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Antinociceptive effects of sinomenine in a rat model of neuropathic pain

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Sinomenine is a principal ingredient of traditional Chinese medicine, *Sinomenium Acutum*, which has been reported to have various pharmacological effects including anti-rheumatism and immunomodulation. This study examined the effects of sinomenine in rats that received chronic constriction injury (CCI), a model of peripheral neuropathic pain. CCI injury on the right sciatic nerve led to long-lasting mechanical hyperalgesia. Acute sinomenine treatment (10–40 mg/kg, i.p.) significantly and dose-dependently reversed mechanical hyperalgesia. In addition, the antinociceptive effects of sinomenine remained stable during repeated daily treatment for up to 2 weeks. Although sinomenine did not alter the duration of immobility in the forced swimming test in healthy animals, it dose-dependently reversed the increased immobility time in rats receiving CCI, suggesting that sinomenine attenuated chronic pain-induced depressive-like behavior. The antinociceptive effects of sinomenine were blocked by the GABAa receptor antagonist bicuculine. The doses of sinomenine studied here did not significantly alter the spontaneous locomotor activity. Together, these results suggested that sinomenine exerts significant antinociceptive effects for neuropathic pain via GABAa-mediated mechanism, which suggests that sinomenine may be useful for the management of chronic painful conditions such as neuropathic pain.

Sinomenine, (+)-4-Hydroxy-3,7-dimethoxy-17-methylmorphin-7-en-6-one, is the major active compound of the traditional Chinese medicine *Sinomenium Acutum*. Scientific investigations of sinomenine can be traced back almost 100 years ago when it was first isolated and its pharmacological effects on blood pressure first described in 1920s[1]. Chemically, sinomenine is a morphinan analog although its pharmacological mechanisms are largely unclear. Mounting preclinical and clinical studies find that sinomenine is effective against rheumatoid arthritis[2,3]. The anti-inflammatory and neuroprotective effects of sinomenine may be partially mediated through inhibition of microglial NADPH oxidase[4]. Sinomenine is effective in modulating the immunological system, which may attribute to its efficacy in the treatment of arthritis and glomerular diseases[5].

Limited evidence also suggests that sinomenine may be able to alleviate pain[6]. In healthy animals, sinomenine only exerts modest antinociceptive effects in animal models of acute nociception including hot plate test and radiant tail flick test. In a mice model of carrageenan-induced inflammatory pain, sinomenine reduces mechanical allodynia and heat hyperalgesia[6]. Importantly, the antinociceptive effects of sinomenine are not attenuated by an opioid receptor antagonist naloxone[6], suggesting that the effects are not mediated through opioid receptors, despite the similarity of the chemical structures between sinomenine and opioids such as morphine.

In order to further examine the antinociceptive actions of sinomenine and understand the potential pharmacological mechanisms of such effects, this study examined the antinociceptive effects of sinomenine in a rat model of chronic constriction injury (CCI)-induced neuropathic pain. Because neuropathic pain usually is long-lasting and pharmacological treatment needs repeated dosing which may lead to the development of antinociceptive tolerance, we also examined the antinociceptive effects during repeated treatment. It is well known that patients with chronic pain often have co-morbid depression[7], this study also examined whether sinomenine could attenuate the depressive-like behaviors in rats with chronic neuropathic pain.

Results

All paw withdrawal data were collected from the von Frey filament test. Before the CCI surgery, paw withdrawal threshold was 17.9 ± 2.8 g in control group and there was no significant differences among the groups (one way ANOVA: $F\ [3, 28] = 0.13$, $P > 0.05$) (Fig. 1). One day after CCI surgery, the paw withdrawal threshold was significantly decreased to 6.0 ± 0.8 g (Fig. 1). Repeated measurement with von Frey filaments did not significantly alter the paw withdrawal threshold (gray circles, Fig. 1). At doses of 10–40 mg/kg, sinomenine dose-dependently increased the paw withdrawal threshold (Fig. 1). Thus, at a dose of 40 mg/kg, the paw withdrawal threshold gradually increased to the maximum 150 min after drug injection and decreased to the pre-drug level

