

## Antinociceptive effects of intrathecally administered huwentoxin-I, a selective N-type calcium channel blocker, in the formalin test in conscious rats

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### Abstract

The present study was undertaken to elucidate the antinociceptive effect of intrathecal administration of huwentoxin-I (HWTX-I), a N-type calcium channel blocker from the venom of the Chinese bird spider *Ornithoctonus huwena* (Wang) [= *Selenocosmia huwena* wang], by comparison with  $\omega$ -Conotoxin-MVIIA ( $\omega$ -CTX-MVIIA) and morphine hydrochloride in the formalin test in conscious rats. Similar to  $\omega$ -CTX-MVIIA and morphine, intrathecal pre-treatment with HWTX-I resulted in suppression of nociceptive behavior in a dose-dependent manner. The ED<sub>50</sub> values of HWTX-I and  $\omega$ -CTX-MVIIA were 0.28 and 0.19  $\mu$ g/kg, respectively. It was also found that, at lower doses (0.1 and 0.5  $\mu$ g/kg), the antinociceptive effect of HWTX-I was identical to that of  $\omega$ -CTX-MVIIA, while  $\omega$ -CTX-MVIIA acted more remarkably than HWTX-I at higher dose (1.0  $\mu$ g/kg). However, the antinociception induced by  $\omega$ -CTX-MVIIA were accompanied with motor dysfunction, and these side-effects became more evident with the doses of  $\omega$ -CTX-MVIIA increasing. In contrast, HWTX-I did not show these side-effects at the doses of 0.5–1.0  $\mu$ g/kg. Compared with HWTX-I and  $\omega$ -CTX-MVIIA, the analgesic effect of intrathecal morphine hydrochloride was initiated faster, but lasted for a shorter time (about 2–3 h at 1.0  $\mu$ g/kg) than that of HWTX-I and  $\omega$ -CTX-MVIIA (about 4–5 h at 1.0  $\mu$ g/kg). Therefore, the present results show that, like  $\omega$ -CTX-MVIIA, the intrathecal administration of HWTX-I is effective in antinociception in the rat model of the formalin test.

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**Keywords:** HWTX-I; N-type calcium channels; Formalin test; Antinociception

### 1. Introduction

It is widely accepted that N-type voltage-sensitive calcium channels (VSCCs) located on primary afferent nerve terminals in the spinal cord and are likely to be involved in transmitter release. The superficial laminae

(I–II) in the dorsal horn of the spinal cord are densely distributed with N-type VSCC-like immunoreactivity, suggesting that N-type VSCCs play an important role in the spinal processing of nociceptive transmission (Malmberg and Yaksh, 1995; Schaible et al., 2000; Vanegas and Schaible, 2000). Among the high-voltage activated VSCCs, N-type channels almost entirely restricted to neurons. In particular, N-type VSCCs in the spinal cord have an important role in modulating the release of key pronociceptive neurotransmitters such as glutamate, substance P, neurokinin A (NKA) and calcitonin gene-related peptide (Takahashi and Momiyama, 1993). Therefore, considerable research in last decade has focused on the potential of N-type VSCCs blockers as novel analgesic

*Abbreviations:* HWTX-I, huwentoxin-I; VSCCs, voltage-sensitive calcium channels; s.c., subcutaneous injection; i.t., intrathecal injection;  $\omega$ -CTX-MVIIA,  $\omega$ -conotoxin-MVIIA.

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drugs (Smith et al., 2002). There is reason to believe that antagonists of N-type VSCCs, such as  $\omega$ -conotoxins, can prevent, and/or attenuate, subjective pain. SNX-111, a 25-amino acid polycationic peptide, is the synthetic equivalent of  $\omega$ -conotoxin MVIIA ( $\omega$ -CTX-MVIIA) isolated from the venom of the predatory marine snail, *Conus magnus* (Olivera et al., 1994), and is a selective, reversible and potent blocker of N-type VSCCs. Studies have demonstrated that  $\omega$ -CTX-MVIIA is an antinociceptive agent in various animal models, including formalin-induced tonic flinch responses, paw pressure, hot-plate, tail immersion and postoperative pain tests, when administered by the intrathecal route (Malmberg and Yaksh, 1994, 1995; Bowersox et al., 1996; Wang et al., 2000).

