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The Rat Chronic Post-Ischemia Pain Model for Complex Regional Pain Syndrome Type-I

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Corresponding Author:	Boyi Liu Zhejiang Chinese Medical University Hang zhou, CHINA
Corresponding Author's Institution:	Zhejiang Chinese Medical University
Corresponding Author E-Mail:	boyi.liu@foxmail.com
Order of Authors:	Qimiao Hu
	Xiaoli Zheng
	Ruixiang Chen
	Boyu Liu
	Yan Tai
	Xiaomei Shao
	Jianqiao Fang
	Boyi Liu
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TITLE:

Chronic Post-Ischemia Pain Model for Complex Regional Pain Syndrome Type-I in Rats

AUTHORS AND AFFILIATIONS:

Qimiao Hu[1*], Xiaoli Zheng[1*], Ruixiang Chen[1], Boyu Liu[1], Yan Tai[2], Xiaomei Shao[1], Jianqiao Fang[1], Boyi Liu[1]

[1]Department of Neurobiology and Acupuncture Research, The Third Clinical Medical College, Zhejiang Chinese Medical University, Key Laboratory of Acupuncture and Neurology of Zhejiang Province, Hangzhou, China

[2]Academy of Chinese Medical Sciences, Zhejiang Chinese Medical University, Hangzhou, China

[*]These authors contributed equally to this work.

Corresponding Authors:

Jianqiao Fang (fangjianqiao7532@163.com)
Boyi Liu (boyi.liu@foxmail.com)

Email Addresses of Co-authors:

Qimiao Hu (huqimiao315@126.com)
Xiaoli Zheng (861260876@qq.com)
Ruixiang Chen (837324951@qq.com)
Boyu Liu (lby-0731@163.com)
Yan Tai (adampha@hotmail.co.uk)
Xiaomei Shao (751257894@qq.com)

KEYWORDS:

pain, complex regional pain syndrome type-I, allodynia, hyperalgesia, transient receptor potential vanilloid 1, chronic post-ischemia pain

SUMMARY:

Provided here is a protocol that details steps to establish an animal model of chronic post-ischemia pain (CPIP). This is a well-recognized model mimicking human complex regional pain syndrome type-I. Mechanical and thermal hypersensitivities are further evaluated, as well as capsaicin-induced nocifensive behaviors observed in the CPIP rat model.

ABSTRACT:

Complex regional pain syndrome type-I (CRPS-I) is a neurological disease that causes severe pain among patients and remains an unresolved medical condition. However, the underlying mechanisms of CRPS-I have yet to be revealed. It is known that ischemia/reperfusion is one of the leading factors that causes CRPS-I. By means of prolonged ischemia and reperfusion of the hind limb, the rat chronic post-ischemia pain (CPIP) model has been established to mimic CRPS-I. The CPIP model has become a well-recognized animal model for studying the mechanisms of CRPS-I. This protocol describes the detailed procedures involved in the establishment of the rat

model of CPIP, including anesthesia, followed by ischemia/reperfusion of the hind limb. Characteristics of the rat CPIP model are further evaluated by measuring the mechanical and thermal hypersensitivities of the hind limb as well as the nocifensive responses to acute capsaicin injection. The rat CPIP model exhibits several CRPS-I-like manifestations, including hind limb edema and hyperemia in the early stage after establishment, persistent thermal and mechanical hypersensitivities, and increased nocifensive responses to acute capsaicin injection. These characteristics render it a suitable animal model for further investigation of the mechanisms involved in CRPS-I.

INTRODUCTION:

Complex regional pain syndrome (CRPS) reprents complex and chronic pain symptoms resulting from fractures, trauma, surgery, ischemia or nerve injury[1-3]. CRPS is classified into 2 subcategories: CRPS type-I and type-II (CRPS-I and CRPS-II)[4]. Epidemiological studies revealed that the prevalence of CRPS was approximately 1:2000[5]. CRPS-I, which shows no obvious nerve damage, can result in chronic pain and dramatically affects the life quality of the patients. Current available treatments show inadequate therapeutic effects. Therefore, CRPS-I still remains an important and challenging clinical problem that needs to be addressed.

