Basic Science

# Rat Model of Trigeminal Neuralgia Using Cobra Venom Mimics the Electron Microscopy, Behavioral, and Anticonvulsant Drug Responses Seen in Patients

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**Background:** A new animal model of trigeminal neuralgia produced by injecting cobra venom into the infraorbital nerve (ION) trunk in rats had been developed. We tested and extended the model by observing the ultrastructural alterations of neurons and ameliorative effect of pregabalin in cobra venom-induced pain behaviors of rats.

**Objectives:** The goal of this study was to prove the feasibility of the cobra venom-induced model of trigeminal neuralgia and to demonstrate the demyelination change of ION and medulla oblongata is the major pathological change of trigeminal neuralgia.

**Study Design:** An experimental study.

**Setting:** Department of Anesthesiology, Pain Medicine, and Critical Care Medicine, Aviation General Hospital of China Medical University.

**Methods:** Experiments were carried out on male Sprague–Dawley rats. Video recordings were taken after the cobra venom injection and pregabalin administration. Ultrastructural alterations of ION and medulla oblongata were observed at the electron microscopic level. We also observed alteration in pain behaviors by analysis of video recordings.

**Results:** Compared to the preoperative and sham-operated group rats, experimental group rats exhibited significant changes in exploratory, resting, face-grooming, and head-shake behaviors on 3, 7, 14 days after operation ($P < 0.01$). The demyelination changes of ION and medulla oblongata were evident after administration of cobra venom to the ION. Compared to the pre-treated (no pregabalin administration) and control group rats, pregabalin group rats showed profound changes in exploratory, resting, face-grooming, and head-shake behaviors throughout the 14 day study period after treatment with drugs ($P < 0.01$).

**Limitations:** Ultrastructural alterations of ION and medulla oblongata were not examined after the treatment with pregabalin.

**Conclusions:** Video recordings of free behaviors and pregabalin application prove the feasibility of the rat model of trigeminal neuralgia. The results of electron micrographs are consistent with a previous study in humans showing that the demyelination change of axons is the major pathological change of trigeminal neuralgia. The central demyelination alterations may explain why the mechanical threshold was found to be decreased on the non-operated side of experimental animals.

**Key words:** Rat model, cobra venom, trigeminal neuralgia, free behavior, pregabalin, demyelination

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Nervous system damage or disease may result in chronic neuropathic pain such as found in trigeminal neuralgia. A variety of animal models have been trialed to elucidate the pathophysiological mechanisms of trigeminal neuralgia (1-4). However definitive models have yet to be demonstrated. Thus, the previous study reported a new animal model of trigeminal neuralgia induced by injecting cobra venom into the infraorbital nerve (ION) trunk of rats (5).

A robust animal model of neuropathic pain should show behavioral and drug responses consistent with that condition. In this study, we extended the cobra venom model by applying video analysis of unrestricted animal behavior and response to anticonvulsant drugs to see if the induced injury was similar to that reported in trigeminal neuralgia. The aim of this study is to test the new model of cobra venom-induced trigeminal neuralgia.

Fig. 1. *The experiment apparatus made for oral gavage.*

## Methods

### Animals

We had obtained Animal Care and Use Committee approval to perform this study (Beijing, China). The entire experimental procedure on conscious animals was consistent with the guidelines of the International Association for the Study of Pain (6). Adult male Sprague–Dawley rats (weighing 200 – 260 g) provided by the Laboratory Animal Center of the Academy of Military Medical Sciences were acclimatized to the laboratory environment for 14 days before initiating the experiment. They were allowed free access to water and food, and kept under a light/dark cycle (12 hours/12 hours) with an ambient temperature of 23°C – 25°C. Thirty rats were randomly divided into 2 groups: (1) cobra venom group (n = 20), and (2) sham-operated group (n = 10). In experimental rats, cobra venom solution was injected into the left sheath of the ION. Twenty rats were chosen in case of accidental deaths to make sure there were 8 rats in both the pregabalin group and control group. Then 19 rats were selected and randomly divided into 2 groups: 1) pregabalin test: n = 8; 2) control group: n = 11. The rats in sham-operated group (n = 10) received all the surgical procedures without cobra venom injection. Three rats were taken from the sham-operated group and cobra venom group for electron microscope examination on day 14 after surgery.