HWTX-I is the most abundant toxic component in the crude venom of the Chinese bird spider *Ornithoctonus huwena* (Wang) [= *Selenocosmia huwena* wang], and consists of 33 residues (Liang et al., 1993; Wang et al., 1993). The three-dimensional structure of HWTX-I was determined as an inhibitor cystine knot motif (Qu et al., 1997), which was similar to that of  $\omega$ -CTX-MVIIA (Kohono et al., 1995). By employing whole-cell patch clamp methods, it was found that HWTX-I selectively inhibits N-typed VSCCs with an  $EC_{50}$  of 100 nM and can block transmitter release from nerve endings by preventing depolarization induced by calcium influx (Pen et al., 2001). Since HWTX-I has been determined to be a selective N-type calcium channel blockers, it was hypothesised that, like  $\omega$ -CTX-MVIIA, HWTX-I given intrathecally may produce antinociception in models of pain.

The purpose of this study was therefore to investigate the antinociceptive effect of intrathecally administrated HWTX-I in formalin test in rats and to compare its efficacy with  $\omega$ -CTX-MVIIA and chemically employed analgesics such as morphine.

## 2. Materials and methods

### 2.1. Animal preparations

Adult Sprague–Dawley rats (either sexes, weighting 180–220 g) were used for this behavioral test. Animals were provided by the Laboratory Animal Center of the Hunan Medical University (HMU) and use of the animal was reviewed and approved by the HMU Animal Care and Use Committee. The IASP's ethical guidelines for experimental pain research in conscious animals were followed (Zimmermann, 1983). Animals were housed in plastic boxes in three groups at 22–26 °C with food and water available ad libitum. A 12:12 h light dark cycle with lights on at 08:00 was maintained and testing was done between 09:00 and 18:30. Rats subjected to subcutaneous injection of 5% formalin into the plantar surface of the hindpaw were randomly divided into four groups: (1) control: intrathecal injection of 0.9% sterile

salt; (2) HWTX-I: intrathecal injection of 0.1, 0.5, 1.0 and 2.0  $\mu$ g/kg; (3) CTX-MVIIA: intrathecal injection of 0.1, 0.5 and 1.0  $\mu$ g/kg; (4) morphine hydrochloride: intrathecal injection of 1.0  $\mu$ g/kg.

### 2.2. Drug and toxin injection

HWTX-I was purified in our laboratory, the purity was over 96%, as judged by analytical rp-HPLC.  $\omega$ -CTX-MVIIA acetate salt, was obtained from Sigma (purification  $\geq$  98%). Morphine hydrochloride was supplied by the Institute of Drug and Biological preparation of China. Injection solutions were prepared in preservative free physiological saline (0.9% w/v NaCl). For intrathecal administration, drugs or toxins were delivered through indwelling catheters in dose volumes of 1.0  $\mu$ l/10 g rat body weight followed by a 10  $\mu$ l saline flush.

### 2.3. Spinal catheterization procedures

For intrathecal administration of the drug, chronic intrathecal catheterization was modified according to previous reports (Yaksh and Rudy, 1976; Chen et al., 1999; Chen and Chen, 2001). Briefly, a PE-10 plastic tube (i.d. 0.23 mm; o.d. 0.61 mm) was inserted from the  $T_2$ – $T_3$  level into the subarachnoid space of the rostral lumbosacral enlargement under ketamine anesthesia (50 mg/kg, i.p.). Experiments were performed at least 5 days after the operation to minimize any influence of the catheterization procedure. Only those without motor disturbances or other abnormal signs were used for formalin test. After tests, each rat was checked for the placement of the end of the catheter, those with incorrect placement or local pathological change were not used for experimentation.