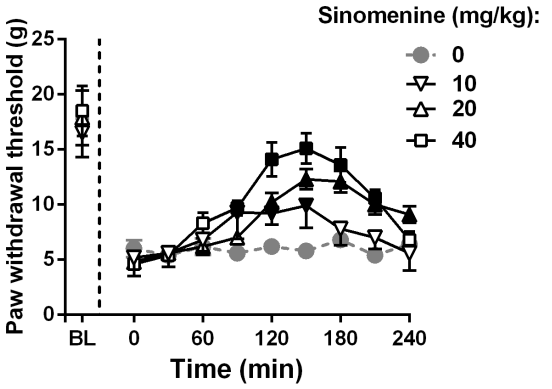

Figure 1 | Effects of sinomenine on CCI-induced mechanical hyperalgesia. Paw withdrawal threshold (g) was plotted as a function of time (min). Data were analyzed using two-way ANOVA followed by *post hoc* Bonferroni' test. Filled symbols indicate data that are significantly different from the corresponding control group (N = 10 per group). Sinomenine dose-dependently reverted mechanical hypersensitivity in rats receiving CCI injury.

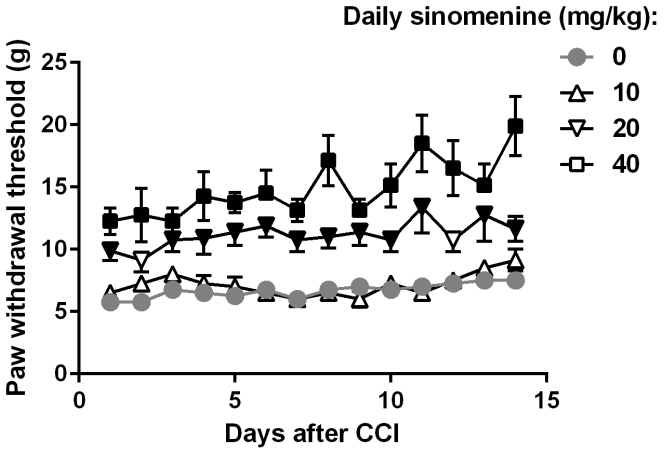

Figure 2 | Effects of repeated treatment with sinomenine on mechanical hyperalgesia in rats receiving CCI surgery (N = 8 per group). The antinociceptive effects of sinomenine remained unchanged across the period of 14 days. See Figure 1 for other details.

4 h after drug administration. Two-way ANOVA analyses found that there were significant main effects of time ($F[8, 324] = 28.9$, $P < 0.0001$), sinomenine dose ($F[3, 324] = 43.0$, $P < 0.0001$) and time × sinomenine dose interactions ($F[24, 324] = 5.9$, $P < 0.0001$). Subsequent *post hoc* analyses revealed that the paw withdrawal threshold was significantly increased between 120–150 min after a dose of 10 mg/kg sinomenine (downward triangles, Fig. 1), 120–140 min after a dose of 20 mg/kg sinomenine (upward triangles, Fig. 1), and 90–210 min after a dose of 40 mg/kg sinomenine (squares, Fig. 1).

Because chronic pain such as CCI-induced neuropathic pain is long lasting and requires repeated drug treatment, next experiment examined the effects of repeated sinomenine in rats with neuropathic pain. Daily treatment with 10–40 mg/kg sinomenine for two weeks induced dose-dependent increase of paw withdrawal threshold, which was consistent with the acute drug effects (Fig. 2 vs. Fig. 1). The baseline of mechanical hypersensitivity did not significantly change over the period of repeated treatment (data not shown). More importantly, the antinociceptive effects did not significantly change during the 2-week daily treatment, suggesting the lack of development for antinociceptive tolerance. Two way ANOVA analysis found significant main effects of time ($F[13, 364] = 3.3$, $P < 0.001$) and sinomenine treatment ($F[3, 28] = 102.0$, $P < 0.0001$). No significant time × sinomenine treatment interaction was identified ($F[39, 364] = 1.07$, $P > 0.05$). *Post hoc* analyses revealed that 20 and 40 mg/kg sinomenine significantly increased the paw withdrawal threshold throughout the 2 weeks except on days 2 and 12 for the 20 mg/kg sinomenine group for which statistical significance was not reached (Fig. 2).