### Drug Preparation

The lyophilized cobra venom (Formosan cobra; Sigma, St. Louis, MO) and pregabalin were dissolved in 0.9% sterile saline, and each model rat was injected 4 uL saline containing 0.4 mg lyophilized whole venom using a 10 υL syringe. Pregabalin was configured to a solution of 5 mg/mL, and then was administered by oral gavage (30mg/kg) using the experiment apparatus made by ourselves (Fig. 1).

### Surgery Procedure

Surgical procedures were performed under pentobarbital sodium (40 mg/kg, ip) anesthesia. As previ-

ously described by An et al (5), the left ION trunk of rats in the experimental group was injected with cobra venom to produce trigeminal neuralgia, and several slivers were set around the injection site to avoid leakage of the cobra venom. In rats of the sham-operated group, the ION was exposed on left side using the same procedure but without cobra venom injection. The incision was closed using 5-0 absorbable sutures.

Three cobra venom-induced model rats and three sham-operated rats were deeply anesthetized with an overdose of pentobarbital sodium and perfused through the aorta with normal saline followed by the mixed solution of 4% paraformaldehyde and 2% glutaraldehyde (German) at postoperative day 14. The left ION and medulla oblongata were removed after one hour at 4°C, post fixed in 3% PFA 2 hours and rinsed with 0.1M phosphate buffer (PB) 3 times. After rinsing in PB, tissue blocks were post fixed in 1% osmium tetroxide for 2 hours and rinsed with 0.1M PB 3 times. Specimens were dehydrated in ethanol at increasing concentrations (50, 70, 80, and 90%), post embedded in araldite one day at 37 °C and 2 days at 60°C. The blocks were stained in uranile acetate for 30 minutes and rinsed with pure water 3 times, post fixed in lead nitrate for 20 minutes and rinsed with sodium hydroxide solution and pure water 3 times. Ultrathin sections (70 nm) of the left ION and medulla oblongata were cut and mounted on slot hole formvar coated grids (Pelco) and viewed in the JEOL JEM-2100 electron microscope.

### Drug Treatments

In drug treatments, 16 model rats were randomly assigned to one of 2 groups. Rats in drug group (n = 8) were administered 30 mg/kg pregabalin once a day orally at postoperative day 14, because free behaviors of model rats had showed significant differences compared to preoperative or sham-operated rats at that time. The rats in control group (n = 8) received no treatments. The drug was repeatedly delivered for 7 days.

### Behavioral Testing

The behavior of rats was tested in a 20×20×20 cm transparent plastic cage with a mirrored back after the rats were habituated to the test room for 15 minutes. A high-definition camera was placed 1 m in front of the cage with the rat's body covering at least one-fourth of the recorded view. The test room was illuminated with a 60W incandescent red bulb which was suspended 1 m above the center of cage with a 45 dB background noise, while the rest of the chamber remained dark. We conducted the testing 3 days before operation, and 3, 7, 14, 15, 16, 17, 18, 19, 20, and 30 days after the operation. Each rat was observed for 7 minute periods using an IBM-microcomputer which was placed outside of the room to avoid interference with their activity. Testing consisted of exploratory (walking, running, climbing, rearing, and sniffing), resting (head resting on flexed forepaws with eyes open/closed), head-shake, licking paws, body-grooming (paws, tongue, or incisors are brought in contact with a body area other than the face or the forepaws), and face-grooming (movement patterns in which paws contact facial areas) body behaviors which were identified as similar to the ones described by Vos et al (7). We recorded the number and duration of each behavior through playing videotapes frame by frame.

### Statistical Analysis

Results were expressed as mean ± SD. The differences of behavioral alterations before and after the operation and drug administration in one group, as well as between groups were analyzed by using Student t-test. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS 13.0.

## Results

### Cobra Venom Induced Behavioral Changes

Cobra venom was successfully injected into the ION in 90% of the experimental animals (n = 20). Compared to preoperative or sham-operated rats, the rats with the injection of cobra venom showed dramatic increases in face-grooming, head-shake, and resting activities, as well as a decrease in exploratory behavior from day 3 to 14 post-operation ($P < 0.05$) (Fig. 2). However the behaviors of licking paws and body-grooming observed after injecting cobra venom remained unchanged compared to preoperative or sham-operated rats ($P > 0.05$).

### Ultrastructural Changes of ION and Medulla Oblongata at Electron Microscopic Level

Fig. 3 illustrates typical demyelination changes of axons in the ION and medulla oblongata of rats after cobra venom injection. Fig. 4 showed the normal ultrastructure of axons in the ION and medulla oblongata of sham-operated group rats. The electron micrographs revealed that the myelins were complete and the density of axons was homogeneous.