### 2.4. Formalin test

Acute and chronic nociceptive responses were measured using the rat paw formalin test as described (Dubission and Dennis, 1977) and modified (Malmberg and Yaksh, 1995). During the test, each rat was placed in a 30  $\times$  30  $\times$  30 cm transparent Plexiglas test box for at least 30 min before administration of the drug or toxins. HWTX-I,  $\omega$ -CTX-MVIIA and morphine hydrochloride were initially dissolved in 0.9% sterile saline at a concentration of 1.0  $\mu$ g/ $\mu$ l before diluting to the working concentration. Intrathecal administration of the test agents was performed using a 50  $\mu$ l microsyringe containing a volume of 1.0  $\mu$ l/10 g (rat body weight) of HWTX-I solution (0.1, 0.5, 1.0 or 2.0  $\mu$ g/kg),  $\omega$ -CTX-MVIIA solution (0.1, 0.5 or 1.0  $\mu$ g/kg), morphine solution (1.0  $\mu$ g/kg) or saline, followed by a flush with 10  $\mu$ l saline. Between the drug solution and flushing saline, 1.0  $\mu$ l air bubble was added to isolate the chemical agent from 10  $\mu$ l flushing saline (Chen et al., 1999; Chen and Chen, 2001).

All agents (drug or toxins) were administered 30 min prior to s.c. intraplantar formalin injection. A volume of 50  $\mu$ l formalin (5%) solution was used as previously described (Malmberg, and Yaksh, 1995) and injected s.c. into the plantar surface of one hindpaw. The rat was then immediately returned to the plexiglas observation chamber and visual pain responses were recorded for a period of 1 h.

### 2.5. Assessment of nociceptive behavior

Assessment of nociceptive behavior was carried out by the method described by Dubission and Dennis (1977). Spontaneous nociceptive response was immediately scored and estimated out a compositive pain score by counting the numbers of flinches [the number of flinches were changed to time (i.e. 1 flinch = 1 s)], the time of lifting, shaking, licking and biting of the injected hindpaw at 5 min intervals for 60 min. A reduction in the weight put on the injected paw was given a rating of 1 ( $T_1$ ), total elevation and flinch of the paw a rating of 2 ( $T_2$ ), and licking, biting and shaking of the paw a rating of 3 ( $T_3$ ). A mean score was calculated with each rating being weighted according to the time spent in each behavioral category.

$$\text{Pain scores (S)} = T_1 + 2T_2 + 3T_3$$

where  $T_1$ ,  $T_2$  and  $T_3$  represent the duration (in seconds) spent in categories 1, 2 or 3, respectively, during each time block. The duration of each time block was 5 min.

### 2.6. Data analysis and statistics

The results were expressed as means  $\pm$  SEM of the value of pain scoring per 5 min. Group differences were considered statistically significant at  $P < 0.05$  using ANOVA post hoc analysis.  $ED_{50}$  values were calculated from dose–response curves using a four parameter logistic function:  $\text{Effect} = E_0 + (E_i - E_0) / (1 + ED_{50}/D)^{nH}$  where  $E_0$  = effect at 0 dose of test article,  $E_i$  = effect at infinite dose of test article,  $D$  = test article dose, and  $nH$  = Hill coefficient.

## 3. Results

### 3.1. Antinociceptive effects of the different doses of HWTX-I

As previously reported (Dubission and Dennis, 1977; Tjølsen et al. 1994), two distinct phases of nociceptive behavior were produced following s.c. formalin injection. The first phase started immediately after injection of formalin and lasted for 3–5 min, the rats show frequent behavioral category 3 or 2 ( $T_3$  or  $T_2$ ) for example flinch, lifting, shaking, licking and biting the injected hindpaw in this phase. It followed by a silence period lasting about 10–15 min. The second phase started approximately

