Original Article

FEMALE CASTRATED RATS WITH NEUROPATHIC PAIN: BEHAVIOURAL AND MORPHOLOGICAL CORRELATES

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Summary

The aim of the study was to investigate the modulatory actions of estradiol on nociceptive thresholds and the activity of analgesic drugs in chronic constriction injury (CCI) model of neuropathic pain in female Wistar rats. Rats were ovariectomised, and 30 days later CCI of the sciatic nerve was induced. They were randomly assigned into control and estradiol-treated animals (estradiol, 0.5 mg/kg, s.c., for 21 days in 11 separate doses). Electron microscopy was applied to study the morphological changes proximal and distal to the sciatic nerve ligatures. The nociceptive thresholds were determined by paw pressure, hot plate, plantar heat tests, von Frey filament test, incapacitance analgesia test. The effects of gabapentin (100 mg/kg), metamizole (150 mg/kg) and tramadol (30 mg/kg) were studied. The myelin and axonal destruction were moderately expressed in ovariectomized females. Estradiol decreased responses to tramadol for mechanical allodynia and hyperalgesia. Gabapentin showed significant efficacy against thermal hyperalgesia where increased activity in estradiol-treated animals was also observed. Our results suggest that estradiol could modulate the activity of analgesic drugs in the CCI model of neuropathic pain.

Key words: chronic constriction injury, female castrated rats, analgesia.

Introduction

Sex differences in pain perception exist due to its differential modulation by estrogens and androgens with females typically reporting higher sensitivity to noxious stimuli and higher incidence of various painful conditions [1,2,3]. Studies on human subjects have demonstrated that sensitivity to pain varied during the menstrual cycle [4] and female reproductive stages [5]. Estradiol could modulate nociceptive transmission and activity of analgesic drugs at different levels and through different mechanisms [6,7]. The exact mechanisms through which sex hormones control pain appear to be complex and there are many unclear points. In recent years, several investigators have examined sex differences in analgesic responses, using experimental pain models. Both human and animal

studies suggest gender differences in pain processing but little is known about influence of estrogens on drug management. Estradiol has been found to have modulatory actions on the analgesic effect of opioids [8]. One possible mechanism by which estrogens might produce this effect is via activation of the opioid system. However estrogen effects can be mediated through modulation of many other systems [9]. Almost all of the animal studies have used acute nociception models, and only a few have addressed the role of gonadal hormones on behavioral responses to persistent and neuropathic pain [10, 11].

The aim of this study was to investigate the modulatory actions of estradiol on nociceptive thresholds and the activity of analgesic drugs in chronic constriction injury (CCI) model of neuropathic pain in female Wistar rats.

Materials and Methods

All procedures were approved by the Ethics Committee of Medical University, Sofia. Female Wistar rats (200-250g) were ovariectomised and 30 days later CCI of the sciatic nerve was induced over the right hind limb [12]. Briefly, after dissection at the middle of the thigh, 3–4 mm of the common sciatic nerve was tied loosely with 2 ligatures spaced by 1 mm. Surgical operations were made under general anesthesia (ketamine 50 mg/kg, i.p. and nembutal 12 mg/kg, i.p.). Rats were randomly assigned to experimental groups (6 to 8 per group): control and estradiol-treated animals. The latter group received estradiol (0.5 mg/kg, s.c.) for 21 days in 11 separate doses.

Nociceptive behavioral tests

Thermal hyperalgesia was assessed by using a plantar heat test apparatus (Ugo Basile, Italy) which measures the paw withdrawal latency in response to radiant heat. The rats were allowed to habituate to the apparatus before testing. Each rat was placed in a separate clear Plexiglas box (23x18x14 cm), positioned on a clear plastic surface. The heat source was then positioned under the plantar surface of the hind paw and activated with an infrared light beam. The heat source was connected to a timer that automatically switched off the heat when the paw was withdrawn. A cut-off time of 20 s was used to prevent tissue damage if response was absent. The mean paw withdrawal latencies (in seconds) for the ipsilateral hind paws were determined as an average of three separate measures. Pain reflexes in response to a thermal stimulus also were measured using a Hot Plate Analgesia Meter (Ugo Basile; hot plate test). The surface of the hot plate was heated to a constant temperature of 51°C, as measured by a built-in digital thermometer with an accuracy of 0.1°C. The latency to respond with either a hindpaw lick was measured. Animals were tested one at a time. A cut-off time of 30 s was used.