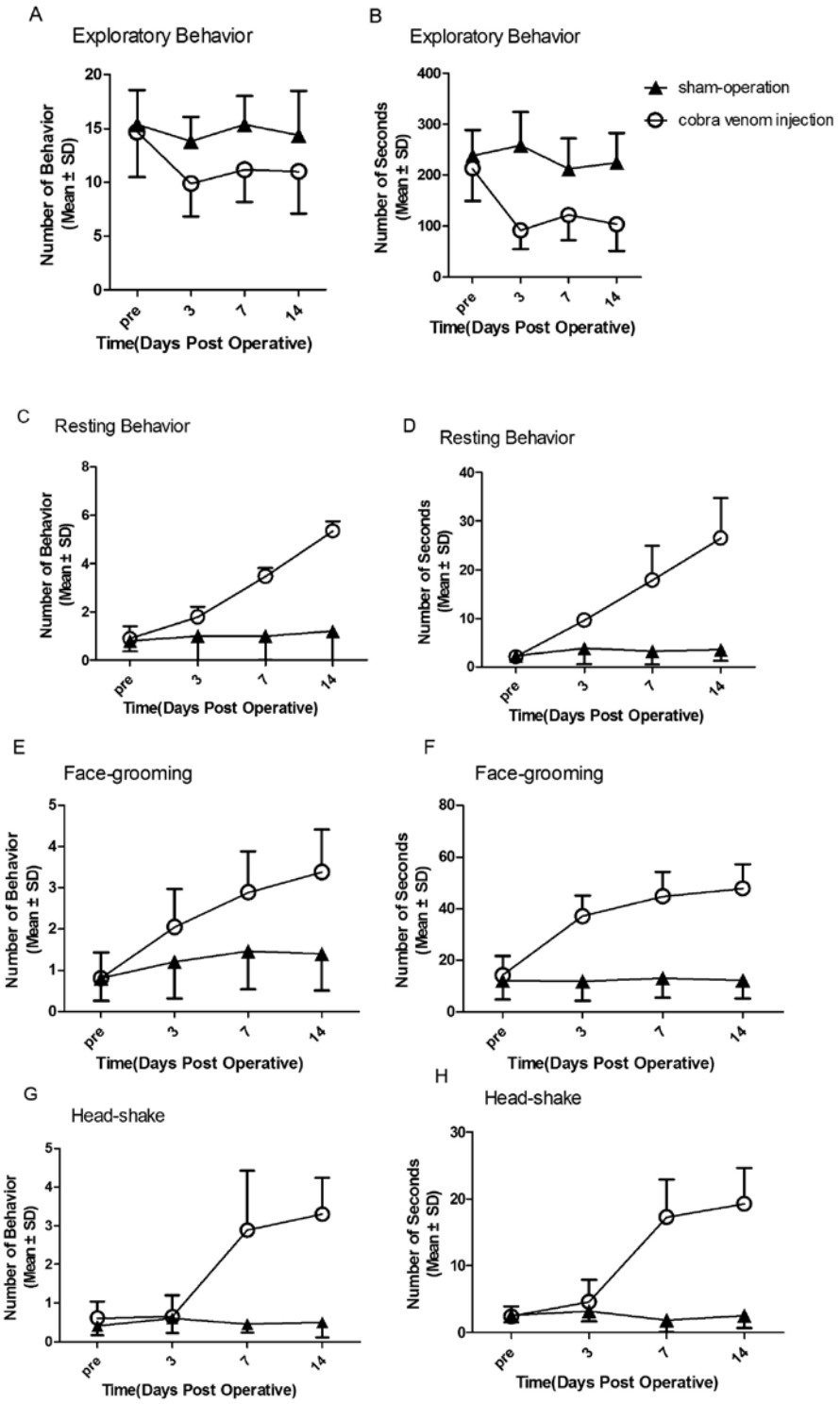


Fig. 2. *(1,2). Time course of changes in behavioral activity. A-H: The behavioral activity of cobra venom injection group rats was different from preoperative or sham-operated group levels at 3 postoperative time points ($P < 0.01$). Behavioral changes of sham-operated rats were never different from preoperative levels ($P > 0.05$). No significant differences between groups were found before operation for each of the behavioral categories ($P > 0.05$). Error bars indicate the standard deviation (SD). Date are presented as mean ± SD.*