15–20 min after formalin injection and lasted for 20–40 min, during this time the behavioral category 3 or 2 reduced gradually, while behavioral category 1 (i.e. a reduction in the weight put on the injected paw) increases gradually. As shown in Fig. 1A, we could see that, in contrast to i.t. saline injection, i.t. pre-treatment with HWTX-I (0.1, 0.5, 1.0 and 2.0  $\mu$ g/kg) resulted in a dose-dependent suppression of the initial and late-phase, induced by s.c. formalin injection. HWTX-I was found to depress both the early and the late phases. The column graph (Fig. 1B) showed that the mean antinociceptive scores over the 60 min period of HWTX-I at 0.1, 0.5, 1.0 and 2.0  $\mu$ g/kg doses were  $59.33 \pm 21.46$  S/5 min ( $n = 6$ ,  $P > 0.05$ ),  $50.74 \pm 4.06$  S/5 min ( $n = 6$ ,  $P < 0.05$ ),  $31.56 \pm 11.31$  S/5 min ( $n = 6$ ,  $P < 0.01$ ), and  $26.95 \pm 9.38$  S/5 min ( $n = 6$ ,  $P < 0.01$ ), respectively, while that of the control group (saline) was  $73.53 \pm 11.45$  S/5 min ( $n = 8$ ). The inhibitory effect of HWTX-I was statistically compared with the control and Group differences were considered statistically significant using ANOVA post hoc analysis.

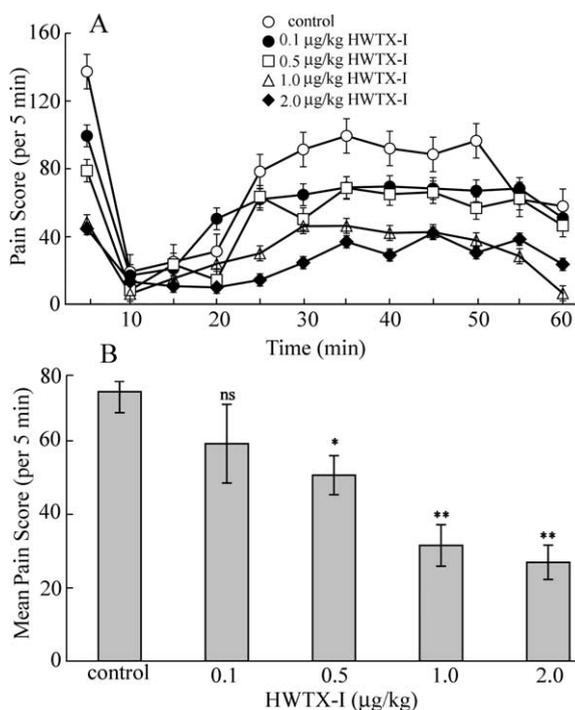


Fig. 1. Effect of i.t. administration of HWTX-I on the both phases of nociceptive behavior induced by injection of formalin into the hindpaw. Saline was used as control. The data are presented as the mean  $\pm$  SEM ( $N = 8$ –10 per dose). (A) Curve graph shows the mean time courses for 60 min after s.c. formalin. (B) Column graph shows the mean numbers of the pain scores per 5 min averaged from 12 time blocks of 60 min after s.c. formalin. Group differences were considered statistically significant at  $P < 0.05$  using ANOVA post hoc analysis: \*\* $P > 0.01$ , \* $P < 0.05$ ; n.s., no significant; vertical bars, mean  $\pm$  SEM.

