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Effects of intramuscular heating-needle stimulation in control of Freund's adjuvant-induced muscle nociception in rats: differential roles of purinergic P2X3 receptors in the thalamus.

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Abbreviations

CFA, complete Freund's adjuvant; i.c., intracerebral; i.m., Intramuscular; i.p., intraperitoneal; GS, gastrocnemius; MD, mediodorsal, VM, ventromedial.

Abstract

Here we investigated effects of intramuscular (i.m.) heating-needle stimulation on persistent muscle nociception evoked by i.m. injection of different doses (50-200 μl) of complete Freund's adjuvant (CFA) in rats. Paw withdrawal reflexes evoked by noxious mechanical and heat stimulation as well as hind limb swelling were determined prior to and two weeks after the CFA injection. The unilateral injection of CFA induced a dose-related and long-lasting (5-14d), bilateral secondary mechanical hyperalgesia and heat hypoalgesia associated with long-term limb swelling. A period of 30-45 min 43 ºC heating-needle stimulation significantly enhanced the i.m. CFA-induced bilateral heat hypoalgesia and alleviated hind limb swelling. In contrast, 30-45min 46 ºC heating-needle stimulation markedly enhanced both mechanical hyperalgesia and heat hypoalgesia, but failed to influence the CFA-induced hind limb swelling. Microinjection of P2X3 receptor antagonist A-317491 (0.5-4.5 nmol/0.5μl) into the thalamic ventromedial (VM) nucleus dose-dependently inhibited the 43 ºC and 46 ºC heating-needle stimulation-induced heat hypoalgesia, whereas the 46 ºC heating-needle stimulation-induced mechanical hyperalgesia was significantly prevented by microinjection of A-317491 into the thalamic mediodorsal (MD) nucleus. In contrast, the hind limb swelling was not affected by the microinjection of A-317491 into the thalamic VM or MD nucleus. The present study indicates that in the CFA-induced persistent muscle nociception condition, 43 ºC heating-needle stimulation selectively increases descending inhibition, which effect is modulated by the thalamic VM nucleus. In addition to the antinociceptive role of P2X3 receptors in

the thalamic VM nucleus, P2X3 receptors within the thalamic MD nucleus participate in the descending facilitation evoked by i.m. 46 ºC heating-needle stimulation.

Keywords: Heating-needle stimulation; Complete Freund's adjuvant; Endogenous descending modulation; Thalamic 'nociceptive discriminator'; P2X3 receptor

INTRODUCTION

Injection of complete Freund's adjuvant (CFA), which is composed of inactivated and dried mycobacteria, into different tissues in experimental animals can elicit and mimic various inflammatory diseases and allow evaluating therapeutic effects of anti-inflammatory drugs (Pearson, 1956; Newbould, 1963; Pircio et al., 1975; Gregory et al., 2013). CFA may also cause long-term nociception as reported by different laboratories (Castro-Costa et al., 1981; Danziger et al., 2001; Walker et al., 2003; Vanegas and Schaible, 2004). For instance, intramuscular (i.m.) injection of CFA into masseter muscle induced mechanical hyperalgesia and limb swelling lasting over 14 days (Simonic-Kocijan et al., 2016). Due to the long time course in the progression of inflammation as well as pain, injection of CFA into muscle has been regarded as a valid model to mimic chronic muscle pain, which is a common clinical problem worldwide causing a high socio-economic burden to the society.

As a warning signal, the perception of pain is a special unpleasant sensation associated with escape behavior, such as a withdrawal reflex, to avoid tissue damage. It is accepted that the spinally-organized nociceptive withdrawal reflex is subject to

endogenous descending controls from different supraspinal structures (Tjølsen, 1991; Heinricher et al., 2009; Pertovaara, 2013). From anatomical and functional perspectives, our previous studies showed specific involvement of two circuits in the discrimination and endogenous modulation of the emotional responses associated with pain: 'the thalamic mediodorsal (MD) nucleus – cingulate cortex- dorsolateral periaqueductal gray (PAG)' circuit and 'the thalamic VM nucleus – insular cortex – ventrolateral PAG' circuit (Lei and You, 2013; You et al., 2013; Lei et al., 2014; Xiao et al., 2015). Importantly, these earlier results revealed critical roles of the thalamic MD and VM nuclei in descending facilitatory and inhibitory control of noxious mechanically and heat-evoked nociception (You et al., 2013). In contrast to tonic modulation of proprioception and sympathetic activities during the physiological state, earlier studies further demonstrated that endogenous modulation of pain (facilitation and inhibition) is, however, inactive or relatively 'silent', if noxious stimulation is insufficient (You et al., 2010, 2013, 2014, 2016). Moreover, peripheral C-fibre afferents were shown to have a key role in initiation of both endogenous inhibition and facilitation. However, the triggering thresholds for activation of the facilitatory and inhibitory circuitries that involve a relay in the thalamic 'nociceptive discriminator', thalamic MD and VM nuclei, are different. The triggering threshold of facilitation is significantly lower than that of inhibition (Lei et al., 2011; Lei & You, 2013; You et al., 2013). Earlier results further revealed that at a painful temperature, i.e. 46 ^{o}C, i.m. heating-needle stimulation recruits both descending facilitation and inhibition, whereas 43 ^{o}C i.m. heating-needle stimulation initiates descending

inhibition alone (You et al., 2014). In addition to the antinociceptive effects of i.m. heating-needle stimulation in animals, it has been demonstrated that 43 ºC i.m. heating-needle stimulation can be considered a promising therapy in control of acute muscle pain in humans (Lei et al., 2017). For clinical applications, exploration of the effect of heating-needle stimulation in various pain models, particularly chronic nociception, has important implications for those developing more effective treatments of intractable pain in various pathophysiological conditions.

ATP has long been recognized as an algogenic agent as indicated by studies, in which administration of ATP evoked pain (Bleehen and Keele, 1977). Both peripheral sensory neurons as well as spinal cord dorsal horn neurons can be depolarized by ATP (Jahr and Jessell, 1985). In terms of the rank order of potency of various ATP congeners, ATP receptors have been classified into two major subsets: the P2X and P2Y receptors (Abbrachio and Burnstock, 1994). To date, seven P2X subunits (P2X1-7) have been further identified, of which P2X3 receptor is considered to be the most potent in the mediation of ATP-driven nociceptive signaling (Barclay et al., 2002). Moreover, there is accumulating evidence indicating that activation of P2X3 receptors at the peripheral or spinal level increases nociceptive behavior in various pain models (Oliveira et al., 2009; Prado et al., 2013; Chen et al., 2016). In contrast, there are studies reporting that activation of P2X3 receptors may have an antinociceptive role at the supraspinal level, i.e. in the thalamus (Gomez-Villafuertes et al., 2001; Fukui et al., 2006; Xiao et al., 2010). To date, potential roles of the P2X3 receptor in endogenous facilitatory and inhibitory modulation of pain induced by

heating-needle stimulation are yet unclear, and remain elusive.

The aim of the present study was twofold. First, we investigated the pain modulatory effect of i.m. heating-needle stimulation in animals with CFA-induced persistent muscle nociception by assessing the noxious stimulation-evoked paw withdrawal reflex as well as hind limb volume. Second, we explored in the CFA model of muscle nociception the role of the purinergic P2X3 receptor in the thalamic MD and VM nucleus in the descending modulation of nociception initiated by i.m. heating-needle stimulation.

EXPERIMENTAL PROCEDURES

Ethical approval and animals

Male Sprague-Dawley rats weighing 260-300 g (10 weeks age) were provided by Animal Center of College of Medicine, Xi'an JiaoTong University, and housed pairwise in plastic boxes under a 12:12 h light dark cycle (lights on at 08:00 AM) at 22-26°C with food and water available *ad libitum*. All experiments were approved by the Animal Care and Use Committee of the University in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The animals were acclimatized to the laboratory and habituated to the test boxes for at least 1 hour each day five days prior to testing. The rats were used only once and sacrificed at the end of the experiment by intraperitoneal (i.p.) injection of an overdose of sodium pentobarbital (200 mg/kg). All efforts were made to minimize the number of animals used and their suffering.

Intramuscular (i.m.) injection of CFA

CFA was injected i.m. at volumes of 50, 100 and 200 μl into the gastrocnemius (GS) muscle of the left (ipsilateral) hind limb in order to induce persistent muscle nociception. The injection site was in the middle part of the GS muscle, and the depth of the injection was about 0.5 cm. The injection procedure was performed manually and lasted more than 30 s. A volume of 200 μl 0.9 % saline served as control in the present study.