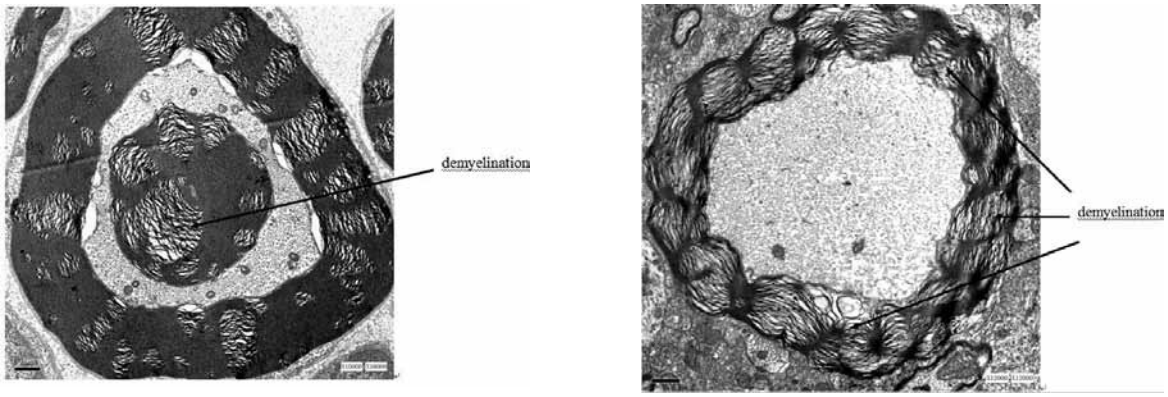


Fig. 3. *(1) The axon of ION in cobra venom injection rat. Electron micrograph showed severe demyelination of axon, a severe breakdown of myelin, and the "formation" of pith ball. (2) The axon of medulla oblongata in cobra venom injection rat. Electron micrograph showed severe demyelination and a mildly inhomogeneous density of axon.*

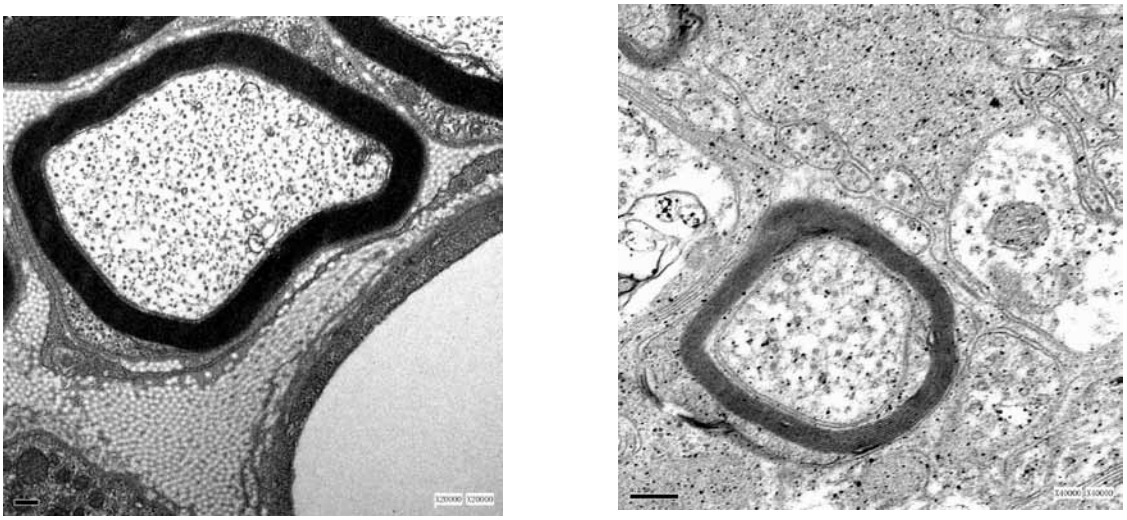

Fig. 4. *(1) The normal axon of ION in sham-operated group rat. (2) The normal axon of medulla oblongata in sham-operated group rat. Electron micrographs (A-B) showed the myelins were entire and the density of axon was homogeneous.*

### Pregabalin Effects on Trigeminal Neuralgia Produced by Cobra Venom

According to video recordings of free behaviors, the rats treated with pregabalin showed decreased face-grooming and head-shake behaviors, decreased resting behavior, and increased exploratory behavior after one, 2, and 3 days of dosing. Those behaviors remained significantly different from that in the pre-treated or control group rats ($P < 0.05$) (Fig. 5). Compared to pre-treated or control group rats, the behaviors of licking paws and body-grooming observed after pregabalin administration remained unchanged ($P > 0.05$).

