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SEX DIFFERENCES IN THE RESPONSES OF SPINAL WIDE-DYNAMIC RANGE NEURONS TO SUBCUTANEOUS FORMALIN AND IN THE EFFECTS OF DIFFERENT FREQUENCIES OF CONDITIONING ELECTRICAL STIMULATION

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Abstract—The purpose of this study was to investigate sex-related differences in nociception elicited by s.c. injection of different concentrations (1–5%) of formalin. S.c. formalin-induced biphasic (early and late phases) persistent nociception was assessed by extracellularly recording the spontaneous activities of single spinal dorsal horn wide-dynamic range neurons in anesthetized male and female rats. The nociceptive responses of the dorsal horn wide-dynamic range neurons following s.c. injection of 5%, but not 1% and 2.5%, formalin in female rats were significantly stronger than the responses obtained in male rats. However, these concentration-dependent differences with respect to different sexes existed only in the late, but not the early, phase of formalin-induced nociception in intact, not spinal rats. The 5% formalin-induced late phase nociception in male rats was significantly depressed by 15 min of repeated conditioning electrical stimulation at a frequency of 5 Hz as well as 50 Hz during and after the period of conditioning electrical stimulation (intensity: 1 mA; pulse duration: 1 ms). In contrast, the inhibitory effect of 50 Hz conditioning electrical stimulation on the 5% formalin-elicited late phase response in female rats was markedly greater in magnitude and longer in duration than that of 5 Hz conditioning electrical stimulation. No significant depressive effects of 5 Hz conditioning electrical stimulation on formalin-induced nociception were found in female rats, indicating that the distinct effects of conditioning electrical stimulation at different frequencies are different in animals of opposite sexes.

In conclusion, s.c. administration of different concentrations of formalin shows a distinct sex-related difference in its late tonic nociception of spinal nociceptive sensory neurons. Sex differences in formalin-induced tonic nociception are stimulus intensity dependent and related to the modulation from the supraspinal regions. S.c. formalin-induced late phase nociception in female rats is only sensitive to depression at a frequency of 50 Hz, but not 5 Hz, of conditioning electrical stimulation. This suggests that the involvement of the central mechanisms in the antinociceptive effects of conditioning electrical stimulation may be different at various frequencies of stimulation.

Key words: formalin test, inflammation, nociception, sex, wide-dynamic range neuron.

During the past decade, the role of different sexes in pain sensitivity and anesthesia has been an intense focus of basic research as well as clinical treatment processes. A higher prevalence of many chronic pain conditions and less tolerance of nociceptive stimuli have been reported in females (for reviews, see Berkley, 1997; Fillingim, 2000; Giles and Walker, 2000; Arendt-Nielsen et al., 2004). At present, sex differences in nociception induced by different noxious stimuli and its underlying central mechanisms remain unclear although a series of genomic studies have been performed (Mogil et al., 2000; Craig et al., 2004). Sex-related differences in acute nociception are believed to vary from case to case, which may be related to the stimulus pattern, intensity, and other factors (Giles and Walker, 2000).

It is generally accepted that the formalin test is a valuable model for the study of peripheral and central mechanisms of pathological nociception. It provides researchers with a special advantage for evaluating not only the transient acute early phase nociception (about 5 min following formalin injection), but also the specific long-term late phase nociception (around 30 min–2 h), which can ultimately mimic persistent pain syndromes such as inflammatory or pathological pain in clinic (Dubuisson and Dennis, 1977; Dickenson and Sullivan, 1987; You and Chen, 1999; You and Arendt-Nielsen, 2005; for reviews, see Tjølsen et al., 1992; Le Bars et al., 2001). To date, few studies using the electrophysiological recording method *in vivo* have focused on the role of sex in formalin-induced tonic nociception although sex-related differences in the formalin test were arguably found by assessing behavioral indices from different laboratories (Aloisi et al., 1994; Kim et al., 1999; Perissin et al., 2003).

The aim of the present study was to 1) investigate and characterize the role of gender in the persistent nociceptive response of dorsal horn (DH) wide-dynamic range (WDR) neurons to s.c. injection of formalin at different concentrations (1–5%), and 2) evaluate the efficacies of different frequencies of conditioning electrical stimulation

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Abbreviations: ANOVA, analysis of variance; cES, conditioning electrical stimulation; cRF, cutaneous receptive field; DH, dorsal horn; EA, electroacupuncture; TENS, transcutaneous electrical nerve stimulation; WDR, wide-dynamic range.

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(cES) in s.c. formalin-induced long-term nociceptive responses of spinal DH WDR neurons.

EXPERIMENTAL PROCEDURES

Age-matched male and female (of mixed estrous phases) Sprague–Dawley rats weighing 220–390 g were used (11 weeks of age; male: 322±6 g, n=56; female: 262±5 g, n=58). The animals were provided by the Experimental Medical Animal Center of Shaanxi Province, China. Animals of the same sex were paired and housed in plastic cages under a 12-h light/dark cycle (lights on at 8:00 AM) with food and water available *ad libitum*. The experiments were approved by the Institutional Animal Care Committee of Xi'an Jiaotong University. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80–23, revised 1986), and efforts were made to minimize suffering and reduce the number of animals used.

Experimental preparation

The rats were initially anesthetized with sodium pentobarbital (50 mg/kg) by i.p. administration. During the surgical procedures, a tracheal cannula and a left jugular vein catheter were inserted in order to ensure adequate breathing and fluid circulation. The electrocardiogram was monitored and maintained within physiological limits throughout the experiment. The rectal temperature was monitored and maintained constant at 37.5±0.5 °C by means of an electronic feedback control heating pad beneath the abdomen of the rat. All vital signs were monitored and kept within physiological range.

