Research

Long-term ovariectomy changes formalin-induced licking in female rats: the role of estrogens

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Abstract

Gonadal hormones have been shown to exert modulatory effects on nociception and analgesia. To investigate the role of gonadal hormones in the response by female rats to both phasic and persistent nociceptive stimulation, we evaluated the effects of long-term ovariectomy (OVX, 6 months) on the thermal pain threshold and on formalin-induced responses. The thermal pain threshold was evaluated with the plantar test apparatus, while persistent pain was induced by a subcutaneous injection of dilute formalin (50 microliter, 10%) in the dorsal hind paw. The formalin test was carried out in an open field apparatus where the animal's spontaneous behavior and formalin-induced responses (licking duration, flinching frequency and flexing duration of the injected paw) were recorded for 60 min. Estradiol and corticosterone plasma levels were determined in blood collected from the anesthetized animals at the end of the test. In OVX females, the duration of formalin-induced licking was longer than in Intact females during both the first and the second phase; flinching and flexing did not differ from Intact. The thermal pain threshold was only slightly affected by OVX. Estradiol and corticosterone were lower in OVX females than Intact ones. These data indicate that long-term depletion of gonadal hormones in female rats modulates the pain-induced behavioral responses related to supraspinal neural circuits (licking of the injected paw) rather than more spinally mediated responses such as formalin-induced flinching and withdrawal latency in the plantar test.

Introduction

Estrogens are gonadal hormones present in both males and females. Their role is complex and involves all body structures including the nervous system [1]. Estrogens have widespread effects throughout the CNS; for instance they modulate enkephalinergic neurons in the spinal cord, catecholaminergic neurons in the brain stem and midbrain, midbrain serotonergic pathways and the basal forebrain cholinergic neurons [1]. These pathways include neural circuits known to participate in pain processes.

Experimental and clinical data strongly suggest a close relationship between estrogens and pain. Indeed, the epidemiology of many chronic painful syndromes indicates important sex differences, with females being more affected than males [2,3]. Moreover, in women, estrogen replacement therapy is associated with an increased probability of developing temporomandibular joint (TMJ) disorders [4]. We have recently shown that intracerebroventricular (i.c.v.) injection of estradiol in male rats induces a female-like behavioral response, with an increase in formalin-induced licking [5]. These data suggest

a pronociceptive role for female gonadal hormones. However, other studies have reported a clear antinociceptive role of estrogens. For instance, in experiments using female and male rats subjected to phasic painful stimulation (i.e. thermal pain), it was demonstrated that estrogens have acute antinociceptive effects when administered i.p. or s.c. [6]. During pregnancy in both humans and rodents, elevated levels of estrogen are accompanied by an increase in the pain threshold (See [7]).

In humans, it is well known that menopause causes depletion of estrogens, while in experimental animals ovariectomy is a common method to deplete animals of their gonadal hormones. Gonadectomized animals undergo a series of changes directly and/or indirectly induced by loss of the gonads. In females the absence of the ovaries induces a drastic decrease of circulating estrogens and progesterone, while indirect changes are mostly due to the loss of estrogen-mediated feedback on the hypothalamic region followed by changes in many releasing hormones, including gonadotrophins. In the present experiment we focused our attention on estrogens, although we know that the behavioral effects observed can result from the interaction of many factors.

Estrogens affect cell functions through their ability to regulate various gene expressions. Thus the depletion of this hormone influences the amount of gene products, including receptors and peptides, required for the modulation of neuronal transmission. However, there are large sex steroid stores in fatty tissue [8] and many weeks are required for altered gene expression to be manifested after removal of estrogens (e.g. rat brain 5HT2a receptor densities were decreased 3 months but not 2 weeks after OVX [9]). Therefore, the several studies conducted after different periods from OVX (a few days, 3–4 months) and the different kinds of painful stimulation used (phasic vs. persistent pain) have not been sufficient to provide a clear idea of the effects of prolonged hormone depletion on pain.

