Attenuation of Neuropathic Pain by Inhibiting Electrical Synapses in the Anterior Cingulate Cortex

Zhi-Yu Chen, M.D., Feng-Yan Shen, Ph.D., Lai Jiang, Ph.D., Xuan Zhao, Ph.D., Xiao-Lu Shen, M.D., Wei Zhong, Ph.D., Sha Liu, Ph.D., Zhi-Ru Wang, Ph.D., Ying-Wei Wang, M.D., Ph.D.

ABSTRACT

Background: Synaptic mechanisms and neuronal oscillations have been proposed to be responsible for neuropathic pain formation. Many studies have also highlighted the important role of electrical synapses in synaptic plasticity and in neuronal oscillations. Thus, electrical synapses may contribute to neuropathic pain generation. However, previous studies have primarily focused on the role of chemical synapses, while ignoring the role of electrical synapses, in neuropathic pain generation.

Methods: The authors adopted microinjection, RNA interference techniques, and behavioral tests to verify the link between connexin 36 (Cx36) and neuropathic pain. They also studied the selective Cx36 blocker mefloquine in rat chronic constriction injury and spared nerve injury model of neuropathic pain. Electrophysiologic recordings were used to further confirm the behavioral data.

Results: The authors found that Cx36, which constitutes the neuron–neuron electrical synapses, was up-regulated in the anterior cingulate cortex after nerve injury (n = 5). Meanwhile, Cx36-mediated neuronal oscillations in the gamma frequency range (30 to 80 Hz) (n = 7 to 8) and the neuronal synaptic transmission (n = 13 to 19) were also enhanced. Neuropathic pain was relieved by disrupting Cx36 function or expression in the anterior cingulate cortex. They also found that mefloquine, which are clinically used for treating malaria, affected gamma oscillations and synaptic plasticity, leading to a sustained pain relief in chronic constriction injury and spared nerve injury models (n = 7 to 12).

Conclusion: The electrical synapses blocker mefloquine could affect gamma oscillations and synaptic plasticity in the anterior cingulate cortex and relieve neuropathic pain. Cx36 may be a new therapeutic target for treating chronic pain.

What We Already Know about This Topic

- Synapses in the brain can be of either the chemical or electrical type
- Electrical synapses have special roles in neuronal oscillations, which may be involved in chronic pain

What This Article Tells Us That Is New

- The electrical synapse protein connexin 36 is up-regulated in the anterior cingulate cortex after nerve injury in rats, and gamma-frequency neuronal oscillations are enhanced as well
- Mefloquine, an electrical synapse inhibitor, reduces allodynia in the same nerve injury model

EUROPATHIC pain, which affects approximately 8% of the population, is a devastating pain syndrome caused by various insults to the nervous system, resulting in chronic spontaneous pain, thermal hyperalgesia, and mechanical allodynia.1,2 Currently available pharmacologic treatments for neuropathic pain have limited therapeutic efficacy. More than two thirds of neuropathic pain patients suffer from insufficient pain relief partially because the underlying mechanisms of neuropathic pain development remain poorly understood. Although many studies have confirmed that chemical synapses are crucial for neuropathic pain formation,3,4 little is known concerning the role of electrical synapses in neuropathic pain pathogenesis.

The electrical synapse is a special transmembrane channel that allows for the fast and efficient transduction of electrical signals and the direct exchange of signals between neurons, thus maintaining and regulating cell activity and stability.5 The electrical synapse is composed of two membrane-docked hexameric hemichannels that consist of connexins (Cx) from two adjacent neurons. Approximately 20 types of synaptic Cx proteins exist, and different Cx proteins are named after their respective Cx molecular weights.6 Connexin 36 (Cx36), which appears in neuron–neuron gap junctions (electrical synapses) but not in the gap junctions between astrocytes and/or oligodendrocytes,9 is expressed throughout the brain.10 When Cx36 is knocked out, electrical coupling between neurons is eliminated or profoundly reduced. Thus, Cx36 is an indispensable constituent of electrical coupling between neurons.7

Previous studies have reported that electrical synapses play an important role in generating neuronal oscillations.11,12 A study regarding Cx36-knockout mice demonstrated that the loss of Cx36 disrupts gamma oscillations.11 Application of the general gap junction blocker carbenoxolone disodium salt (Cbx) also significantly reduced the power of gamma

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Corresponding article on page 13. The first three authors contributed equally to this work.

Submitted for publication February 16, 2015. Accepted for publication September 4, 2015. From the Department of Anesthesiology and Intensive Care Medicine, Xinhua Hospital, College of Medicine, Shanghai Jiaotong University, Shanghai, China (Z.-Y.C., F.-Y.S., L.J., X.Z., X.-L.S., Y.-W.W.); and Laboratory of Neural Circuit Plasticity & Memory, Institute of Brain Functional Genomics, East China Normal University, Shanghai, China (W.Z., S.L., Z.-R.W.).

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neuronal oscillations. Moreover, painful stimuli induce the activation of neuronal oscillations in the portion of the cortex associated with pain. Therefore, electrical synapses may participate in pain processing. Furthermore, deleting Cx36 impairs long-term potentiation of synaptic transmission in the hippocampus, and Cbx induces a long-lasting depression of neurotransmission, indicating that the function of electrical synapses is associated with synaptic plasticity. Synaptic plasticity that contributes to central sensitization is the key mechanism for neuropathic pain. Thus, we hypothesized that electrical synapses are crucial for neuropathic pain formation. In the current study, we aimed to study the role of electrical synapses in neuropathic pain pathophysiology.

**Materials and Methods**

**Animals**

Sprague–Dawley rats aged 9 to 11 weeks and weighing 240 to 340 g (Shanghai Sipper; BK Laboratory Animals Co., Ltd., China) were housed in cages at 24°C and at 50 to 60% humidity with a 12/12 h light/dark cycle and a sufficient food and water supply. Sample size determination was based on previous experience. Rats were randomly divided into different groups. All of the surgical procedures were performed under anesthesia with intraperitoneal injection of pentobarbital sodium (40 mg/kg). The Animal Use and Care Committee for Research and Education, Shanghai Jiao Tong University, Shanghai, China, approved this research protocol.