The presence of mechanical allodynia was assessed using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy von Frey filament test). A mechanical stimulus was applied to the plantar surface of one hind paw by a stainless steel filament (0.5mm in diameter) with increasing force (2.5g/s). The threshold for paw withdrawal was calculated by taking the average of 3-4 repeated stimuli (in g), which induced a reflex paw withdrawal.

Mechanical hyperalgesia was measured as the threshold of response to increasing pressure by the paw-pressure test (analgesimeter, Ugo Basile, Italy). A constantly increasing pressure was applied to the dorsal surface of the right hind paw of the rats to determine the nociceptive response (paw withdrawal). The nociceptive threshold was determined as the force, in grams. A 500-g cut off value was used to prevent tissue damage.

To evaluate neuropathic pain, the rats were placed in the box of an incapacitance meter (Ugo Basile, Italy). The bearing force of each hind limb was quantified by two mechanotransducers, separately placed below the two hind limbs: the left on the normal, and the right – on the operated limb (CCI). The bearing force of each hind limb was estimated as a 3 average, and the mean bearing force was calculated as the ratio between weight borne by ipsilateral limb (R) and the weight borne by contralateral limb (L). As the pain progressed, the balance of weight was disrupted (~ 1 for a healthy rat), resulting in a reduction of the R/L ratio.

Pharmacological treatment

The effects of gabapentin (100 mg/kg, p.o., Pfizer), metamizole (150 mg/kg, i.p., Sopharma) and tramadol (30 mg/kg, p.o., Heumann Pharma) were studied.

Electron microscopy

Thirty days after CCI, 3 rats per group were deeply anesthetized with intraperitoneal injection of thiopental (40 mg/kg). Thereafter they were transcardially perfused through the ascending aorta with a freshly prepared fixative containing 2% paraformaldehyde and 2,5% glutaraldehyde in 0.1M phosphate buffer pH 7.4. Small tissue pieces from the following peripheral nerve segments were taken: 5 mm proximal and 5 mm distal to the ligation of the sciatic nerve; the approximately same place of the contralateral noninjured sciatic nerve; sural nerve of the injured side; sural nerve of the contralateral noninjured side. They were postfixed overnight in the same fixative at 4° C and in 1% OsO_4 in phosphate buffer for 1 hour, dehydrated in graded series of ethanol and embedded in Durcupan (Fluka, Buchs, Switzerland). Thin transverse and longitudinal sections were cut with an ultramicrotome (LKB, Stockholm-Bromma, Sweden), counterstained with uranyl acetate and lead citrate, and then examined with a Hitachi H-500 electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

The data are expressed as means ± SEM. The obtained data were evaluated by the one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test; $p < 0.05$ was considered significant.

Results

Rats showed signs of neuropathy on the operated paw 12 days after induction of injury. The neuropathic behavior included abnormal positioning of the paw and signs of spontaneous pain such as shaking and/or licking of the injured paw. Manifestations of the neuropathic pain were evident from the behavioral pain tests. Both groups developed mechanical and thermal hyperalgesia and allodynia, compared to baseline pain thresholds before constrictive injury (Table 1). No difference was found between ovariectomised and estrogen-treated rats in regard to nociceptive thresholds.

Metamizol 150 mg/kg i.p. alleviated tactile allodynia (von Fray filament test) 30 and 60 min after application (Fig.1). No difference was established between the groups treated with metamizol. Tramadol (30 mg/kg) displayed significant analgesic effects in all behavioral pain tests. Both groups of rats showed significant decrease in mechanical sensitivity measured by von Frey and paw pressure tests. In both tests, analgesic activity of tramadol was pronounced in ovariectomized rats, and the magnitude of change in groups was significant at 90 min in paw pressure test (Fig.2). Gabapentine (100 mg/kg) increased thermal withdrawal latency (hot plate test) and mechanical paw threshold (von Frey filament test). No statistically significant difference between groups was found in alleviation of allodynia. The attenuation of thermal sensitivity was more prominent in the estrogen treated group (Fig. 3), statistically significant on 90 min.