## Discussion

### Cobra Venom-induced Behavioral Changes

After the injection of cobra venom into the ION trunk, rats revealed quantitative changes in free behavioral activity. Behavioral changes developed in 2 main stages. In an early postoperative period (1 – 3 days postoperative), changes in free behavior were maximal: Rats showed a dramatic decrease in exploratory behavior and spent more observation time in resting behavior, they groomed their faces, especially the territory of the injured nerve very frequently and

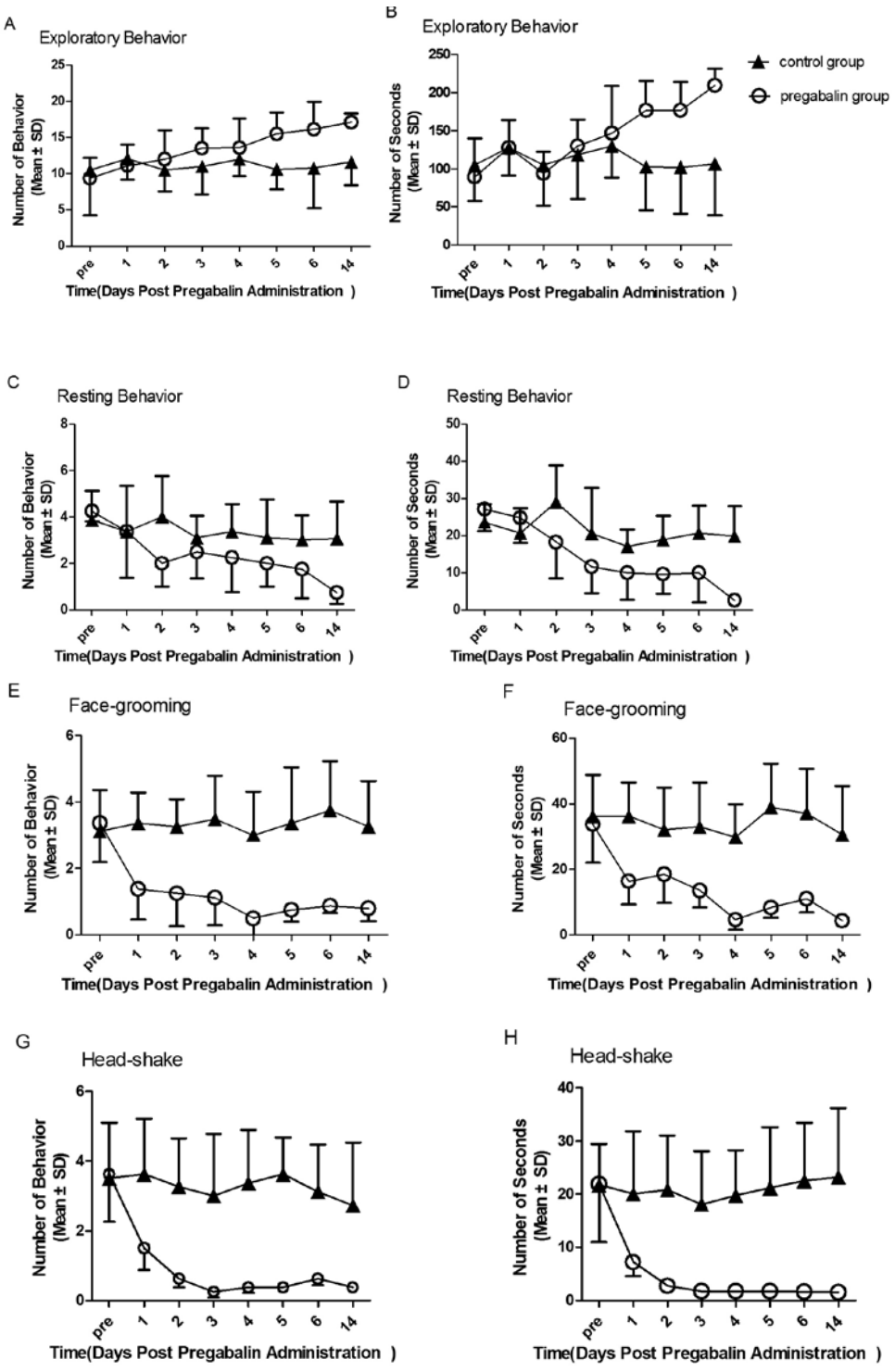


Fig. 5. *(1,2). Time course of the change in behavioral activity in control group rats (n = 8) or pregabalin group rats (n = 8). The changes of pregabalin group rats were decreased face-grooming and head-shake behaviors (E-H), decreased resting behavior (C-D), and increased exploratory behavior (A-B) after one, 2, 3 days of dosing respectively, and they were different from pre-treated or control group levels (P < 0.01). No significant differences between groups were found before pregabalin administrated for each of the behavioral categories (P > 0.05). Error bars indicate the standard deviation (SD). Data are presented as mean ± SD.*

