Pain Physician 2013; 16:E577-E589 • ISSN 2150-1149

Experimental Trial

Chronic Constriction Injury Induced Long-Term Changes in Spontaneous Membrane-Potential Oscillations in Anterior Cingulate Cortical Neurons in Vivo

Li Ning, MD[1], Li-Qing Ma, MD[1], Zhi-Ru Wang, PhD[2], and Ying-Wei Wang, MD, PhD[1]

From: [1]Department of Anesthesiology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China; [2]Institute & Key Laboratory of Brain Functional Genomics of Shanghai and Chinese Ministry of Education, East China Normal University, Shanghai 200062, China.

Address Correspondence: Ying-Wei Wang, MD, PhD, Department of Anesthesiology, Xinhua Hospital Shanghai Jiaotong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China. Email: wangyingwei@yahoo.com.

Disclaimer: This work was supported by the grants from the National Natural Science Foundation of China (No.81171169 and 91132711) and Shanghai New 100-Talent Program Grant (No.XBR2011023). Conflict of interest: Each author certifies that he or she, or a member of his or her immediate family, has no commercial association, (i.e., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might post a conflict of interest in connection with the submitted manuscript.

Manuscript received: 04-05-2013
Revised manuscript received: 05-16-2013
Accepted for publication: 05-17-2013

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Background: Neuropathic pain induction by nerve injury has been shown by in vitro studies to be accompanied by synaptic strengthening in the anterior cingulate cortex (ACC) and has been shown by pharmacological studies in vivo to be prevented by blocking *N*-methyl-D-aspartate (NMDA) receptor-dependent ACC plasticity. These findings indicate that ACC neurons undergo nerve injury-induced synaptic modifications and further raise a new question about neuropathic pain-associated changes in neuronal activity in the ACC in vivo, particularly spontaneous neuronal oscillations – a process believed to be fundamental for many forms of brain function.

Objective: In this study, we examined the change of spontaneous membrane-potential (MP) oscillations in the ACC in vivo in a neuropathic pain animal model of chronic constriction injury (CCI), which may account for neuropathic pain development, as well as pain hypersensitivity and spontaneous pain.

Study Design: Experimental trial in rats.

Methods: Neuropathic pain rats were produced by CCI surgery on the common sciatic nerve. Neuropathic pain-related behaviors were accessed by evoked responses to both mechanical and thermal stimuli, as well as spontaneous pain indicated by spontaneous foot lifting. In vivo whole-cell recording was performed in both control and neuropathic pain rats under anaesthesia. MP and action-potential (AP) changes of layer II/III ACC pyramidal cells were measured in current-clamp mode. The level of anaesthesia was evaluated by monitoring respiratory and heart rates in some experiments.

Results: Within 7 to 14 days after CCI surgery, the frequency of MP oscillations of ACC neurons was found to be significantly higher than that in control rats. Such an increase in oscillation frequency after surgery was not due to periphery transmission via the sciatic nerve subjected to CCI surgery and was indicated to be accounted for by neuronal modifications in the central nervous system. Furthermore, this increase was found to result in a higher overall level of MP excitation as well as an increase in spontaneous AP firing.

Limitations: Our findings in MP and AP changes were obtained in anaesthetized brains; this issue remains to be further examined by using whole-cell recording in awake behaving animals.

Conclusions: Neuropathic pain is accompanied by the increase in rates of spontaneous oscillations of ACC neurons. This change may be critical for neuropathic pain development, as well as pain hypersensitivity and spontaneous pain in neuropathic pain animals.

Key Words: Neuropathic pain, anterior cingulate cortex, spontaneous activity, neuronal oscillation, chronic constriction injury

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Neuropathic pain is prevalent in the patient subjected to nerve injury; these patients suffer from pain hypersensitivity to external sensory stimuli and spontaneous pain in the absence of sensory stimuli (1-3). The neural and molecular mechanism of neuropathic pain has been extensively studied in terms of nerve injury-induced neuronal modifications in the peripheral nervous system (4,5) and spinal cord (6-8). However, much less is known about the change in brains.

Previous studies have shown that pain processing in the brain involves 2 major pathways – the lateral pain system and the medial pain system (9), which are responsible for the sensory-discriminative and affective-motivational pain perceptions, respectively. The lateral pain system relays spinal nociceptive inputs to the somatosensory cortex for the sensory dimension of pain, including the intensity, location, and quality of pain stimuli (10). The medial pain system relays spinal nociceptive inputs to the limbic cortex for the affective dimension pain, i.e., the emotional outcome of unpleasantness for pain perception (9,10). In the study of neuropathic pain, the limbic cortex – in particular the anterior cingulate cortex (ACC) receives increasing attention. Studies have shown that local lesions in the ACC can largely eliminate the unpleasant emotion of pain perception without significantly impairing the sensory-discriminative perception of the stimulus (11). Basic research in both animals and humans has further provided evidence for the contribution of the ACC to neuropathic pain (12), as well as psychological and social pain (13). Social pain is defined as the distressing experience arising from the perception of actual or potential psychological distance from close others or a social group. Recently, it has been found by in vitro studies that the nerve injury causing neuropathic pain induces synaptic strengthening in the ACC neurons (14), i.e., long-term potentiation (LTP) at these synapses. More importantly, pharmacologically blocking this synaptic potentiation, such as applying *N*-methyl-D-aspartate (NMDA)-receptor blocker in the ACC, can prevent the development of neuropathic pain (15).

Although it has been clear that neuronal plasticity induced by nerve injury in the ACC is critical for neuropathic pain development, little is known about the alteration of ACC activity accounting for the functional disorder in neuropathic pain subjects. Given that neuropathic pain is induced by short-period nerve injury but can last for a long-period after injury (16), an open question is about the possible change in spontaneous activity of ACC neurons in vivo accompanying neuropathic pain. It has been well documented that spontaneous activity is prevalent in various brain regions in vivo and usually occurs in a rhythmic pattern commonly referred to as oscillations (17). Neuronal oscillations are believed to be fundamental for many forms of brain function (18), for example, for memory consolidation with the effect on synaptic modifications (19) and for sensory perception with the effect on feature binding during perceiving an object (20). Considering the similarity between neuropathic pain development and memory consolidation in the underlying mechanism of synaptic plasticity (21), as well as the similarity between pain hypersensitivity and sensory perception in processing non-noxious stimuli, neuronal oscillations in ACC neurons would be expected to play important roles in neuropathic pain, similar to that found in other forms of brain function. However, this issue remains largely unknown.

Using a method of in vivo whole-cell recording from layer II/III ACC neurons in anaesthetized adult rats, we investigated the change in spontaneous membrane-potential (MP) oscillations and action-potential (AP) firing within one to 2 weeks after chronic constriction injury (CCI) surgery.

