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Effects of neonatal testosterone treatment on sex differences in formalin-induced nociceptive behavior in rats

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Abstract

There are sex differences in nociceptive behavior induced by formalin in rats. To determine whether these sex differences are the result of the sexual differentiation of the brain, that is masculinization and defeminization [A.P. Arnold, R.A. Gorski, Gonadal steroid induction of structural sex differences in the central nervous system, Annu. Rev. Neurosci. 7 (1984) 413–442; M.M. McCarthy, A.T.M. Konkle, When is a sex difference not a sex difference? Front Neuroendocrinol. 26 (2005) 85–102], some female rats were injected with testosterone propionate (TP, 100 μg/25 μl/rat) on the day of birth and on the following day. As controls, other female rats and all male rats were injected with the same volume of sesame oil. They were castrated at the age of 8 weeks, and implanted with a silicon tube containing 20% of 17β-estradiol or cholesterol. Two weeks after the implantation, rats were injected with 50 μl of 2% formalin in the right hind paw and their behavioral changes were observed for 1 h. In cholesterol-implanted rats, all rats exhibited three typical phases of pain response and there were no significant differences in the scores of nociceptive behavior. In 17β-estradiol implanted rats, female and TP-treated female rats had a significantly higher score of nociceptive behavior than male rats. These results indicate that estrogen produces sex differences in nociceptive behavior induced by formalin, and suggest that these differences are not due to the sexual differentiation of the brain, since the dose and the timing of the TP treatment effectively defeminize and masculinize female rats. Alternatively, sexual differentiation of the brain response to formalin-induced nociceptive behavior may be different from ordinary sexual differentiation.

Keywords: Formalin; Sexual differentiation; Testosterone; Pain; Nociceptive behavior; Estrogen

Experimental as well as clinical studies have suggested that there are sex differences in pain and pain-related phenomena: females are in general more sensitive to pain than males [2,6,18,20]. The formalin test is widely used as a model of tonic pain since it is comparable to the human clinical situation [11,24]. Studies in rats and mice show that females exhibit greater nociceptive behavior response than males [1,8,12,15,23]. In addition, Fos expression induced by formalin in the hippocampus of female rats is greater than in male rats [3]. Although gonadal steroid hormones play a major role in these sex differences, the effects of the hormones on formalin-induced pain are complicated and contradictory [2,18]. For example, estrogen and progesterone replacement in ovariectomized rats reverses the ovariectomy-induced decline in the nociceptive responses, while estrogen replacement alone in ovariectomized rats is without effect, showing a hyperalgesic effect of ovarian hormones [13]. In contrast, it was reported that estrogen alone decreases the nociceptive responses, showing a hypoalgesic effect of estrogen [18].

In rats, the sexual differentiation of the brain occurs during a prenatal and postnatal period, the so-called critical period, and the increment in testosterone secretion during this period from the testis induces functional and morphological changes (organizational effects) to produce the male brain type [5,19]. Later in adult life, gonadal steroid hormones exhibit a variety of physiological functions (activational effects). Thus, it would appear that sex differences in the response to nociceptive stimuli, such as formalin are the result of the sexual differentiation of the brain. In support of this view, it has been shown that sex differences in morphine-induced analgesia are due to the organizational effects of testosterone [9,17].

The aim of this study was to determine whether or not neonatal treatment with testosterone in female rats affected the formalin test in adults. Concurrently, we also examined the effects of estrogen on the formalin test in castrated rats.

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Intact Wistar male and female rats (7–8 weeks of age), and pregnant Wistar female rats were obtained from Charles River (Yokohama, Japan). They were maintained at a constant temperature of 24–26 °C under controlled lighting conditions (lights on 5:00–19:00) with food and water available *ad libitum*. Daily vaginal smears were taken in intact female rats, and those exhibiting 2 or more consecutive 4-day estrous cycles were used on the day of proestrus in the present study. Intact male rats were handled on a time course similar to that of intact female rats. Next, some female pups were injected subcutaneously with 100 μg testosterone propionate (TP) in 25 μl sesame oil on the day of birth and on the next day (designated as the masculinized group). The other female and male pups were injected with 25 μl of sesame oil in the same manner as for TP treatment. In order to avoid possible cross-contamination with TP-containing oil, when female pups were treated with TP, the remaining male littermates were not used. After weaning, the vaginal opening was checked in female rats to confirm the success of the TP treatment. The dose and the number of TP injections to produce masculinization in females have been determined elsewhere [4,21]. All rats treated neonatally with oil or TP were orchidectomized (ORX) or ovariectomized (OVX) at the age of 8 weeks to achieve a similar environment of gonadal hormones.