I.m. heating-needle stimulation at different temperatures

Rats tested in the present study were anesthetized initially by mask inhalation of isoflurane (4 % isoflurane in 96 % oxygen), followed by 1 % isoflurane in 99 % oxygen for the maintenance of anaesthesia during the i.m. heating-needle stimulation. The heating-needle stimulation was induced by a concentric needle (diameter: 1.05 mm, length: 30 mm) which was connected to and feedback-controlled by an inner heating therapeutic device (Model: NWX-1, Acuceuticals Co., Ltd., Shanghai, China) (Figure 1). The heating-needle was inserted unilaterally into the upper-middle part of GS muscle, and the depth of the insertion was about 0.5 cm. As described previously, for the temperature of heating-needle stimulation was chosen either 43 °C or 46 °C. The 43 °C heating-needle stimulation has been demonstrated to be effective in evoking descending inhibition in animals as well as in humans (You et al., 2014; Lei et al., 2017). By contrast, both descending facilitation and inhibition can be initiated by 46 °C heating-needle stimulation (You et al., 2014). During the experiment, heating-needle stimulation was only performed 4 h after the i.m. injection of CFA, and the durations of heating-needle stimulation were 15 min, 30 min and 45 min at each temperature. Note that the insertation of the concentric needle was about 1.5 cm distance away from the injection site of CFA.

Intracerebral microinjection with P2X3 receptor antagonist

As described elsewhere (You et al., 2013), a craniotomy was made with a dental drill in order to perform the intracerebral (i.c.) cannulation. One guide cannula (OD:

0.35 mm; ID: 0.25 mm; RWD Life Science Co., Shenzhen, China) was advanced stereotactically into the thalamic mediodorsal (MD) or ventromedial (VM) nucleus at the following coordinates: MD nucleus: anteroposterior – (2.3-2.8) mm from bregma, lateral 0.75 mm from midline, dorsoventral 5.2-5.4 mm from the cranium; VM nucleus: anteroposterior –(2.3-2.8) mm, lateral 1.2-1.5 mm from midline, dorsoventral 7.1-7.2 mm from the cranium (Paxinos and Watson, 1998; You et al., 2013). After installation of the guide cannula, the cannula was fixed in the skull with dental cement, the wound was washed with sterile 0.9 % saline, closed and treated with antibiotics. The animals were then put back to the box for a 7 days' recovery, during which the animals' behavior and motor function were monitored. Animals showing severe permanent neurological deficits or motor dysfunction were excluded.

It has been reported that intracerebroventricular administration of P2X3 receptor antagonist A-317491 at dose of 1 nmol reverses antinociceptive effects of a P2X3 agonist (Fukui et al., 2006). In the current study, P2X3 receptor antagonist (Sigma-Aldrich Chemie Gmbh, Taufkirchen, Germany) was freshly prepared at doses of 0.5-4.5 nmol and dissolved in 0.9 % saline. Thirty minutes after the i.m. heating-needle stimulation, a bolus of 0.5 μl solution containing A-317491 was injected through the intrathalamic cannula using a 1 μl microsyringe while the rat was gently restrained by the experimenter's hand. All drugs were slowly infused at a constant speed over a 30 s period. Effects of the drug were tested within 4 h. The mean responses within the 4 h observation period were compared among groups receiving different treatments. After the experiment, the drug injection site was

marked by a microinjection with Pontamine Sky Blue dye (0.5 μl; 2 % in 0.5 M sodium acetate acid).

Experimental design

Animals were randomly divided into different experimental groups, each consisting of 10 rats. The investigator conducting the behavioral measurements was blinded to the experimental group; i.e., the experimenter was not aware whether the studied animal belonged to the "heating-needle stimulation" group or the "needle insertion without heating" group, or whether the animal had received i.c. P2X3 receptor antagonist or 0.9 % saline.

Measurement of mechanical and heat sensitivity

For the measurement of paw withdrawal response evoked by noxious mechanical stimulation, the rat was placed in a plexiglass chamber with a mesh floor underneath and with a transparent top cover (20 × 20 × 25 cm). A hand-held electronic von Frey device (2290 Electrovonfrey®, IITC, Woodland Hills, CA, USA) with a rigid filament was used to detect the mechanical paw withdrawal threshold. According to the mapping of the withdrawal field of the GS muscle (You et al., 2003, 2010), the filament was applied to the heel part of the hind paw until a foot-withdrawal response was elicited, indicating the mechanical threshold (g) or until the cut-off force of 90 g had been achieved.

Noxious heat evoked paw withdrawal response was determined using a 390G plantar stimulator Analgesia Meter (IITC, Woodland Hills, CA). Rat was placed individually into a plexiglass cubicle placed on a constant temperature-controlled transparent glass used to avoid temperature sink from the tested hind paws. The radiant heat stimulus was a high-intensity beam (setting = 30-40 % intensity) aimed at the heel part of the hind paw. The beam intensity selected from the 30-40 % intensity was based on preliminary experiments showing that around 90-95 % rats were in response to such intensity and have 10-11 s withdrawal reflex latency. The same individually-defined intensity was used in all experiments when testing the same rat. The hind paw withdrawal latency was determined as the time from the onset of noxious heat stimulation to withdrawal of the tested hind paw. Latency of the paw withdrawal reflex was initially around 10-11 s, which was considered the baseline response. When the heat stimulus of 10-11 s duration was applied to the experimenter's hand, it generated a painful, but tolerable sensation. To avoid excessive tissue injury, cut-off latency was 20 s.

The paw withdrawal thresholds to noxious mechanical and heat stimuli were tested for both the ipsilateral and contralateral heel part of the hind paw 30 minutes prior to and 30 minutes, 1-4 hours, and 1-14 days after the i.m. administration of CFA with or without accompanying heating-needle stimulation. At each time point, hind paws were tested bilaterally three times, with at least a 30-s interval between subsequent trials. The mean of the three test trials at each time point represented the mechanical paw withdrawal threshold (g) and thermal paw withdrawal latency (s). A reduced

mechanical threshold was considered to represent mechanical hyperalgesia and an increased mechanical threshold was considered to represent mechanical hypoalgesia. With heat-evoked withdrawal latency, an increase in latency was considered to represent heat hypoalgesia and a decrease heat hyperalgesia.

Since mechanical and heat nociception were determined in a skin area (hind paw) that was not directly influenced by CFA injection into the GS muscle, the CFA-induced changes in mechanical and heat sensitivity were considered to be centrally mediated. Therefore, the changes in nociception observed by assessing noxious test stimulation-induced withdrawal reflexes in the present study represent secondary hypo- or hyperalgesia.

Assessment of hind limb volume

In the present study, change in the limb volume was used as an index of the CFA-induced inflammatory response. Based on the principle of Archimedes, the limb volume was measured at knee level by water displacement using a plethysmometer (Woodland Hills, CA, USA). Limb volume measurements were performed bilaterally at 30 min prior to and 30 min, 4 h and 1-14 days after the unilateral i.m. injection of CFA.

Assessment of motor function

Briefly, animals were placed on a Rota-Rod treadmill (Model 755, IITC, Woodland Hills, CA, USA) rotating at a gradually increasing speed from 5 to 30 rpm

for 30 s and maintained for another 120 s at 30 rpm. Rats with a motor dysfunction (drop latency < 5 s) after the implantation of i.c. guide cannula were excluded from the remaining experiments.

Histology for identification of the placement of the cannula

At the end of behavioral testing, the animals receiving implantation of i.c. cannula were anaesthetized by sodium pentobarbital (50 mg/ kg, i.p.) and transcardially perfused with 10 % formalin. The brains were then isolated and stored in 30 % sucrose for 2 days. Freezing serial sections (50 μm thickness) were cut in the coronal plane and stained with Nissl stain, and were screened under a microscope (Leica, Germany). Schematic reconstruction of the injection sites was drawn according to the stereotaxic atlas of rats (Paxinos and Watson, 1998). In brain, microinjection of 0.5 μl solution of Pontamine Sky Blue spread from the injection site about 0.4 mm. Nearby structures in the brain were checked in both the coronary and the sagittal plane. The histological determination of the location of the cannula tip was performed without knowledge of the behavioral results.

Statistical analysis

All results were expressed as means ± SEM. The data were analyzed using SigmaStat^{TM} (Systat Software Inc., California, USA) and using either one-way analysis of variance (one-way ANOVA) or two-way repeated measures ANOVA (two-way RM ANOVA) followed by a post-hoc Bonferroni t-test. $P < 0.05$ was

considered statistically significant.