Methods

Animals

Male Sprague-Dawley rats (10- to 14-weeks-old; 300–380 g) were used in this study. All experiments were performed under protocols approved by the Animal Care and Use Committee of East China Normal University and Shanghai Jiaotong University School of Medicine.

Surgery

The CCI model was performed similar to a method previously described (22). Briefly, the rats were anaesthetized with sodium pentobarbital. The common sciatic nerve was then exposed and dissected from the surrounding connective tissue. Three loosely constrictive ligatures (5–0 chromic gut suture) were tied around the nerve with a 1–1.5 mm distance between ligatures. The muscle and skin were closed in layers by using suture lines; as a surgery procedure before the electrophysiological experiments using local lidocaine treatments on the sciatic nerve, these lines were cut off to expose the nerve. All operations were conducted by the same person. After surgery, the rats were individually housed.

Behavioral Studies

For mechanical allodynia tests, the rats were placed on a mesh floor, which was covered by a transparent Plexiglas chamber. The animals were habituated for 2–3 days in the test environment before the test. After about 30-minute habituation, a series of 9 calibrated von Frey filaments (bending forces: 0.6, 1, 1.4, 2, 4, 6, 8, 10, and 15 g) (Stoelting) were delivered in an incremental order of bending forces to the central region of the plantar surface of the hindpaw ipsilateral to the sciatic nerve subjected to CCI surgery. Each filament was applied with sufficient force to bend for 5 seconds, which was repeated at an interval of 6 minutes. The withdrawal threshold was defined as the smallest filament size which evoked at least 3 withdrawal responses in 5 measurements. Since filaments with force exceeding 15 g could cause the paw to be lifted, 15 g was defined as the cutoff value.

Thermal hyperalgesia was evaluated as the threshold of withdrawal responses to noxious heat stimuli, which was measured by using a radiant heat method (23) in the same group of animals used in mechanical pain tests. For the tests, the rats were placed in a Plexiglas chamber, positioned over an elevated glass surface and habituated to the environment for about 30 minutes before testing. During the tests, a light beam with a high and constant intensity generated by a mobile radiant heat source stimulator (BME-410 thermal radiation, Peking Union Medical College Institute of Biomedical Engineering) at 10 V and 30 W was focused onto the midplantar region of the hindpaw. The paw withdrawal latency was referred to as the mean value from 3 measurements (intervals; 6 minutes). A cutoff time of 40 seconds was used to prevent any possible tissue damage.

To access spontaneous pain-related behaviors, the rats were placed in a Plexiglas chamber above a wire mesh floor and allowed to habituate for about 20 minutes. Spontaneous pain-related behaviors were observed for 5 minutes and evaluated by using a numerical scale modified as in a previous study (24). The scale was defined as follows: 0, the paw of the operated side was pressed normally on the floor; 1, the paw rested lightly on the floor; 2, only the internal edge of the paw was pressed on the floor; 3, only the heel was pressed on the floor, and the hindpaw was in an inverted position; 4, the whole paw was elevated; 5, the animal licked the lesioned paw.

Electrophysiological Recording and Stimulation

Before electrophysiological recording in CCI rats, hypersensitivity of mechanical pain, which developed together with the hypersensitivity of thermal pain and spontaneous pain-like behaviors (see experiments shown in Fig. 1), was tested and found to be successfully induced in nearly all (> 90%) of these rats. The CCI rats not showing this hypersensitivity were not considered for further recording.

In vivo whole-cell recording was performed as described previously (25). In brief, the animals were anaesthetized with sodium pentobarbital (initially with ~80mg/kg; supplemented 2–4 hours later with ~20 mg/kg/hr; i.p.), and the anaesthesia was maintained at a light level just below the threshold of body movements consisting of licking or scratching. Rectal temperature was maintained at 37.3–37.8°C by using a heating blanket placed beneath the animal. To measure the heart and respiratory rates, pressure sensors, customized with the use of piezoceramics, were placed under the chest and abdomen, respectively, and signals were sampled at 4 kHz by a data acquisition card (Digidata 1440, Axon Instr.). After a tracheotomy, the head of the rat was restrained with a stereotaxic apparatus (David Kopf Instr.), and a hole (2–3 mm diameter) on the skull was drilled (0.3–0.6 mm lateral to the midline, 2–3 mm anterior to the Bregma) for recording. A small piece of dura mater was partially removed. For histological analysis, animals were perfusion fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS and the brain was removed and fixed in PFA solution for 12 hours at 4°C.

A patch pipette with a tip opening of 2.5–3.0μm was pulled from borosilicate glass tubing (Kimble Glass Inc.) with a resistance of 3.0–4.5 MΩ. The internal solution contained (in mM) 136.5 K-Gluconate, 17.5 KCl, 9.0 NaCl, 1.0 MgCl2, 10.0 HEPES, 0.2 EGTA, and Amphotericin B (0.5 mg/ml). The pH value of the internal solution was adjusted to 7.3 with KOH. For histological staining, 0.5%–1% neurobiotin was included in the pipette solution to label recorded neurons. A positive pressure was applied to the pipette while it was advanced in the brain by a motor-driven manipulator (Siskiyou MMX7630, Siskiyou Corp.) at a speed of 15–30μm/s. Signals were acquired with a patch-clamp amplifier (Axopatch 200B, Axon Instr.) and sampled at 5 kHz by a data acquisition card (Digidata 1440, Axon Instr.), with 1, 2, or 5 kHz low-pass filtering. In the neuron included for analysis, membrane resistance was 56 ± 5 MΩ, and series resistance (not compensated) was 55 ±11 MΩ.

For DC electric shock stimulation, 2 mA and 2 seconds DC currents were applied via an electric stimulator (Master8, A.M.P.I) to 2 metal wiring rings surrounding around the hindpaws (inter-stimulus-intervals, 7 seconds).

Histological Analysis

After fixation, the brain was cut into 100-μm thick slices with a vibratome (Vibratome 3000, Vibratome Corp.). Slices were then incubated in 0.3% H_2O_2 for 30 minutes, followed by a one-hour treatment of 0.3%–0.5% Triton X-100 (Sigma), and then 5-hour incubation in PBS containing an avidin-biotinylated horseradish peroxidase complex (1:100; Vectastain ABC Elite kit) with 0.3% Triton X-100. The reaction was visualized with the tris-buffered saline containing 0.06% daminobenzidine (DAB), 0.03% H2O2, and 0.08% nickel chloride.

Statistical Analysis

Neurons with resting potentials between –66 mV and –87 mV (–75 ± 0.5 mV; mean ± SEM) were considered for further analysis, and recordings with > 15% changes in access resistance were further discarded. Liquid junction potentials (–13 mV) were corrected in this study. Unless otherwise specified, all data are presented as the mean ± SEM, and P-values were calculated by the Student's t-test.