Electron microscopy findings established were not uniform (Fig. 4A, B). Distal to the ligation, the sciatic nerve and the sural nerve that branched off from it showed considerable changes.

*In o*variectomized females, *both preserved and* differently damaged nerve fibers were exposed. Closely to unchanged profiles of myelinated and unmyelinated nerve fibers, large myelinated nerve fibers with small and large destructions of the myelin were often seen. Instead of the parallel appearance of the myelin lamellae, at these places they tended to be fused or distanced, building vortex figures. In some instances these figures were considerably thickened and homogenized, giving the impression of spotted appearance and a generally thickened myelin sheath. The axoplasm was partly detached from the axolemma. It contained different vacuolar profiles and its electron density was either increased or decreased. The basal lamina was thickened. The characteristic amyelin fibre bundles often showed a damaged structure and many fibres were swollen, with lightened axoplasm, containing vacuolar profiles.

In ovariectomized estrogen treated females, small and medium sized nerve fibres with normal appearance were also seen. However, most of the large myelinated fibres showed focal splitting of their myelin sheath. Differently sized destructions of the myelin with forming of vortex figures were often visible. However, they were concentrated in one part of the myelin sheath rather than occupy it entirely. The axoplasm had a changed electron density and vacuolar degeneration appearance. The amyelin fibre bundles looked either unchanged or some of them showed vacuolar changes in their axoplasm.

In both groups, the sciatic and the sural nerves of the noninjured side showed normal structure of

peripheral nerve. The same structure was also exposed by the sciatic nerve proximal to the CCI. However, rarely in these nerve zones some slightly damaged axons could be seen.

Table 1. Development of neuropathy after induction of CCI in female rats

Test	Ovariectomised rats (Co)	Estrogen treated rats (E)
Paw pressure (g/cm^2 x20)	6.17±0.58 – 4.8±0.49*	5.52±0.47 – 4.08±0.27*
von Frey filament (g)	31.36±1.26 – 23.2±3**	31.3±0.78 – 20.25±1.65***
Hot plate (s)	17.38±1.05 – 12.47±2.4	19.6±1.95 – 18.69±4.2
Plantar heat (s)	10.34±0.74 – 7.79±0.6**	11.91±0.47 – 9.16±0.9*
Incapacitance (ratio R/L paw)	1.04±0.06 – 0.8±0.11*	0.97±0.01 – 0.91±0.1

The nociceptive thresholds (R- operated paw) in different behavioral tests were compared vs. level before injury. The data are presented as means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (n=6-8).

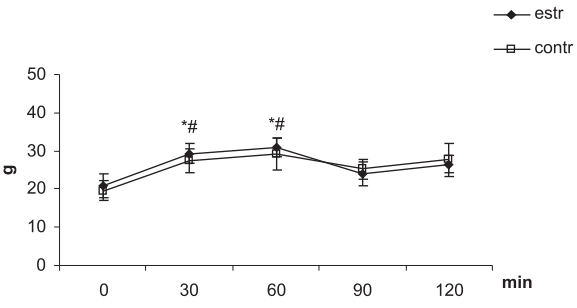

Fig. 1. Effect of metamizol on mechanical allodynia (von Fray filament test). $p < 0.05$, estradiol treated group vs. 0 min (before treatment); # $p < 0.05$, control (ovariectomized) group vs. 0 min; n=6-8.

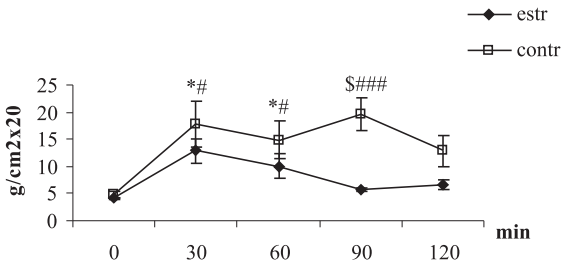

Fig. 2. Effect of tramadol on mechanical hyperalgesia *$p < 0.05$, estradiol treated group vs. 0 min (before treatment); # $p < 0.05$, ### $p < 0.001$, control (ovariectomized) group vs. 0 min; $ $p < 0.05$, estradiol treated vs. ovariectomized group; n=6-8.

