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Acute Estrogen Surge Enhances Inflammatory Nociception Without Altering Spinal Fos Expression

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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 10μg/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 two hours 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.

Keywords

Pain; Rat; Formalin Test; Spinal cord; Behavior; Estrogen

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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 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 hours 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.

Materials & Methods

A total of 106 adult female Sprague-Dawley rats (~11 weeks old, 200–230 g, Harlan, Indianapolis, IN) were housed on a 12-hour 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 hours 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 minutes 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 hours after formalin injection [30]. Accordingly, two hours 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 hours 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.

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].

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 hour 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 4^{th}–5^{th} 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.

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.

Results

Measurement of uterine weights of E2-treated rats revealed that rats receiving E2 (OVX + E2; n = 18) had significantly higher uterine weight (197.7 ± 8.5 g) than controls receiving vehicle (OVX + Veh, n = 17; 130.5 ± 4.1 g; Student's t-test, Welch-corrected).

Behavioral analyses of formalin-injected OVX rats revealed that, for 1.25% formalin, the total number of late-phase flinches (30–40 min) for rats receiving E2 (67.2 ± 6.0) were not different than controls receiving vehicle (79.6 ± 7.2; Student's t-test, n = 10 per group, Figure 1). In contrast, for 5% formalin, rats receiving E2 (140.3 ± 9.4) flinched significantly more than controls (112.0 ± 9.4; Student's t-test, n = 12 per group, Figure 1). More detailed temporal analyses were performed to examine the effect of E2 on formalin-evoked hindlimb flinches 0–60 minutes post-injection for 1.25% formalin. Total flinches over the entire 60 minutes were: Vehicle, 286.4 ± 15.1; E2, 289.6 ± 16.1. When flinching behaviors were broken down into Early (0–5 min), Inter- (5–15 min) and Late (15–60 min) phases, total flinches for rats receiving E2 were not different than controls receiving vehicle for any time period or for the entire 60 minutes (Data not shown; Student's t-test, n = 10 per group).

Hindpaw formalin injection elicited robust Fos expression in neurons throughout the ipsilateral lumbar dorsal horn after two hours (Figure 2). Formalin-evoked Fos expression was visible throughout the upper laminae of the ipsilateral dorsal horn; Fos staining in the contralateral dorsal horn was sparse. Fos-positive neurons were more prevalent in the medial part of the dorsal horn than laterally. Additionally, the number of Fos-positive neurons in the ipsilateral spinal cord dorsal horn was proportional to the stimulus intensity (Figure 3).

Comparison of rats receiving E2 (OVX + E2) with those receiving vehicle (OVX + Veh; n = 8 each) showed E2 administration did not alter the number of Fos-positive neurons in spinal cord laminae I–II, III–IV, V–VI, or the total across laminae I–VI evoked by 1.25% formalin (Figure 3A). At 5 % formalin, results also revealed no difference due to E2 for any of the

laminar regions (Figure 3B; n = 5–6 per group). The hypothetical possibility of a ceiling effect on the number of Fos-positive neurons at 5% formalin initially investigated prompted further investigation of the same comparison at 1.25% formalin. This experiment revealed that 1.25% formalin also elicited robust Fos expression in the ipsilateral dorsal horn, with a pattern similar to that observed with 5% formalin, except lower in magnitude.

Discussion

For confirmation of systemic efficacy of E2, measurement of uterine weights revealed that ovariectomized rats receiving a single dose of E2 mimicing the proestrus surge of estrogen in intact female rats [23, 24, 34–36] had higher uterine weights than controls. Consistent with a previous report [24], these data demonstrated that the single dose of E2 produced systemic estrogenic (uterotrophic) effects evident 24 hours after E2 injection. These results confirmed the efficacy of this estrogen dose and timing regimen.