The aim of this study was to compare intact female rats to OVX ones to determine if long-term hormonal depletion (6 months) could significantly modify the behavioral responses to phasic or persistent nociceptive stimuli. The plantar test [10] was used to determine the thermal pain threshold in free-moving animals; the formalin test, a well known method to induce tonic, long-lasting pain, was also used since it is particularly useful to record different behavioral responses mediated at different levels of the CNS (i.e. flinching, a phasic short contraction of the leg, present also in spinalized animals; flexing, a tonic contraction of the injected paw; licking, mediated at higher supraspinal levels) (see [11]). Estradiol and corticosterone plasma levels were determined in blood samples collected from the abdominal aorta at the end of the
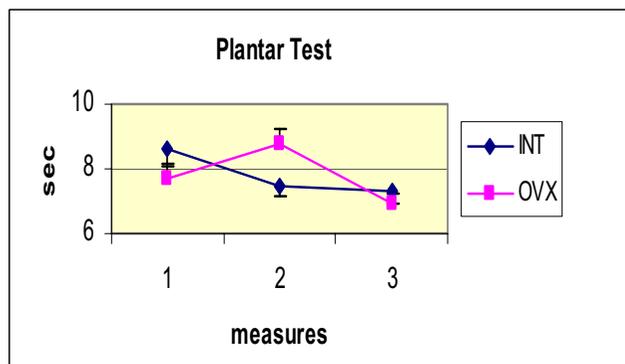
Figure 1
Plantar test Latency of paw withdrawal after administration of a thermal pain stimulus to the plantar surface of the rat's paw with an infra-red source (set at an intensity of 190 mWatt/cm^2). The measures were determined three times, with two minutes in between.

experimental session to evaluate the effects of OVX on activation of the hypothalamus-pituitary-gonadal (HPG) and hypothalamus-pituitary-adrenal (HPA) axes.

Material and Methods
Subjects
Twenty-eight adult female Sprague-Dawley rats, obtained from Charles River Italia (Calco, Lecco, Italy) and weighing 220–240 g on arrival, were used. The animals were housed in groups in a temperature- and humidity-controlled room with a light-dark cycle (lights on at 19.00 h, off at 07.00 h) and with free access to food and water. In all experiments, attention was paid to the regulations of local authorities for handling laboratory animals, the European Communities Council Directive (86/609/EEC) and the Ethical Guidelines for investigation of experimental pain in conscious animals issued by the ad-hoc Committee of the International Association for the Study of Pain [12].