Results

Pain Hypersensitivity and Spontaneous Pain Induced by CCI Surgery

We first examined the development of neuropathic pain-like behaviors by carrying out experiments on different days after CCI surgery, with the use of 10 rats subjected to CCI surgery (CCI rats) and 10 littermate controls (naive rats not receiving any surgery). In our measurements from CCI rats, the threshold of hindpaw withdrawal responses to mechanical stimuli (measured by von Frey filament stimulation; see Methods) was significantly reduced to 4.4 ± 0.3 g ($P < 0.001$) on day 3 and further reduced to 2.0 ± 0.4 g ($P < 0.001$) on day 7, as compared with the normal pain threshold measured in naive rats (13.4 ± 0.1 g) (Fig. 1A). In addition, all the CCI rats exhibited thermal hyperalgesia, which was indicated by a significantly shorter onset latency of hindpaw withdrawal responses to a thermal stimulus (see Methods) than that measured in naive rats (Fig. 1B). The pain hypersensitivity to both mechanical and thermal stimuli could last for at least 3 weeks after CCI surgery (Fig. 1A and 1B).

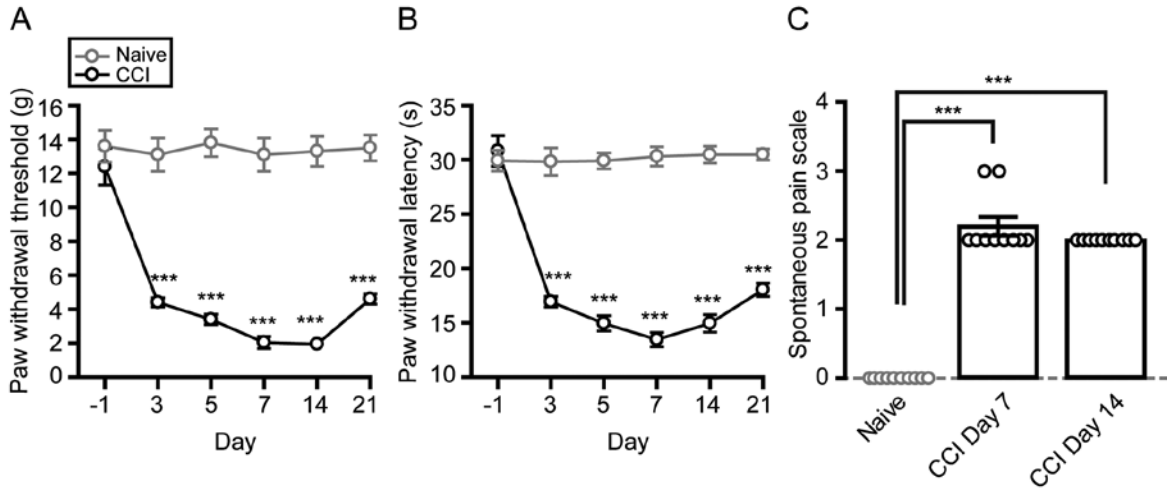

Fig. 1. *Pain hypersensitivity and spontaneous pain in CCI rats. (A) Thresholds of hindpaw withdrawal responses to von-Frey filament stimulation in naive (n = 10) and CCI (n = 10) rats. Data from both groups of rats were obtained on the same day, which was conducted one day before and within 21 days after the surgery in CCI rats. (B) Same as in (A), for the latencies of hindpaw withdrawal responses to a thermal stimulus in the same groups of naive and CCI rats shown in (A). (C) The degree of spontaneous pain-related behavior was evaluated using a numerical scale (see Methods). Scale number of the positioning of the hindpaw for naive (n = 10) and CCI (n = 10) rats shown in (A), which were measured at the injury side on day 7 (CCI day 7) and 14 (CCI day 14). Histograms, mean; bars, ± SEM; circles, data from individual rats. ***P < 0.001.*

Likewise, the CCI rats showed spontaneous pain-related behaviors, as indicated by spontaneous foot lifting (26). In our measurements of the positioning (referred to as scale numbers [0 to 5]; see Methods) of the hindpaw at the injury side, a scale number of 0 (normal positioning) was observed in all naive rats (n = 10). Whereas, abnormal positioning was found in all CCI rats – scale number at 2 or 3 (median at 2.2) on day 7 (n = 10 rats, $P < 0.001$) and at 2 on day 14 (n = 10 rats, $P < 0.001$) after surgery (Fig. 1C).

In Vivo Whole-cell Recordings from Layer II/III ACC Pyramidal Cells in Anaesthetized Adult Rats

Perforated whole-cell recording in current-clamp mode (no current injection without otherwise noted; see Methods) was performed from ACC neurons to monitor MP and AP changes. The recording site and a recorded pyramidal cell (stained by conventional whole-cell recording; see Methods) are illustrated in Figs. 2A and 2B. In some experiments, we further identified the