RESULTS

Paw withdrawal reflex to mechanical and heat stimulation, and limb swelling following i.m. injection of CFA

As shown in figure 2, paw withdrawal reflexes to noxious mechanical and heat stimuli were evaluated bilaterally 30 minutes prior to and 30 min, 1-4 h, 1-7 days, 10 days, and 14 days after the i.m. injection of CFA at volumes of 50-200 μl. The unilateral injection of 50 μl CFA into the GS muscle failed to cause any significant changes in mechanically evoked paw withdrawal reflexes ipsi- or contralaterally, whereas i.m. injections of 100 μl and 200 μl CFA induced bilaterally long-lasting (14 days) secondary mechanical hyperalgesia (Fig. 2a. $P < 0.05$, two-way ANOVA). The onset of secondary mechanical hyperalgesia was 1 h or 2 h after the i.m. injection of 200 μl or 100 μl CFA, respectively. In contrast, significant bilateral prolongations of the heat-evoked paw withdrawal latency (secondary heat hypoalgesia) were observed after the i.m. injections of CFA at 3 different volumes. The earliest onset (1 h) and the longest (14 days) duration in the prolongation of the heat-evoked paw withdrawal latency was found in rats treated i.m. with 200 μl of CFA (Fig. 2b. $P < 0.05$, two-way ANOVA).

After the i.m. injection of either 100 μl or 200 μl, but not 50 μl of CFA, significant limb swelling with an onset of one day and with the duration over 14 days was observed in the injected limb (Fig. 3. $P < 0.05$, two-way ANOVA). No significant changes in limb volume were found at the contralateral side following the i.m. injection of saline or CFA at different volumes (Fig. 3. $P > 0.05$, two-way ANOVA).

In the saline control group, neither secondary mechanical hyperalgesia, secondary heat hypoalgesia nor limb swelling was found at any time point after the i.m. injection of 200 μl of 0.9 % saline (Figs. 2-3).

Effects of 43℃ and 46℃ heating-needle stimulation on noxious stimulation-induced withdrawal responses in animals with persistent CFA-induced muscle nociception

In this experiment, persistent muscle nociception was induced unilaterally by i.m. administration of CFA at a volume of 200 μl. Heating-needle stimulation at 43 ºC for 15-45 min in the limb ipsilateral to the CFA treatment had no significant effects on secondary mechanical hyperalgesia induced by CFA either in the ipsi- or contralateral hind paw (Fig. 4a; $P > 0.05$, two-way ANOVA). In contrast, significantly enhanced secondary heat hypoalgesia was observed following i.m. heating-needle stimulation at 43 °C for 30-45 min. The enhancement of heat hypoalgesia occurred 1 day after the heating-needle stimulation and lasted for 5 days (Fig. 4b; $P < 0.05$, two-way ANOVA).

Figure 5 illustrates the effects of 46 °C heating-needle stimulation of varying duration on nociceptive withdrawal reflexes in animals with persistent CFA-induced muscle nociception. Heating-needle stimulation of 46 ºC for 15 min had no significant effect on secondary mechanical hyperalgesia in either the ipsi- or contralateral test site (Fig. 5a; $P > 0.05$). In contrast, 46 °C heating-needle stimulation lasting for 30-45 min significantly enhanced secondary mechanical hyperalgesia bilaterally, and this facilitatory effect lasted for 6 days during the 14 days observation period (Fig. 5a; $P < 0.05$, two-way ANOVA).

Heating-needle stimulation of 46 ºC for 15-45 min enhanced secondary heat hypoalgesia bilaterally in animals with persistent CFA-induced muscle nocicepton

(Fig. 5b; $P < 0.05$, one-way ANOVA). When the heating needle stimulation of 46 ºC lasted 30-45 min, a significant enhancement of secondary heat hypoalgesia was observed bilaterally up to 5 days (Fig. 5b).

In the present study, heating-needle stimulation for a period of 45 min, but without heating, was used as a control stimulus. The insertion of the needle without heating had no effects on the CFA-induced bilateral mechanical hyperalgesia or heat hypoalgesia (see "CFA + 45 min N.S."-group in Figs. 4 & 5).

Effects of 43℃ and 46℃ heating-needle stimulation on hind limb swelling induced by i.m. administration of CFA

The effect of heating-needle stimulation at 43 ºC and 46 ºC on the CFA-induced increase in the limb volume was assessed with a plethysmometer. Limb volume was assessed bilaterally, while heating-needle stimulation was applied unilaterally in the limb treated with 200 µl of CFA. Heating-needle insertion without heating was used as a control stimulus for a period of 45 min. CFA caused swelling only in the ipsilateral limb. Control stimulation (heating-needle without heating), heating-needle stimulation of 43 ºC for 15 min, or heating-needle stimulation of 46 ºC for 15-45 min had no effects on the CFA-induced swelling (Fig. 6). In contrast, heating-needle stimulation of 43 ºC for 30-45 min significantly reduced swelling in the CFA-treated limb from the first post-treatment day until the 14th post-treatment day (Fig. 6a; $P < 0.05$, two-way ANOVA).

Influence of i.c. microinjection of P2X3 antagonist into the thalamic MD and VM nuclei on the 43 °C/46 °C heating-needle stimulation-induced effects in animals with CFA-induced persistent nociception

Figure 7 shows the schematic reconstructions of microinjection sites of thalamic MD (n=80) and VM nuclei (n=80), which were contralateral to the heating-needle stimulation as well as to the CFA treatment (200 µl). As shown in figure 8a, i.c. microinjection of P2X3 receptor antagonist A-317491 into the contralateral thalamic MD nucleus of CFA-treated animals dose-dependently (0.5-4.5 nmol/0.5 µl) reduced the facilitation of secondary mechanical hyperalgesia elicited by 46 °C heating-needle stimulation of 30 min duration ($P < 0.05$, one-way ANOVA). In contrast, administration of A-317491 (0.5-4.5 nmol/0.5µl) into the MD nucleus of CFA-treated animals had no effect on the enhancement of secondary heat hypoalgesia induced by heating-needle stimulation at the temperature of 43°C or 46 °C for 30 min (figure 8b).

Microinjections of A-317491 into the contralateral VM nucleus failed to influence secondary mechanical hyperalgesia, independent whether the CFA-treatment was accompanied by heating-needle stimulation at the temperature of 43 ºC or 46 ºC for 30 min (Figure 8c; $P > 0.05$, one-way ANOVA). However, the enhancement of secondary heat hypoalgesia caused by heating-needle stimulation at the temperature of 43 °C and 46 °C for 30 min was attenuated by administration of A-317491 into the VM nucleus of CFA-treated animals in a dose-dependent manner (Figure 8d. $P < 0.05$, one-way ANOVA).

We also investigated whether microinjection of A-317491 into the thalamic MD and VM nuclei influences CFA-induced hind limb swelling. In contrast to the effects of A-317491 on modulation of nociceptive behavior, pharmacological blocking of the P2X3 receptor in the VM or MD nucleus had no effects on modulation of the CFA-induced hind limb swelling by heating-needle-stimulation at the temperature of 43 °C or 46 °C at any time point during the observation period (data not shown).

DISCUSSION

The present study showed that i.m. injection of CFA elicited dose-dependently and bilaterally persistent secondary mechanical hyperalgesia and secondary heat hypoalgesia that were accompanied by unilateral limb swelling. These effects lasted at least for 2 weeks. Unilateral i.m. heating-needle stimulation at the temperature of 43 ºC significantly enhanced the CFA administration-induced secondary heat hypoalgesia bilaterally, whereas heating-needle stimulation at the temperature of 46 ºC strengthened both secondary mechanical hyperalgesia and secondary heat hypoalgesia bilaterally. The present pharmacological intervention further showed that purinergic P2X3 receptors within the thalamic MD nucleus and VM nucleus have distinct roles in the enhanced descending inhibition of heat nociception induced by heating-needle stimulation at the temperature of 43 ºC or 46 ºC, and in the enhanced descending facilitation of mechanically evoked nociception induced by heating-needle stimulation at the temperature of 46 ºC. In contrast to the changes in descending modulation of nociception, pharmacological blocking of thalamic P2X3 receptors did not influence the decrease in the CFA-induced limb swelling induced by heating-needle stimulation at the temperature of 43°C. This finding suggests that the anti-inflammatory-like effect of heating-needle stimulation on limb swelling dissociates from central mechanisms underlying modulation of nociception by heating-needle stimulation.

Intramuscular CFA-induced muscle nociception and limb swelling

Injection of adjuvant-induced tissue inflammation is a common and validated technique in arthritis research (Pearson, 1956; Gregory et al., 2013). In the current study, i.m. injections of 100 µl or 200 µl CFA into the GS muscle induced dose-dependently a significant and long-lasting limb swelling at the injection side. The currently used i.m. CFA-induced inflammation model was similar to that in rats exposed to intradermal, subcutaneous or i.m. injections of CFA in earlier studies (Glenn and Grey, 1965; Holmdahl et al., 1992; Almarestani et al., 2008; Schomburg et al., 2015). Following the i.m. injection of CFA into the GS muscle, limb swelling was found only at the ipsilateral injection side, but not the contralateral side. A dose of CFA that is considerably higher than used in the present study leads initially to unilateral paw inflammation and acute arthritis, followed by contralateral paw swelling and a rheumatoid arthritis-like disease that affects multiple joints and organs (Philippe et al., 1997; Schoft et al., 2006). When a low dose of CFA has been injected, as in the present study, hind paw edema and arthritis have been strictly unilateral, with no obvious signs of contralateral or systemic changes (Bertorelli et al., 1999).