Surgery
One-two weeks after their arrival, half of the rats were bilaterally ovariectomized (OVX) under pentobarbital anesthesia (40 mg/kg, i.p.) by removal of the ovaries and ovarian fat with a dorsal incision. The other rats were sham operated: the ovaries were exteriorized but not removed. The subjects were allowed 7–8 days to recover from surgery, during which they were kept alone in a cage. Groups were then reformed and the animals remained in groups till the end of the experiment.
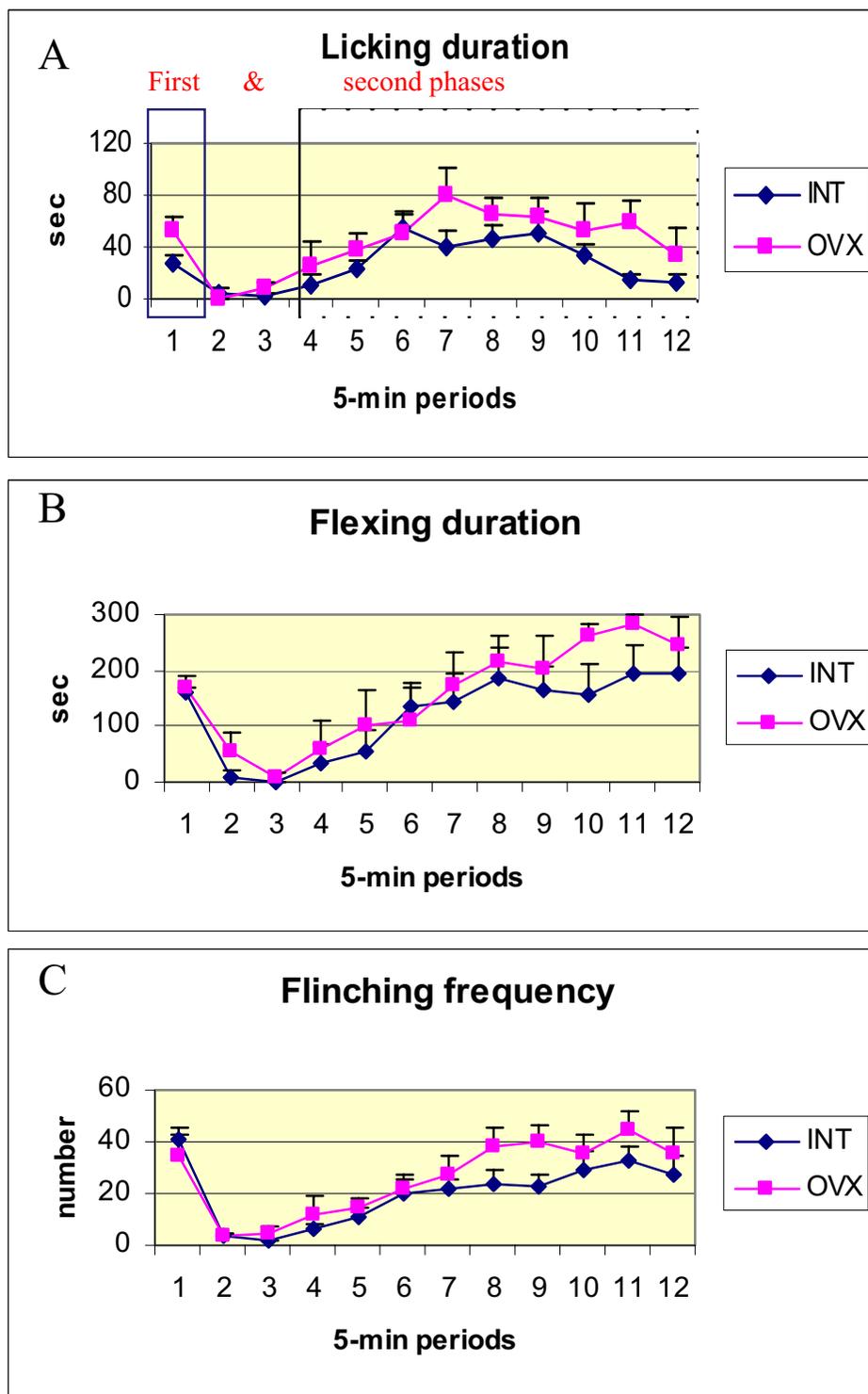
Figure 2
Formalin test A, Licking duration (sec), B, Flexing duration (sec) and C, Flinching frequency (num) were scored for a 1-hr period