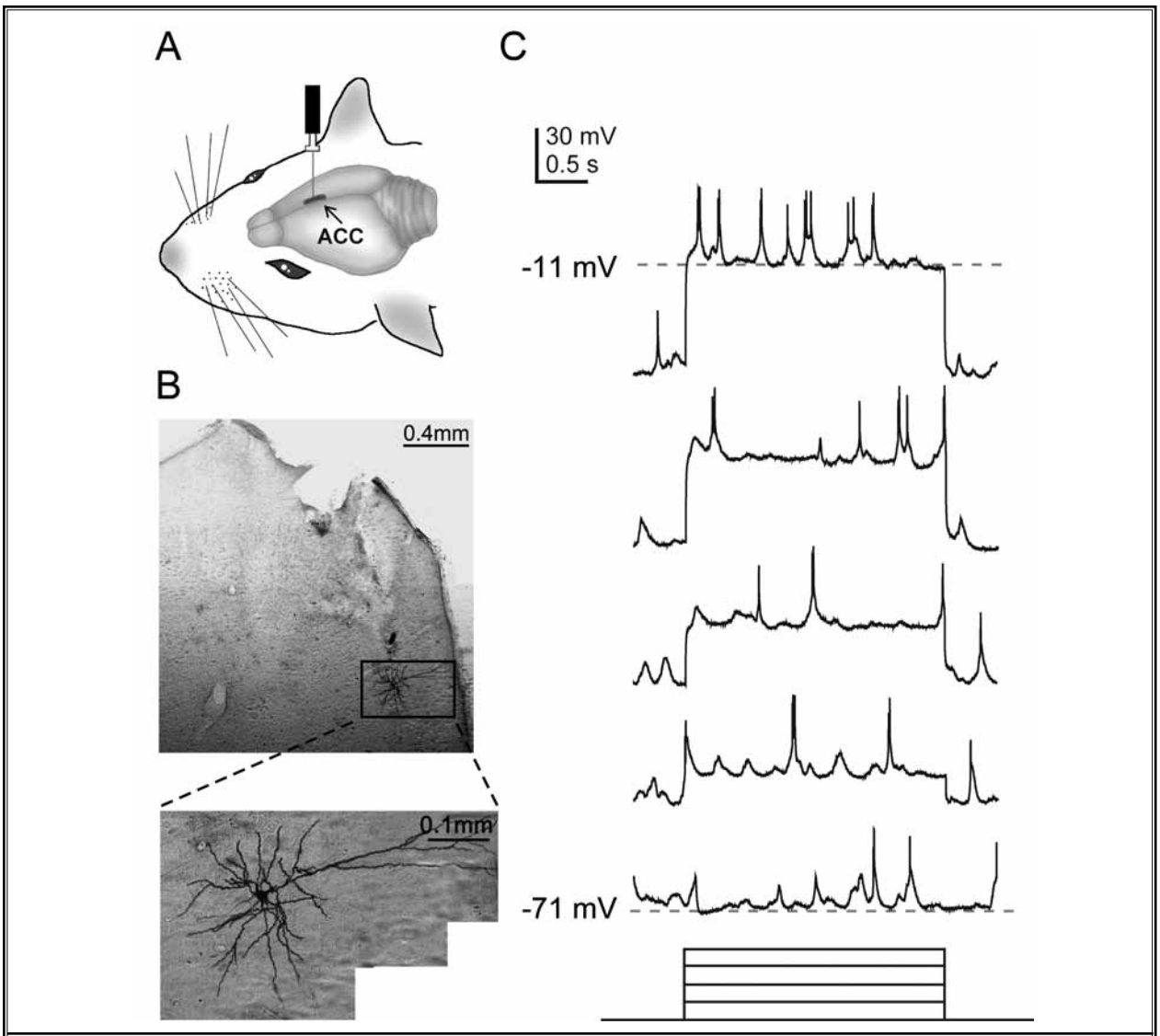

Fig. 2. *In vivo whole-cell recording on ACC pyramidal cells. (A and B) Schematic of the electrophysiological recording (A) and an ACC pyramidal cell labeled by conventional whole-cell recording (B). (C) For one example cell, firing pattern at resting potentials (–71 mV) and 4 levels (up to –11 mV) of baseline MP potentials depolarized by current injection (70 pA in increments; duration, 2.5 sec), as illustrated by the inset at bottom.*

cell type by depolarizing MPs at 4 different levels (up to –11 to –28 mV in individual cells; 10–16 mV increments [70 pA increments in current injection] between successive steps). An irregular spiking pattern similar to that observed in pyramidal cells by in vitro studies (27) was found in all cells (n = 40 neurons) (see Fig. 2C for one example recording). Thus, our data mainly reflect the observation in ACC pyramidal cells.

Increase in the Frequency of Spontaneous MP Oscillations after CCI Surgery

In the absence of external stimulation, ACC neurons exhibited large spontaneous MP depolarizations in an oscillatory pattern, as shown by the 2 example cells recorded in naive rats and auto-correlograms of their MP oscillations (Fig. 3A). Considering the possible role of spontaneous MP oscillations involved in the development of neuropathic pain, as well as pain hypersensitivity and spontaneous pain, we set out to investigate whether CCI surgery could cause long-term changes in these MP events of ACC neurons, a possible effect along with the symptom of neuropathic pain. For this purpose, we measured spontaneous MP changes from 40 ACC neurons in 18 naive rats and from 46 ACC neurons in 21 CCI rats within 7 to 14 days after CCI surgery.

In our observations, ACC neurons in CCI rats exhibited a spontaneous MP oscillation at a frequency significantly higher than that found in naive rats. As

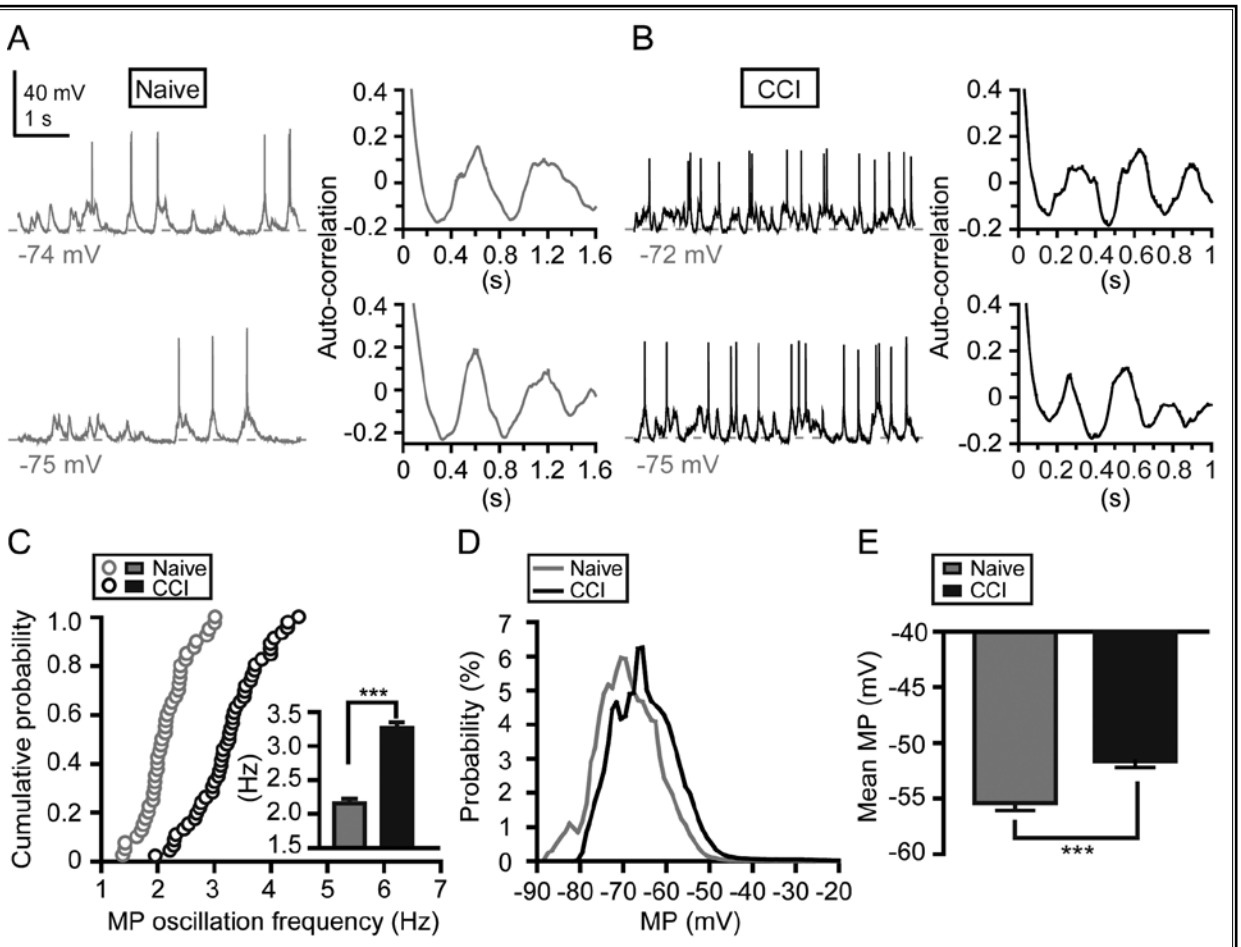

*Fig. 3. Increase in MP oscillation frequencies and overall MP levels after CCI surgery. (A) Spontaneous MP oscillations (left) and auto-correlograms (right) for 2 example recordings in naive rats. Gray dashed line at left: resting potentials. (B) Same as in (A), for recordings in CCI rats. (C) Cumulative distributions of MP oscillation frequencies (measured from auto-correlograms) for naive (n = 40 neurons) and CCI (n = 46 neurons) rats. Each circle represents one cell. Inset: mean ± SEM. (D and E) MP distributions (D) and mean MP levels (E) for the same groups of cells shown in (C). ***P < 0.001.*

