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Sex differences in a Murine Model of Complex Regional Pain Syndrome

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ABSTRACT

Complex Regional Pain Syndrome (CRPS) is a major cause of chronic pain after surgery or trauma to the limbs. Despite evidence showing that the prevalence and severity of many forms of chronic pain, including CRPS, differ between males and females, laboratory studies on sex-related differences in animal models of CRPS are not available, and the impact of sex on the transition from acute to chronic CRPS pain and disability are unexplored. Here we make use of a tibia fracture/cast mouse model that recapitulates the nociceptive, functional, vascular, trophic, inflammatory and immune aspects of CRPS. Our aim is to describe the chronic time course of nociceptive, motor and memory changes associated with fracture/cast in male and female mice, in addition to exploring their underlying spinal mechanisms. Our behavioral data shows that, compared to males, female mice display lower nociceptive thresholds following fracture in the absence of any differences in ongoing or spontaneous pain. Furthermore, female mice show exaggerated signs of motor dysfunction, deficits in fear memory, and latent sensitization that manifests long after the normalization of nociceptive thresholds. Our biochemical data show differences in the spinal cord levels of the glutamate receptor NR2b, suggesting sex differences in mechanisms of central sensitization that could account for differences in duration and severity of CRPS symptoms between the two groups.

1. Introduction

The sex and gender of clinical patients are important variables in the pain equation since the prevalence and severity of many forms of chronic pain differ between males and females. Examples include the female preponderance of patients suffering from migraine headache, fibromyalgia, irritable bowel syndrome (Racine et al., 2012a,b), various forms of neuropathic pain (Butler, Jonzon, Branting-Ekenback, Wadell, & Farahmand, 2012), and chronic postoperative pain (van Gulik et al., 2011). Furthermore, sex- and gender-related differences to experimental pain in humans have been reported (Alabas, Tashani, Tabasam, & Johnson, 2012; Dannecker et al., 2012; Racine et al., 2012a,b). Despite the clinical evidence, only a limited number of preclinical studies have addressed these differences, and with varying results. For example, the severity of neuropathic pain was shown to be less in female rats after sciatic nerve lesion (Wagner, DeLeo, Coombs, & Myers, 1995), although allodynia was shown to be greater in female rats after spinal nerve transection (DeLeo & Rutkowski, 2000) and in female mice after chronic nerve constriction injury (Vacca et al., 2014). Likewise, inflammatory stimuli have different pain-related effects in male and female rats (Tall & Crisp, 2004). Both intrinsic differences in nociceptive neurons and estrogen effects may be responsible for these sex-related responses (Chaban, Li, McDonald, Rapkin, & Micevych, 2011; Dina, Aley, Isenberg, Messing, & Levine, 2001; Hendrich et al., 2012). For instance, estradiol receptors are expressed in dorsal root ganglion neurons and interact with metabotropic glutamate receptors to mediate intracellular signaling (Dewing et al., 2007), a mechanism that is very relevant to nociception.

Complex regional pain syndrome (CRPS) is a debilitating condition characterized by severe pain usually confined to one limb. It encompasses a wide range of signs and symptoms including the

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sensory, motor and autonomic nervous systems, bone demineralization, skin growth changes and vascular dysfunction. Additionally, CRPS patients often display deficits in executive functioning, memory, and even global cognitive impairment (Libon et al., 2010) that could further exacerbate overall patient well-being. With an estimated 50,000 new cases in the US annually, CRPS exhibits a high prevalence in female patients, with females affected at least 3 times more than males (de Mos et al., 2007). Despite the need for studies targeting sex differences in CRPS, data from animal models of CRPS is not available, and the impact of sex on the transition from acute to chronic CRPS pain and disability are unexplored, thereby limiting patient-specific selection of treatments and the rational design of new therapies.

For the present studies, we made use of the tibia fracture/cast model of CRPS. Characterized both in mice and rats, this model recapitulates the nociceptive, functional, vascular, trophic, inflammatory, and immune aspects of CRPS observed in patients, including unilateral limb warmth, edema, allodynia, unweighting, and the associated peripheral (e.g. neurogenic inflammation) and central (brain neuroplasticity) alterations (Gallagher et al., 2013; Guo, Offley, Boyd, Jacobs, & Kingery, 2004; Li, Guo, Li, Kingery, & Clark, 2010; Tajerian et al., 2014; Wei, Sabsovich, et al., 2009). Our main goal was to describe the timecourse of nociceptive, motor, and memory changes associated with fracture/cast in male and female mice, in addition to exploring their underlying spinal mechanisms. It is our hope that studies such as ours exploring disease etiology and mechanism in both sexes will identify gender specific mechanisms contributing to the development and maintenance of CRPS, and ultimately explain the preponderance of female CRPS patients.