In non-CCI healthy rats, sinomenine at the dose range of 10–40 mg/kg did not change the immobility behavior in the forced swimming test (left panel, Fig. 3). Interestingly, rats demonstrated significantly increased duration of immobility 3 weeks after CCI surgery, suggesting the presence of depressive-like behavior (right, panel, Fig. 3). Repeated treatment with sinomenine after the CCI surgery dose-dependently and nearly completely reversed the increased immobility time, suggesting the prevention of depressive-like behavior in these rats (one-way ANOVA: $F[5, 37] = 13.7$, $P < 0.0001$). *Post hoc* analyses suggested that 20 and 40 mg/kg sinomenine significantly prevented the development of depressive-like behavior ($P < 0.05$).

In order to understand the underlying receptor mechanisms of sinomenine-induced antinociception, different receptor antagonists were studied in combination with an effective dose of sinomenine (40 mg/kg) and the paw withdrawal threshold was measured. As can be seen in Figure 4 (left panel), 1 mg/kg serotonin 5-HT_{1A} receptor antagonist WAY100635 or 2 mg/kg opioid receptor antagonist naltrexone did not significantly alter the antinociceptive effects of 40 mg/kg sinomenine. Two-way ANOVA found neither significant main effects of antagonist treatment ($F[2, 21] = 0.5$, $P > 0.05$) nor time × antagonist treatment interaction ($F[16, 168] = 0.5$, $P > 0.05$). In contrast, the $GABA_A$ receptor antagonist bicuculine dose-dependently blocked the antinociceptive effects of sinomenine (right panel, Fig. 4). Two-way ANOVA revealed significant main effects of time ($F[8, 168] = 17.0$, $P < 0.0001$), bicuculine treatment ($F[2, 21] = 6.7$, $P < 0.01$) and time × bicuculine treatment interaction ($F[16, 168] = 4.9$, $P < 0.0001$). *Post hoc* analyses indicated that 0.67 mg/kg bicuculine significantly attenuated the effects of sinomenine between 120–150 min and 2 mg/kg bicuculine significantly attenuated the effects of sinomenine between 120–180 min.

In order to examine whether the observed antinociceptive effects of sinomenine were behaviorally specific, the effects of different doses of sinomenine were examined for its effects on the spontaneous locomotor activity. Sinomenine at doses lower than 40 mg/kg did not significantly change the locomotor activity, although further increasing the dose to 80 mg/kg led to significant locomotor suppression (one-way ANOVA: $F[3, 28] = 4.1$, $P < 0.05$). *Post hoc* analyses indicated that 80 mg/kg of sinomenine significantly reduced the rats' locomotor activity ($P < 0.05$) (Fig. 5).

Discussion

Although the immunomodulation effects of sinomenine are well studied[5], the potential antinociceptive effects of sinomenine are relatively unknown[6]. In addition, because chronic pain often leads to psychiatric disorders such as depression[7], it is unknown of whether sinomenine can prevent pain-induced depression-like effects even if it is effective against chronic pain. This study was the first to examine the effects of sinomenine on the combined issue of chronic pain and depression. Here we report that sinomenine was able to significantly reduce neuropathic pain, prevent the development of depressive-like behavior in rats with neuropathic pain, and the effects did not seem to develop tolerance over repeated treatment. Moreover, our pharmacological analysis revealed for the first time that the antinociceptive effects of sinomenine were mediated through $GABA_A$ receptors.

