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Acute estrogen surge enhances inflammatory nociception without altering spinal Fos expression

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HIGHLIGHTS

- Effect of an estrogen surge on inflammatory nociception was studied in female rats.
- Estrogen increased formalin-induced pain-related behavior.
- Estrogen did not alter formalin-induced Fos-positive neuron counts in spinal cord.
- Pronociceptive effects of acute estrogen may occur via non-spinal mechanisms.

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ABSTRACT

Chronic pain is a major neurological disorder that can manifest differently between genders or sexes. The complex actions of sex hormones may underlie these differences; previous studies have suggested that elevated estrogen levels can enhance pain perception. The purpose of this study was to investigate the hypothesis that acute, activational effects of estradiol (E2) increase persistent inflammatory nociception, and anatomically where this modulation occurs. Spinal expression of Fos is widely used as a marker of nociceptive activation. This study used formalin-evoked nociception in ovariectomized (OVX) adult female rats and measured late-phase hindlimb flinching and Fos expression in the spinal cord, and their modification by acute estrogen supplementation similar to a proestrus surge. Six days after ovariectomy, female rats were injected subcutaneously (s.c.) with 10 μg/kg E2 or vehicle. Twenty-four hours later, 50 μL of 1.25% or 100 μL of 5% formalin was injected into the right hindpaw; hindlimb flinches were counted, and spinal cords removed 2 h after formalin injection. The numbers of Fos-expressing neurons in sections of the lumbar spinal cord were analyzed using immunohistochemistry. Formalin-induced inflammation produced a dose-dependent increase in late-phase hindlimb flinching, and E2 pretreatment increased flinching following 5%, but not 1.25% formalin injection. Despite the modification of behavior by E2, the number of spinal Fos-positive neurons was not altered by E2 pretreatment. These findings demonstrate that an acute proestrus-like surge in serum estrogen can produce a stimulus-intensity-dependent increase in inflammation-evoked nociceptive behavior. However, the lack of effect on spinal Fos expression suggests that this enhancement of nociceptive signaling by estrogen is independent of changes in peripheral activation of, expression of the immediate early gene Fos by, or signal throughput of spinal nociceptive neurons.

1. Introduction

Notable sex differences exist in the prevalence of pain disorders and the experience of pain [1–6]. Sex hormones (e.g., estrogens) are thought to contribute to these differences through organizational and/or activational effects. Estrogens appear to have complex activational – often pro-nociceptive – effects on innervation, synapse formation, and sensory function. Previous reports demonstrated that elevated serum estrogen levels enhance persistent inflammatory nociception [3,7–14]. However, the literature is complicated by

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studies that do not directly address acute sensory modification by a single estrogen (i.e., E2) in female subjects. Studies of female hormone effects on nociception have investigated longer time scales than estrous cycle fluctuations [15,16], directly manipulated multiple hormones [17], manipulated a hormone other than E2 [18], were conducted in males [19,20], observed effects of endogenous hormones over the estrous cycle [7], or employed pain models other than formalin.

Therefore, the purpose of this study was to determine whether the acute, activational effects of E2 increase persistent inflammatory nociception. We postulated that sex differences in pain sensation and the disproportionate burden of inflammatory pain in women are due, at least in part, to direct, acute effects of E2. Thus, the working hypothesis was that acute administration of E2 would increase nociceptive behavior evoked by persistent inflammation in female rats. The model chosen was the intraplantar injection of dilute formalin, which is widely used to evoke spontaneous pain-related behaviors [21,22]. For this study, acute fluctuations in serum estrogen levels on a time scale modeling the proestrus phase of the estrous cycle were produced by s.c. injection of E2 [23,24] as a pretreatment 24 h before the induction of inflammatory nociception with formalin.

This study also aimed to investigate the primary site(s) where E2 modifies nociception, hypothesizing that these acute, activational effects of E2 occur in peripheral, spinal and/or supraspinal sites involved in the transmission and perception of pain. There have been no reports of direct, systematic investigation into identification of the anatomical sites of action in the nervous system of the enhancement of pain by estrogens. As a first step in determining where this modulation occurs, this study addressed spinal nociceptive activation with the hypothesis that the acute modulatory effects of E2 target the peripheral and/or spinal nervous system and would manifest as increased numbers of spinal Fos-positive neurons. Previous studies demonstrated stimulus-intensity dependence of spinal Fos immunoreactivity following formalin injection [25–27]. The distribution of spinal Fos-positive neurons following noxious stimulation has been widely studied [26–30]. The number Fos-positive neurons in deeper spinal laminae correlated with the intensity of nociception-evoked behavior [31], suggesting the rationale for quantification of Fos within the dorsal laminae used in the current study.