shown in Fig. 3A–C by the example cells and summary data, the oscillation frequency in naive rats ranged from 1.4–3.0 Hz with a mean value at 2.2 ± 0.1 Hz, whereas this frequency was increased to 2.0–4.5 Hz with a mean value at 3.3 ± 0.1 Hz in CCI rats ($P < 0.001$). In addition, the CCI surgery-induced changes in MP oscillations is accompanied by an elevation in the overall level of MP excitation, as can be discerned from the distribution of MPs of the cells recorded in naive and CCI rats (Fig. 3D) and mean MP values averaged across all cells (Fig. 3E).

We further tested whether CCI surgery could change the intrinsic membrane properties of ACC neurons, which is involved in processing dendritic inputs to determine the generation of AP output in individual neurons (28). In our comparisons of the data obtained from naive and CCI rats, no significant difference was found in the resting potential (–75.3 ± 0.7 mV [n = 40 neurons] for naive and –75.2 ± 0.6 mV [n = 46 neurons] for CCI rats; $P = 0.93$) (Fig. 4A), the spiking threshold (from resting potentials; 22.7 ± 0.9 mV for naive and 21.8 ± 0.7mV for CCI rats; $P = 0.43$) (Fig. 4B), or the membrane resistance (56 ± 5 MΩ for naive and 55 ± 4 MΩ for CCI rats; $P = 0.45$) (Fig. 4C). Thus, the CCI operation-induced changes in MP oscillations may primarily result from the change of excitatory synaptic transmission in ACC circuits rather than the modification of intrinsic properties of individual neurons.

Independence of CCI Surgery-induced Changes in Spontaneous ACC Oscillations on Peripheral Transmission

In following experiments, we determined whether the observed increases in the frequency of spontaneous MP oscillations depends on sensory transmission at the sciatic nerve which had been subjected to CCI surgery (CCI nerve). For this purpose, we first measured spontaneous MP oscillations of ACC neurons in CCI rats (on day 7–14 after surgery) for 5–6 minutes using the same method in the above experiments, and then locally applied lidocaine (0.1 mL, 1%; see Methods) on the CCI nerve for the following measurement (Fig. 5A). In the same group of cells (n = 15 neurons), no significant difference was detected in oscillation frequencies measured before (3.0 ± 0.2 Hz) and within 10–16 minutes after lidocaine application (3.0 ± 0.1 Hz) ($P = 0.54$; paired t-test) (Fig. 5B and 5C).

To verify the blocking effect of the lidocaine treatment on sensory transmission at the sciatic nerves, we measured MP responses of ACC neurons to a DC electric stimulus (2 mA, 2s; see Methods) in CCI rats (on days 7–14), which was applied at the hindpaw ipsilateral to the CCI nerve. In the absence of lidocaine treatments, the electric stimulus evoked excitatory responses in all ACC neurons we recorded (n = 8 neurons from 5 rats). Unlike the lack of changes in spontaneous MP oscillations, 10 min. after the same lidocaine applica-

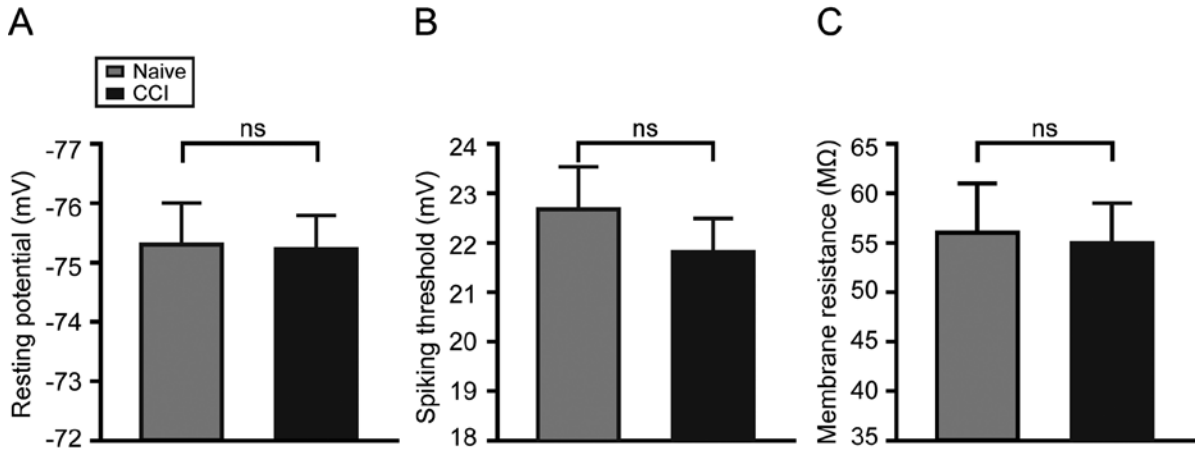

Fig. 4. *No significant difference in the resting potential (A), spiking threshold (from resting potentials) (B), and membrane resistance (C) between the cells recorded in naive and CCI rats. n.s., not significant.*

