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Higher pain perception and lack of recovery from neuropathic pain in females: A behavioural, immunohistochemical, and proteomic investigation on sex-related differences in mice

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ABSTRACT

In experimental and clinical pain studies, the sex of subjects was rarely taken into account, even if nociceptive inputs appear to be processed and modulated by partially distinct neural mechanisms in each sex. In this study we analysed, in male and female mice, behavioural and neuronal responses in developing, maintaining, and recovering from neuropathic pain. Experiments were carried out in adult CD1 mice by using Chronic Constriction Injury (CCI) as neuropathic pain model. We investigated the temporal trend of mechanical nociceptive threshold together with functional recovery of the injured paw, and the immunofluorescence staining of proteins associated with nerve injury and repair and with spinal gliosis, 7 and 121 days after CCI. A proteomic analysis on proteins extracted from sciatic nerves was also performed. Male mice showed a gradual decrease of CCI-induced allodynia, the complete recovery occurring 81 days after the sciatic nerve ligation. On the contrary, in female mice, allodynia was still present 121 days after CCI. Sex-dependent differences also resulted from immunofluorescence experiments: in sciatic nerve, the expression of P0 and Neu200 is greater in neuropathic males than in neuropathic females, suggesting faster nerve regeneration. Proteomic analysis confirmed sex-related differences of proteins associated with nerve regenerative processes. In addition, the reactive gliosis induced by CCI at day 7, as revealed by colocalization of glial fibrillary acidic protein (astrocytes) and CD11b (microglia) with phosphorylated p38, disappeared 121 days after CCI in male but not in female mice. These results may have important therapeutic implications for the treatment of neuropathic pain.
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1. Introduction

In recent years the study of pain-related sex differences has received increased attention both in humans and in animals [5,9,19,27,34,72]. Epidemiological studies demonstrated higher prevalence of painful disorders such as migraine, temporomandibular joint disorder, fibromyalgia, arthritis, and interstitial cystitis, as well as higher susceptibility to nociceptive stimuli and more frequent use of analgesic medications in women than in men [26,46]. From an experimental point of view, differences in structural and functional features of pain pathways between sexes have been observed, with female rats being more susceptible than male rats to inflammatory pain [3,20,35] and neuropathy development [15,16,42,68]. However, the effects of sex on nociceptive processing and pain responsiveness have not been deeply investigated, and mechanisms underlying these differences, mainly as concerns neuropathic pain, are complex and far from understood.

Although genetic, environmental, and sociocultural factors should be considered [56], gonadal hormones undoubtedly play an important and multifaceted role [9,17,27,28]. Oestrogens, in particular, have received considerable attention. There are contradictory results showing oestrogens displaying pro- or antinociceptive effects as well as neuroprotective action [6,14,17,26,31,32,34,58].

Evidence for a relationship between glial and sex hormones exists. Glial cells are affected by sex hormones that are involved in maintaining their physiological homeostasis, and gonadal hormones influence reactive gliosis [6,7,29,30]. In fact, Schwann cells (SCs) in the peripheral nervous system, and oligodendrocytes,

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astrocytes, and microglia in the central nervous system, are targets of oestrogens. The reaction of glial cells includes a mixture of positive and negative responses for neuronal survival and regeneration. A key role of SCs, satellite cells, microglia, and astrocytes has increasingly been recognized in the development and maintenance of neuropathic pain [67,69].

The identification of factors that regulate reactive glial cells together with the investigation of sex-related hormonal influences on pain would be of great interest for the development of potential therapeutic strategies to reduce neural damage and promote regeneration after central and peripheral nervous system injuries. To this aim, the purpose of the present study was to investigate sex-related differences in behavioural manifestations and in developing, maintaining, and recovering from neuropathic pain. Furthermore, to assess potential differences in the mechanisms involved, we analysed the expression of some proteins associated with peripheral nerve de/regenerative processes and with spinal glial activation in neuropathic animals by using immunofluorescence (IF) staining and proteomic analysis.

2. Methods

2.1. Animals

CD1 male and female mice, about 3 months old from Charles River Labs (Como, Italy) were used. Animals were housed in standard transparent plastic cages, in groups of 4, lined with sawdust under a standard 12/12-hour light/dark cycle (7:00 AM/7:00 PM), with food and water available ad libitum. Testing was performed blind as to which treatment group each subject belonged. After behavioural testing, the oestrous cycle was analysed in females by means of vaginal smears. Because we did not observe any difference in the behavioural responses, we included all females in the same experimental group independently from the oestrus cycle. All procedures were in strict accordance with the Italian National law (DL116/92, application of the European Communities Council Directive 86/609/EEC) on care and handling of the animals and with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain [75].