2. Materials and methods

A total of 106 adult female Sprague-Dawley rats (~11 weeks old, 200–230 g, Harlan, Indianapolis, IN) were housed on a 12-h light/dark cycle, fed Harlan Teklad 8604 chow, and all procedures were performed during the light cycle. Rats were housed one per cage for days 1–4 post-surgery and two per cage otherwise. All procedures were approved by the KUMC Institutional Animal Care and Use Committee and followed the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals and the *Guide for the Care and Use of Laboratory Animals* [32].

The effects of estrogen on behavioral responses to formalin were investigated using two randomized groups of rats: (1) ovariectomized (OVX) receiving E2 (OVX + E2), and (2) OVX receiving an equivalent volume of vehicle (OVX + Veh). Six days after OVX, a surge in E2 produced by bolus s.c. injection was followed by nociceptive behavioral evaluation. Approximately 24 h after E2 or vehicle injection (seven days after OVX), randomly-selected rats received a unilateral injection of 100 μL of 5% or 50 μL of 1.25% formalin into the right plantar hindpaw. Spontaneous hindpaw flinches were quantified 30–40 min post-injection (during the peak of the late-phase behaviors) in randomized order by an observer blind to E2 status/treatments.

In experiments assessing the spinal expression of Fos, a separate cohort of rats received identical E2 and formalin treatment as the behavioral cohort. A previous time-course study showed that maximum spinal Fos staining occurred 2 h after formalin injection [30]. Accordingly, 2 h after formalin injection, rats were anesthetized with ketamine/xylazine and perfused transcardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS, pH 7.4. Lumbar spinal cords were subsequently removed by laminectomy and post-fixed in paraformaldehyde at 4 °C, then in 30% sucrose at 4 °C before freezing.

In a separate control experiment to assess the efficacy of estrogen injection, 24 h after E2 injection (seven days after OVX), rats were weighed then decapitated, adipose tissue was removed from uteri, and uteri were excised at the base and weighed wet.

2.1. Hormone manipulation

For ovariectomy (OVX), rats were anesthetized with ketamine (64 mg/kg, i.p.; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5.3 mg/kg, i.p.). Under aseptic conditions, both ovaries were externalized and excised. Muscle and skin layers were individually closed. Six days later, rats received a single s.c. injection of 10 μg/kg E2 benzoate (Sigma, E-9000) at 10 μg/mL, or an equivalent volume of vehicle (10% ethanol/90% corn oil). By this time point after OVX, serum E2 levels were shown to be below non-proestrus levels by a previous study [33]; this dose of E2 was chosen to mimic the surge in E2 observed during proestrus in rats [23,24,34–36] as previously described [23,24].

2.2. Immunohistochemistry for Fos

For fluorescent immunohistochemistry, frozen lumbar spinal cords were cut into 20 μm-thick transverse sections and placed on charged glass microscope slides. Slides were washed in 0.4% Triton X-100 in PBS, pH 7.4, blocked with 5% normal donkey serum plus 1% bovine serum albumin in the same buffer, and incubated with primary rabbit anti-Fos antibody (1:1000, Calbiochem, Ab-5, Cat. No. PC38) overnight at 4 °C. They were then incubated with secondary fluorescein (FITC)-conjugated donkey anti-rabbit antibody (1:200, Jackson ImmunoResearch, Code No. 711-095-152) for 1 h at room temperature, then mounted with Vectashield Hard Set mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Cat. No. H-1500). Sections were fluorescently co-labeled for NeuN to verify neuronal identification. NeuN co-labeling with Fos was not quantified, but Fos-positive cells appeared to be exclusively co-labeled with NeuN. Slides were then viewed on a Nikon 80i fluorescent microscope system by an observer blind to E2 status/treatments, and Fos-positive neurons counted in ipsilateral laminae I–VI of sections at least 60 μm apart in spinal lumbar 4th–5th vertebral segments, with at least five sections counted and averaged for each rat. Laminar divisions and spinal segments were based on gross landmarks of the lumbar enlargement [37]. Comparison of Fos immunoreactivity with DAPI staining during counting was used to confirm that only neurons displaying intact nuclei were quantified.

2.3. Data analyses

All data represent the mean ± SEM. Data were analyzed by Student's *t*-test (SigmaPlot 10.0, Systat Software, Inc.). Significance was set at $p \leq 0.05$ throughout all analyses.

3. Results

Measurement of uterine weights of E2-treated rats revealed that rats receiving E2 (OVX + E2; $n = 18$) had significantly higher uterine