after formalin injection (50 μl, 10%) in Intact (INT) and Ovariectomized (OVX) female rats. The time after formalin injection (in 5-min periods) is indicated on the abscissa and the time spent licking or flexing or the frequency of flinching is indicated on the ordinate. Indications of significance for single time points were not included in order to maintain the clarity of the graph.
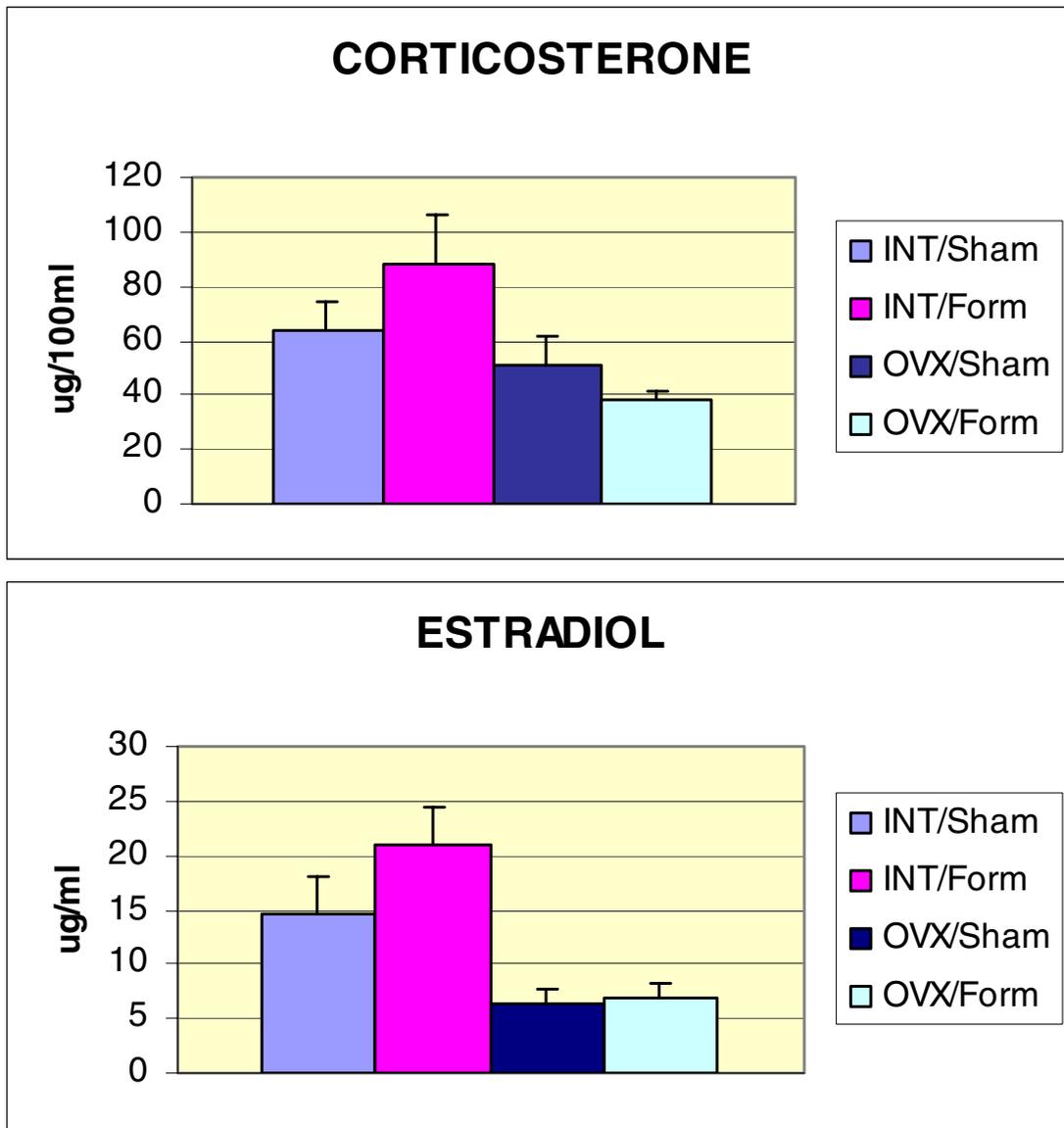
Figure 3
Hormone assays. Corticosterone and estradiol plasma levels were determined in all groups at the end of the experimental tests. Corticosterone and estradiol were lower in ovariectomized animals, independently of the formalin treatment.