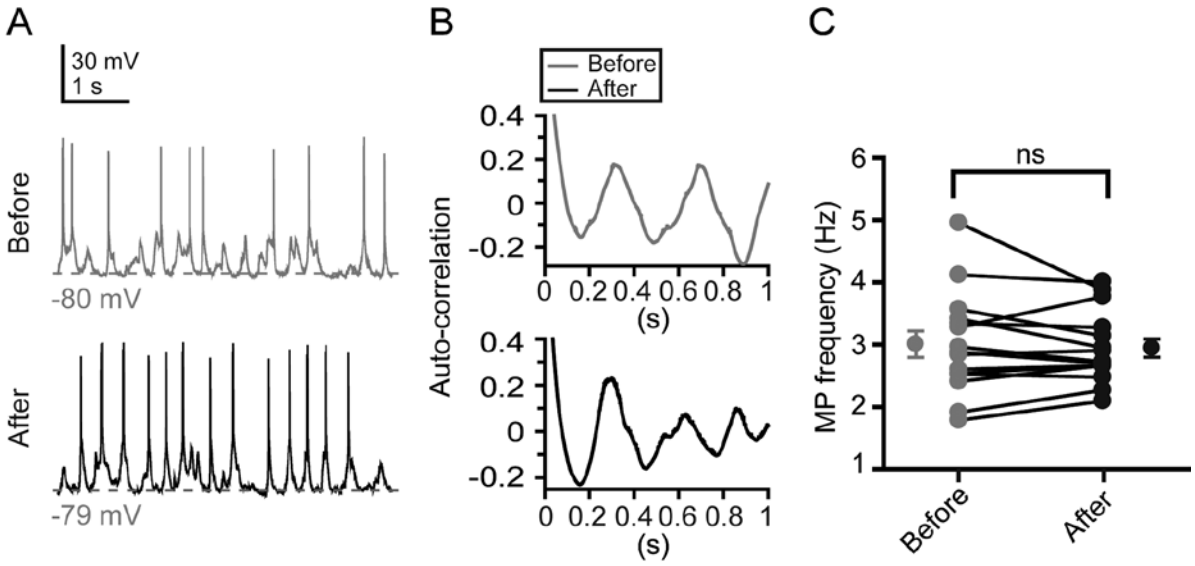

Fig. 5. *Independence of CCI surgery-induced increase in MP oscillation frequencies on periphery transmission at the sciatic nerve. (A and B) For an example, spontaneous activities (A) and its auto-correlograms (B) before and after lidocaine application at the sciatic nerve. (C) Summary for the experiments shown in (A and B) (n = 15 neurons), no significant difference (P = 0.54; paired t-test) was detected in the oscillation frequency before and after lidocaine application.*

tion at the CCI nerve, a remarkable reduction in these responses was observed, as shown by the multi-trial averages and time course of responses measured from 2 example cells (Fig. 6A and 6B). In the 8 cells measured, the responses were reduced from 4.3 ± 0.6 mV to 1.4 ± 0.4 mV (~3-fold reduction; ***$P < 0.001$) after lidocaine application, and in 3 cells no responses could be detected in the presence of lidocaine (Fig. 6C and 6D; data points in Fig. 6D at 0 mV indicate no responses). These findings revealed the blocking effect of the lidocaine treatment on sensory transmission at CCI nerves. Thus, the observed changes in spontaneous ACC oscillations induced by the CCI operation resulted from the change in associated regions in the central nervous system and were not caused by sensory transmission at the sciatic nerve subjected to CCI surgery.

Increase in the Frequency of Spontaneous AP Firing after CCI Surgery

In addition to MP oscillations, almost all ACC neurons in naive and in CCI rats exhibited spontaneous AP firing. Furthermore, in both naive and CCI rats, the rate of AP firing was positively correlated with the mean MP level (Fig. 7A), although no clear correlation was found between the rates of AP firing and MP oscillations (Fig. 7B). Due to the changes of MP events found in CCI rats (Fig. 3), these results suggested that CCI surgery could increase the rate of spontaneous ACC firing in addition to MP oscillations. Indeed, in our analysis, the rate of spontaneous firing was found to be increased from 1.4 ± 0.3 Hz (measured in naive rats) to 3.0 ± 0.4 Hz after CCI surgery ($P = 0.003$) (Fig. 7C). The increased firing rate observed in ACC neurons in the absence of sensory stimuli is reminiscent of the symptom of spontaneous pain perceived without external stimulation (29).

A Similar Anaesthesia Level for Recordings in Naive and CCI Rats

In the above electrophysiological recordings, the anaesthesia level was maintained as light as possible (see Methods) for both naive and CCI rats for comparing the neuronal activities between groups. To further exclude the possibility whether the observed difference in ACC activity between naive and CCI rats resulted from the difference in anaesthesia levels, we monitored the respiratory (see Fig. 8A) and heart (see Fig. 8B) rates in some of the above experiments to evaluate the anaesthesia depth (see Methods). In naive and CCI rats, similar respiratory rates (96 ± 2.7 per minute in naive (n = 13) and 95 ± 2.8 per minute in CCI (n = 13) rats; $P =$