Establishing a preclinical animal model mimicking CRPS-I is crucial for exploring the mechanisms underlying CRPS-I. In order to address this issue, Coderre et al. designed a rat model by applying prolonged ischemia and reperfusion to the hind limb to recapitulate CRPS-I[6]. It is known that ischemia/reperfusion injury is among one of the major causes of CRPS-I[7]. The rat CPIP model exhibits many CRPS-I-like symptoms, which include hind limb edema and hyperemia in the early stage after model establishment, followed with persistent thermal and mechanical hypersensitivities[6]. With the aid from this model, it is proposed that central pain sensitization, peripheral TRPA1 channel activation and reactive oxygen species generation, etc. contribute to CRPS-I[8-10]. We recently successfully established the CPIP rat model and performed RNA-sequencing of the dorsal root ganglia (DRGs) that innervate the affected hind paw[11]. We discovered some potential mechanisms that are possibly involved in mediating the pain hypersensitivities of CRPS-I[11]. We further identified transient receptor potential vanilloid 1 (TRPV1) channel in DRG neurons as an important contributor to the mechanical and thermal hypersensitivities of CRPS-I[12].

In this study, we described the detailed procedures involved in the establishment of the rat model of CPIP. We further evaluated the rat CPIP model by measuring the mechanical and thermal hypersensitivities as well as its responsiveness to acute capsaicin challenge. We propose that the rat CPIP model can be a reliable animal model for further investigation of the mechanisms involved in CRPS-I.

PROTOCOL:

The animal protocols were approved by Zhejiang Chinese Medical University Animal Ethics Committee.

1. Animals

1.1. Obtain male Sprague-Dawley (SD) rats (280–320 g, 8-10 weeks of age) from Shanghai Laboratory Animal Center. Breed the animals in Zhejiang Chinese Medical University Laboratory Animal Center. Note that the breeding conditions should include 12 h/ 2h light/dark cycles and keep temperature constant at 24 °C. Provide water and food *ad libitum*. Note that a total of 48 rats areused in this study.

2. CPIP model establishment

2.1. Anesthetize all rats (including sham and CPIP model groups) with sodium phenobarbital (50 mg/kg, intraperitoneal injection [i.p.]). Maintain anesthesia with up to 20 mg/kg/h phenobarbital, if necessary. Check the reflexes of each animal by pinching its hind paw or tail tip using forceps. Make sure that the rats are not responsive before model establishment. Place vet ointment on eyes to avoid dryness during the procedure. Place the anesthetized rats on a heated pad maintained at 37 °C for the following procedure.

2.2. Ischemia and reperfusion of the hind paw

2.2.1. Lubricate the right hind paw and ankle with glycerol once the rat is anesthetized.

2.2.2. Slide a Nitrile 70 Durometer O-ring with a 7/32” internal diameter into the larger side of a 1.5 mL Eppendorf tube (with the snap-cap cut off before use). Carefully insert the hind paw into the hollow Eppendorf tube until reaching the bottom.

2.2.3. Gradually slide the O-ring from the tube to the right hind limb near the ankle joint and place for 3 h. Apply the same treatment to a sham group of rats, except that a broken O-ring, which is cut off and should not induce ischemia, should be placed around the ankle.

2.2.4. Cut off the O-ring 3 h after the ischemia step. Carefully watch the rat until it recovers enough consciousness to maintain sternal recumbency. Note that the rat that received anesthesia should not be placed back to the company of other rats until it fully recovered.

3. Nocifensive behavioral tests

3.1. Place the rat in a transparent Plexiglas chamber that is sitting on a mesh floor. Habituate the rat for 0.5 h before any behavioral testing.