General Procedure

About six months after ovariectomy, all animals were subjected to the plantar and formalin tests, with a week in between. The experiments were carried out in an isolated experimental room supplied with red light and white noise. Starting one week before testing, estrous cyclicity was monitored by daily vaginal lavages in both intact and OVX animals. Intact animals were subjected to the plantar and formalin tests while in the proestrous phase of the estrous cycle since during this cycle phase the estrogens reach their maximum plasma levels. Nociceptive tests were conducted during the dark, active phase of the light:dark cycle.

Plantar test

The rats were subjected to the plantar test [10] to measure the thermal pain threshold (Intact n = 14; OVX n = 14). The plantar test apparatus (Ugo Basile, Comerio, Italy) consisted of three perspex boxes (23 × 18 × 14 cm, each) with a transparent floor, through which it was possible to check the correct position of the infra-red source (set at an intensity of 190 mWatt/cm^2) under the plantar surface of the rat's paw. The latencies of paw withdrawal were directly measured by the apparatus itself. The animals were left undisturbed in the box for 5 min to familiarize them with the new environment. They were then subjected to 3 consecutive stimulations of the right hind paw, with 2-min periods in between.

Formalin test

Intact and OVX female rats were randomly divided into a no-pain group (Sham, the animals were only pricked in the dorsal hind paw with a syringe needle without injection of a substance) and a formalin-pain group (Form, the animals received a subcutaneous injection of 50 μl of 10% formalin in the dorsum of the hind paw). The following groups were obtained: Intact/Sham (n = 6), Intact/Form (n = 8), OVX/Sham (n = 6) and OVX/Form (n = 8). Immediately after injection, each animal was exposed to the open field apparatus for 1 hr; the animal's behavior was video-recorded for subsequent analysis. Formalin is an irritant that induces a series of behavioral responses indicative of pain which can be easily measured and quantified: flinching, flexing and licking of the injected paw as previously described [13]. In the present experiment we divided the formalin-induced responses in two parts, the first (0–5 min) and second (15–60 min) phase depending on their time course that shows a pick of activity in the first minutes after injection, a period of silence and than a second, longer phase, starting around 10–15 minutes after injection and lasting till the end of the test. Then, the animals were deeply anesthetized to collect blood from the abdominal aorta.

Hormonal assays

Corticosterone and estradiol plasma levels were determined by radioimmunoassay after extraction with methylene chloride using tritiated corticosterone and estradiol (NEN Life Science Products, Boston, USA) as tracers and the corresponding antibody (Analytical Antibodies, Milan Italy) according to the method of Abraham et al. [14] with modifications. Briefly, sixteen hours after incubation at 4°C, the free and bound hormones were separated with a charcoal-dextran mixture. After centrifugation, the radioactivity of the supernatant was determined with a β-scintillation counter. We employed a quality control procedure using tritiated trace hormones for extraction recovery and known samples to check the efficiency of the method. The inter-assay and intra-assay coefficients of variation were 8% and 5%, respectively, for all hormonal determinations.

Statistical analysis

All results are presented as mean ± SEM. The significance of differences between groups was determined by analysis of variance (ANOVA) with one between-subject factor, Ovariectomy (2 levels: OVX, Intact); for the plantar test data, the within-subject factor Measure (3 levels: Measure 1, M1; Measure 2, M2; Measure 3, M3) was also used; for the formalin test data, the factors Formalin (2 levels: Sham, Form) and Time (12 levels: 12 5-min intervals) were used. Fisher's Least Squares Differences test was used for post-hoc comparisons. Differences were considered significant when $p < 0.05$. Pearson's Correlation Coefficient was used to test for correlations among the different hormonal and behavioral measures.

Results

Two females belonging to the OVX group were found to cycle and were excluded from the analysis. Post-mortem dissection of these animals did not show any sign of ovaries. Ovariectomy did not significantly affect the body weight (ANOVA, p = 0.1) although the average weight of the OVX animals tended to be higher than the Intact ones (477 g vs. 437 g).

Plantar test

ANOVA applied to the latency of paw withdrawal revealed a significant interaction Ovariectomy × Measure ($p < 0.01$) due to the time course of the latencies in the three measures. In Intact animals the latencies progressively decreased from the first to the third measure, while in OVX animals the second measure was significantly higher than the first ($p < 0.05$) and third measures ($p < 0.001$) (Fig. 1).

Formalin test
Formalin-induced responses (Fig. 2)
ANOVA applied to licking duration recorded during the first phase (0–5 min) and the second phase (15–60 min)

Table 1: Formalin test: Spontaneous behaviors. Total duration (d, sec) or frequency (f, num) of the spontaneous behaviors recorded during the formalin test (60 min) in the groups subjected to Sham or Formalin treatment. INT: intact animals; OVX: ovariectomized animals. Data are means ± SEM.