At the time of castration, some rats were implanted subcutaneously with a silicone tube (inside diameter, 2.0 mm; outside diameter, 3.0 mm; length, 1.5 mm) containing cholesterol alone (ORX, OVX, and masculinized + OVX groups) as controls. The remaining rats were implanted subcutaneously with a silicone tube (length, 1.5 mm) containing 20% 17β-estradiol crystals (ORX + E2, OVX + E2, and masculinized + OVX + E2 groups). The silicone tubes had been first soaked in saline for more than 24 h to facilitate rapid hormone release.

To confirm the effects of the steroid replacement, serum concentrations of estradiol were determined by EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA) in some rats implanted with 17β-estradiol. Serum samples were extracted once with diethyl ether and reconstituted with assay buffer.

The rats were injected with 50 μl of 2% formalin into the right hind paw and their behavioral changes were examined for 1 h. Immediately after the injection, a rat was placed in a transparent Plexiglas box (30 cm × 30 cm × 30 cm) with a transparent floor positioned over a mirror at an angle of 45 degree to allow the observation of nociceptive behavior. Assessment of nociceptive behavior was carried out on a single parameter by scoring the time spent licking the injected paw [24]. The total time that the paw was kept elevated from the floor was calculated from observations at 5-min intervals and designated as foot-lifting duration.

Data were analyzed by repeated measure ANOVA and one way ANOVA followed by Fisher's protected least significant difference post hoc test for comparison of treatments. Differences were considered significant at $p < 0.05$.

As shown in Fig. 1, both sexes of rats exhibited typical pain-related behavior, showing two phases as previously reported: phase 1 and phase 2 showed a high score separated by an interphase [11,24]. That is, phase 1 started immediately after formalin injection and lasted for approximately 5 min followed by a relatively low level of nociceptive behavior for 10 min (interphase). Thereafter, phase 2 of nociceptive behavior appeared and lasted for the entire observation period. However, the foot-lifting duration in intact female rats appeared to be longer than in intact male rats at interphase. Repeated measure ANOVA showed a significant effect of time ($p < 0.0001$) with a significant interaction ($p < 0.05$), but no effect of sex ($p > 0.3$, males versus females). Since the interaction was significant, the data were further analyzed by one way ANOVA at each time point, and found that the foot-lifting duration in intact female rats was significantly longer than in intact male rats at 10 and 15 min after formalin injection ($p < 0.05$), showing a sex difference in the formalin test.

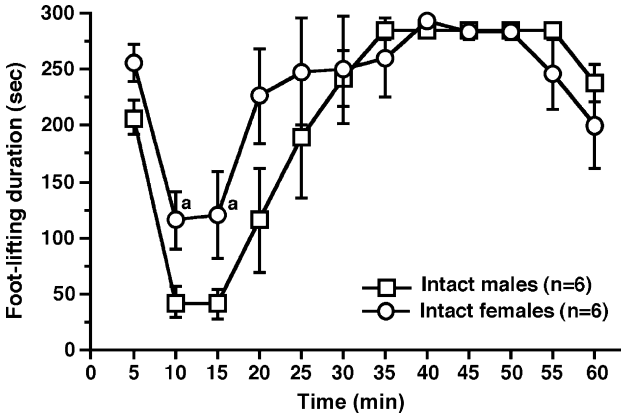

Fig. 1. Formalin tests in the intact male and female groups. Each data point represents the amount of time the animals spent elevating the injected paw at each 5-min bin during 1 h of observation. There was no significant difference between the groups for phase 1 and phase 2, but the intact female group presented more nociceptive responses than the intact male group for the interphase. Each point and vertical line indicates the mean and ±S.E.M., respectively. Numbers in brackets refer to the number of animals. "a" indicates $p < 0.05$ vs. intact male rats. See the text for further statistical details.

Female rats treated with TP neonatally (masculinized group) did not show a vaginal opening at the time of experiment, suggesting the success of the TP treatment, as shown in a previous report [25]. In the castrated rats, there were no significant differences among the ORX, OVX, and masculinized + OVX groups in the formalin test (Fig. 2, ANOVA, $p > 0.5$), showing that there were no sex differences in the absence of the gonads, and that neonatal TP treatment in female rats did not affect the response to formalin in the absence of the ovaries in adults.

No significant differences in serum concentrations of estradiol were observed among the groups (Table 1, ANOVA, $p > 0.9$).

Table 1
Serum concentrations of estradiol

Group	Number	pg/ml
ORX + E2	4	116.1 ± 74.4
OVX + E2	7	119.3 ± 75.0
Masculinized-OVX + E2	7	113.5 ± 45.7

ORX, orchidectomized rats; OVX, ovariectomized rats; E2, 17β-estradiol replaced. All data are presented as the means ± S.E.M.