**Medications and Reagents**

First, mefloquine hydrochloride (Meflo; Sigma-Aldrich, USA, Catalog #M2319) and its isomers were suspended in Tween-80 and then dissolved in normal saline. Once-daily intraperitoneal injections of Meflo or Meflo isomers were administered to the rats on days 0, 1, 2, 3, and 4 after the chronic constriction injury (CCI) or the spared nerve injury (SNI). Both the sham group and the vehicle group received 1% Tween-80 saline. For microinjection, Cbx (100 mM; Sigma-Aldrich, Catalog #C4790) and Meflo (100 mM; dimethyl sulfoxide stock solution) were dissolved in 0.1 M sterile phosphate-buffered saline (PBS), followed by ultrasonification for 10 min. Vehicle animals received similar volumes of 0.1 M sterile PBS or 0.1 M sterile PBS with 1% dimethyl sulfoxide for the Cbx and Meflo vehicles, respectively. (+)-(11S,12R)-Meflo and (−)-(11R,12S)-Meflo were synthesized by the Shanghai Engineering Research Center of Molecular Therapeutics and New Drug Development, Shanghai, China.

**CCI Surgery**

Chronic constriction injury model was established in accordance with the method described by Bennett and Xie. In brief, the left sciatic nerves were exposed unilaterally after a skin incision was made at the mid-thigh level, followed by blunt dissection at the biceps. Four chronic gut (5-0) ligatures were tied loosely around the nerve proximal to its trifurcation at 1-mm intervals. Sham surgery was performed by exposing the sciatic nerve without ligation.

**SNI Surgery**

Spared nerve injury model was established according to the method described by Decosterd and Woolf. In brief, an incision was made on the upper edge of the left hind leg of the rat. The muscle was isolated longitudinally to expose the sciatic nerve trunk and its subordinate tibial, peroneal, and sural nerves. The tibial and peroneal nerves were ligated and cut transversely, whereas the thin sural nerve was preserved. Care was taken to avoid excessively stretching the nerves.

**Cannulation and Microinjection**

Each rat was anesthetized with pentobarbital sodium and secured on a stereotaxic apparatus. A median incision was made to expose the skull of the rat. In accordance with the position determined by the stereotaxic atlas, 28-gauge guide cannulas were bilaterally implanted into the anterior cingulate cortex (ACC) at the following coordinates: 2.7 mm anterior from bregma, ± 0.6 mm lateral to midline, and 2.0 mm ventral from the dura; 0.4 mm anterior from bregma, ± 0.6 mm lateral to midline, and 2.0 mm ventral from the dura. Three stainless steel screws were implanted into the skull around the guide cannulas and fixed with dental acrylic. Dummy cannulas were inserted after surgery and left in place until the infusion day. The animals were housed individually after surgery. Penicillin (80,000 units) was administered for infection prophylaxis 1 day before surgery and 2 days consecutively after surgery. Cerebral microinjection was performed 7 days after cannulation. The dummy cannulas were removed before microinjection. Internal injection cannulas (32 gauge), which extended 1 mm beyond the guide cannulas, were inserted. The solution was injected at a constant rate for longer than 120 s using a microliter injector. The injection cannulas were left in place for 3 additional min before being withdrawn. The animals were allowed 30 additional minutes before beginning behavior experimentation. Once-daily, Cbx or Meflo was microinjected into the ACC region of rat brains on days 0, 1, 2, 3, and 4 after CCI.

**Mechanical Allodynia Test**

The animals were habituated for 2 to 3 days in the test environment before each test. The rats were placed in a plexiglass box with a metal net bottom for 30 min. After habitation to the environment, the hind paw was stimulated using one of a series of von Frey hairs with logarithmically increasing stiffness (0.6, 1, 1.4, 2, 4, 6, 8, 10, and 15 g) (Stoelting Co., USA), which was presented perpendicular to the plantar surface (5 to 6 s for each hair). A positive performance was recorded when the rat escaped the mild pressure from von Frey hairs or raised the hind leg. Dixon up–down method was used to determine the 50% withdrawal threshold. The experimenters who conducted the various assessments were blinded to the treatment conditions.
Thermal Hyperalgesia Test
The rats were placed in a plexiglass box on a 3-mm-thick glass plate. After habituation in the box for 30 min, the sole skin of each animal was irradiated with light within a 0.5-cm-diameter circle using a BME-410 thermal radiation stimulator (Institute of Biomedical Engineering, Peking Union Medical College, Tianjin, China) at 10 V and 30 W. The time from irradiation initiation to paw withdrawal was recorded as the paw withdrawal latency value. A cutoff time of 40 s was used to avoid local burn injury. Three measurements were taken for each animal, with a 6- to 8-min interval allowed between trials, and the mean value was used for the analysis.

Open-field Test
The open-field test system was applied to pair sets of photo beams to detect movement. All of the animals were acclimated to the testing room for 30 min before the start of the session. The rats were placed individually in the center of the observation box, which had four transparent walls, an open top, and a gray floor. The total distance that each animal traveled in 15 min was measured using a tracking analysis system (Coulbourn Instruments, USA). This distance was used as a parameter for rat locomotion.

Rotarod Test
Motor coordination and balance were tested using an accelerating rotarod (Shanghai Jiliang Software Technology, Shanghai, China). This apparatus is composed of a base platform and a rotating horizontal rod with a nonskid surface. The rats were habituated to the apparatus for 30 min to avoid stress during testing. Before testing, the rats were acclimatized to acceleration in six training runs over 3 days. The rod was set to accelerate from 8 to 25 rpm in a 5-min period. The locomotor function was assessed as the performance time on the rod from the start of rotation until the animal fell to the base platform. The mean performance time of the test runs was recorded.

Western Blotting
The rats were deeply anesthetized with pentobarbital sodium (50 mg/kg). The bilateral ACC was removed, homogenized in a lysis buffer containing 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% sodium dodecyl sulfate, and a cocktail of protease inhibitors and phosphatase inhibitors, lysed on ice for 30 min, and centrifuged at 4°C for 10 min. The supernatant was removed completely and fixed in 4% paraformaldehyde for 1 to 8 h. To ensure equal loading, the membranes were also probed with a 1:3,000 dilution of anti-actin antibody (Sigma Life Science, USA, Catalog #A2668). The results of the Western blotting were quantified using ImageJ software (National Institutes of Health, USA).