### 3.2. Comparison of antinociceptive effects of HWTX-I with $\omega$ -CTX-MVIIA and morphine

In order to determine the antinociceptive efficacy of HWTX-I, we compared the antinociception of HWTX-I with  $\omega$ -CTX-MVIIA and morphine.  $\omega$ -CTX-MVIIA (0.1, 0.5 and 1.0  $\mu\text{g}/\text{kg}$ ) produced a similar significant reduction in the spontaneous nociceptive scores for both phases of the formalin test. As shown in Fig. 2, the dose–response curves for HWTX-I and  $\omega$ -CTX-MVIIA at 0.1 and 0.5  $\mu\text{g}/\text{kg}$  were almost identical (Fig. 2A,B), which suggested that they have similar antinociception. It was interesting that, at a dose of 1.0  $\mu\text{g}/\text{kg}$ , the antinociception of  $\omega$ -CTX-MVIIA was slightly greater than that of HWTX-I (Fig. 2C). However, at this dose,  $\omega$ -CTX-MVIIA caused marked tail or whole body muscle tremor, while no evident side-effects were seen with HWTX-I.

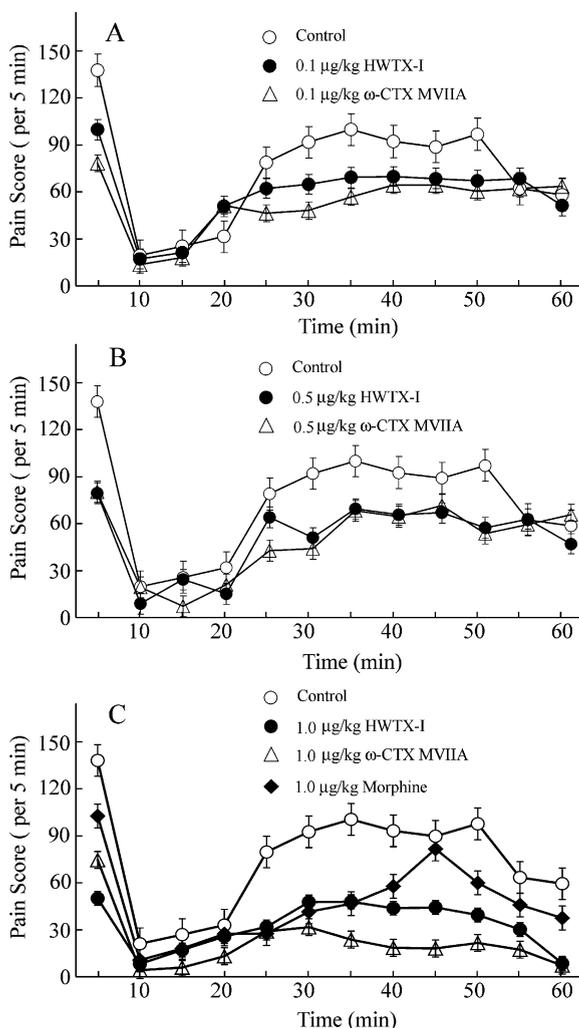


Fig. 2. Comparison of antinociceptive effects of HWTX-I with  $\omega$ -CTX-MVIIA and morphine. (A) 0.1, (B) 0.5, (C) 1  $\mu\text{g}/\text{kg}$ . The data are presented as the mean  $\pm$  SEM ( $N=8-10$  per drug).

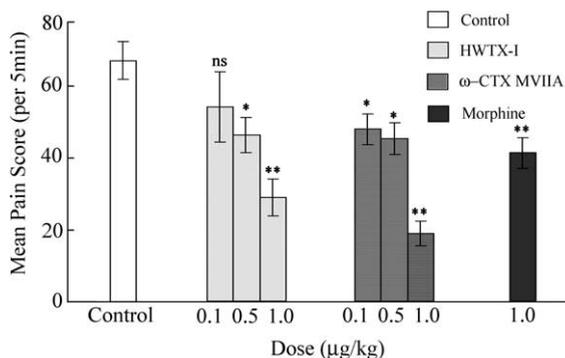


Fig. 3. Column graph showed the mean numbers of pain scores per 5 min averaged from 12 time blocks of 60 min after s.c. formalin. Group differences were considered statistically significant at  $P < 0.05$  using ANOVA post hoc analysis: \*\* $P > 0.01$ , \* $P < 0.05$ ; n.s., no significant; vertical bars, mean  $\pm$  SEM.