		Inner crossing (f)	Outer crossing (f)	Exploratory activity (dur)	Grooming (dur)	Rearing (f)	Crouch (dur)
INT	Sham	147.2 ± 38.2	538.0 ± 79.6	1821 ± 220	501.9 ± 83.8	108.2 ± 24.0	83.7 ± 72.3
	Form	142.9 ± 28.5	415.4 ± 46.8	1527 ± 180	376.5 ± 72.2	100.4 ± 23.8	378.1 ± 111.2
OVX	Sham	29.5 ± 5.9	242.7 ± 29.0	1067 ± 173	763.8 ± 156.5	38.2 ± 5.8	592.5 ± 247.6
	Form	75.3 ± 36.7	416.5 ± 81.1	1110 ± 175	321.2 ± 61.8	38.3 ± 9.84	450 ± 177.7

revealed the significance of the factor Ovariectomy ($p < 0.03$, $p < 0.04$ respectively) due to OVX animals showing longer licking than Intact. Flexing duration and flinching frequency did not differ between the two groups (Fig. 2B and 2C).

Spontaneous behaviors (Table 1)
Ovariectomy changed all spontaneous behaviors. In particular, ANOVA showed that exploratory activity duration, inner crossing, outer crossing and rearing frequencies were lower in OVX than Intact animals ($p < 0.002$, $p < 0.006$, $p < 0.004$, $p < 0.002$); the significant interaction OVX × FORM × Time ($p < 0.01$) in exploratory activity duration was due to the higher formalin-induced decrease in Intact than OVX animals starting from the second half of the test. Grooming duration was higher in OVX than Intact animals ($p < 0.05$) only in sham-treated subjects.

All groups showed periods during which they spent most of their time motionless; crouch, the relaxed sleeping-like posture, appeared in both groups at around 35 minutes and reached the highest levels at the end of the test. Crouch was higher in OVX than Intact animals ($p < 0.05$).

Hormones
ANOVA applied to estradiol and corticosterone plasma levels revealed a significant effect of ovariectomy for both hormones ($p < 0.001$ and $p < 0.02$, respectively) due to the higher levels in Intact than OVX animals (Fig. 3). As for corticosterone, formalin treatment appears to differently modulate its levels since in Intact animals it induce higher levels than sham-treated animals, while in OVX animals formalin tended to decrease it.

Correlations
Pearson's Correlation Coefficient revealed a positive correlation between corticosterone and estradiol plasma levels ($n = 21$, $r = 0.53$ $p < 0.05$). In addition to being positively correlated with corticosterone, estradiol was negatively correlated with the total amount of formalin-induced licking ($n = 10$, $r = -0.63$ $p < 0.05$) (Fig. 4).

Discussion
In this study, we have shown that licking of the paw induced by subcutaneous injection of formalin was higher in OVX female rats than Intact ones. This difference can be explained by long-term depletion of gonadal hormones and their direct and/or indirect modulatory effects on nociceptive pathways.

In this and other studies, we have shown that formalin-induced licking, flexing and flinching are rarely affected in the same way by a particular treatment [5,13], suggesting that the neural circuits involved in their modulation are affected differently. In this case, the clear increase of licking in OVX animals with respect to Intact ones during both the first and second phase, and the lack of effects on flexing and flinching, support the hypothesis that gonadal hormone depletion affects supraspinal circuits more than spinal ones.