3.2. Mechanical allodynia

3.2.1. Use von Frey filaments (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, 15.0, and 26.0 g filaments) for the test. Begin the test from the middle filament (4.0 g). Vertically apply the filaments to the middle plantar surface of the hind paw. Slightly apply suitable force to bend the filament for up to 5 s. A sudden retraction of the hind paw in response to the stimuli is considered a nocifensive

behavior. Conduct the mechanical allodynia test on days -3, -2, -1, and every other day until day 13.

3.2.2. Apply the up-down testing method to test the threshold. Apply a nonparametric Dixon test for calculating 50% paw withdrawal threshold (PWT)[13-15].

3.3. Thermal hyperalgesia

3.3.1. Use Hargreaves' method to examine thermal hyperalgesia. Directly aim the light beam emitted from a bulb (50 W) to the hind paw to measure the paw withdrawal latency (PWL). Set 20 s as the cut-off threshold to avoid excessive injury from the heating.

3.3.2. Repeat each test 3x in 5 min intervals for each hind paw. Take the average of these three tests as the PWL of each rat[16]. Conduct the thermal hyperalgesia test on days -3, -2, -1, and every other day until day 13.

3.4. Capsaicin-induced acute nocifensive behavior

3.4.1. Prepare capsaicin stock solution (200 mM) using dimethyl sulfoxide (DMSO) and further dilute to 1:1000 in phosphate-buffered saline (PBS) for hind paw injection. The final DMSO concentration in PBS is 0.1% (vehicle contains 0.1% DMSO in PBS only). Inject capsaicin or vehicle into the hind paw (intraplantar injection) at a volume of 50 µL using a 30 G needle attached to 1 mL syringe.

3.4.2. Record the nocifensive behavior (i.e., licking, biting, or flinching of the injected paw) using a video camera for 10 min right after the injection and quantified thereafter as previously described[17-19].

3.5. Hind paw edema evaluation: Evaluate the hind paw edema by measuring the increase in paw diameter. Measure with a digital caliper and calculate the difference between the basal value and the test value observed at different time points. Assess the changes in paw thickness at 15 min, 24 h, 48 h, and 72 h after model establishment.

REPRESENTATIVE RESULTS:

After placing the O-ring on the ankle, the ipsilateral hind paw skin showed cyanosis, an indication of tissue hypoxia (**Figure 1A**). After cutting the O-ring, the ipsilateral hind paw began to fill with blood and showed robust swelling, which demonstrated an intense sign of hyperemia (**Figure 1A**). The paw swelling gradually diminished and returned to normal 48 h after the ischemic/reperfusion procedure (two-way ANOVA with Sidak post-hoc test, **Figure 1B**). All of these signs are consistent with previous studies[6,12].

Then, mechanical allodynia was measured using a von Frey hair test. The ipsilateral hind paw of the CPIP group exhibited obvious mechanical allodynia 1 day after model establishment compared to the sham group. The mechanical allodynia of the ipsilateral hind paw persisted

until 13 days of the observation timeframe (two-way ANOVA with Sidak post-hoc test, **Figure 1C**). The contralateral hind paw of the CPIP group also displayed mechanical hyperalgesia similar to the ipsilateral hind paw, lasting for 13 days (two-way ANOVA with Sidak post-hoc test, **Figure 1D**).

Thermal hyperalgesia was then measured using a Hargreaves' test. Bilateral hind paws of CPIP rats exhibited significantly reduced withdrawal latency in response to noxious thermal stimuli, a sign of thermal hyperalgesia, compared to the sham group rats (two-way ANOVA with Sidak post hoc test, **Figure 1E,F**). This observation is consistent with previous studies[11,12]. The thermal hyperalgesia of the ipsilateral hind paw persisted until the end of the observation timeframe, whereas the thermal hyperalgesia of the contralateral hind paw lasted for 7 days (two-way ANOVA with Sidak post-hoc test, **Figure 1E,F**). The above results suggest that CPIP rats develop robust and persistent mechanical and thermal hypersensitivities, consistent with previous observations[11,12].