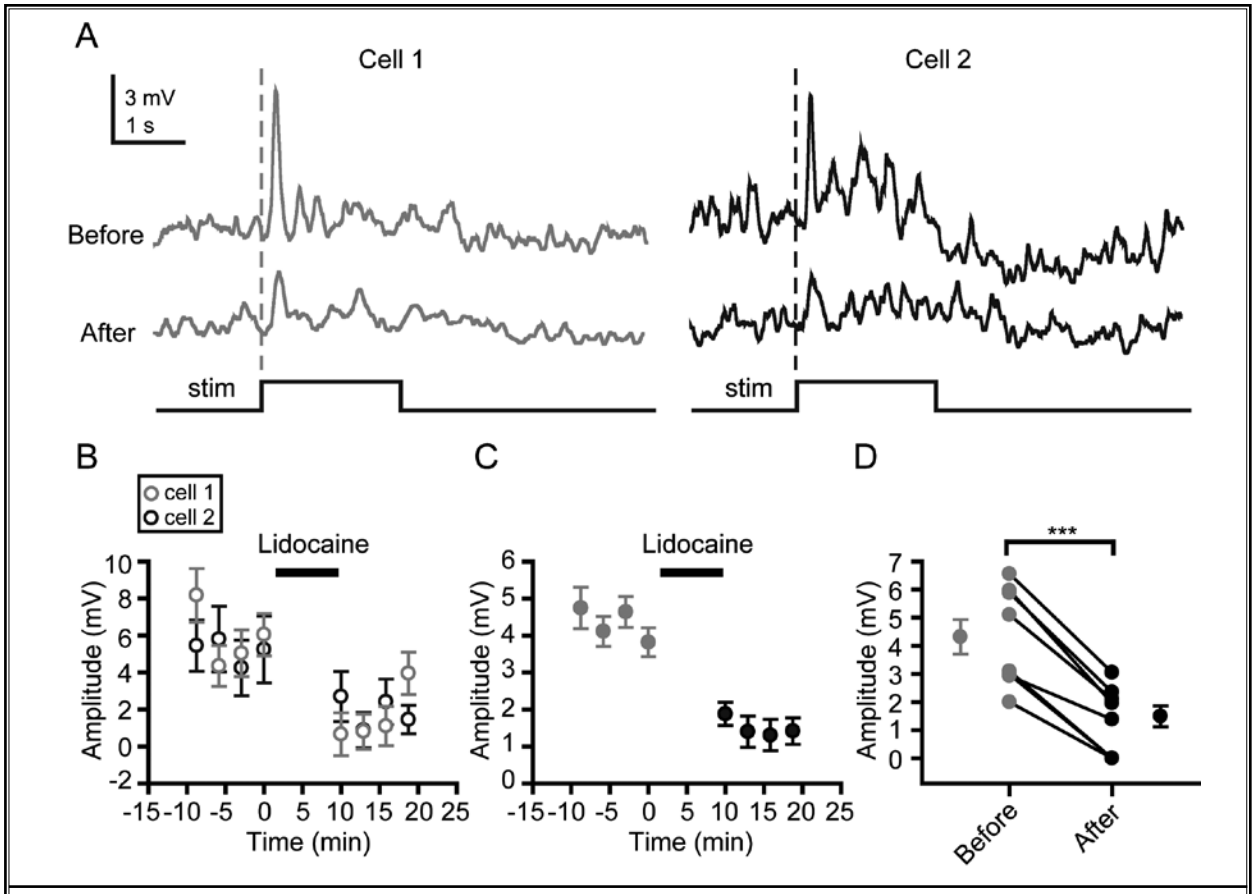

Fig. 6. *Blocking effect of lidocaine application on electric stimulus-evoked ACC responses. (A) For 2 example cells, multi-trial (100) averages for MP responses to an electric stimulus applied at the ipsilateral hindpaw, which were measured before and 10 minutes after lidocaine application at the sciatic nerve. (B) Time course of responses before and after lidocaine treatments for the 2 cells shown in (A). (C) Same as in (B), for all cells (n = 8 neurons). (D) For the cells shown in (C), amplitudes of responses before and after (10 min.) lidocaine application; data points at 0 indicate no responses. ***P < 0.001 (paired t-test).*

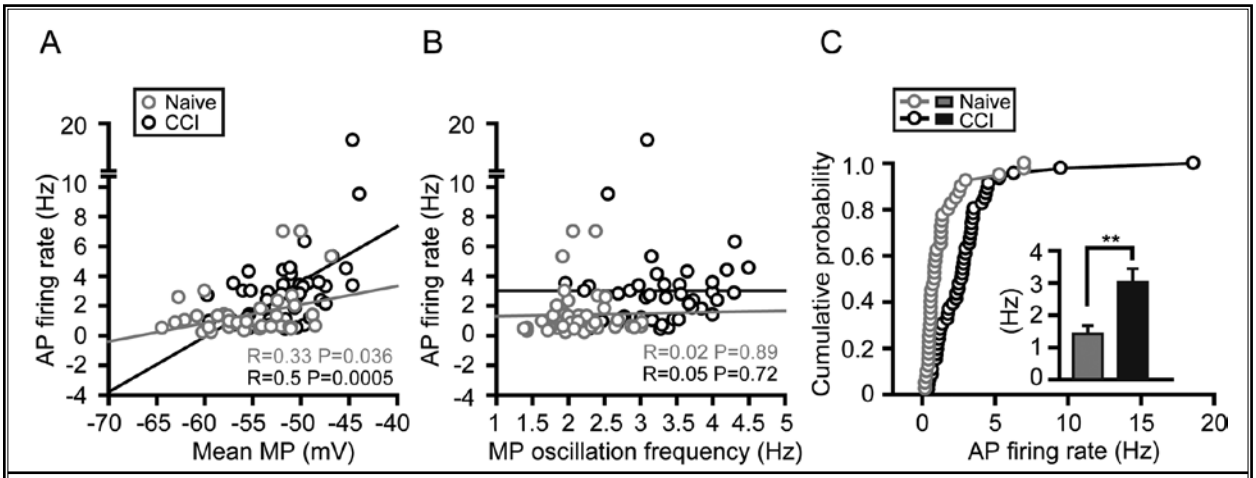

Fig. 7. *Changes in spontaneous firing rates after CCI surgery. (A and B) For cells recorded in naive and CCI rats, spontaneous firing rates plotted as the functions of mean MP levels (A) and MP oscillation frequencies (B). (C) Cumulative distributions of spontaneous firing rates for cells recorded in naive (n = 40 neurons) and CCI (n = 46 neurons) rats. Each circle represents one cell. Inset: mean ± SEM. **P = 0.003.*