Neuropathic pain is a chronic and debilitating condition that affects millions of patients worldwide, with estimates of prevalence rates ranging from 1% to 8.9% in the general population depending

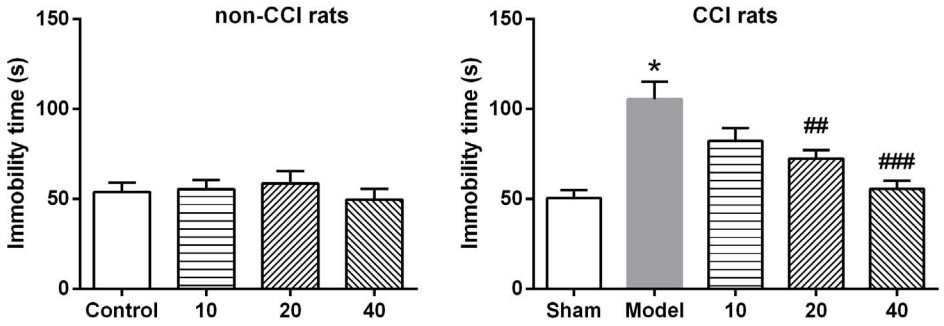

Figure 3 | Effects of sinomenine on the duration of immobility in the forced swimming test in non-CCI healthy (left) or CCI rats (right). * $P < 0.001$ as compared to sham group; ##, ### $P < 0.01$ and $P < 0.001$ as compared to model group (N = 6–10 per group). Sinomenine did not alter the immobility time in healthy rats but dose-dependently reversed prolonged CCI-induced increase of immobility.

on the studies[8]. Although many medications are used for the treatment of neuropathic pain clinically, their efficacies are generally limited and side effects are common[9]. This fact highlights the necessity of developing novel analgesics for the management of neuropathic and neurogenic painful conditions. This study examined the effects of the plant-derived compound sinomenine in a well-studied rodent model of peripheral neuropathic pain CCI and we found that sinomenine was very effective in reversing mechanical hyperalgesia. Importantly, because pharmacotherapy of neuropathic pain most likely needs long term use of the medication, we examined whether repeated treatment with sinomenine could lead to possible development of antinociceptive tolerance. No significant tolerance was observed during the period of two weeks of daily treatment. Tolerance is a pharmacological phenomenon that often occurs during prolong drug treatment. For example, daily treatment with the opioids tramadol or morphine at an antinociceptive dose for 4 to 7 days is sufficient to induce significant tolerance to their antinociceptive effects[10,11]. Twice daily treatment with the cannabinoid delta9-tetrahydrocannabinol for 9 days leads to greater than 4-fold rightward shift of the delta9-tetrahydrocannabinol dose-effect curve in its antinociceptive effects[12]. In the present study, daily treatment with sinomenine for 2 weeks failed to show a trend of antinociceptive tolerance is a strong evidence that little tolerance should be expected during prolong sinomenine use for the management of pain. These findings are consistent with recent studies using different animal models of neuropathic pain and shorter treatment period (twice daily for 5 days)[6,13]. It is worth noting that the effects of sinomenine appeared to be behaviorally specific as the doses used in the study did not impact the general locomotor activity (Fig. 5). Given the limited half-life time of sinomenine, drug accumulation during daily treatment also seems unlikely.