In the present study, unilateral i.m. administration of CFA induced muscle nociception that was accompanied bilaterally by secondary mechanical hyperalgesia and heat hypoalgesia as shown by paw withdrawal reflexes evoked by noxious mechanical stimulation and heat. These results are consistent with those from our previous studies showing that i.m. hypertonic saline (5.8 %) in the tibialis anterior muscle in humans and i.m. 5.8 % saline or 2.5 % formalin in the GS muscle in rats induce bilaterally secondary mechanical hyperalgesia and secondary heat hypoalgesia

(Lei et al., 2008, 2011, 2017; Lei and You, 2012, 2013; You et al., 2010, 2013). From physiological perspective, it is well established that the somatosensory and autonomic nervous systems are under tonic control by various supraspinal centers (Jänig, 2006). With regard to control of nociception, a series of studies by us revealed that both descending facilitation and inhibition of nociception are inactive or relatively 'silent' during the physiological state, and need to be triggered by sufficient activity in C-fiber afferents (Lei et al., 2011, 2017; You et al., 2010; 2013, 2016). Importantly, the triggering threshold of endogenous descending facilitation is significantly lower than that of descending inhibition (You et al., 2010, 2013, 2014). Following i.m. administration of 5.8 % saline, descending facilitation causing bilaterally secondary mechanical hyperalgesia had early onset (5-10 min), whereas descending inhibition leading bilaterally to secondary heat hypoalgesia was observed not earlier than 1 day after i.m. injection of 5.8% saline (Lei et al., 2011; You et al., 2010, 2013). Electrophysiological studies have demonstrated that peripheral C-fiber afferents are recruited by CFA (Djouhri et al., 2006) as well as by hypertonic saline (Kumazawa et al., 1987). It may be speculated that the afferent barrage in high-threshold C-fibers that presumably triggers the descending inhibition is stronger after i.m. treatment with CFA than hypertonic saline. This would explain the shorter onset in the enhancement of secondary heat hypoalgesia following CFA than hypertonic saline treatment (1 h in the present study versus 1 day in You et al., 2010, 2013, respectively).

Effects of heating-needle stimulation induced descending modulation on nociception and limb swelling in CFA-treated animals

Assuming that C fibers were critical for recruiting descending pain inhibition, it might not be possible to activate descending inhibition alone without initiation of descending facilitation, since descending facilitation has a lower triggering threshold. However, a subpopulation of C fibers consists of low-threshold mechanoceptors and thermoceptors, which can be activated by innocuous stimuli (Besson and Chaouch, 1987). Using simultaneous electrophysiological recordings at multiple levels of the neuraxis, we recently explored responses in the circuitry consisting of "the spinal dorsal horn neuron – the thalamic MD/VM nucleus neuron – the gastrocnemius (GS) muscle electromyography (EMG)". The recordings were performed with and without i.m. heating-needle stimulation at the temperature of 43°C and 46°C. We found that the activity of spinal wide-dynamic range (WDR) and that of thalamic MD/VM nuclei neurons associated with the GS muscle EMG responses during heating-needle stimulation at the temperature of 46 °C. In contrast, spinal WDR neurons and thalamic VM nucleus neurons responded to i.m. heating-needle stimulation at the temperature of 43°C (unpublished data). Our previous studies demonstrated that i.m. heating-needle stimulation at temperatures lower than 45 °C (e.g., 43 °C) may bypass the lower triggering threshold of descending facilitation, and thereby selectively recruit descending inhibition that otherwise has a higher triggering threshold in humans as well as in animals (You et al., 2014; Lei et al., 2017). The current results are in line with this by showing that a period of 30-45 min of 43 °C heating-needle

stimulation prolonged noxious heat-evoked paw withdrawal latencies bilaterally, without any significant changes in mechanically induced paw withdrawal reflexes. In contrast, a period of 30-45 min of 46 °C heating-needle stimulation enhanced bilaterally both secondary mechanical hyperalgesia and secondary heat hypoalgesia. These findings are consistent with the hypothesis that 43 °C heating-needle stimulation could be an effective way for activation of primary afferent C-fibers and triggering selectively thalamus-mediated descending inhibitory controls. From clinical perspective, the present results are in line with the proposal that warm heating-needle stimulation or moxibustion, a traditional Chinese therapy method presumably activating a similar mechanism as warm heating-needle stimulation, may provide useful alternative treatments for alleviating pathophysiological pain, without adverse effects of analgesic drugs.

One interesting finding from the present study is that the CFA-induced hind limb swelling was significantly attenuated by i.m. heating-needle stimulation at the temperature of 43 °C, but not 46 °C. In addition, neither the CFA-induced limb swelling nor its reduction by heating-needle stimulation at the temperature of 43 ºC was influenced by the thalamic administration of a P2X3 receptor antagonist, unlike descending modulation of nociception by heating-needle stimulation. This finding indicates that the mechanisms underlying descending modulation of nociception by heating-needle stimulation dissociate, at least partly, from those underlying the reduction of limb swelling (anti-inflammatory-like effect) induced by the heating-needle stimulation. It remains to be studied whether the

anti-inflammatory-like effect of heating-needle stimulation on limb swelling involves central mechanisms, such as hypothalamus-mediated control of sympathetic activity (Jänig, 2006; Lei et al., 2008), or whether the decrease of limb swelling may be explained by peripheral mechanisms.

Central P2X3 receptor in heating-needle stimulation-induced descending modulation of nociception

It is well known that Adenosine 5'-triphosphate (ATP) is an intracellular energy source and an important neurotransmitter released by various neuronal and non-neuronal cells (Koles et al., 2007). The purinergic P2X3 receptor is one subunit of the seven P2X channels (P2X1-P2X7). It is mainly expressed on primary afferent neurons and especially on nociceptive C-fibers (Burnstock, 2015). The release of ATP induced by cell stress, lysis or stimulation may activate P2X3 receptors on sensory afferents to induce pain (Kennedy and Leff, 1995). Activation of P2X3 receptors at the peripheral or spinal levels has increased nociceptive behavioral responses (Jarvis et al., 2002; Prado et al., 2013; Chen et al., 2015). While the P2X3 receptor has predominantly a pronociceptive role in the periphery, activation of the P2X3 receptor at supraspinal levels has been reported to be involved in descending inhibition and exerting an analgesic effect (Fukui et al., 2004; Xiao et al., 2010). In the current study, microinjection of A-317491, a P2X3 receptor antagonist, into the thalamic MD nucleus significantly attenuated descending facilitation induced by heating-needle stimulation at the temperature of 46 °C, whereas administration of A-317491 into the

thalamic VM nucleus reduced descending inhibition induced by heating-needle stimulation both at the temperature of 43 °C and 46 °C. It has been demonstrated that descending facilitatory and inhibitory modulation of nociception are strengthened by glutamate and weakened by GABA microinjected into the thalamic MD and VM nuclei, respectively (You et al., 2013). These results together with the present findings suggest that the role of thalamic P2X3 receptors in modulation of pain or nociception varies with the specific thalamic nucleus and its function (You et al., 2013).

From signaling perspective, there are reports showing complex roles of noradrenergic, serotonergic, and non-dopaminergic (i.e. opioid) systems in the control of thalamus-organized descending modulation of nociception (Lei et al., 2011; You et al., 2010, 2014, 2016). To elucidate this issue, additional experiments addressing interactions between purinergic P2X3 receptor and classic signalling pathways in descending modulation of pain are required in the future.

CONCLUSION

I.m. injection of CFA induced in a dose-related fashion persistent muscle nociception that was accompanied by hind limb swelling. Heating-needle stimulation both at the temperature of 43 °C and 46 °C increased bilaterally secondary heat hypoalgesia (descending inhibitory effect on heat nociception), while heating-needle stimulation at the temperature of 46 °C increased bilaterally secondary mechanical hyperalgesia (descending facilitatory effect on mechanical nociception). Thalamic P2X3 receptors were involved in both descending facilitation and inhibition, thereby

showing a paradoxical role in control of nociception. Concerning potential clinical applications, i.m. heating-needle stimulation at a non-painful temperature induced descending inhibition through a circuitry that involves thalamic P2X3 receptors, providing a promising alternative therapy for control of pathological intractable pain, in particular soft tissue pain, without adverse effects of analgesic drugs.

Conflict of interest statement

The authors declare no personal or financial conflict of interests.