To explain the hyperalgesic action induced by long-lasting OVX, we can hypothesize that one of the neural systems involved includes opioids and their receptors. Indeed, a region-specific loss of µ-opioid receptor labeling following long-term OVX was shown in mice [15], while Quinones-Jenab [16] reported that estrogens increase µ-opioid receptor mRNA levels in the ventromedial and arcuate hypothalamic nuclei of OVX female rats. Moreover, estrogens affect the proopiomelanocortin (POMC) system; indeed, it is known that a subset of POMC neurons contains estrogen receptors [17]. However, it is also known that estrogens can produce potent effects on other neurotransmitter systems (e.g. dopaminergic and/or noradrenergic systems); for instance, Bosse [18] observed a decrease in D1 receptors in the frontal cortex of OVX rats together with an alteration of GABA receptors in other motor nuclei. Interestingly it was shown that modulation of catecholaminergic systems can change POMC mRNA concentrations [19]. Estrogen receptors are present not only in supraspinal structures but also in the superficial layers of the spinal dorsal horn, i.e. the area of the CNS where the initial processing of pain sensation occurs. Estrogen administration to OVX female rats increases enkephalin transcription in the spinal cord [20], in
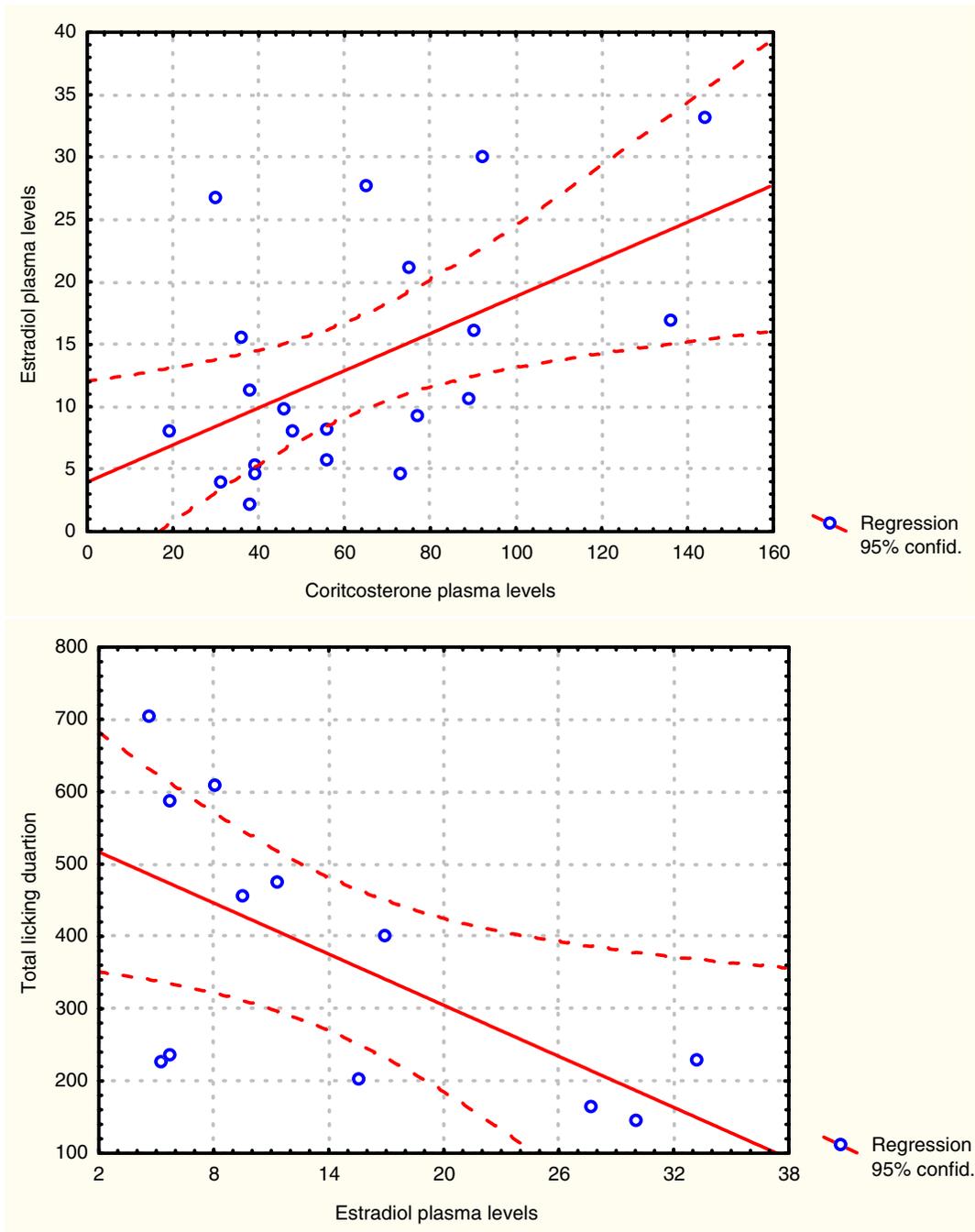
Figure 4
Correlations. Graphic representation of the linear correlation between estrogen and corticosterone plasma levels (n = 21, r = 0.53, p < 0.05) and between estrogen plasma levels and the total amount of formalin-induced licking (n = 10, r = -0.63 p < 0.05).