We also compared the antinociceptive effects of HWTX-I and  $\omega$ -CTX-MVIIA with that of morphine. Fig. 2C indicated that all the three drugs could suppress the nociceptive behavior responses at the dose of 1.0  $\mu\text{g}/\text{kg}$  when intrathecally administered 30 min prior to formalin injection. However, it was found that the antinociception of HWTX-I or  $\omega$ -CTX-MVIIA was greater than that of morphine (Fig. 2C). Furthermore, HWTX-I and  $\omega$ -CTX-MVIIA lasted two-fold longer time (about 4–5 h) than morphine (about 1–2 h) for antinociception.

As shown in Fig. 3, the mean pain scores of HWTX-I and  $\omega$ -CTX-MVIIA at three doses were  $59.33 \pm 21.46$  S/5 min ( $n=6$ ,  $P > 0.05$ ) and  $52.45 \pm 9.47$  S/5 min ( $n=6$ ,  $P < 0.05$ ) at 0.1  $\mu\text{g}/\text{kg}$ ;  $50.74 \pm 4.06$  S/5 min ( $n=6$ ,  $P < 0.05$ ) and  $49.58 \pm 9.05$  S/5 min ( $n=6$ ,  $P < 0.05$ ) at 0.5  $\mu\text{g}/\text{kg}$ ; and  $31.56 \pm 11.31$  S/5 min ( $n=6$ ,  $P < 0.01$ ) and  $20.64 \pm 7.05$  S/5 min ( $n=6$ ,  $P < 0.01$ ) at 1.0  $\mu\text{g}/\text{kg}$ , respectively, while that of morphine at 1.0  $\mu\text{g}/\text{kg}$  was  $45.18 \pm 9.55$  min ( $n=6$ ,  $P < 0.01$ ), compared with the control group ( $73.53 \pm 11.45$  S/5 min  $n=8$ ). Group differences were considered statistically significant at  $P < 0.05$  using ANOVA post hoc analysis.

## 4. Discussion

The formalin test is widely used in behavioral and pharmacological pain studies, because the formalin test is different from most models of pain in that it is possible to assess the way an animal responds to moderate, continuous pain generated by injured tissue. An important feature of the formalin test in rodents is that the animals show two phases of nociceptive behavior (Figs. 1A and 2), which seem to involve two distinctly different stimuli (Dubission and Dennis, 1977; Cowan et al., 1989; Malmberg, et al., 2003). The first phase starts immediately after injection of formalin and lasts for 3–5 min. It

is probably due to direct chemical stimulation of nociceptors, and experimental data indicate that formalin predominantly evokes activity in C fibres, and not in A $\delta$  afferents (Heapy et al., 1987). Subsequently, there is a period of 10–15 min when the animals display very little behavior suggestive of nociception. The second phase starts approximately 15–20 min after formalin injection and lasts for 20–40 min, while the late phase appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord (Cowan et al., 1989).

Spinal opioids, such as morphine produced antinociception by two mechanisms. First, activation of opioid receptors inhibit neuronal activity by hyperpolarisation of post-synaptic neurons through an increase in potassium conductance (North et al., 1987). Second, occupation of pre-synaptic opioid receptors suppresses neurotransmitter release from primary afferents (Go and Yaksh, 1987). Both these processes are G protein dependent.

Calcium channels are believed to be essential for pre-synaptic neurotransmitter release (Augustin and Charlton, 1987). The N-type channel is an important channel in regulating neurotransmitter release in the nervous system and  $\omega$ -conopeptides have been shown to inhibit neurotransmitter-release from both peripheral and central nerve terminals. There is some evidence that opioid receptors are connected to the N-type VSCC, through a G protein (Bean, 1989), and it is possible that some of the acute effects produced by opioids are mediated by inhibition of N-type calcium channels.