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Legends to figures

Fig. 1. Schematic diagram of composition of heating-needle controlled by an inner heating therapeutic device. The heating-needle (about 1.05 mm in diameter) designed for rats is made of stainless steel, and filled with heating element and temperature probe, which detects variations of temperature precisely (± 0.25°C).

Fig. 2. Paw withdrawal responses evoked by mechanical or heat stimulation of the hind paw at various time points after intramuscular (i.m.) injection of various doses of CFA or 0.9 % saline (200 μl) into the GS muscle. Left panel shows responses ipsilateral to the CFA treatment and right panel shows responses contralateral to the CFA treatment. * $P < 0.05$ and # $P < 0.05$ compared with rats treated with 0.9 % saline. (B: baseline response before the i.m. injection) (n = 10 for each group).

Fig. 3. Hind limb volume before and at various time points after unilateral intramuscular (i.m.) injection of either 0.9 % saline or different doses of CFA into the GS muscle. The left graph shows limb volume ipsilateral to the CFA treatment, and the right graph shows limb volume contralateral to the CFA treatment. * $P < 0.05$ and # $P < 0.05$ compared with rats treated with 200 μl 0.9 % saline. (B: baseline response before the i.m. injection) (n = 10 for each group).

Fig. 4. Effect of heating-needle stimulation (H.N.S.) at the temperature of 43 ºC on mechanically (upper graphs) and heat-evoked (lower graphs) hind paw withdrawal responses in animals treated with intramuscular (i.m.) administration of 200 μl of

CFA. Left panel shows responses ipsilateral to the CFA treatment and heating needle stimulation, and right panel shows responses contralateral to the CFA treatment and heating-needle stimulation. The duration of the heating-needle stimulation was 15 min, 30 min, and 45 min. * $P < 0.05$ compared with rats treated by the i.m. injection of CFA alone. (B: baseline response before the heating-needle stimulation; N. S.: needle stimulation without heating) (n = 10 for each group).

Fig. 5. Effect of heating-needle stimulation (H.N.S.) at the temperature of 46 ºC on mechanically (upper graphs) and heat-evoked (lower graphs) hind paw withdrawal responses in animals treated with intramuscular (i.m.) administration of 200 µl of CFA. Left panel shows responses ipsilateral to the CFA treatment and heating needle stimulation, and right panel shows responses contralateral to the CFA treatment and heating-needle stimulation. The duration of the heating-needle stimulation was 15 min, 30 min, and 45 min. * $P < 0.05$ compared with rats treated by the i.m. injection of CFA alone. (B: baseline response before the heating-needle stimulation; N. S.: needle stimulation without heating) (n = 10 for each group).

Fig. 6. Effects of heating-needle stimulation (H.N.S.) at the temperature of either 43 °C or 46 °C on the hind limb volume. Heating-needle stimulation was given ipsilateral to the intramuscular (i.m.) treatment with 200 µl of CFA. * $P < 0.05$ compared with the group of CFA rats. (B: baseline response before the heating-needle stimulation; N.S.: needle stimulation without heating) (n = 10 for each group).

Fig. 7. Reconstructions of the locations of 160 microinjection sites within the thalamic mediodorsal (MD) and ventromedial (VM) nuclei. The spread of microinjections is marked by gray areas. Effective sites for microinjection of 0.9 % saline, 0.5 nmol, 1.5 nmol, and 4.5 nmol of the P2X3 receptor antagonist A-317491 are indicated by circles, up-triangles, squares, and diamonds, respectively. Note that some microinjection sites overlap with others in this schematic drawing.

Fig.8. Effects of microinjections with 0.9 % saline, and 0.5, 1.5 and 4.5 nmol of the P2X3 receptor antagonist A-317491 into the contralateral thalamic MD nucleus (panels ‘a’ and ‘b’) or VM nucleus (panels ‘c’ and ‘d’) on modulation of the mechanically and heat-evoked paw withdrawal responses by 30 min of heating-needle stimulation at the temperature of 43 °C. In each graph, '% of response' means a percentage change from the baseline response obtained in each experimental condition one day after the intramuscular (i.m.) treatment of GS muscle with 200 µl of CFA (see for comparison, time point 1d in Figs. 3-5). Decreases in the responses below 100 % represent changes towards antinociception (or attenuation of hyperalgesia), whereas increases in the responses above 100 % represent changes towards pronociception (or attenuation of hypoalgesia). Graphs show medians, the boxes extend from the 25th to the 75th percentile, and the whiskers extend from the smallest to the largest value (n = 10 in each group). * $P < 0.05$ and ** $P < 0.001$ compared with CFA rats treated by the heating-needle stimulation (H.N.S.) alone.