CRPS patients exhibited an obvious increased response to capsaicin-induced pain in affected areas[20]. It was then examined whether the CPIP rat model can recapitulate this phenomenon. Nocifensive behavior in CPIP rats was observed in response to intraplantar capsaicin (a TRPV1 agonist) injection into the ipsilateral hind paw. First, the nocifensive responses of the rats when a vehicle was injected were tested. The sham group showed a slight nocifensive response to vehicle injection, whereas the CPIP group showed a significantly higher response compared to the sham group (one-way ANOVA with Sidak post-hoc test, **Figure 2**). Furthermore, capsaicin injection resulted in robust nocifensive response in the sham group (**Figure 2**). More importantly, CPIP rats showed significantly higher responses to capsaicin injection than the sham group (**Figure 2**). These data suggest that CPIP rats exhibited enhanced nocifensive responses to capsaicin, a phenomenon mimicking human patients with CRPS-I.

FIGURE AND TABLE LEGENDS:

Figure 1: The CPIP rat model showed thermal and mechanical pain hypersensitivities in bilateral hind limbs. (**A**) Typical images taken during different timepoints (during ischemia, 10 min after reperfusion, and 7 days later). (**B**) Ipsilateral hind paw thickness measurements of both CPIP and sham groups. (**C,D**) 50% paw withdraw threshold (50% PWT, index of mechanical hyperalgesia) of ipsilateral (C) and contralateral (D) hind paws of rats. (**E,F**) Paw withdrawal latency (PWL, index of thermal hyperalgesia) of ipsilateral (E) and contralateral (F) hind paws of rats (n = 8 rats per group, **p < 0.01). Results are expressed as mean ± SEM (two-way ANOVA followed by Sidak post-hoc test).

Figure 2: CPIP rats exhibited more nocifensive behaviors compared to sham rats in response to intraplantar capsaicin injection into ipsilateral hind paws. The cumulated time the rats spent licking, biting, or flinching the hind paws was calculated over 10 min after vehicle (0.1% DMSO in PBS, intraplantar) or capsaicin (10 nmol in 50 µL volume, intraplantar) injection (n = 8 rats per group, **p < 0.01). Results are expressed as mean ± SEM (one-way ANOVA followed by Sidak post-hoc test).

DISCUSSION:

This protocol describes the detailed methods for establishing a rat CPIP model by applying ischemia/reperfusion to hind limbs of the rats. It involves the evaluation of hind limb appearance, edema, mechanical/thermal hypersensitivities, and acute nocifensive behaviors in response to capsaicin injection.

Limb ischemia/reperfusion is a common factor contributing to CRPS-I in human patients[12]. This protocol describes how to establish the rat CPIP model, which is a commonly used animal model to recapitulate human CRPS-I[6]. Ischemia was induced in the rat's hind limb with a tight O-ring applied to the ankle for 3 h under anesthesia. The O-ring was removed, and reperfusion occurred. After the model establishment, the CPIP model rat developed early hyperemia and edema in the hind limb. The model also displays neuropathic pain behaviors, including persistent mechanical/thermal hypersensitivities. These symptoms all mimic the typical characteristics of human CRPS-I [21].

CRPS patients exhibit obvious increased responses to capsaicin-induced pain in the affected limbs[20]. However, it remains unclear whether CPIP model animals exhibit similar responses. Thus, in this study, nocifensive behaviors of CPIP rats were examined in response to intraplantar capsaicin injection into the ipsilateral hind paw. It was found that CPIP model rats showed significantly higher nocifensive responses to capsaicin injection compared to sham rats. To our knowledge, this is the first report of this phenomenon in CPIP model rats.