2.2. Surgery

Following the procedure originally proposed by Bennett and Xie [8] adapted to the mouse, the Chronic Constriction Injury (CCI) model was used as a model of neuropathic pain. CCI of sciatic nerve was performed under anaesthesia with chloral hydrate (500 mg/kg intraperitoneally; Sigma-Aldrich, Milan, Italy); the middle third of the right sciatic nerve was exposed through a 1.5-cm longitudinal skin incision. Three ligatures (5-0 chromic gut; Ethicon, Rome, Italy) were tied loosely around the sciatic nerve. The wound was then closed with 4-0 silk sutures. In the following, the injured right hind paw will be named as ipsilateral paw and the uninjured left hind paw will be named as contralateral paw.

2.3. Behavioural testing

2.3.1. Mechanical nociceptive threshold (Dynamic Plantar Aesthesiometer test)

The onset of neuropathy was assessed by measuring the sensitivity of both ipsilateral and contralateral hind paws to normally nonnoxious punctuate mechanical stimuli at different time intervals from postoperative day 3 (D3) to day 121 (D121). The nerve injury-induced mechanical allodynia was tested by using a Dynamic Plantar Aesthesiometer (Model 37400; Ugo Basile, Comerio, Italy), an apparatus that generates a mechanical force linearly increasing with time. The force is applied to the plantar surface of the mouse hind paw, and the nociceptive threshold is defined as the force, in grams, at which the mouse withdraws its paw. On each day of testing, the mechanical withdrawal response of ipsilateral and contralateral hind paws was recorded for 3 consecutive trials with at least 10 seconds between each trial. The withdrawal threshold was taken to be the mean of the 3 trials.

2.3.2. Weight bearing (incapacitance test)

Hind limb weight bearing was determined using an incapacitance test (Linton Instrumentation, Norfolk, UK) consisting of a dual weight averager. The apparatus is adapted for mice with a strain gauge/amplifier resolution of 0.03 g and strain gauge/amplifier accuracy of 0.1 g. Weight distribution was measured between ipsi- and contralateral hind limbs as previously described [12]. The weight percent distribution onto the ipsilateral hind limb was calculated by the following equation: (ipsilateral weight/[ipsilateral weight + contralateral weight]) $\times$ 100.

2.4. Immunohistochemical analysis

Sciatic nerve and lumbar spinal cord (L4–L5) of mice belonging to each experimental group (n = 3/group) were harvested for IF analysis. Animals were sacrificed with lethal doses of chloral hydrate and perfused with saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). Sciatic nerve and spinal cord were removed and kept in immersion for 48 hours in 4% paraformaldehyde in PBS (pH 7.4), after cryoprotection with solution of 30% (w/v) sucrose in PBS and maintained at −80 °C. Cryostat sections of sciatic nerve and spinal cord, 20 and 40 μm, respectively, were taken.

For sciatic nerve, IF analysis was made in naïve (no-CCI) and in CCI mice (CCI D7). For double IF staining, sections were first incubated overnight with: (1) the antibody against Neurofilament 200 (Neu200, rabbit polyclonal, 1:100, Sigma-Aldrich: N4142), the intermediate filament that constitutes a structural cytoskeletal element of axons, permits one to visualize regenerating fibres, and plays an important physiological role for the formation and maintenance of the multilamellar structure of the myelin in the peripheral nervous system; and (2) the antibody against myelin protein zero (designated as P0 or MPZ, chicken polyclonal, 1:100, AB9352; Millipore, Billerica, MA, USA), the major structural protein of peripheral myelin that determines the thickness of myelin [37,62]. Both antibodies were diluted in Triton 0.3% (Sigma-Aldrich).

For spinal cord, IF analysis was made in naïve animal groups (no-CCI) and in CCI mice at 2 different time points (D7 and D121 after CCI). For double IF staining, sections of lumbar spinal cord were incubated for 48 hours at room temperature with: anti-GFAP (glial fibrillary acidic protein, astrocyte marker) antibody (mouse monoclonal, 1:100, G6171; Sigma-Aldrich); anti-NeuN (neuronal marker) antibody (mouse monoclonal, 1:100, MAB377; AbD Serotec, Kidlington, UK); anti-CD11b (complement receptor 3/cluster of differentiation 11b, microglia marker) antibody (rat anti-mouse, 1:100, MCA711; Millipore), and anti-phosphorylated (p)-p38 antibody (rabbit polyclonal, 1:100, P1491; Sigma-Aldrich) in Triton 0.3%.

After 3 washings in PBS, sections were incubated for 2 hours at room temperature with fluorescein-conjugated goat anti-mouse (1:100; Jackson ImmunoResearch, West Grove, PA, USA), fluorescein-conjugated rat antimouse (FITC, 1:100; Jackson ImmunoResearch) or rhodamine-conjugated goat antirabbit (TRITC, 1:100; Jackson ImmunoResearch) secondary antibodies in 0.3% Triton. After 2 washings in PBS, sections were incubated for 10 minutes with bisbenzimide, DNA-fluorochrome (Hoechst, 1:1000, Sigma-Aldrich) in PBS.

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