The lumbar enlargement of the spinal cord was exposed by laminectomy at the vertebrae T13 to L1 for DH neuron recordings. The dura mater and the arachnoid membrane of the lumbar spinal cord were carefully removed under a dissection microscope. In some experiments, a separate laminectomy was also performed at T8–T9, and the spinal cord was subsequently transected with a surgical knife under the microscope in order to evaluate whether supraspinal descending modulation is involved in sex-related difference in the formalin-induced nociceptive responses of the spinal DH neurons. After these procedures the animal was placed in a stereotaxic frame. The vertebral column was rigidly fixed in the frame with clamps, and a paraffin pool was made with ambient skin flaps around the exposed wound area of the lumbar spinal cord and filled with warm paraffin oil (37 °C) to prevent drying.

One hour post-surgery, anesthetic was administered continuously by i.v. infusion of sodium pentobarbital at a constant rate (15–20 mg/kg/h) to maintain the anesthesia at a level where noxious pinch stimuli did not produce flexion reflexes or changes in heart rate. During the extracellular recordings the animals were paralyzed with gallamine triethiodide (flaxedil, 40–50 mg/kg, i.v.; supplemental dose 8–10 mg/kg/h) and artificially ventilated with room air at a tidal volume of 15 ml/kg. The rats were used only once and killed at the end of the experiment by an overdose of sodium pentobarbital (200 mg/kg, i.v.).

Extracellular recording procedure

The electrophysiological responses of the single spinal DH neurons with the cutaneous receptive field (cRF) located on the hind paw were recorded extracellularly using glass micropipettes (0.5 M NaAc+2% Pontamine Sky Blue, 10–15 MΩ). All neurons were found in the spinal DH area while the hind paw was touched by innocuous searching stimuli such as the gentle touch by a finger. Sometimes an electrical stimulus with low intensity (intensity: 0.5 mA; pulse duration: 0.5 ms; frequency: 0.3–0.5 Hz) applied to the hind paw was used instead of touching in order to find high threshold neurons without background activities. It has been demonstrated that no windup and central sensitization can be elicited by this repeated electrical stimulation with low intensity (You and Chen, 1999; You et al., 1999; Wang et al., 2003; You and Arendt-Nielsen, 2005). The depth of the recorded neurons was 200–1250 μm (Rexed's I–VI) from the dorsal surface of the spinal cord and was estimated by microdrive readings, both on descent and during withdrawal to the spinal cord surface (Paxinos and Waston, 1998). After a neuron was found in the spinal DH area, the cRF related to the recorded neuron was carefully determined by mechanical innocuous pressure and noxious pinch stimuli using different fine surgical forceps. Each neuron was characterized by its responses to natural stimuli (brush, pressure, and pinch). Only WDR neurons, which showed a graded response to stimuli ranging from peripheral innocuous stimuli to noxious stimuli, were used in the rest of the experiment. The DH WDR neurons showing habituation or strong spontaneous background firing (>five spikes/s) were not used for the remainder of the study. This criterion followed the previous recommendations in the formalin test (You and Chen, 1999).

The DH WDR neuronal signals were amplified by a microelectrode amplifier (MEZ-8201, Nihon Kohden, Japan) and displayed on an oscilloscope. Data acquisition was performed using data processing software (Spike 2, UK) via a data acquisition interface (CED 1401, UK) on a PC.

Experimental protocol

Experiment 1: s.c. formalin-induced pathological nociception in male and female rats. The formalin-induced persistent pathological nociception was established mainly based on previous experiments (You and Chen, 1999; You et al., 1999; You and Arendt-Nielsen, 2005). As shown in Fig. 1A and 1B, 50 μl of 1%, 2.5% and 5% formalin (0.37%, 0.925% and 1.85% formaldehyde diluted in 0.9% saline, respectively) were injected subcutaneously into the most sensitive area of the cRF of the recorded DH WDR neurons, respectively. After that, s.c. formalin-induced nociceptive responses of the DH WDR neurons were investigated and recorded for 60 min. Some experiments were performed in spinalized rats in order to investigate the role of supraspinal descending modulation in sex-related differences in s.c. formalin-induced DH WDR neuron activities. Two hours after the spinalization surgery, recordings began in order to avoid any influence from completely acute spinal injuries such as spinal shock (Sherrington, 1906).

The effects of the vehicle (0.9% saline) were tested for control. Each DH WDR neuron only received one formalin injection in a one-day experiment.

Experiment 2: Effects of different frequencies of cES on formalin-induced nociception in male and female rats. In order to avoid the involvement of spinal segmental mechanisms (You et al., 1999), the conditioning stimulation electrodes consisted of two stainless-steel needles with a diameter of 0.15 mm diameter which were inserted ipsilaterally into the medial area between the first and the second toe of the forepaw, and 1 cm proximal s.c. inner side of the forelimb to the carpal joint overlying the median nerve trunk corresponding to 'Hegu' and 'NeiGuan' acupoints in humans, respectively (Fig. 1A). The inserting depth of the electrodes was about 0.3 cm. Based on previous studies (You et al., 1999; Wang et al., 2003), the cES consisted of repeated standard, constant current pulses (pulse duration: 1 ms; intensity: 1 mA) at a frequency of either 5 Hz or 50 Hz. Peripheral A-fibers, but not C-fibers, have been proven to be recruited by this kind of cES (You et al., 1999; Wang et al., 2003). Twenty-five minutes after injection of formalin, the cES was applied and lasted for 15 min in both male and female rats. The effects of the cES at different frequencies were investigated continuously during and after the treatment with cES. Each rat only received the treatment of cES at either 5 Hz or 50 Hz during a one-day experiment.