In a previous study, TRPV1 channel expression was shown to be upregulated in ipsilateral hind paw tissues and DRG neurons that innervated the hind paw[12]. Furthermore, it has been found that spinal glial cells are activated in CPIP model rats, resulting in central pain sensitization and facilitation of pain perception and transmission[9,12]. Therefore, peripheral and central pain sensitization may both be involved in the increased nocifensive behaviors in response to capsaicin injection in CPIP model rats. Future studies are needed to test whether capsaicin injection results in enhanced responses in contralateral hind paws of CPIP model rats, as well. In the tests performed here, we did not observe autotomy in the CPIP model rats. This contrasts with rats that have undergone sciatic sectioning, who usually develop autotomy in addition to chronic pain hypersensitivities[22,23]. In addition, no referred pain response has been reported in CRPS-I patients or CPIP mode animals.

One of the critical steps in the model is to maintain anesthesia throughout the procedure. In this study, the anesthesia was initiated by intraperitoneal injection of sodium phenobarbital (50 mg/kg). This dosage is frequently used in many studies involving rat anesthesia, including studies with CPIP rat model establishment[9,12,24]. Besides, anesthesia should be maintained via 20 mg/kg/h (i.p.) sodium phenobarbital when necessary to guarantee successful implement of the procedure[12,24].

In the initial CPIP model established by Coderre et al., the O-ring was actually slid off from the outside of a 3 cm^3 syringe (cut in half) when the hind paw was placed into the barrel of the syringe as far as possible[25]. Here, in our study, we made minor modifications to this method. We

used a 1.5 mL Eppendorf tube (with the snap-cap cut off beforehand) instead of the syringe, since this material was more accessible and fit just well with the diameter of the rat's hind paw. The O-ring can be easily placed on the ankle part of the rats in this modified method without injuries to allow for model establishment.

At present, the CPIP rat model was exclusively established in male rats based upon previous studies, including our present study[25]. However, it should be noted that the incidence of CRPS-I is more frequent in female than in male patients according to epidemiological analysis[26,27]. This is further supported by the findings that female animals exhibited more pain responses than male animals when CPIP was established[28]. Therefore, it will be necessary to establish the CPIP model in both male and female animals in the future. This will be important for studying the translational significance of this model.

In conclusion, described here are methods for establishing a rat CPIP model. This CPIP rat model recapitulates many clinical characters of human CRPS-I, and its properties may render it a suitable animal model to study CRPS-I.

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DISCLOSURES:

The authors declare no conflicts of interest in this work.