The current study tested the effects of E2 on nociception by using pain-related behavioral analysis. This study modeled effects of a proestrus E2 surge in modifying persistent inflammatory pain similar to that of chronic pain disorders. Results of E2 manipulation showed that a single injection of E2 enhanced late-phase formalin-induced hindpaw flinching at 5% formalin (Figure 1B); in contrast, no difference was observed at 1.25% (Figure 1A), even upon further, more detailed investigation of flinching for the entire hour post-injection. At the higher stimulus intensity, the proestrus-like E2 surge increased inflammatory pain-related behavior. This observation is consistent with several previous reports suggesting that elevated serum estrogen levels enhance persistent inflammatory nociception [3, 7–14]. However, the current results are difficult to directly compare or contrast with many previous studies that differed in the hormone manipulation, formalin dose, age and sex of rats, or pain model used. Previous reports have investigated the effect of gonadal hormones on persistent inflammatory nociception, but few have directly manipulated E2. In a gonadectomy study performed in rats, Aloisi et al. [38] reported that gonadal hormones in both sexes inhibited paw flexion duration following formalin, but did not affect flinching. In other studies [39, 40], females had higher flexion and licking duration than males following formalin. Another study showed gonadectomy for 3 months in female rats increased rubbing of a formalin-injected lip, but there was no change in flinches of an injected paw [41]. A different study demonstrated that E2 increased formalin-induced paw licking behavior in rats [19] –possibly corroborating the current findings – but was performed in male, not female subjects. A few studies showed an anti-nociceptive effect of E2 on formalin-related behaviors, but measured a different metric of paw movement and used longer E2 supplementation in younger rats [16, 42, 43]. This is the first report to demonstrate that an acute E2 manipulation previously shown to alter E2 levels and uterine innervation [24], is pronociceptive in the formalin model.

The observation that E2 increased flinching at 5%, but not 1.25% formalin indicates E2 is pronociceptive in a stimulus-intensity-dependent manner, and may require a threshold level of nociception. Stimulus-intensity dependence for sex differences in behavioral responses to formalin [44] and other noxious stimuli have been described [45]. This stimulus-intensity dependence likely extends to sex-hormone modulation of nociception – which current

results suggest. The current results add to the existing knowledge of sex hormone modulation of persistent nociception by demonstrating that acute, direct manipulation of E2 in female rats increased nociceptive behavior evoked by 5% formalin.

This study, however, indicates a lack of effect of E2 on nociception-evoked spinal Fos expression. Results of immunohistochemical analysis revealed that formalin injection elicited robust Fos expression in neurons throughout the ipsilateral lumbar spinal cord dorsal horn after two hours (Figure 2). The number of Fos-positive neurons was directly related to the stimulus intensity (Figure 3), corroborating previous reports establishing Fos expression as a biomarker of nociceptive activation [25–27]. However, E2 did not alter the number of Fos-positive neurons in the ipsilateral dorsal spinal cord evoked by either formalin concentration (Figure 3), despite enhancing formalin-evoked flinching at the higher stimulus intensity. Together, these observations support the conclusion that, whereas an acute estrogen surge enhances pain-related behaviors evoked by persistent inflammatory nociception, estrogen may not significantly modulate peripheral or spinal nociceptive neuronal activation, and that these sites may not be primary targets for the pronociceptive effects of E2. Estrogen may enhance nociception at supraspinal sites where pain sensation is modified subsequent to spinal transmission. The increased flinching in E2-treated subjects may result from modification of nociceptive sensation or subsequent motor responses at sites subsequent to the first synaptic connection in the spinal dorsal horn.

Alternatively, the number of Fos-expressing spinal neurons may not fully reflect the degree of spinal activation by a persistent stimulus. There may be a ceiling on the number of Fos-expressing spinal nociceptive neurons available for activation above that evoked by 5% formalin or for “recruitment” under the effects of estrogen. Also, Fos expression may represent activation of both inhibitory and excitatory neurons, and both may respond to E2. Furthermore, immunohistochemical “positivity” quantified in this study may not reflect the intracellular *degree* of activation of individual neurons. E2 enhancement of neuronal function could be manifested as a cause or consequence of modification of expression of other genes without modulation of Fos. For example, a previous study showed that nociception-evoked gene expression in spinal cord dorsal horn was modulated by estrogen status [15], but this study used longer, constant E2 exposure and measured gene expression at later time points. Nonetheless, the current results suggest that behavioral enhancement by an E2 surge may be independent of spinal Fos modulation and/or the neuronal activation that it represents.

Conclusion

This study demonstrates that acute, direct manipulation of E2 in female rats increased formalin-evoked hindlimb flinching in a stimulus intensity-dependent manner. However, the numbers of nociception-evoked Fos-positive neurons in the spinal cord were not modified by an acute estrogen surge. Together these results suggest that non-spinal targets or mechanisms independent of spinal Fos modulation are responsible for the pronociceptive, acute activational effects of estrogen. These findings further the understanding of the modulation of pain by sex hormones, and how estrogen may contribute to the disproportionate burden of chronic inflammatory pain in women.