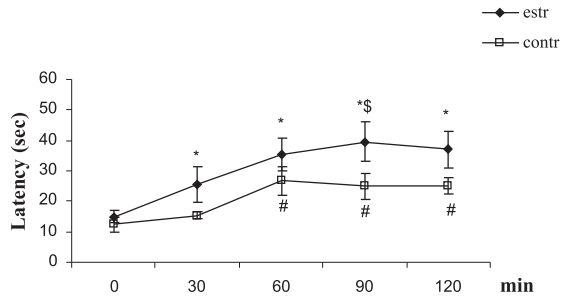

Fig. 3. Effect of gabapentin on thermal hyperalgesia *$p < 0.05$, estradiol treated group vs. 0 min (before treatment); # $p < 0.05$, control (ovariectomized) group vs. 0 min; $ $p < 0.05$, estradiol treated vs. ovariectomized group; n=6-8.

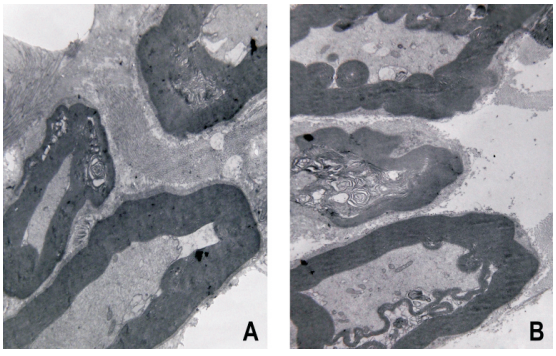

Fig. 4. Sciatic nerve distal to the ligation in ovariectomized female rat (A) and in ovariectomized and estrogen treated female rat (B). For explanation see text. A – x 11 500; B – x 9 500.

Discussion

Neuropathic pain is a common clinical syndrome associated with nerve injuries. CCI model has been widely used in studying many aspects of pain-related behaviors [13]. In the present study, ovariectomy lowered nociceptive threshold relative to right paw before CCI and contralateral paw during the study, and replacement with estrogen (0.5 mg/kg, s.c.) did not show significant difference. This result confirms the data of Tall and al. [10], but the other investigation reported that, in ovariectomized

females, the duration of formalin-induced licking was longer than in intact females [14]. Abundant evidence suggests that pain treatment responses may be different in women versus men [15]. In our experiments, estradiol decreased responses to tramadol in the dynamic plantar and paw pressure test. At the same time, gabapentin showed significant activity in the hot plate tests where increased activity in estradiol-treated animals was also noted. The differences in analgesic responses probably depend on complex mechanisms: estrogens can influence nociceptive activity through genomic and nongenomic effects but the pathogenesis of experimental model and painful stimulus also moderated analgesic responses.

The morphological correlates of the CCI model of neuropathic pain in gonadally intact rats are mainly the degenerative changes of the myelin sheath and axoplasm of large nerve fibers as well as the changes of the amyelinated fibers [16]. In view of the growing body of evidence for the sexual dimorphism of pain perception and the role of estrogens in this process, the great advantage of the present study is to investigate simultaneously the behavioral and morphological correlates of CCI model in ovariectomized animals with or without additional estradiol treatment. Notwithstanding the latter, the ultrastructural changes distal to the injury were basically the same. Moreover, their presence in the 100% sensory sural nerve on the injured side can explain the impairment of the sensory function. The damaged nerve fibers proximal to the injury and of the noninjured side may be related to the dramatic decrease of bone innervation after ovariectomy [17]. Other mechanisms may also be responsible. This is suggested by the similarity of the changes without and with estradiol treatment as well. Obviously, the addition of estradiol cannot compensate the lack of gonadal influence on the normal nerve function. Therefore further research is needed to unravel the ovarial compounds possibly modulating the sensory function.

Conclusion

In conclusion, our results suggest that estradiol (5 mg/kg) could modulate the activity of analgesic drugs in the CCI model of neuropathic pain. The modulatory effects of estradiol were differentially expressed depending on analgesic compound and experimental model. The morphological equivalents of CCI at ovariectomy with or without estradiol treatment were similar.

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