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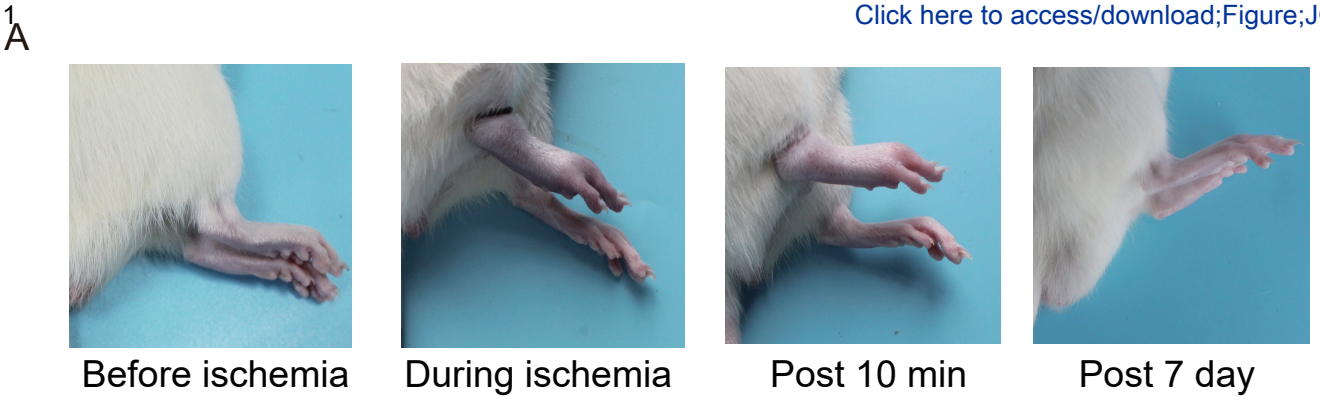
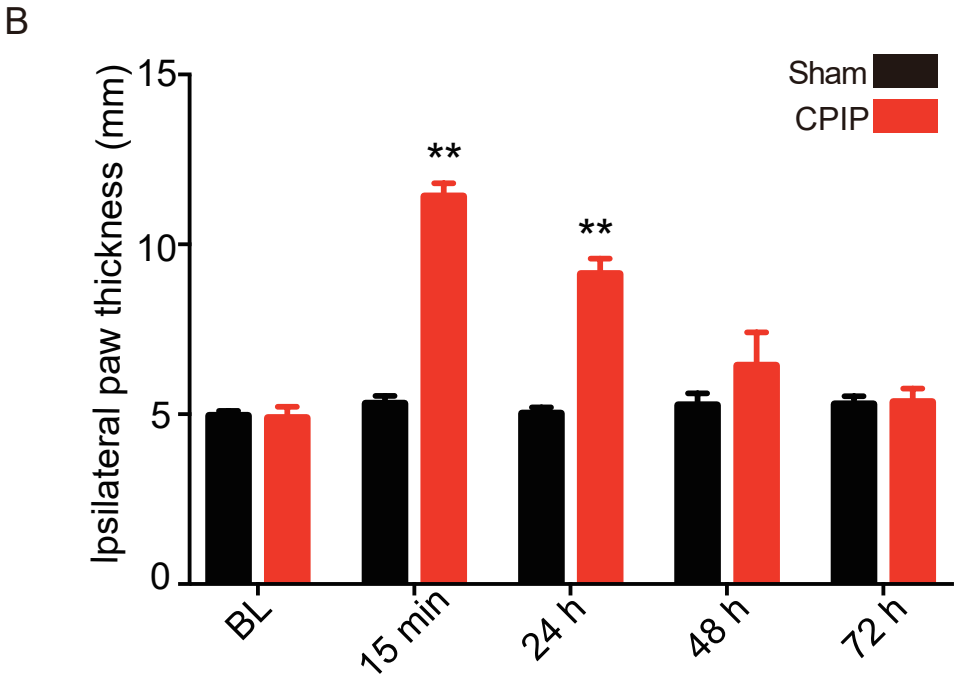
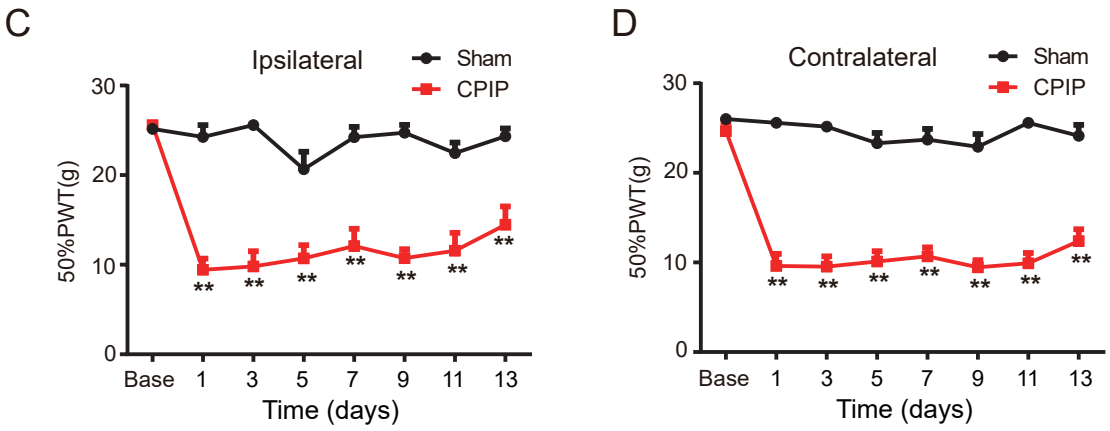
Fig. 1