Increasing studies suggest that chronic painful conditions such as inflammatory and neuropathic pain often accompany with depressive-like behaviors in animals and depression in humans[7]. Whether analgesics also attenuate affective pain and psychiatric disorders due to chronic pain besides their effects against pain sensation is an emerging field in pain research[7]. Thus, drugs that are able to reduce the comorbidity of pain and depression should be expected to have better clinical consequences. In the current study, we found that the presence of CCI-induced neuropathic pain for 3 weeks dramatically led to depressive-like behaviors as demonstrated by markedly increased immobility time in the forced swimming test, consistent with the literature[14,15]. Interestingly, we found that sinomenine treatment during the process of chronic pain successfully prevented the development of depressive-like behaviors. Importantly, sinomenine did not demonstrate similar antidepressant-like behavior in healthy rats that did not receive CCI surgery. Therefore, it seems that sinomenine was particularly effective against chronic neuropathic pain-induced depressive-like effects. If the data can be extrapolated to clinical reality, this may suggest that sinomenine is an effective analgesic that can both reduce chronic pain and also prevent the development of depressive disorders due to the presence of chronic pain.

The receptor mechanisms of sinomenine underlying its behavioral actions have been largely unknown. Sinomenine is reported to bind to mu opioid receptors and produce antinociception in a mice model of acute nociception through mu opioid receptors[16]. However, this result was not replicated in another study[6]. In the latter study, it was found that the opioid receptor antagonist naloxone failed to block the antinociceptive effects of sinomenine in a mice model of neuropathic pain[6]. With these results in mind, we first examined the possible involvement of opioid receptors in sinomenine-induced antinociception. A large dose of 2 mg/kg opioid receptor antagonist naltrex-

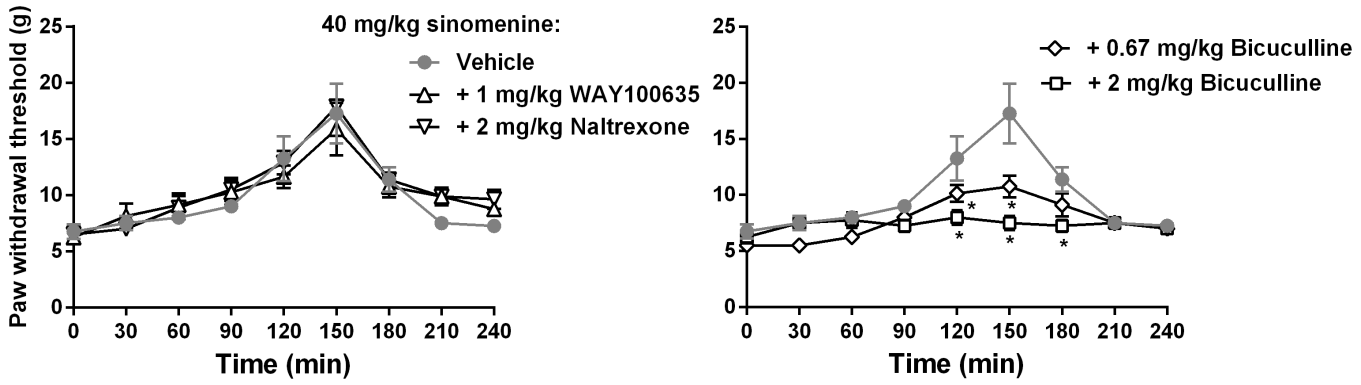

Figure 4 | Effects of antagonists on the anti-hyperalgesic effects of sinomenine in CCI rats. * $P < 0.05$ as compared to vehicle control group (N = 8 per group). The $GABA_A$ receptor antagonist bicuculline but not the opioid receptor antagonist naltrexone nor the 5-HT_{1A} receptor antagonist WAY100635 blocked the antinociceptive effects of 40 mg/kg sinomenine.

