expression of CX3CL1 in the spinal cord slices. It was previously reported that intrathecal injection of CX3CL1 induced a delayed pain behavior.[48] Here, we further found that blockade of CX3CL1 signaling with the neutralizing CX3CL1 antibody largely inhibited the increase of eEPSC and sEPSCs amplitude in NK1R+ projection neurons induced by oxaliplatin. Furthermore, we also found that inhibition of NF-κB p65 with PDTC or specific siRNA significantly prevented the up-regulation of CX3CL1 and pain-related behavior induced by oxaliplatin. Similarly, we previously reported that treatment with paclitaxel also induced NF-κB p65–mediated epigenetic up-regulation of CX3CL1 in the dorsal horns, thus contributing to the mechanical allodynia induced by systemic administration of paclitaxel.[28] This study further demonstrated that the low concentration of chemotherapeutic agents, *e.g.*, oxaliplatin, crossing the BBB exhibited the sufficient potency to induce such epigenetic modification and central sensitization in dorsal horn and pain behavior. Recent studies showed that oxaliplatin, a DNA-damaging agent, activated NF-κB through acting on protein IκB kinase (IKK)-γ in normal cells and tissues.[49–51] In addition to their affinity for DNA, platinum analogs or metabolites may undergo spontaneous chemical reactions and irreversibly bind to protein and low-molecular-weight nucleophiles. These protein-bound and low-molecular-weight platinum complexes might contribute to the toxic effect of these drugs.[11,52] Altogether, our data strongly suggested that oxaliplatin entering the CNS may directly activate the NF-κB/CX3CL1 pathway and consequently affect the normal function of central neurons and mediate the pain-related behaviors.

In general, this study, for the first time, reported that oxaliplatin entering the CNS directly activated the NF-κB/

CX3CL1 signaling pathway and consequently upregulated the excitability of NK1R projection neurons and contributed to the acute painful neuropathy induced by systemic administration of oxaliplatin. Our findings revealed a novel mechanism for chemotherapy-induced acute painful behavior and proposed the potential targets for the development of interventions to prevent acute CNS-related symptoms, such as acute painful neuropathy or encephalopathy, during chemotherapy in the cancer patients.

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Competing Interests

The authors declare no competing interests.

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