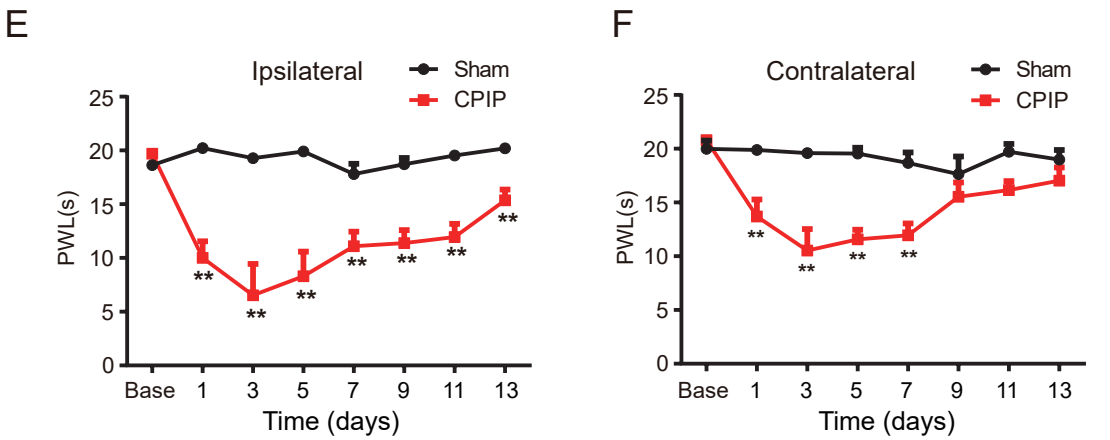


Fig. 2

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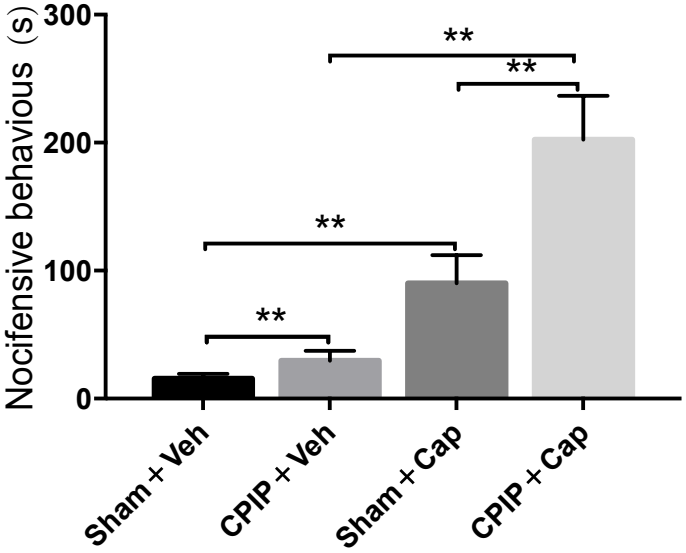

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
1.5 ml Eppendorf tube	Eppendorf	22431021	
DMSO	Sigma-Aldrich	D1435	
Capsaicin	APEXBIO	A3278	
Digital caliper	Meinaite	NA	
O-ring	O-Rings West	Nitrile 70 Durometer	7/32 in. internal diameter
Plantar Test Apparatus	UGO Basile, Italy	37370	
von Frey filaments	UGO Basile, Italy	NC12775	

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4. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

Answer: Done. This can be found in 2.2.4.

5. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

Answer: Done. This can be found in 2.2.4.

6. Please define all abbreviations before use, e.g., DMSO, PBS, etc.

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7. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
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CORRESPONDING AUTHOR

Name:	Boyi Liu
Department:	Dept. Neurobiology and Acupuncture Research, the Third Clinical Medical College
Institution:	Zhejiang Chinese Medical University
Title:	Professor
Signature:	[signature]
Date:	8.28.2019

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