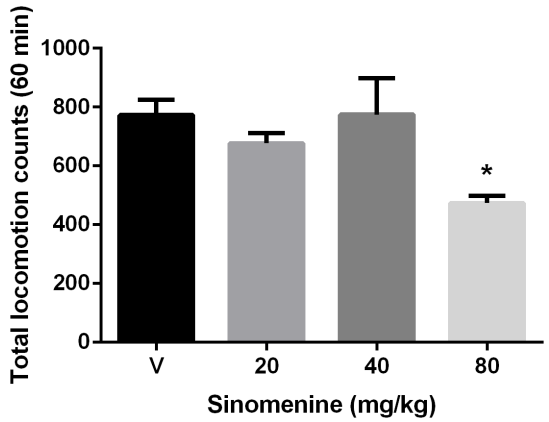

Figure 5 | Effects of sinomenine on the general locomotor activity in non-CCI healthy rats. Data represent the cumulative locomotion data of the 60 min test session (N = 8 per group).

one failed to block the anti-hyperalgesic effects of 40 mg/kg sinomenine. This dose of naltrexone is sufficient to occupy the majority of opioid receptors in rats and the lack of blockade strongly suggests that opioid receptors do not play a major role in sinomenine-induced antinociception. Serotonergic system plays an essential role in pain modulation and drugs acting on specific 5-HT receptors such as 5-HT_{1A} receptors have been shown to be effective analgesics in animal models of neuropathic pain[17]. To test the hypothesis that the antinociceptive effects of sinomenine may be mediated through 5-HT_{1A} receptors, we utilized a selective 5-HT_{1A} receptor antagonist WAY100635. At a dose that sufficiently blocks 5-HT_{1A} receptors, WAY100635 did not significantly alter the antinociceptive effects of sinomenine, thus excluding the possible involvement of 5-HT_{1A} receptor in sinomenine-induced antinociception. Increasing evidence indicates the involvement of $GABA_A$ receptors in pain processing[18]. We next examined this possibility by combining a selective $GABA_A$ receptor antagonist bicuculine with sinomenine. We found a dose-dependent and significant blockade of sinomenine-induced antinociception by bicuculine. In fact, 2 mg/kg bicuculine completely prevented a dose of 40 mg/kg sinomenine-induced antinociception. Combined, these results strongly suggest that sinomenine produces antinociception via activating $GABA_A$ receptors.

In conclusion, the present study demonstrated that sinomenine is effective against neuropathic pain by reducing both sensory hyperalgesia and chronic pain-induced depression. In addition, the effects do not develop tolerance during repeated treatment and are mediated through $GABA_A$ receptors. These results represent a significant extension of a previous study on sinomenine-induced antinociception[6] and support further examination of sinomenine as a novel analgesic for the treatment of chronic peripheral mononeuropathy.

Methods

Animals. Male Sprague-Dawley rats weighing of 250–300 g (Laboratory Animal Center, Nantong University, China) were group housed and habituated to the housing environment (12 h light/dark cycle, lights on at 7:00 AM) for at least 3 days before the initiation of behavioral studies. Subjects were fed *ad libitum* except during the periods of behavioral tests. Experimental protocols were approved by the local Institutional Animal Care and Use Committee, Nantong University. Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (8th edition, Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences, Washington DC). All pain tests were conducted in accordance with the recommendations of the International Association for the Study of Pain and efforts were made to minimize animal suffering.

Drugs. Sinomenine ((+)-4-Hydroxy-3,7-dimethoxy-17-methylmorphin-7-en-6-one) was purchased from Aladdin Reagents (Shanghai, China). WAY100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]- *N*-(2-pyridyl)cyclohexanecarboxamide), naltrexone hydrochloride and bicuculline were purchased from Selleck Chemicals (Houston, TX, USA). All drugs were dissolved in 0.9% physiologic saline. Drug was administered intraperitoneally (i.p.) in a volume of 1 ml/kg of body weight.

CCI surgery. CCI procedure was performed according to published procedure[19]. Specifically, rats were anaesthetized under isoflurane anesthesia (2% isoflurane mixed with 100% oxygen at a flow rate of 5 L/min) during surgery. The right sciatic nerve was surgically exposed and four loosely tied ligatures (4.0 chromic gut) were placed around the nerve (1 mm apart) proximal to the trifurcation to prevent the interruption of blood circulation through the epineural vasculature. For animals that received sham operations, the right sciatic nerve was exposed but no ligature was applied. After surgery, the skin was closed through standard suture and the animals were allowed to recover in their home cages. Soft bedding was used to minimize suffering of the animals after surgery.