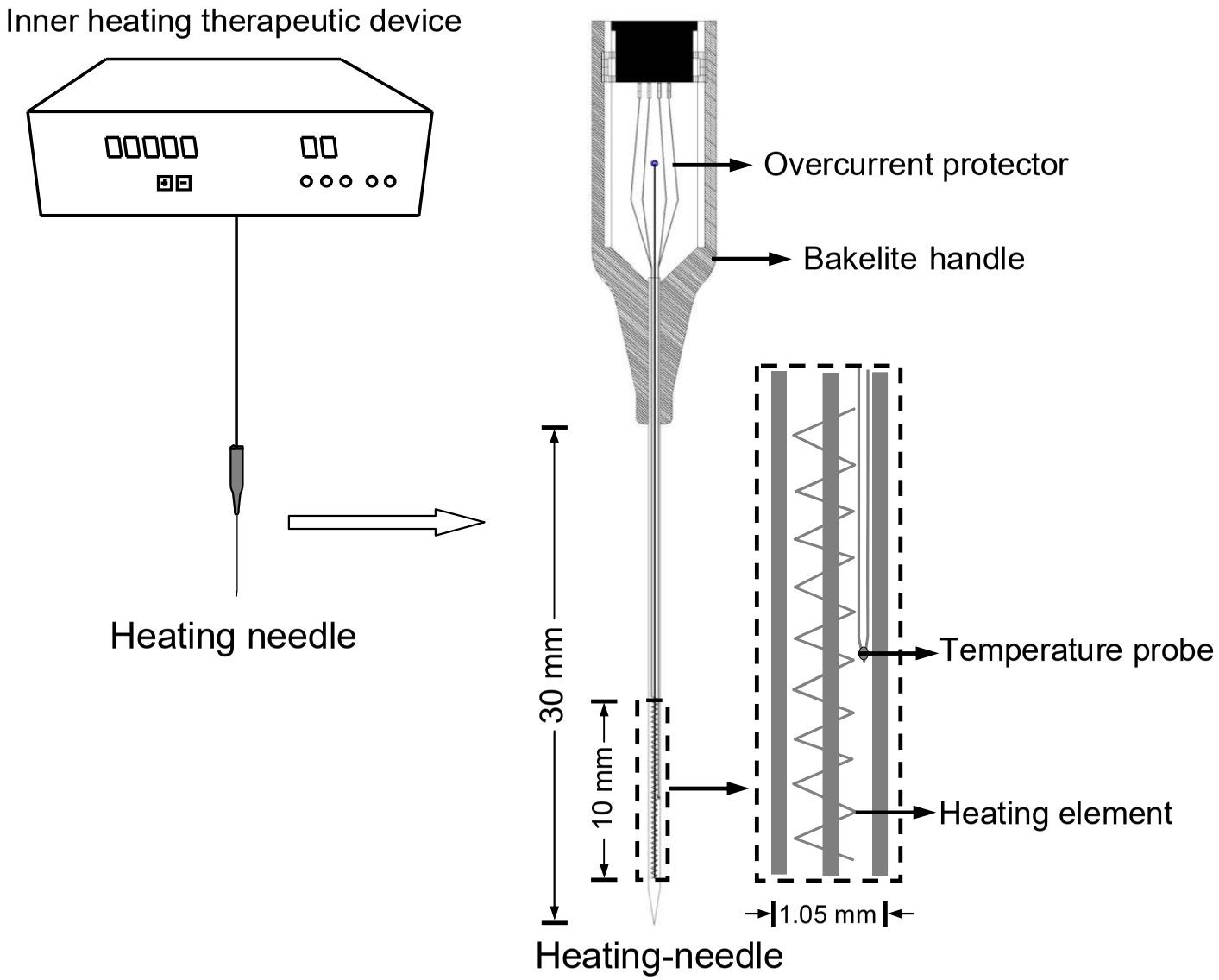


Figure 1. Lei et al., 2019

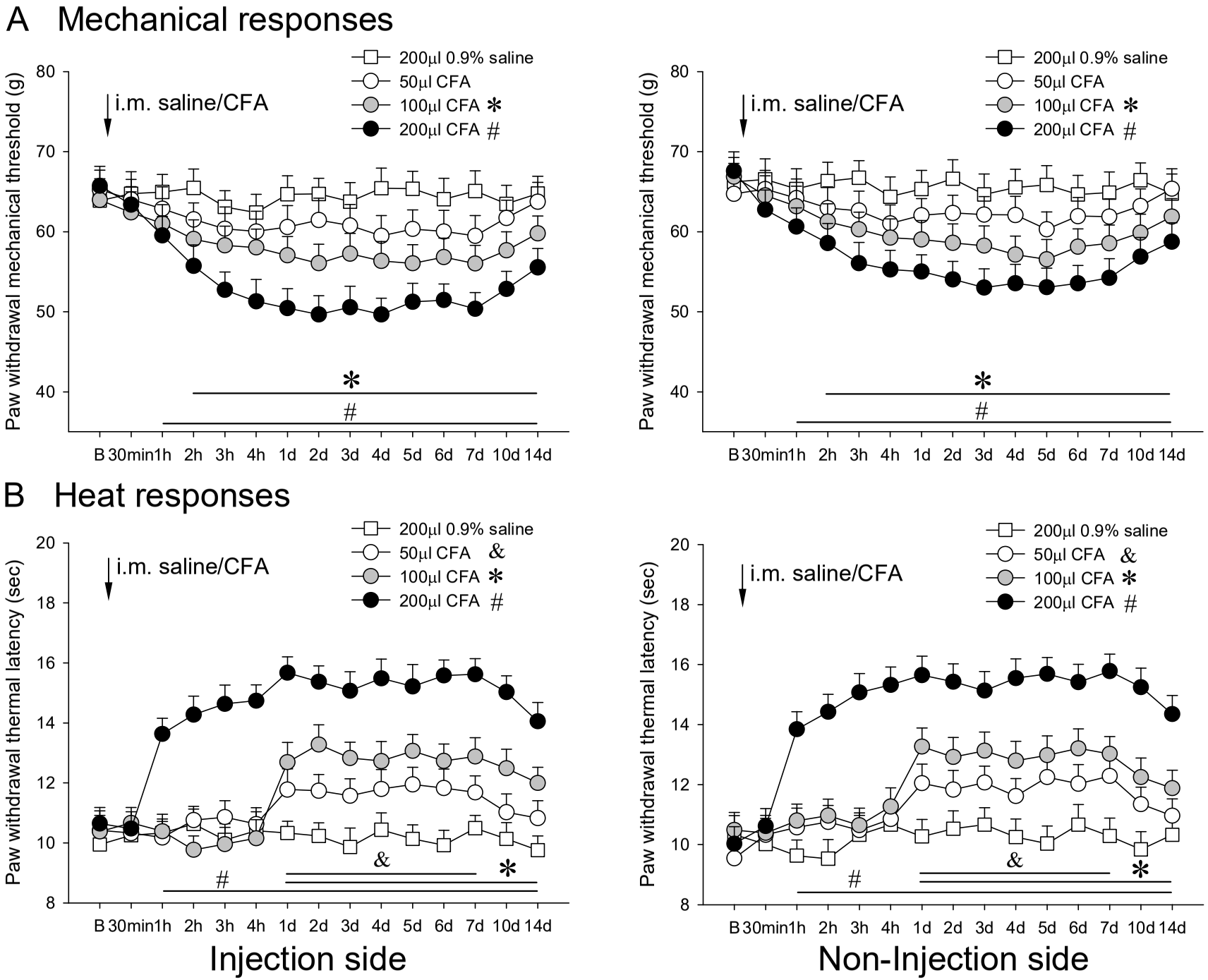
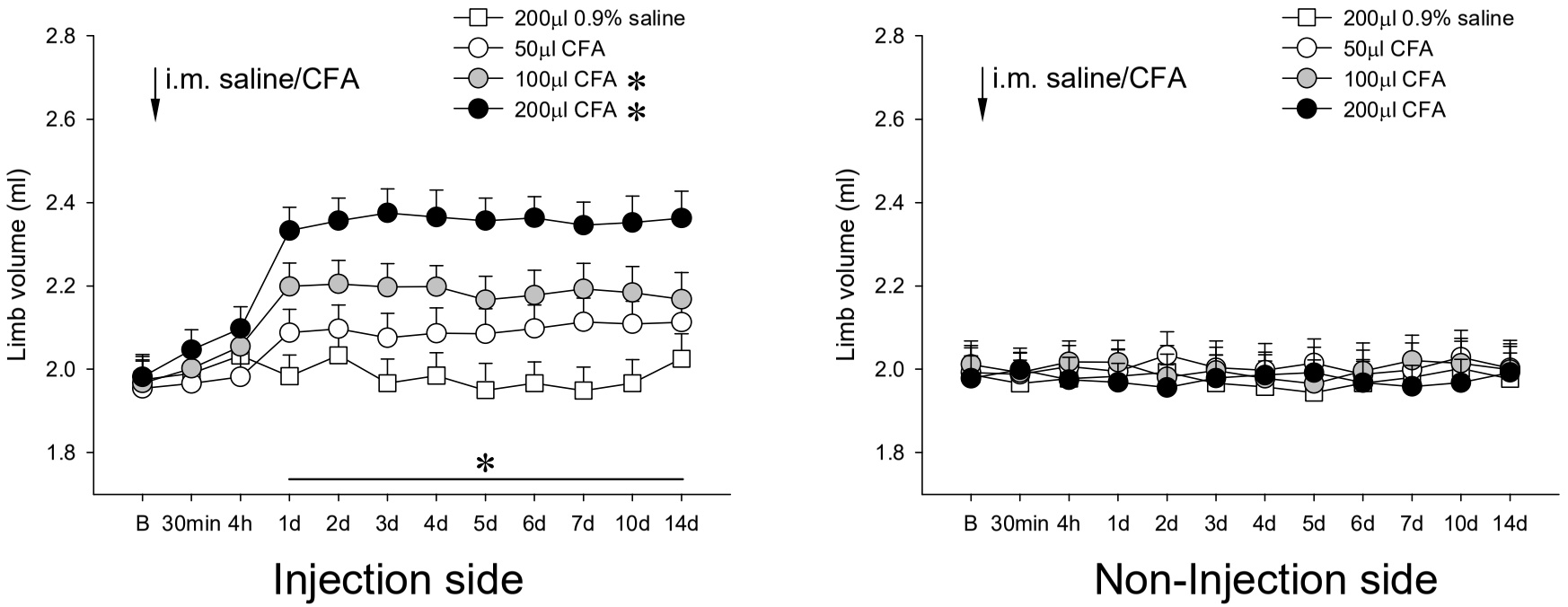


Fig. 2 Lei et al., 2019



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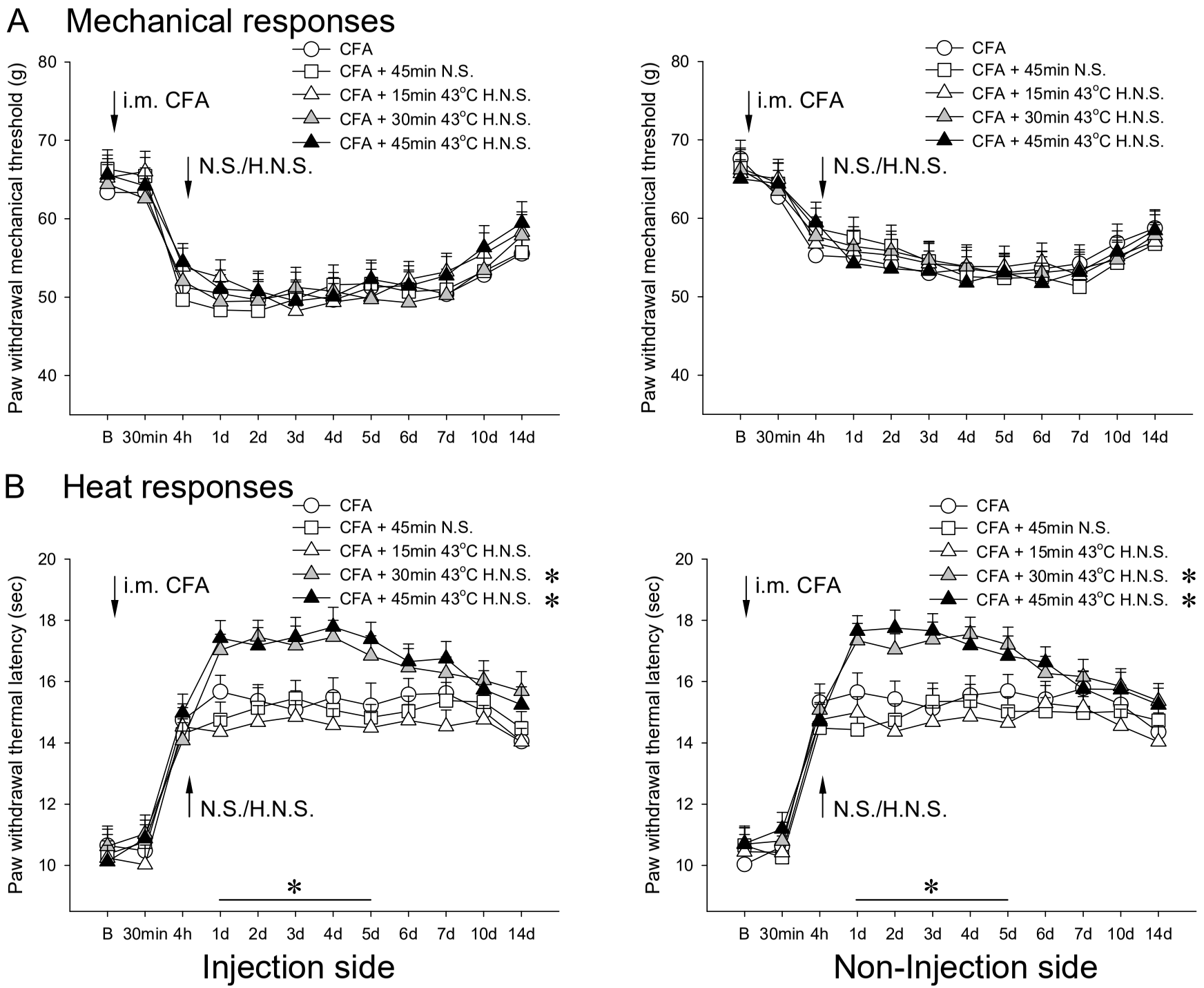