contrast to the reduction of this peptide in OVX rats. Enkephalinergic neurons in the superficial dorsal horn are crucial components of the endogenous pain-inhibitory system since they mediate the inhibition of nociceptive relay cells [21].

There have been several studies of the effects of OVX on pain thresholds, although different measures were used and different results were obtained. For instance, it was shown that tail withdrawal latency was lower in OVX rats than Intact ones. The animals were repeatedly tested and there was a slow increase in latency in Intact rats but not in OVX ones during the 7 weeks of testing [22]. In contrast, Dawson-Basoa and Gintzler [23] reported that pre- and post-ovariectomy baseline pain thresholds were indistinguishable.

In the present experiment the differences between Intact and OVX groups in thermal pain threshold was only due to a different time course and flinching frequency did not show any difference between the two groups. This indicates that spinal cord circuits were not affected or had completely recovered their functions after such a long time. Indeed, although estrogen plasma levels were lower in OVX rats than Intact ones, they were present at detectable levels, indicating the important role played by the adrenals in secreting steroid hormones once the ovaries no longer produce hormones. It appears that only high levels of estrogens, like those reached during proestrus, can affect supraspinal structures. This is well known for gonadotropin regulation, indicating once more the close relationship between reproductive and sensory (including pain) systems.

Corticosterone is one of the primary hormones produced by the adrenal cortex. It is a central component of the stress response and interacts with sympathetic and opioid mechanisms that can influence central pain processing. There is good evidence that gonadal steroids modulate the HPA axis. Corticosterone levels are lower in ovariectomized rats than in intact ones, as observed in the present experiment. Moreover, formalin treatment tends to increase corticosterone in Intact but not in OVX animals. In humans, reduced adrenocortical activity has been implicated in chronic pain conditions, such as low back pain, rheumatoid arthritis and chronic pelvic pain (see [24]). Interestingly, research on experimental animals suggests the involvement of CRF in pain sensitivity [25].

Alterations of female gonadal hormones are associated with anxiety and mood changes. In rodents, grooming behavior can represent a displacement behavior due to a stressful environmental situation and/or a behavioral pattern whose expression is affected by specific damage to its neural substrates (see ref. [26] for a review). In the present experiment the higher levels of grooming in OVX rats can probably be attributed to the lower levels of gonadal hormones, i.e. estrogens, but also of progesterone which, as a precursor of neurosteroids, can play a crucial role in determining the levels of anxiety [27]. In addition, it has been suggested that some of these effects may be mediated by serotonin system modulation. It is interesting that Cyr et al. [9] found that OVX for 3 months decreased 5-HT2a receptor mRNA and receptor binding in the frontal cortex, whereas estradiol supplementation for 2 weeks restored receptor expression to control levels; this research, showing a correlation between cognitive functions and pain, indicates the possibility of indirect effects of gonadal hormones on pain through anxiety and mood processes.

In summary, we have shown that there are differences in formalin-induced responses between intact and OVX female rats. Moreover, these modifications are long-lasting and refer to supraspinal circuits mediating complex pain responses. Further experiments are required to determine whether the effects are directly induced by the estrogen depletion or are due to the lack of modulation of gonadal hormones on other neural systems.

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