Immunohistochemistry
We deeply anesthetized the animals with pentobarbital sodium and transcardially perfused the rats with saline, followed by 4% paraformaldehyde. The brain tissues were removed completely and fixed in 4% paraformaldehyde for 4 h, followed by submersion in a 20% cane sugar solution until the tissues sank to the bottom. Then, the brain tissues were sliced into 30 μm sections in a cryostat. The sections were blocked with 5% normal goat serum at room temperature and incubated overnight at 4°C with a polyclonal rabbit antibody against Cx36 (1:50; Santa Cruz Biotechnology, Catalog #sc-25715). Then, the sections were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated donkey anti-rabbit immunoglobulin G (1:300; Life Technologies, USA, Catalog #A-11008) in the dark. The slices were coverslipped with a mixture of 50% (v/v) glycerol and 2.5% (w/v) triethylenediamine in 0.05 M PBS. 4′,6-Diamidino-2-phenylindole was also included in the mounting medium for covisualization of nuclei. The sections were observed under a confocal microscope.

Brain Slice Preparation
Brain slices at the level of the ACC were prepared using standard methods. In brief, the animals were anesthetized with pentobarbital sodium, and their brains were quickly excised from the skull and submerged in an ice-cold artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 25 mM NaHCO₃, 1 mM NaH₂PO₄, and 10 mM glucose). After 1 to 2 min of chilling, each brain was trimmed to a block containing the anterior half of the brain. Using a vibroslicer (VT1000 S; Leica Microsystems, USA), coronal slices (350 to 400 μm) were cut from the tissue block in ice-cold ACSF. The slices containing the ACC were transferred to a gas interface-type recording chamber perfused with aerated (95% O₂–5% CO₂) ACSF (26° to 28°C) at a rate of 1 to 1.5 ml/min using a peristaltic pump-driven or gravity-fed bath perfusion system.

Whole Cell Patch Clamp Recordings
Voltage clamp recordings were obtained from neurons in ACC slices equilibrated for 1 to 8 h in the recording chamber. The micropipettes (tip diameter: 1.5 to 2.0 μm; resistance: 4 to 6 MΩ) were pulled from borosilicate capillaries (P-97; Sutter Instruments, USA) and filled with an internal solution (adjusted to pH 7.2 with KOH) composed of the following: 145 mM K-gluconate, 5 mM NaCl, 1 mM MgCl₂, 0.2 mM EGTA, 10 mM HEPES, 2 mM Mg-adenosine triphosphate, 0.1 Na₄-guanosine-5′-triphosphate, and 10 phosphocreatine disodium. Evoked excitatory postsynaptic currents (EPSCs) were recorded from layer II/III neurons, and the
Field Potential Recordings

Local field potentials were recorded using ACSF-filled glass microelectrodes (tip diameter: 2 to 4 μm; resistance: 3 to 5 MΩ) that were placed into layer II/III of the ACC. The data were collected using a MultiClamp 700B amplifier and a low-pass filtered at 2 kHz. Kainate (Sigma Life Science, Catalog #K0250) was bath-applied for 20 min to stabilize the neuron signals, which were amplified using an Axoclamp-700B amplifier (bandwidth filter set at 10 kHz for current clamp and 1 kHz for voltage clamp recordings) were digitized and sampled at 50 μs intervals (Digidata 1440A, pClamp 10.1; Molecular Devices, USA). The series resistance used was 12 to 25 MΩ and was monitored throughout the experiment. Data were discarded when the access resistance changed by more than 15% during the experiment.5

Synthesis of the Meflo Isomers

Meflo hydrochloric acid (200 g, 0.48 mM) was suspended in 1 M of NaOH solution (2 l) in a separatory funnel. Ethyl acetate (1 l) was added, and the layer was separated after shaking. The aqueous layer was extracted using ethyl acrylate (EA), and the combined organic layers were dried over Na2SO4. A solution of 1-O, O-di-p-toluoyl tartaric acid (180 g, 0.48 mM) was added to a solution of mefloquin in EA (5 l). The resulting solution was stoppered and stirred at room temperature overnight. The resulting solid was filtered and washed using EA (4 l) to yield (−)-(11R,12S)-mefloquine 1-O, O-di-p-toluoyl tartaric acid salt that was then triturated with boiling EA (4 l) and dried to obtain the solid. The solid (154 g) was suspended in 500 ml methyl alcohol, and 1.25 l of 1 M NaOH was added, followed by 1.25 l water. The mixture was stirred for 1 h. The solid was filtered off, washed with 2 l water, and dried in vacuo to obtain a white solid (74.6 g).
The mother liquor was reduced to dryness and neutralized with a 1 M NaOH solution (1.5 l). After drying over Na₂SO₄, ω-O,O-di-p-toluoyl tartaric acid (111 g, 0.288 mM) in EA (5 l) was added. Then, the solution was stirred at room temperature and left overnight, generating a white solid, which was filtered and washed in EA (2 l) to yield (+)-(11S,12R)-mefloquine ω-O,O-di-p-toluoyl tartaric acid salt. This solid was suspended in 500 ml methyl alcohol, and 1.25 l of 1 M NaOH was added, followed by 1.25 l water. The mixture was stirred for 1 h. The solid was filtered off and washed with 2 l of water and dried in vacuo to obtain a white solid (81 g).

**Statistical Analysis**

The data are expressed as the means ± SEM. A one-way ANOVA with Tukey multiple comparison post hoc tests were used when more than two groups of data were compared, or Student’s t test was used when only two groups were compared. The brain slices were treated as though they were independent of one another when analyzing the electrophysiology data. Behavioral data were analyzed by using a two-way repeated-measures ANOVA that group as a between-groups factor and time as a repeated-measures factor, followed by the Bonferroni post hoc test. SigmaStat 3.5 (Jandel Scientific Software, USA) was used to carry out statistical analyses. The value of the criterion for statistical significance was P less than 0.05 (two-tailed test).

**Results**

**Cx36 Up-regulation and Gamma Oscillation Activation in the ACC after Nerve Injury**

The ACC is a key cortical region engaged in neuropathic pain formation. To explore the role of electrical synapses in the pain pathway, we examined the biochemical and electrophysiological changes in the ACC after nerve injury. Cx36 expression in the ACC was observed on days 3 and 7 after CCI in our study. Both Western blotting and immunohistochemistry assay showed that Cx36 expression in the ACC was significantly up-regulated 7 days after CCI (fig. 1, A and B). Western blotting in the sham group, 0.78 ± 0.08, n = 5; Western blotting 3 days after CCI, 0.90 ± 0.13, n = 5; Western blotting 7 days after CCI, 1.18 ± 0.20; one-way ANOVA, P = 0.005). To investigate whether the changes in Cx36 expression were a specific phenomenon found only in the ACC, we also examined Cx36 expression levels in an adjacent cortex (i.e., the primary motor cortex) and in a pain-associated area (i.e., the hippocampus). These data demonstrated that Cx36 expression in the primary motor cortex (fig. 1, C and D) and in the hippocampus (fig. 1, E and F) did not change significantly 7 days after CCI.