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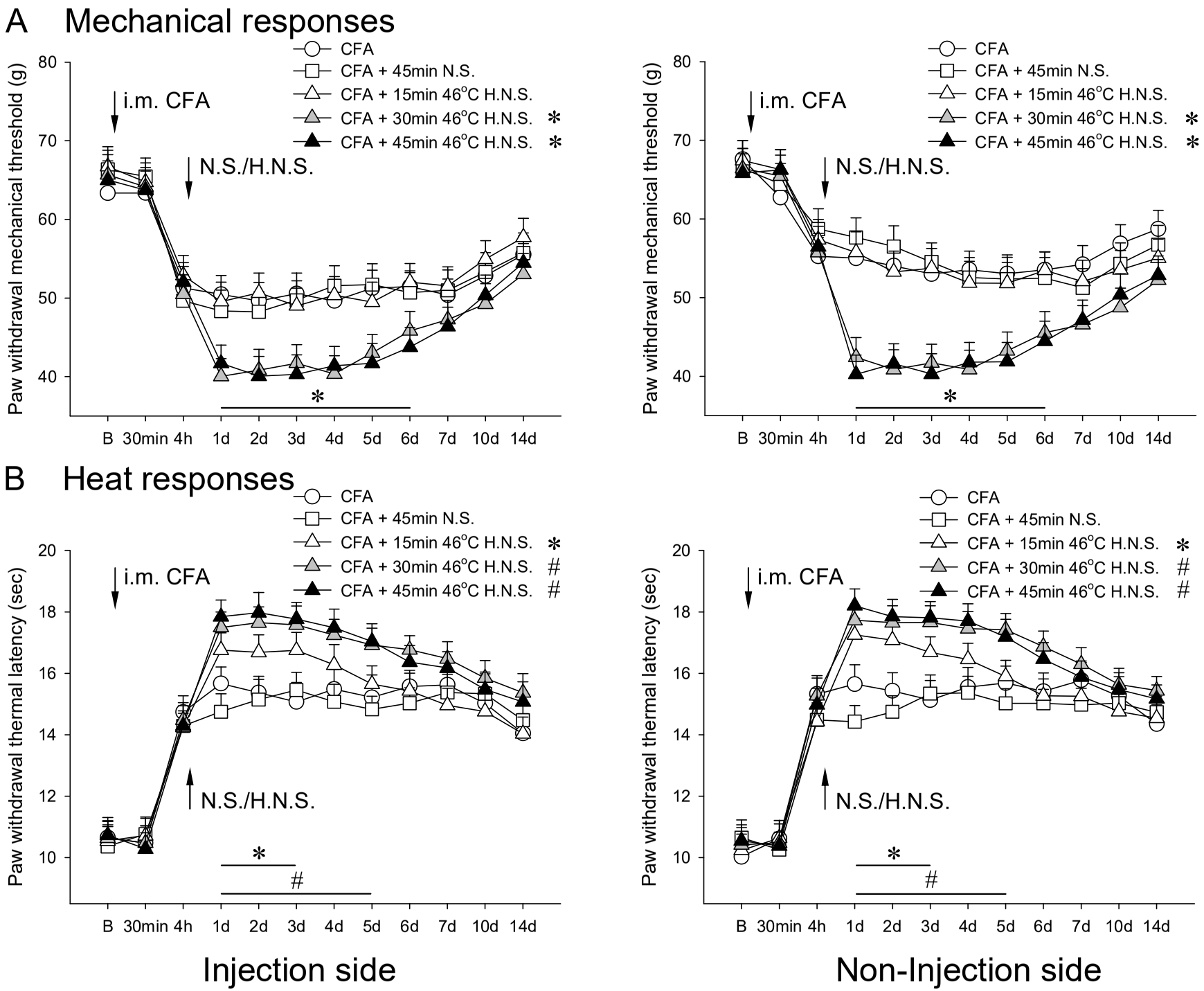

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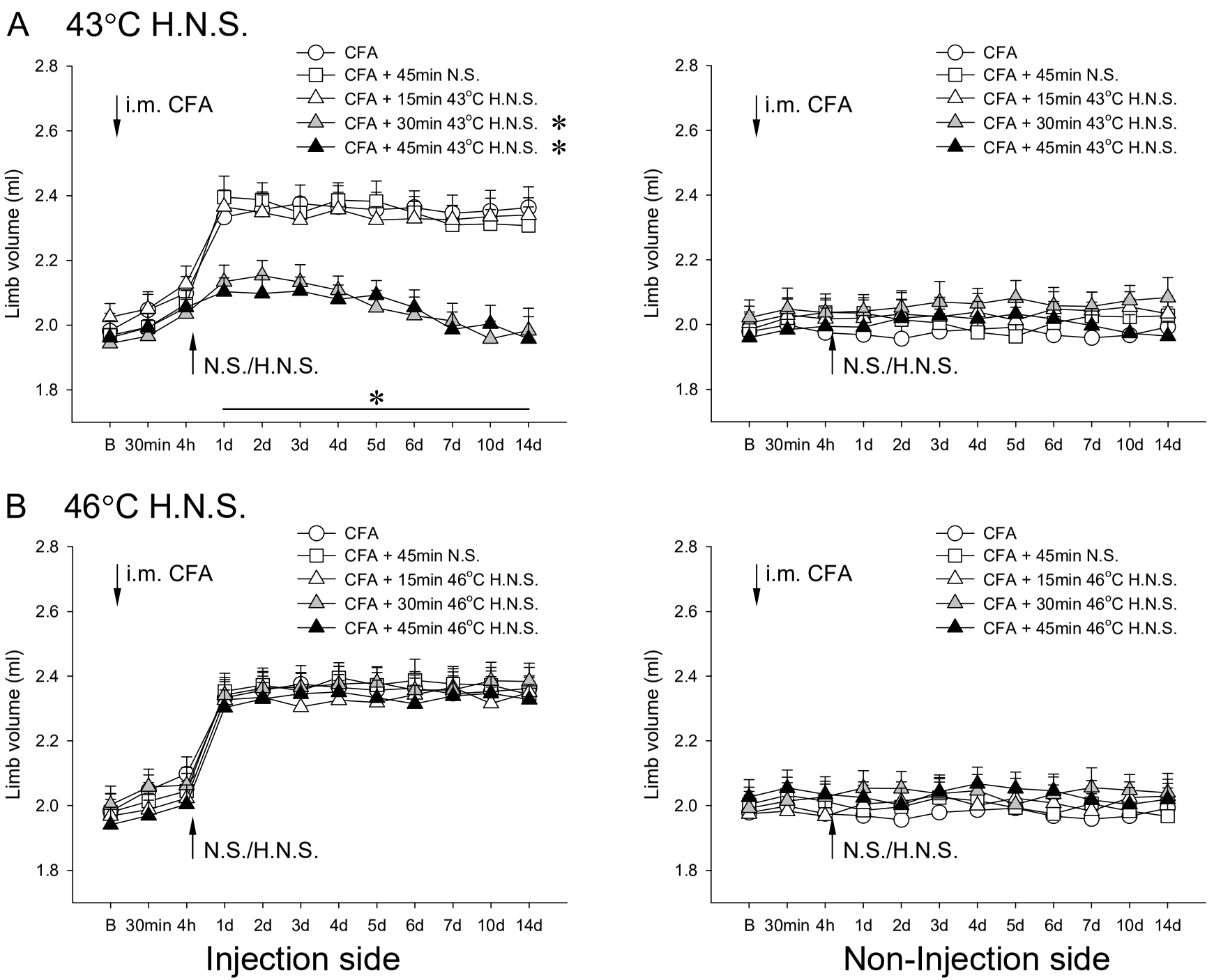