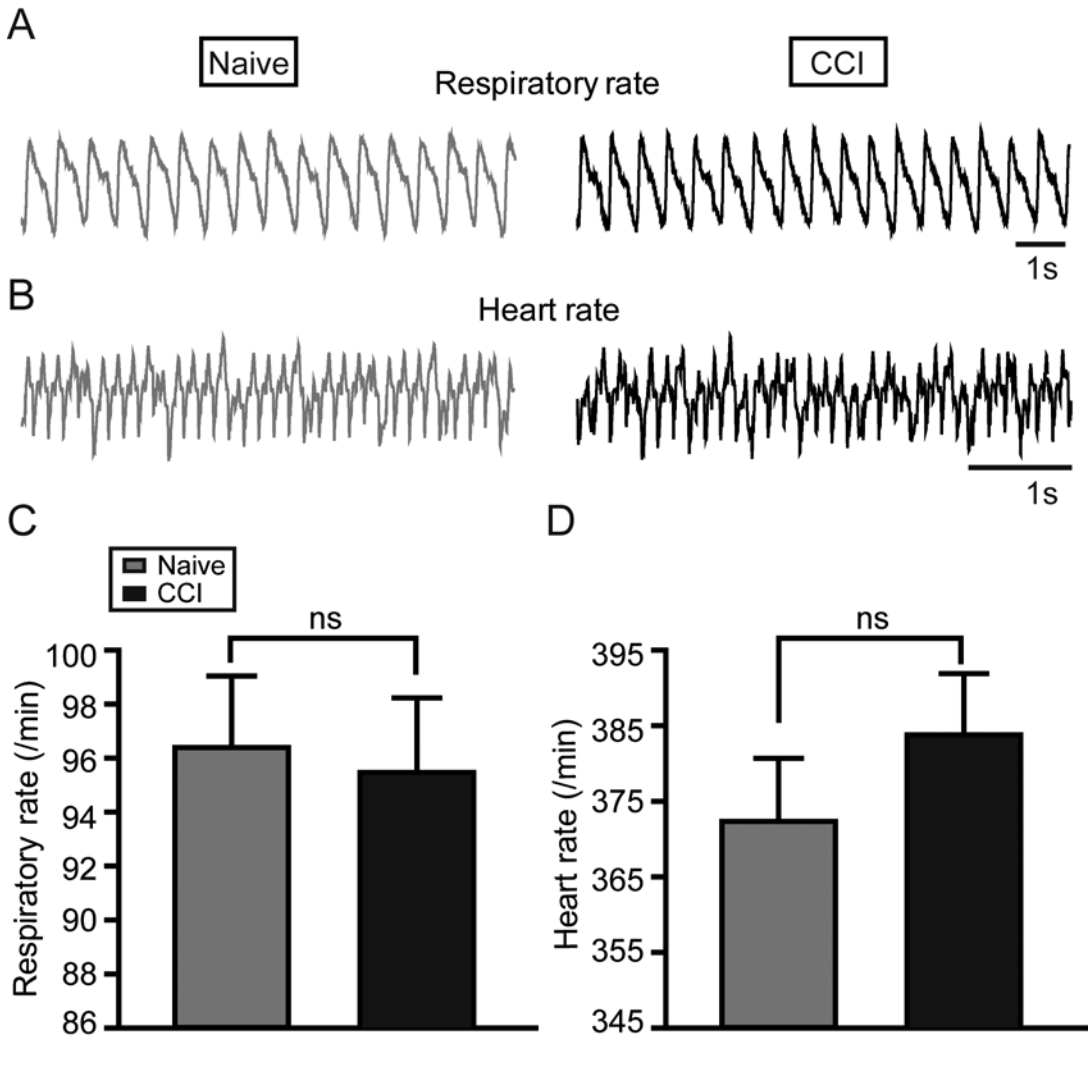

Fig. 8. *Similar anaesthesia levels for recordings in naive and CCI rats. (A and B) Example recordings of respiratory (A) and heart (B) rates from naive and CCI rats. (C and D) Summary of all experiments shown in (A and B), no difference was detected in respiratory (C) (P = 0.8) and heart (D) (P = 0.34) rates between naive (n = 13) and CCI (n = 13) rats.*

0.8) and heart rates (372 ± 8.4 per minute in naive and 384 ± 8.1 per minute in CCI rats; $P = 0.34$) were observed (Fig. 8C and 8D), indicating a similar level of anaesthesia for these 2 groups of recordings. Thus, the higher frequency of spontaneous ACC oscillations after CCI surgery was not due to the difference in anaesthesia levels between groups. These findings further indicate that the change in spontaneous neuronal activity accompanying neuropathic pain could be retained in the ACC under anaesthesia.

Discussion

Using in vivo whole-cell recording in anaesthetized rats, we have shown a CCI surgery-induced increase in the frequency of spontaneous MP oscillations entrained in ACC neurons, accompanying the symptom of neuropathic pain in awake behaving rats. This change was independent of periphery sensory transmission at the sciatic nerve. We also observed a CCI surgery-induced increase in the overall level of MP excitation and the increase in spontaneous firing rates of ACC neurons.

Moreover, CCI surgery did not induce detectable changes in the intrinsic membrane properties of these cells.

Possible Mechanisms Underlying CCI Surgery-induced Changes in Spontaneous ACC Oscillations

Spinal nociceptive inputs project to the ACC via pain-related regions in the thalamus (midline and intralaminar thalamic nuclei) and somatosensory cortex (10). In the absence of sensory stimulation, all these regions have been shown to exhibit neuronal activity occurring spontaneously (30,31). Therefore, changes in spontaneous activity possibly induced by CCI surgery in any of these projection regions, as well as intrinsic changes in ACC, likely account for the observed increases in the rate of spontaneous ACC oscillations.

Previous in vitro studies have shown that nerve injury can induce synaptic modifications, including an increase in the presynaptic vesicle release probability and recruitment of postsynaptic AMPA subtype of glutamate receptors, at the input to ACC neurons (32), a process similar to that underlying learning-induced synaptic strengthening (33) in other brain regions. Recently, it was reported in a study using a rat model of lumbar radicular pain (29) that spontaneous synaptic activity in the spinal cord can be changed by nerve injury, which was demonstrated by using a similar method of in vivo whole-cell recording. Both of the changes found in the ACC and the spinal cord may account for, at least partially, the increase in the rate of spontaneous MP oscillations in the ACC region.

Possible Roles of the Changes in Spontaneous ACC Oscillations in Neuropathic Pain

Neuronal oscillations play important roles in various forms of brain function, including sensory perception, cognition, and learning and memory (34,35). For instance, in the visual cortex, oscillations have been shown to be critical for feature combination when perceiving a visual object (20), and in the hippocampus oscillations have been shown to be critical for memory consolidation (19). However, the involvement of ACC oscillations in neuropathic pain has not been well understood.

In the present study, we showed the increase in MP oscillation frequency in anaesthetized rats, along with the symptom of neuropathic pain induced by CCI surgery. These changes may account for pain hypersensitivity by allowing weak synaptic transmission in association with a non-noxious stimulus to discharge a large population of ACC neurons, leading to ACC responses similar to those elicited by the noxious stimulus in normal subjects. Furthermore, the increased frequency of oscillations entrained spontaneously would be expected to contribute to the development of chronic pain, by inducing synaptic strengthening at ACC synapses, in a manner similar to that involved in memory consolidation (36). In addition, because the ACC is a key cortical area involved in affective-motivational pain perception (37), the increase in spontaneous AP firing rates in ACC neurons could be responsible for the perception of spontaneous pain in neuropathic pain subjects.

Possible Roles of the Changes in Spontaneous ACC Oscillations in Other Forms of ACC Function

As a multimodal brain area, the ACC is involved in a variety of brain functions in addition to pain perception, including learning and memory, emotion, and attention (38,39). The change in spontaneous ACC oscillations, as found in the present study, may cause disorders of all these ACC functions. Indeed, clinical studies have shown that neuropathic pain patients exhibit cognitive deficits involving weakened memory and attention, as well as mood disorders such as depression and anxiety (40). For instance, about two-thirds of chronic pain patients have been found to show disruption in working memory and the consequent disorder in intellectual ability (41). Thus, rescuing the change in ACC oscillations in neuropathic pain subjects would be critical for the therapy of not only the disease of pain but also the disruption in other brain functions.

Limitations of the Present Study

The change of spontaneous MP events in ACC neurons in association with neuropathic pain was investigated in the present study by using whole-cell recording under anaesthesia, during which the awareness of pain should be largely abolished by the anaesthetic. Although we have found the difference in ACC oscillations between naive and CCI rats under anaesthesia, more aspects and/or different extents of neuropathic pain-associated changes in these MP events could be observed in awake ACC. A new method of whole-cell recording in awake animals remains to be developed for this concern.

Conclusion

In summary, we have shown an increase in spontaneous MP oscillation frequencies as well as the

consequent increase in overall levels of MP excitation and spontaneous AP firing rates in ACC neurons in neuropathic pain animals. These results provide a new mechanism for neuropathic pain by suggesting that neuronal oscillations in the ACC are involved in this disease, a process probably similar to that has been extensively studied in other forms of brain function. Further studies on the contribution of ACC oscillations to neuropathic pain may shed light on our understanding of the neural substrates of neuropathic pain as well as our search for clinical treatments for this disease.

Funding/Support

This work was supported by grants from the National Natural Science Foundation of China (No.81171169 and 91132711) and Shanghai New 100-Talent Program Grant (No.XBR2011023).

Role of the Sponsor

The sponsors had no role in the design and conduct of the study; in the collection, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript.

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