Cx36 was reported to be responsible for generating gamma-frequency neuronal oscillations. Given the importance of gamma oscillations in the perception of pain, we further assessed the consequences of the increase in Cx36 expression on gamma oscillations in the ACC. Previous studies have shown that kainate application resulted in persistent gamma oscillations in the hippocampus and in the entorhinal cortex in vitro. Similarly, we used ACC slice preparations, and the typical pattern of gamma oscillations was evoked in the presence of 150 nM kainate (fig. 2A). After CCI, the peak frequency of gamma oscillations remained unaffected (fig. 2B). However, a significant increase in the power of gamma oscillatory activity remained 7 days after CCI compared with those values from sham rats (fig. 2C; sham group, 5.09 ± 1.04 × 10⁻¹² V²/Hz, n = 7 slices of four rats; 3 days after CCI, 7.54 ± 1.12 × 10⁻¹⁴ V²/Hz, n = 7 slices of four rats; 7 days after CCI, 11.29 ± 1.49 × 10⁻¹² V²/Hz; one-way ANOVA, P = 0.006, n = 8 slices of four rats). Thus, both Cx36 expression and gamma oscillation power in the ACC increased after CCI.

**Disruption of Cx36 Expression in the ACC Attenuates Nerve Injury-induced Neuropathic Pain**

To further examine whether Cx36 up-regulation and gamma oscillation activation contributed to neuropathic pain formation, we investigated the relation between electrical synapses and neuropathic pain by altering Cx36 gene expression. Given that changes in motor and cognitive abilities were observed in Cx36 knockout mice, we used the local RNA interference (RNAi) technique. By using the Cx36 gene sequences as references, three RNAi sequences were designed to establish a lentiviral vector system to infect shRNA-Cx36 (fig. 3A). shRNA-Cx36 and shRNA-control were microinjected into the ACC before the CCI surgery (fig. 3B). The transfection efficiency of lentivirus was 69.8 ± 2.23% (n = 5). Immunohistochemistry (fig. 3C) and Western blotting (fig. 3D) results indicated that the shRNA-Cx36 group experienced a significant reduction in Cx36 expression in the ACC after CCI compared with the rats in the shRNA-control (control) group (Western blotting in control group, 0.76 ± 0.07, n = 5; Western blotting in shRNA-Cx36 group, 1.26 ± 0.09, n = 5; Student’s t test, P < 0.001). The behavioral test further demonstrated that both the paw withdrawal threshold and the paw withdrawal latency significantly increased in the rats in the shRNA-Cx36 group (fig. 3, E and F).

Furthermore, we interfered with the electrical coupling in the ACC using a microinjection of electrical synapse blockers. Both the selective Cx36 blocker mefloquine (Meflo) and the general gap junction blocker Cbx were used in the current study. We microinjected Meflo and Cbx locally into the ACC immediately after CCI, and the administration continued for the following 4 days (fig. 4, A and B). Pain behaviors were investigated on days 3, 5, 7, 14, and 21 after CCI. We found that both Meflo (100 mM, 1.6 μl) and Cbx (100 mM, 1.6 μl) were successful in attenuating thermal hyperalgesia and mechanical allodynia when injected locally into the ACC (fig. 4, C–F). Collectively, these data suggest that Cx36 up-regulation in the ACC may be responsible for neuropathic pain formation after CCI.
Fig. 1. Nerve injury induces up-regulation of connexin 36 (Cx36) in the anterior cingulate cortex (ACC). (A) Cx36 immunohistochemistry in the ACC of sham (day 7 after surgery) and chronic constriction injury (CCI) (days 3 and 7 after surgery) rats. Scale bar, 50 μm. (B) Cx36 Western blot of the ACC of sham (day 7 after surgery) and CCI (days 3 and 7 after surgery) rats. Bottom, quantification of Cx36 band intensity (black, sham group; green, day 3 after CCI surgery; and red, day 7 after CCI surgery, n = 5); **P < 0.01 versus sham group. (C) Cx36 immunohistochemistry in the primary motor cortex of sham (day 7 after surgery) and CCI (day 7 after surgery) rats. Scale bar, 50 μm. (D) Cx36 Western blot of the primary motor cortex of sham (day 7 after surgery) and CCI (day 7 after surgery) rats. Bottom, quantification of Cx36 band intensity (n = 5). (E) Cx36 immunohistochemistry in the cornu ammonis region 3 region of hippocampus from sham group (day 7 after surgery) and CCI (day 7 after surgery) rats. Scale bar, 50 μm. (F) Cx36 Western blot of the hippocampus of sham (day 7 after surgery) and CCI (day 7 after surgery) rats. Bottom, quantification of Cx36 band intensity (n = 5). DAPI = 4′,6-diamidino-2-phenylindole.
**Fig. 2.** Nerve injury induces activation of gamma-frequency neuronal oscillations in the anterior cingulate cortex. (A) Perfusion with nanomolar concentrations of kainate (150 nM) resulted in the appearance of persistent gamma oscillations. Right, sample traces of kainate-induced gamma oscillations in anterior cingulate cortex slices from sham group and chronic constriction injury (CCI) (days 3 and 7 after surgery) rats. Left, corresponding power spectra of the traces (black, sham group; green, day 3 after CCI surgery; and red, day 7 after CCI surgery.) Scale bars represent 50 μV and 500 ms. (B) Peak frequency from sham group and CCI (days 3 and 7 after surgery) rats. (C) Summary results of the gamma oscillation power from sham group (black, n = 7 slices of four rats), day 3 after CCI surgery (green, n = 7 slices of four rats), and day 7 after CCI surgery (red, n = 8 slices of four rats). **P < 0.01 versus sham group.