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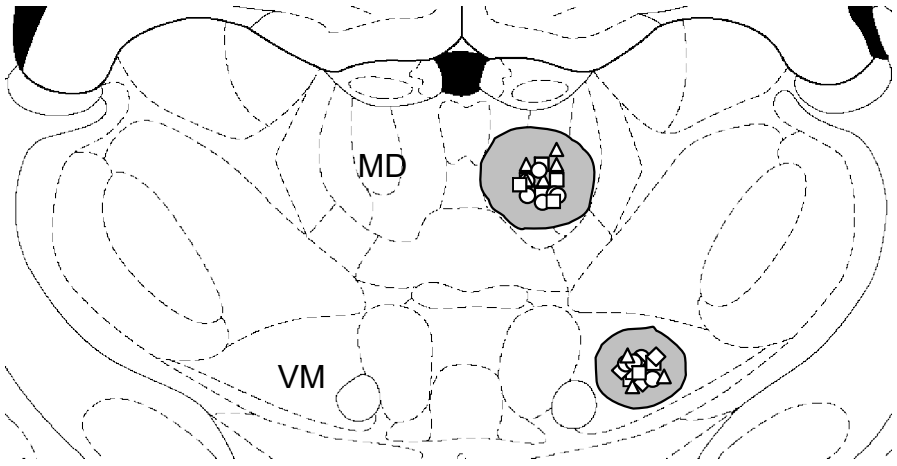
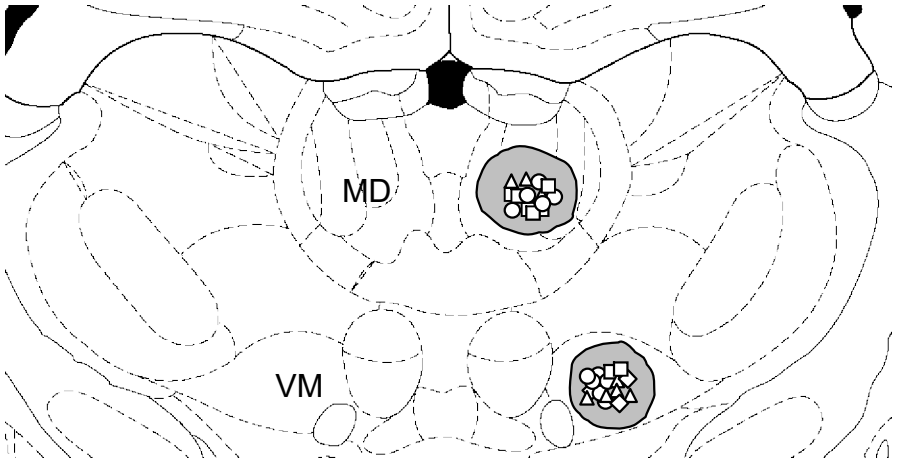
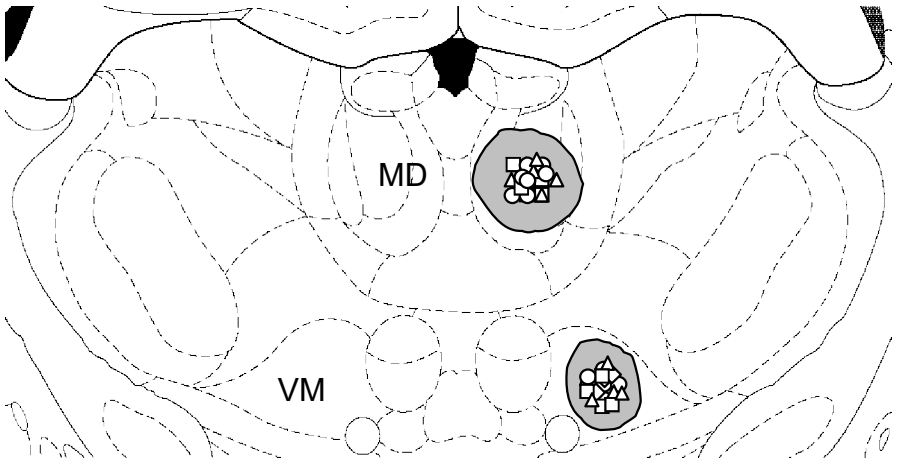


Bregma -2.3 mm



Bregma -2.56 mm



Bregma -2.8 mm

1 mm

Fig. 7 Lei et al., 2019

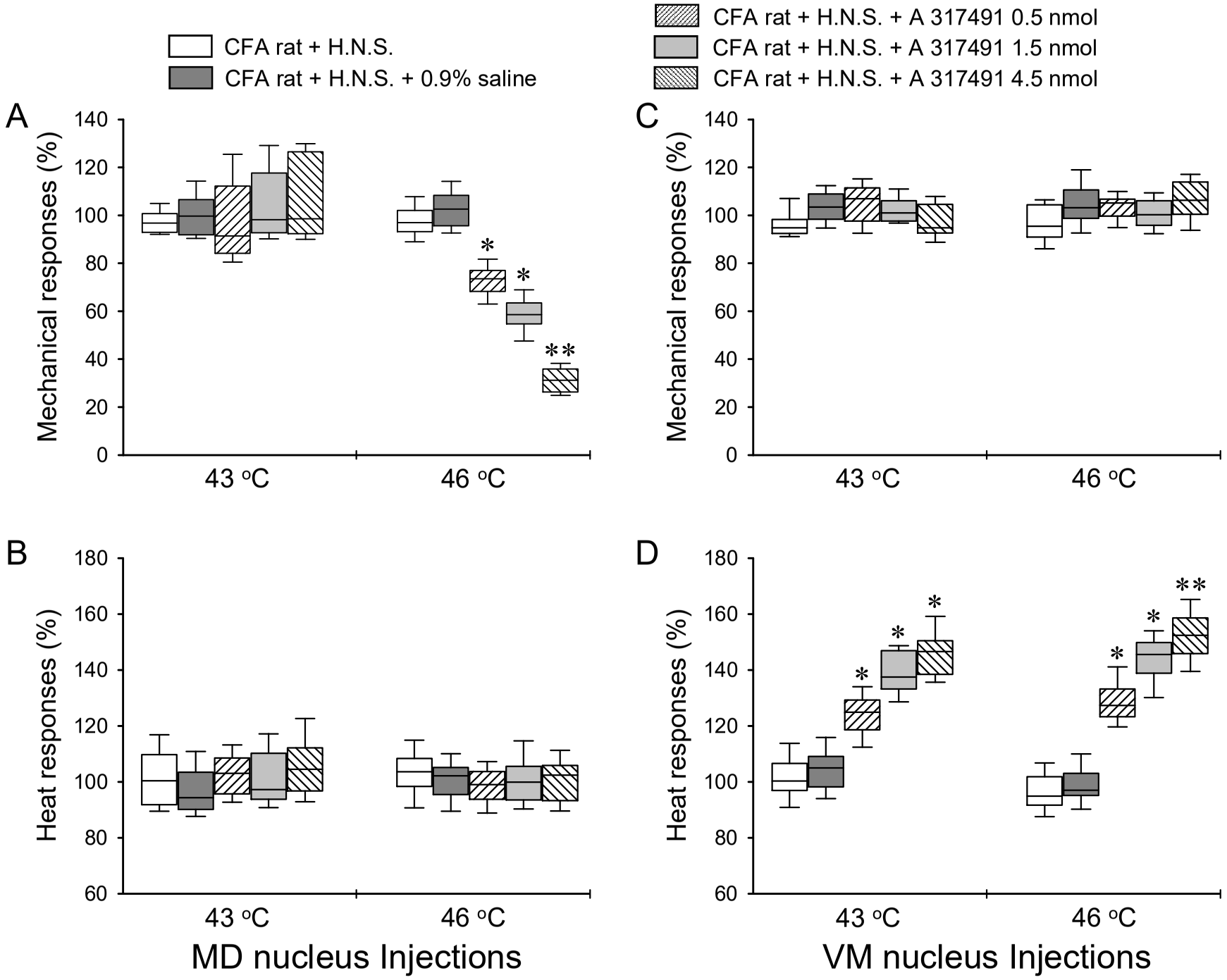


Figure 8 Lei et al., 2019