2. Materials and methods

2.1. Animals

A total of 3 cohorts of mice were used. Cohort 1 was used for the longitudinal measurements of mechanical sensitivity and physiological measures of hindpaw edema and temperature, in addition to measures of rotarod and latent sensitization. Cohort 2 was used for conditioned place preference and fear memory, while cohort 3 was used for tissue collection. Male and female C57/B6J mice aged 12–14 weeks were purchased from the Jackson Laboratory (Sacramento, CA, USA) and were allowed to habituate to the animal facility for a minimum of 10 days prior to the experiments. Mice were housed in groups of 4 on a 12-h light/dark cycle and an ambient temperature of 22 ± 3 °C, with food and water available *ad libitum*. All animal procedures and experimental designs were approved by the *Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee* (Palo Alto, CA, USA) and followed the "animal subjects" guidelines of the *International Association for the Study of Pain.*

2.2. Limb fracture and cast immobilization

Mice were randomly allocated to the control or the fracture/cast group. Mice were anesthetized with 1.5% isoflurane and underwent a distal tibia fracture in the right leg. Briefly, a hemostat was used to make a closed fracture of the right tibia just distal to the middle of the tibia. Then the hindlimb was wrapped in casting tape (Scotchcast™ Plus) so the hip, knee and ankle were all fixed. The cast extended from the metatarsals of the hindpaw up to a spica formed around the abdomen. A window was left open over the dorsal paw and ankle to prevent constriction when post-fracture edema developed (Guo et al., 2012). After the procedure, the mice were given subcutaneous buprenorphine (0.05 mg/kg) and enrofloxacin (5 mg/kg) for the next two days, as well as normal saline (1.5 ml once) for post-operative analgesia, prevention of infection and prevention of dehydration. Mice were inspected daily to ensure the cast was positioned properly through the 3-week period of cast immobilization. Mice were provided with chow pellets postoperatively *ad libitum*; dietary gels were also made available on the cage floor for mice having undergone surgery. At 3 weeks after surgery, the mice were briefly anesthetized using isoflurane and the casts were removed.

A summary of the allocation of cohorts in addition to the experimental timeline is illustrated in Fig. 1.

2.3. Physiological measures

All measurements were conducted 3 days prior to fracture and weekly after cast removal at 3 weeks post-fracture.

2.3.1. Hindpaw volume

A laser sensor technique was used to determine the dorsal–ventral thickness of the hindpaw, as we have previously described (Li et al., 2009). The sensor device has a measurement range of 200 mm with a 0.01 mm resolution (cat. #4381-Precicura, Limab, Göteborg, Sweden).

2.3.2. Hindpaw temperature

The temperature of the hindpaw was measured using a fine wire thermocouple (Omega, CT, USA) applied to the paw skin, as described previously (Li et al., 2009). The investigator held the wire using an insulating Styrofoam block. 3 sites were tested over the dorsum of the hindpaw: the space between the 1st and 2nd metatarsals (medial), the 2nd and 3rd metatarsals (central), and the 4th and 5th metatarsals (lateral). After a site was tested in one hindpaw, the same site was immediately tested in the contralateral hindpaw. The testing protocol was medial dorsum right then left, central dorsum right then left, lateral dorsum right then left, medial dorsum left then right, central dorsum left then right, and lateral dorsum left then right. The six measurements for each hindpaw were averaged for the mean temperature.

2.4. Behavioral testing

The experimenter was blind to the identity and experimental condition of the animals throughout behavioral experiments and data analysis. Mice were habituated to handling by the experimenter for a few minutes each day for 7 days before initiation of the behavioral tests. Male and female mice were tested separately.

2.4.1. Mechanical hypersensitivity

Calibrated monofilaments (Stoelting Co., IL, USA) were applied to the plantar surface of the hindpaw and the 50% threshold to withdraw (grams) was calculated as previously described (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). The stimulus intensity ranged from 0.004 to 1.7 g, corresponding to filament numbers (1.65, 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17, and 4.31). For each animal, the actual filaments used within the aforementioned series were determined based on the lowest filament to evoke a positive response followed by 5 consecutive stimulations using the up-down method. The filament range and average interval were then incorporated along with the response pattern into each individual threshold calculation. Mechanical sensitivity was assessed on the plantar surface of the hindpaw (response = flexion reflex).

2.4.2. Conditioned place preference

To assess ongoing pain associated with fracture, a single trial counter balanced conditioned place preference (CPP) test was employed starting at 8 weeks after fracture; similar to techniques