**Fig. 3.** Reversal of chronic constriction injury (CCI)-induced neuropathic pain by short hairpin RNA (shRNA)-connexin 36 (Cx36). (A) Schematic diagram shows the lentiviral shRNA expression vector system. Three RNA interference sequences were designed to establish a lentiviral vector system shRNA-Cx36. (B) The schematic view of knockdown experiments. (C) Cx36 immunohistochemistry and 4',6-diamidino-2-phenylindole (DAPI) stain of shRNA-control–infected and shRNA-Cx36–infected anterior cingulate cortex. Scale bar, 50 μm. (D) Western blotting of shRNA-control–infected (n = 5) and shRNA-Cx36–infected (n = 5) anterior cingulate cortex lysates with antibodies against Cx36 and β-actin. The protein expression of Cx36 was quantified and normalized by β-actin. ***P < 0.001 compared with lentiviral-shRNA-control–infected group. (E) Reversal of CCI-induced mechanical allodynia by shRNA-Cx36 (two-way ANOVA followed by Bonferroni post hoc test, F(1,90) = 109.134, P < 0.0001; *P < 0.05, compared with shRNA-control, n = 8 to 9). (F) Attenuation of CCI-induced thermal hyperalgesia by shRNA-Cx36 (two-way ANOVA followed by Bonferroni post hoc test, F(1,90) = 85.671, P < 0.0001; *P < 0.05, compared with shRNA-control, n = 8 to 9). CMV = cytomegalovirus promoter; delta2 = the gene of Cx36; GFP = green fluorescent protein; Gjd2 = gap junction protein; U6 = U6 promoter.
Systemic Administration of Meflo Attenuates Nerve Injury–Induced Neuropathic Pain

Given that the method of local microinjection is not applicable for clinical practice, we further explored whether systemic administration of Meflo can relieve neuropathic pain. Following the same protocol of ACC microinjection, Meflo was intraperitoneal administered to the rats for 5 days (fig. 5A). We found that Meflo (20 and 30 mg/kg) was able to significantly increase the paw withdrawal threshold to mechanical stimuli and the paw withdrawal latency to heat stimuli compared with the vehicle 3 and 21 days after CCI (fig. 5B). Furthermore, this electrical synapses blocker relieved the chronic pain induced by CCI in a dose-dependent manner. To exclude the possibility that the electrical synapse blocker can affect normal pain perception in rats, Meflo was intraperitoneal administered to animals after sham surgery for 5 days consecutively (fig. 5C). We found that Meflo administration did not affect the paw withdrawal threshold to mechanical stimulus or vehicle on CCI-induced mechanical allodynia (two-way ANOVA, F(1,150) = 43.281, P < 0.0001; post hoc tests revealed that CCI-induced mechanical allodynia was significantly reduced by Cbx treatment; *P < 0.05, compared with PBS vehicle, n = 8).

Fig. 4. Microinjection of mefloquine (Meflo) or carbenoxolone disodium salt (Cbx) to the anterior cingulate cortex (ACC) could relieve chronic constriction injury (CCI)-induced neuropathic pain. (A) The schematic view of ACC microinjection experiments. (B) The image shows an example of the location of microinjection site in one ACC slice with hematoxylin and eosin staining. Scale bar, 1 mm. (C) Reversal of CCI-induced mechanical allodynia by ACC microinjection of Meflo (100 mM, 1.6 μl) (two-way ANOVA followed by Bonferroni post hoc test, F(1,144) = 60.174, P < 0.0001; *P < 0.05, compared with phosphate-buffered saline [PBS] with 1% dimethyl sulfoxide vehicle [Veh], n = 12 to 14). (D) Attenuation of CCI-induced thermal hyperalgesia by ACC microinjection of Meflo (two-way ANOVA followed by Bonferroni post hoc test, F(1,84) = 112.141, P < 0.0001; *P < 0.05, compared with PBS with 1% dimethyl sulfoxide vehicle, n = 7 to 9). (E) Effects of ACC microinjection of Cbx (100 mM, 1.6 μl) or vehicle on CCI-induced mechanical allodynia (two-way ANOVA, F(1,150) = 43.281, P < 0.0001; post hoc tests revealed that CCI-induced mechanical allodynia was significantly reduced by Cbx treatment; *P < 0.05, compared with PBS vehicle, n = 13 to 14). (F) Reversal of thermal hyperalgesia after CCI by ACC microinjection of Cbx (two-way ANOVA followed by Bonferroni post hoc test, F(1,84) = 79.52, P < 0.0001; *P < 0.05, compared with PBS vehicle, n = 8).
Fig. 5. Systemic administration of mefloquine (Meflo) could relieve neuropathic pain induced by chronic constriction injury (CCI) or spared nerve injury (SNI). (A) The schematic view of intraperitoneal (ip) injection experiments. (B) Left, reversal of mechanical allodynia after CCI by different dose of Meflo ip administration (two-way ANOVA followed by Bonferroni post hoc test, $F(1,114) = 13.952, P < 0.0001$ for 10 mg/kg group, $F(1,120) = 101.577, P < 0.0001$ for 25 mg/kg group, and $F(1,102) = 132.204, P < 0.0001$ for 35 mg/kg group; *$P < 0.05$, compared with 1% Tween-80 saline vehicle [Veh], $n = 7$ to 12). Right, the attenuation of thermal hyperalgesia after CCI by different dose of Meflo ip administration (two-way ANOVA followed by Bonferroni post hoc test, $F(1,90) = 2.301, P = 0.133$ for 10 mg/kg group, $F(1,84) = 87.804, P < 0.0001$ for 25 mg/kg group, and $F(1,78) = 96.488, P < 0.0001$ for 35 mg/kg group; *$P < 0.05$, compared with 1% Tween-80 saline vehicle, $n = 7$ to 9). (C) The schematic view of pain perception function experiments in sham animals. (D) 30 mg/kg Meflo for 5 consecutive days did not affect paw withdrawal threshold of von Frey hair stimulation ($n = 10$ to 12). (E) 30 mg/kg Meflo did not affect paw withdrawal latency to heat stimuli ($n = 10$ to 12). (F) 30 mg/kg Meflo for 5 consecutive days did not affect the total distance traveled during the 15-min recording time in open field ($n = 8$). (G) 30 mg/kg Meflo did not reduce the rotorad time of the rats when compared with the vehicle group ($n = 8$). (H) The attenuation of SNI-induced mechanical allodynia by Meflo (20 mg/kg) at days 3, 5, 7, and 14 after surgery; however, no pain relief was found at day 21 after SNI (two-way ANOVA followed by Bonferroni post hoc test, $F(1,96) = 36.032, P < 0.0001$, *$P < 0.05$, compared with 1% Tween-80 saline vehicle, $n = 8$ to 10).
Systemic Administration of Meflo Decreases Gamma Oscillation Power and Synaptic Transmission in the ACC