The results of the previous study (Malmberg and Yaksh, 1995) have shown that continuous spinal infusion of SNX-111, a synthetic  $\omega$ -CTX-MVIA, resulted in a significant reduction in the first and second phase of the formalin test. Measurement of responses to the formalin test revealed no difference between the antinociception generated on day 2 or 7, suggesting that no tolerance developed to  $\omega$ -CTX-MVIA. In contrast, continuous infusion of morphine was shown to have a significant depression of the formalin response on day 2. However, this effect was significantly reduced by 7 days of infusion, suggesting the development of tolerance. The loss of morphine analgesia may be interpreted in terms of a receptor specific tolerance (Stevens and Yaksh, 1989).

The present study indicates that HWTX-I injected intrathecally, like  $\omega$ -CTX-MVIA, produces a dose-dependent suppression of both the acute and tonic phases of nociceptive behavior. This means that, although there are different mechanisms involved in the biphasic pattern of nociceptive behavior, N-type VSCCs should also play an important role in the two phases. In addition, at lower doses (0.1 and 0.5  $\mu$ g/kg), the antinociceptive effect of HWTX-I was identical to that of  $\omega$ -CTX-MVIA, while  $\omega$ -CTX-MVIA acted more effectively than HWTX-I at higher dose (1.0  $\mu$ g/kg). However, the antinociception induced by  $\omega$ -CTX-MVIA were accompanied with motor dysfunction, such as whole body shaking, circling, ataxia and tail wiggling and so on characteristic body shaking behaviors as described

previously (Malmberg and Yaksh, 1995) during the 60 min observation period. These side-effects became more evident with the doses of  $\omega$ -CTX-MVIA increasing. In contrast, HWTX-I did not have those side-effects at the doses of 0.5–1.0  $\mu$ g/kg. Compared with HWTX-I and  $\omega$ -CTX-MVIA, the analgesic effect of intrathecal morphine hydrochloride was initiated faster, but lasted for a shorter time than those of HWTX-I and  $\omega$ -CTX-MVIA.

In the present study, intrathecal injection of HWTX-I and  $\omega$ -CTX-MVIA had appreciable suppression of formalin-evoked persistent spontaneous pain, which is consistent with the results as described previously (Malmberg and Yaksh, 1994; Wang et al., 2000). N-type VSCCs were almost entirely restricted to neurons, where they play a major role in the release of synaptic mediators such as glutamate, acetylcholine, dopamine, etc. The function of N-type VSCCs has been studied in several neural structures including the spinal cord, as well as in DRG (Takahashi and Momiyama, 1993). HWTX-I and  $\omega$ -CTX-MVIA could inhibit the influx of Ca<sup>2+</sup>, which is believed widely to play an important role in neurotransmitter release, by blocking N-type calcium channels on neuronal pre-synaptic membrane. Subsequently, the toxin could stop the transmission of exciting signal in the junction point of two neurons. However, there are some questions remaining to be studied further, for example,  $\omega$ -CTX-MVIA caused motor problems such as whole body shaking, circling, ataxia and tail wiggling 30–60 min after injection while HWTX-I did not, although both of them selectively targets neuronal N-type VSCCs. It suggested that the underlying mechanism for HWTX-I might be slightly different from that of  $\omega$ -CTX-MVIA. This conclusion could be confirmed by the study of a new conotoxin, CVID, a selective blocker of N-type VSCCs.  $\omega$ -Conotoxin CVID had similar potency to N-conotoxin MVIA, but showed less toxicity in the therapeutic range in acute and neuropathic pain in the rat (Scott et al., 2002).

The present study confirms that intrathecal administration of HWTX-I, like  $\omega$ -CTX-MVIA and morphine, has antinociceptive effect in the rat model of formalin-evoked persistent spontaneous pain, suggesting that it may be a potential drug in clinical control of pain. As the new calcium channel antagonists, perhaps both HWTX-I and  $\omega$ -CTX-MVIA possess more extensive applicative outlook for the treatment of pain, hyperalgesia and allodynia in clinical than classical drugs.

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