Von Frey filament test. As described in our previous study[20], the von Frey filament test was used to assess the existence of mechanical hypersensitivity by measuring paw withdrawal thresholds to applied mechanical stimuli (Stoelting, USA). A series of ten von Frey filaments were used in a sequential order, which have approximately equal logarithmic incremental bending forces (equivalent to 1, 1.4, 2, 4, 6, 8, 10, 15, 26, 60 g force, respectively). Rats were situated in transparent Perspex boxes with wire mesh floors, and at least 15 min habituation time was applied before behavioral tests. The filaments were individually applied to the plantar surface of each hind paw in ascending orders. At each force, behavior was tested three times per paw, and the hyperalgesia mechanical threshold was defined as the minimal force that caused at least two withdrawals observed out of three consecutive trials[21].

Forced swimming test. Forced swimming test was conducted according to published procedure with minor modifications[22]. Briefly, rats were gently placed in a custom-made acrylic cylinder (20 cm diameter and 45 cm high) filled with warm tap water (25°C, 30 cm deep). On the first day of the experiment, a training period was performed during which rats were placed in the water for 15 min; on the second day animals were placed in the cylinder for a 5-min test. For the acute sinomenine effect, saline or sinomenine was administered (i.p.) three times (i.e., 23, 9 and 1 h before the 5-min test). For the CCI-treated rats, animals were treated daily with different doses of sinomenine for 3 weeks and the forced swimming test was performed 24 h after the last treatment. During the test rats were videotaped for retrospective scoring. The cumulative time of the individual behaviors (immobile, swimming or climbing) was recorded using a stopwatch according to reported methods[23].

Locomotor activity. The rats' general locomotor activity was assessed using commercially available apparatus (YLS-1B, Shandong Academy of Medical Sciences, China) consisting of a control panel and 4 circular black acrylic locomotion recording units (30 cm in diameter and 30 cm in height). The spontaneous locomotor activity of the rats was recorded through the photocell sensor located in the center of the cover. The beam breaks (counts) indicate the general locomotor activity and a 60-min test period was used in this study.

Experimental design. Mechanical hyperalgesia was measured 24 h after the surgery for acute experiments and daily thereafter for repeated drug treatment studies. Daily baseline measures prior to surgery were also conducted for 3 days to facilitate the animals to habituate to the test. In CCI-treated rats, forced swimming test was conducted 3 weeks after CCI surgery while in control studies the test was performed in non-CCI healthy rats after matched days of normal housing. For the duration of action of acute sinomenine study, different doses of sinomenine (10–40 mg/kg) were administered 1 day after surgery and then paw withdrawal threshold was measured every 30 min for 4 hours. For the study involving daily sinomenine treatment, mechanical hyperalgesia measure was performed 3 h after daily drug treatment. For antagonist studies, antagonists were given 10 min prior to 40 mg/kg sinomenine administration.

Data analyses. For acute and repeated sinomenine-induced antinociception studies, paw withdrawal threshold (gram) was plotted as a function of time (min or days) and data were analyzed using two-way repeated measures analysis of variance (ANOVA) using time as within group factor and treatment dose as between group factor, which was followed by *post hoc* Bonferroni analysis. For antagonist studies, data were analyzed using two-way ANOVA using time as within group factor and antagonist dose as between group factor, which was followed by *post hoc* Bonferroni analysis. For the locomotor activity and forced swimming tests, data were analyzed using one-way repeated measures ANOVA followed by *post hoc* Bonferroni analysis.

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Author contributions

Q.Z., Y.S. and J.L. conceived and designed the project and prepared the manuscript. Q.Z., Y.S., J.Z. and T.F. conducted the experiments. Q.Z., W.Z. and J.L. analyzed the data. All authors read and approved the manuscript.

Additional information

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