Gamma oscillations in the pain-associated cortex are particularly related to the experience of pain. To further confirm the connection between gamma oscillations and neuropathic pain, we examined the effects of Meflo on gamma oscillations in the ACC. The gamma-frequency oscillatory activity in ACC slices was observed after Meflo administration (fig. 6A). The field recordings demonstrated that Meflo application had no effect on the peak frequency of gamma oscillations (fig. 6B). However, we found that the activation of gamma-frequency oscillatory activity after CCI was almost completely reversed by the selective Cx36 blocker Meflo (fig. 6, A and C; sham group, 5.09 ± 1.04 × 10⁻¹² V²/Hz, n = 7 slices of four rats; CCI group, 11.29 ± 1.49 × 10⁻¹² V²/Hz, n = 8 slices of four rats; Meflo group, 5.11 ± 0.53 × 10⁻¹² V²/Hz, n = 8 slices of four rats; one-way ANOVA, P = 0.002). Thus, gamma oscillation activation in the ACC after CCI may contribute to neuropathic pain generation and Meflo may relieve the pain by reversing the gamma oscillation activation.

Studies have also shown that ACC synaptic plasticity is responsible for neuropathic pain generation. To verify whether Cx36 contributes to the synaptic transmission potentiation in the ACC after nerve injury, we also used brain slices to investigate the probability of glutamate release from the presynaptic membranes and AMPA receptor-mediated responses in the postsynaptic membranes of ACC neurons. Consistent with previous studies, significant enhancements were found in the amplitude (P = 0.0022) and frequency (P = 0.0159) of mEPSCs in the ACC neurons after CCI (d 7 to 14) (fig. 6, D–F and table 1; sham group, n = 14 neurons, four rats; CCI group, n = 15 neurons, four rats, one-way ANOVA). Furthermore, the amplitude (P = 0.0005) and frequency (P = 0.0018) of mEPSCs significantly decreased in the Meflo group (consecutively Meflo intraperitoneal injection for 5 days after CCI and performed on days 7 to 14) compared with the CCI group (fig. 6, D–F and table 1; Meflo group, n = 16 neurons, five rats, one-way ANOVA).

To further determine whether the presynaptic and postsynaptic transmissions were decreased by Meflo, paired-pulse facilitation (PPF) and inward rectification were examined in ACC neurons in the current study. PPF is a form of short-term synaptic plasticity commonly used as a measure of presynaptic function in which the response to the second stimulus is increased as a result of residual calcium in the presynaptic terminal after the first stimulus. PPF was observed at stimulus intervals of 30, 60, and 120 ms in this study. A significant reduction in PPF was observed in the ACC neurons after nerve ligation. Inward rectification indicated better Ca²⁺ permission of AMPA receptors and enhanced postsynaptic transmission. Previous studies have demonstrated that inwardly rectifying properties of AMPA receptors occurred as a result of an alteration to their subunit composition in ACC neurons after nerve injury. Taken together, these results suggested that Meflo normalized gamma oscillation activation and decreased potentiated synaptic transmission in CCI animal model.

Effects of the Isomers of the Antimalarial Drug Meflo on Neuropathic Pain

Meflo is composed of two isomers, (+)-(11S,12R)-Meflo and (−)-(11R,12S)-Meflo (fig. 7A). We further examined which isomer was responsible for the pain relief by Meflo injection. The rats were intraperitoneal administered (+)-(11S,12R)-Meflo or (−)-(11R,12S)-Meflo immediately after CCI for 5 days consecutively (fig. 7B). We discovered that (−)-(11R12S)-Meflo (20 mg/kg) was able to significantly decrease the paw withdrawal threshold to mechanical stimuli and the paw withdrawal latency to heat stimuli (fig. 7, C and D). However, (+)-(11S,12R)-Meflo (20 mg/kg) had no effect on neuropathic pain induced by CCI (fig. 7, C and D). Thus, (−)-(11R,12S)-Meflo may have potential as a new therapeutic for neuropathic pain.

Discussion

The results presented in this study confirm the relation between the electrical synapses of the central nervous system and the formation of neuropathic pain. Evidence of the association between gap junctions and neuropathic pain formation is quite sparse. Previous studies primarily focused on gap junctions in the peripheral nerves and in glial cells. Cx37 mRNA levels have been reported to increase proximally and distally to injured locations in rat sciatic nerves after injury,
Fig. 6. Mefloquine (Meflo) reverses the activation of gamma-frequency neuronal oscillations and the potentiation in synaptic transmission in the anterior cingulate cortex (ACC). (A) Perfusion with nanomolar concentrations of kainate (150 nM) resulted in the appearance of persistent gamma oscillations. Right, sample traces of kainate-induced gamma oscillations in ACC slices from sham group, chronic constriction injury (CCI) (day 7 after surgery) rats, and Meflo group (consecutive Meflo intraperitoneal injection for 5 days after CCI and performed on day 7 after surgeries). Left, corresponding power spectra of the traces (black, sham group; red, day 7 after CCI surgery; and blue, Meflo group). Scale bars represent 50 μV and 500 ms. (B) Peak frequency from sham group, CCI rats, and Meflo group. (C) Summary results of the gamma oscillation power from sham group (black, n = 7 slices of four rats), day 7 after CCI surgery (red, n = 8 slices of four rats), and Meflo group (blue, n = 8 slices of four rats). **P < 0.01, versus CCI group. (D) Representative miniature excitatory postsynaptic currents (mEPSCs) recorded in the ACC neurons in slices from different groups at a holding potential of −70 mV. Scale bars represent 10 pA and 500 ms. (E) Amplitude of mEPSCs recorded in slices from sham rats (black; n = 14 neurons of four rats), rats after CCI (red; n = 15 neurons of four rats), and Meflo group (blue; n = 16 neurons of five rats). **P < 0.01, ***P < 0.001 versus CCI group. (F) Frequency of mEPSCs recorded in slices from sham rats, rats after CCI, and rats after CCI with Meflo injection. *P < 0.05, **P < 0.01 versus CCI group.
and this increase lasted for 21 days.\textsuperscript{36} Several studies have demonstrated that a close association exists between interglial connexin (Cx43) and neuropathic pain formation. An orofacial neuropathic pain model was used to prove that the selective silencing of Cx43 expression on the dorsal root ganglion of the trigeminal nerve could attenuate neuropathic pain after infraorbital nerve injury.\textsuperscript{57} Another study reported that heat hyperalgesia and mechanical allodynia induced by spinal cord injury were prevented in transgenic mice with Cx43/ Cx30 deletions but were fully developed in transgenic mice with only the Cx30 deletion.\textsuperscript{38} Whether electrical synapses contributed to neuropathic pain formation remains unclear.