for long periods. In the late postoperative period (7 – 14 days postoperative), the most obvious change was a profound increase in head-shake behavior. The changes of face-grooming, exploratory, and resting behaviors were mildly reduced, however they remained significantly different from sham-operated group rats. As reported previously (8-10), the major behavioral performance of trigeminal neuralgia was face-grooming behavior. Furthermore, the behavioral changes investigated in our study were consistent with that in CCI-induced animal models of trigeminal neuralgia (7). According to the results observed in our study, we confirm that the model of trigeminal neuropathic pain produced by administration of cobra venom to the ION in the rat is reasonable.

### Ultrastructure of Axons

The axons of the trigeminal ganglion and nerve root in patients with trigeminal neuralgia exhibited severe demyelination and myelin proliferation (11,12). As reported (7,13), the electron and light micrograph of the ligated ION in rats showed a similar picture of axonal pathology as found after CCI to the sciatic nerve. It had not been reported that there was ultrastructural changes of the medulla oblongata both in patients and rats. In this study, we found that there were demyelination changes of the ION and medulla oblongata, which was consistent with that in previous studies on humans. Idanpaan-Heikkila and Guilbaud (14) pointed that the mechanical thresholds were found to be decreased also on the contralateral (non-operated) side of experimental animals, but there was no specific explanation for this phenomenon. However the demyelination of the medulla oblongata in our study might support the hypothesis that central processes participate in this phenomenon. In addition, our findings also provided further insights into the central mechanism of trigeminal neuralgia.

### Pregabalin can Attenuate the Free Behaviors of Rats with Cobra Venom-Induced Trigeminal Neuropathic Pain

Pregabalin was generally considered to be one of the most effective anticonvulsant drugs for treating neuropathic pain (15-22). In this study nociceptive responses were reduced in our model of neuropathic pain at a varying doses of 3 to 30 mg/kg orally (23). Additionally, the rats showed marked behavioral changes including decreased face-grooming and head-shake, decreased resting posture, and increased exploratory behavior after the administration of pregabalin (30 mg/kg orally). These free behaviors remained significantly different from those in pre-treated or control group rats. The ameliorative effects of pregabalin seen in our results suggests that this rat model of trigeminal neuropathic pain correlates with clinical findings.

## Conclusion

In conclusion, the study confirmed our previous work that the administration of cobra venom can reliably mimic trigeminal neuralgia in the rats. Video recordings of free behaviors and improvement after pregabalin application by oral gavage further verified the usefulness of this model. The axons of the ION and medulla oblongata after the administration of cobra venom to the ION of rats showed significant demyelination changes which were consistent with that in previous studies on humans. The central demyelination alterations of the medulla oblongata might explain the reason for the decrease of the mechanical threshold on the contralateral side of experimental animals. To our knowledge, this is the first observation reported of the structural pathology in the medulla oblongata after inducing a model of neuropathic pain.

### Limitations

Although the present result verifies the feasibility of the cobra venom-induced model of trigeminal neuralgia, we do not know whether the demyelination change in axons after the administration of pregabalin would change electromicroscopic findings or limit pathology if administered at any time frame during our study consistent with the behavioral changes we observed. Ultrastructural alterations of the ION and medulla oblongata after treatment with pregabalin could be examined in future studies or even prior to damage to limit pathology.

### Contributions

Xiao Yan Qian and Qian Qian Zhao contributed equally to this work.

Qian Qian Zhao helped design and conduct the study, collect and analyze the data, and prepare the manuscript. Xiao Yan Qian helped design and conduct the study. Jian Xiong An helped design the study, analyze the data, and prepare the manuscript. Cai Cai Liu helped conduct the study and prepare the manuscript. Qi Wu Fang and Yong Wang helped conduct the study and analyze the data. Yi De Jiang helped conduct the study and collect the data. Doris K Cope and John P.

Williams approved the final manuscript. We acknowledge the neurobiology and electron microscopy laboratory of Capital Medical University for their contribution on producing the electron microscope images.

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