In the current study, we demonstrated that systemic application of Meflo relieved neuropathic pain by both behavioral tests and electrophysiologic experiments. Meflo was invented at the Walter Reed Army Institute of Research in the 1970s and was marketed as Lariam and Mephaquine. Meflo has been used as an antimalarial drug since its approval by Food and Drug Administration in 1989 for systemic oral use in humans; thus, its safety has been well established. Meflo is effective on various types of human and animal malaria and can control \textit{Plasmodium falciparum} that are resistant to chloroquine.\textsuperscript{39} In summary, the current study attempted to provide evidence regarding the new clinical indications for this drug. Moreover, we found that (−)-(11R, 12S)-Meflo, which is a Meflo isomer, is promising as a new drug candidate for neuropathic pain. However, its effectiveness and the adverse effects under the effective dosage must be confirmed in further clinical studies. Once established after nerve injury, the symptoms of neuropathic pain are often resistant to conventional approaches of pain treatment. Thus, we applied the electrical synapse blocker during the early phase of neuropathic pain development. Our research may provide a new therapeutic strategy that could prevent the development of this disease during the early phase after nerve injury.

Our data demonstrated that Cx36 expression increased in the ACC region after nerve injury. Cx36, which is the major neuronal connexin, has been shown to modulate the gamma-frequency (30 to 80 Hz) oscillations.\textsuperscript{11} The gamma oscillations in cortical brain regions may be a possible mechanism for various cognitive processes, such as pain perception, attention, and memory formation.\textsuperscript{14,40,41} However, the involvement of ACC gamma oscillations in nerve injury-induced neuropathic pain has not been elucidated. Using brain slice experiments, we confirmed that ACC gamma oscillations could be evoked in a manner similar to the entorhinal cortex and hippocampus in the presence of nanomolar concentrations of kainate. We found that a significant increase in ACC gamma oscillation power was found after nerve injury, along with the up-regulation of Cx36 and an increase in the symptoms of neuropathic pain. Electrical coupling enhancement between neurons in the ACC might lead to a better synchronization of the functions of Cx36 gap junction channels, resulting in improved precision of action potential timing and of the rhythmicity of oscillation.\textsuperscript{13} The outcome of these changes would be reflected by gamma oscillation activation in the ACC. We further demonstrated that the selective Cx36 blocker Meflo could reverse the activation of gamma-frequency oscillatory activity and relieve the behavioral sensitization induced by CCI. Thus, ACC gamma oscillations might play a prominent role in the pathogenesis of nerve injury-induced neuropathic pain.

Our present results also provide some specific evidence explaining how Cx36 and gamma oscillations participate in neuropathic pain development. Gamma oscillation has been supposed to facilitate the neuronal communications by the temporal precision of coincident synaptic inputs,\textsuperscript{40,42,43} which may result in synaptic transmission strengthening. Moreover, potentiated synaptic transmission in the ACC was found to be the key mechanism for neuropathic pain. Thus, given the close link between synaptic transmission and pathological pain, we further demonstrated that the specific electrical synapse blocker Meflo could decrease the chemical excitatory synaptic transmission by electrophysiologic techniques, which might lead to the attenuation of neuropathic pain. Both presynaptic and postsynaptic changes were observed in excitatory synapses within the ACC after CCI. The changes in PPF and in the frequencies of mEPSCs in ACC neurons after nerve injury represent enhanced presynaptic transmitter release. However, after Meflo injection, these alterations were primarily restored. In addition, the postsynaptic functions of excitatory synapses within the ACC, including the amplitude of mEPSCs and the function of AMPA receptors, were also evaluated. Similarly, Meflo prevented the increase in mEPSC amplitudes after CCI. The biophysical properties of AMPA receptors depend on their subunit composition.\textsuperscript{44,45} Long-term potentiation of synaptic transmission was absent from the hippocampus in the adult GluA1 knockout mouse.\textsuperscript{46} Because of nerve injury, the AMPA receptor GluR1 subunit increased in the membrane fraction.\textsuperscript{5} This alteration of subunit composition in ACC neurons after nerve injury led to an increase in the rectification

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{(Continued) (G) Paired-pulse ratio (the ratio of excitatory postsynaptic current 2/excitatory postsynaptic current 1) was recorded at intervals of 30, 60, and 120 ms from sham rats (\textcolor{black}{black}; \(n = 19\) neurons of four rats), rats after CCI (\textcolor{red}{red}; \(n = 18\) neurons of four rats), and Meflo group (\textcolor{blue}{blue}; \(n = 19\) neurons of five rats). Upper current traces represent the examples of paired-pulse stimulation (stimulus interval: 60 ms) evoked responses. \textit{Scale bars} represent 40 pA and 100 ms. *\(P < 0.05\), Meflo versus CCI group. (H) Rectification of \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) receptor–mediated peak currents at −65, 0, and +35 mV holding potentials were recorded in slices from sham rats (\textcolor{black}{black}; \(n = 14\) neurons of four rats), rats after CCI (\textcolor{red}{red}; \(n = 13\) neurons of four rats), and rats after CCI with Meflo injection (\textcolor{blue}{blue}; \(n = 13\) neurons of four rats). Rectification index \(= (amplitude\ at\ −65\ mV\ holding\ potential)\ /\ (amplitude\ at\ +35\ mV\ holding\ potential)\). \textit{Scale bars} represent 10 pA and 50 ms. *\(P < 0.05\), versus CCI group. Veh = vehicle.}
\end{figure}
index of AMPA receptor–mediated transmission,\(^5\) which may denote better Ca\(^{2+}\) permission of AMPA receptors and potentiation in synaptic transmission. Thus, the rectification index was a reflection of synaptic plasticity. In this study, we identified whether the inwardly rectifying properties of AMPA receptors in ACC neurons after nerve injury could be restored by inhibiting electrical synapses. Our results demonstrated that the specific electrical synapse blocker Meflo could significantly decrease the rectification index of AMPA receptors in ACC neurons after nerve injury, which might result in less Ca\(^{2+}\) permission of the AMPA receptors and decreased synaptic transmission.

In addition, we interfered with Cx36 expression in the ACC region using the RNAi technique and found that neuropathic pain was relieved after blocking Cx36 expression in the ACC region. Therefore, the increased Cx36 expression in the ACC after nerve injury might lead to gamma oscillation activation and then to synaptic transmission potentiation, which might further result in neuropathic pain generation after nerve injury.

### Table 1

<table>
<thead>
<tr>
<th>Parameters of Synaptic Transmission</th>
<th>Sham Group</th>
<th>CCI Group</th>
<th>Meflo Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude of mEPSCs (Pa)</td>
<td>7.69 (7.05–8.34)</td>
<td>10.71 (8.77–12.65)</td>
<td>7.40 (6.74–8.05)</td>
</tr>
<tr>
<td>Frequency of mEPSCs (Hz)</td>
<td>0.96 (0.64–1.28)</td>
<td>1.75 (1.16–2.34)</td>
<td>0.78 (0.55–1.01)</td>
</tr>
<tr>
<td>Inward rectification</td>
<td>1.72 (1.54–1.89)</td>
<td>2.06 (1.80–2.33)</td>
<td>1.74 (1.59–1.88)</td>
</tr>
</tbody>
</table>

CCI = chronic constriction injury; Meflo = mefloquine; mEPSCs = miniature excitatory postsynaptic currents.

### Table 2

<table>
<thead>
<tr>
<th>PPR</th>
<th>30 ms</th>
<th>60 ms</th>
<th>120 ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>1.53 (1.34–1.71)</td>
<td>1.39 (1.20–1.57)</td>
<td>1.18 (1.05–1.30)</td>
</tr>
<tr>
<td>CCI group</td>
<td>1.15 (0.99–1.32)</td>
<td>1.06 (0.95–1.16)</td>
<td>0.99 (0.90–1.08)</td>
</tr>
<tr>
<td>Meflo group</td>
<td>1.52 (1.37–1.68)</td>
<td>1.26 (1.09–1.42)</td>
<td>1.13 (1.03–1.22)</td>
</tr>
</tbody>
</table>

CCI = chronic constriction injury; Meflo = mefloquine; PPR = paired-pulse ratio.

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**Fig. 7.** Effects of the isomers of antimalarial drug mefloquine (Meflo) on neuropathic pain. (A) Chemical structures of (−)-(11R, 12S)-Meflo and (+)-(11S, 12R)-Meflo. (B) The schematic view of the isomers of Meflo intraperitoneal (ip) injection experiments. (C) Alleviation of mechanical allodynia after chronic constriction injury (CCI) by (−)-(11R, 12S)-Meflo (30 mg/kg) ip injection; however, no analgesic effect was found when (+)-(11S, 12R)-Meflo (30 mg/kg) was injected intraperitoneally (two-way ANOVA followed by Bonferroni post hoc test, \(F(1,60) = 39.581, P < 0.0001\) for (−)-(11R, 12S)-Meflo group, \(F(1,64) = 4.196, P = 0.045\) for (+)-(11S, 12R)-Meflo group, \(^*P < 0.05\), compared with 1% Tween-80 saline vehicle [Veh], \(n = 8\) to 9). (D) The attenuation of CCI-induced thermal hyperalgesia by (−)-(11R, 12S)-Meflo ip injection; however, no pain alleviation was found when (+)-(11S, 12R)-Meflo was injected intraperitoneally (two-way ANOVA followed by Bonferroni post hoc test, \(F(1,60) = 49.096, P < 0.0001\) for (−)-(11R, 12S)-Meflo group, \(F(1,64) = 2.288, P = 0.135\) for (+)-(11S, 12R)-Meflo group, \(^*P < 0.05\), compared with 1% Tween-80 saline vehicle, \(n = 8\) to 9).
A recent study showed decreased Cx36 expression in spina
dorsal horn resulted in the development of neuropathic
pain. It is very interesting that the change of Cx36 in spi
nal dorsal horn and ACC is different during the neuropathic
pain. According to their results, Cx36 was colocalized with
glycinergic neurons but not GABAergic neurons in spinal
dorsal horn. However, Ma et al. indicated that Cx36 was
expressed almost specifically in GABAergic neurons of the
adult rat neocortex. Therefore, the function of Cx36 in spi
nal dorsal horn and neocortex may be different. We speculate
that the up-regulation of Cx36 in ACC is a high-level central
regulation for peripheral pain. As we discussed in our study,
the persistent up-regulation of Cx36 may lead to a potentia
 tion of chemical synapses and result in central sensitization
finally. This may be one of the reasons why available treat
ments for neuropathic pain have limited therapeutic efficacy.

There are also some limitations of the study need to be
addressed. We did not conduct studies of neuropathic pain
using the oral form of Meflo. For Meflo to have any utility
in clinical settings, an oral application is necessary. Intragas
tric administration of Meflo could ensure the accuracy of
the administered dose. However, injuries caused by gavage
are not uncommon. Moreover, repeated gavage may lead to
stress in rats that would affect the function of the ACC.
Thus, accurate behavioral data of oral administration could
not provide by our study. We found that Meflo could relieve
mechanical pain in CCI model for 21 days at least, whereas it
could not in SNI model. The two nerve injury models exam
ined in our study differ considerably. Previous study also
reported the excitatory synaptic transmission changed dif
ferently in CCI and SNI models. These facts may account for
the different pain relief effects of Meflo on two animal
models. Moreover, our future research will try to figure out
the distinct effects of Meflo on the synaptic transmission in
different animal models. This may further explain how Cx36
involve in the synaptic transmission and neuropathic pain.

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Competing Interests
The authors declare no competing interests.

Correspondence
Address correspondence to Dr. Wang: Department of An
esthesiology and Intensive Care Medicine, Xinhua Hospital,
College of Medicine, Shanghai Jiaotong University, No. 1665
Kongjiang Road, Yangpu District, Shanghai, 200092, China.
wangy ingwei@yahoo.com. Information on purchasing re
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