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- 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

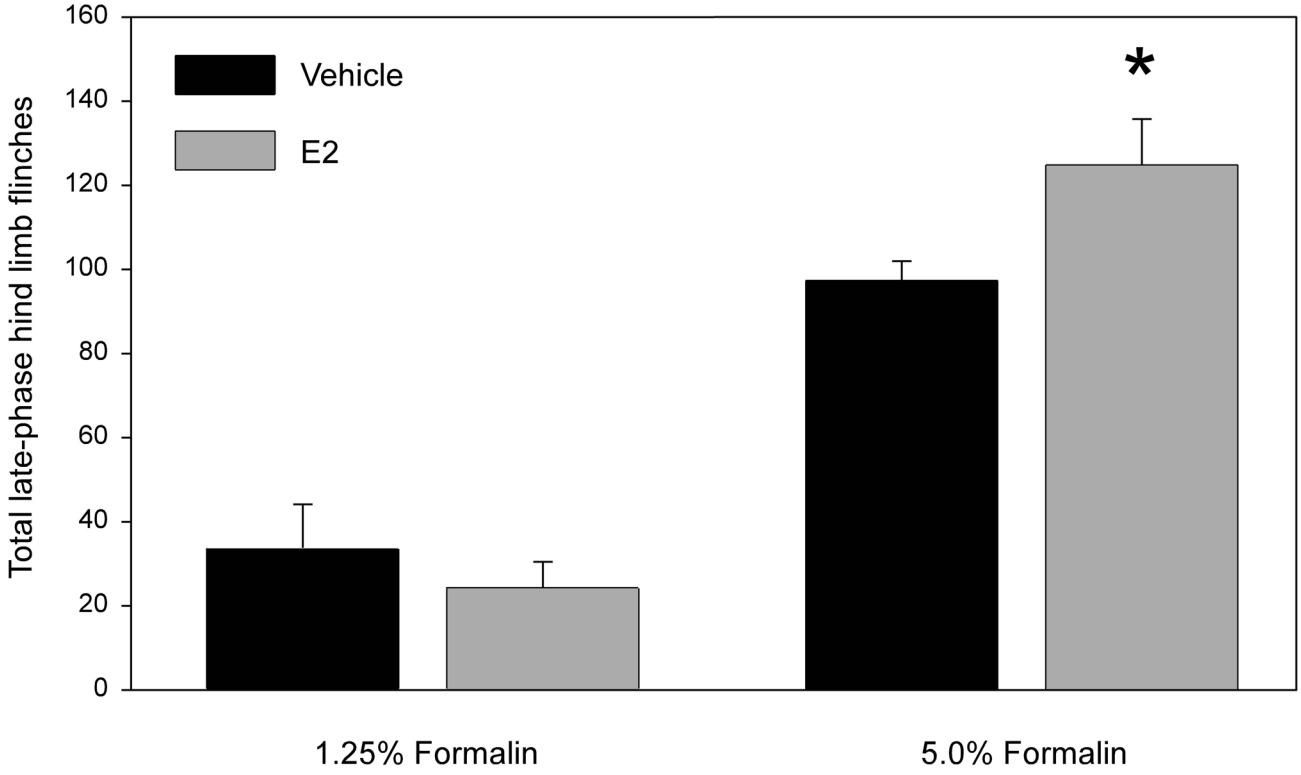

Figure 1.

Effect of E2 on (**A**) 1.25% or (**B**) 5% formalin-evoked flinching behavior 30–40 minutes post-formalin. Data represent the mean ± SEM ($p \leq 0.05$, unpaired Student's t-test; 1.25% n = 10; 5% n=12).

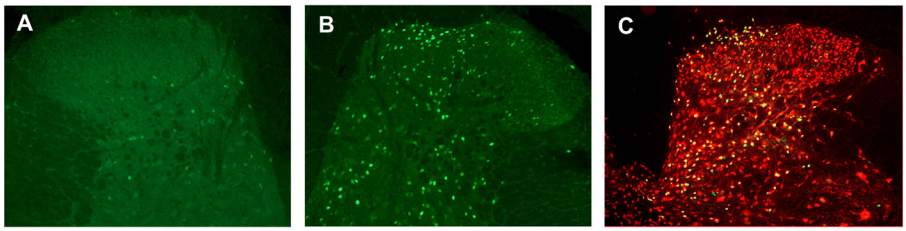

Figure 2.

Representative photomicrographs of Fos fluorescent immunohistochemistry in lumbar spinal cord dorsal horns following formalin. Note that formalin-evoked Fos expression is visible throughout the upper laminae of the ipsilateral (right) dorsal horn (**B**) but is sparse in the contralateral dorsal horn (**A**). Fos-positive nuclei were verified as neuronal via co-labeling for NeuN (**C**). 10× magnification.

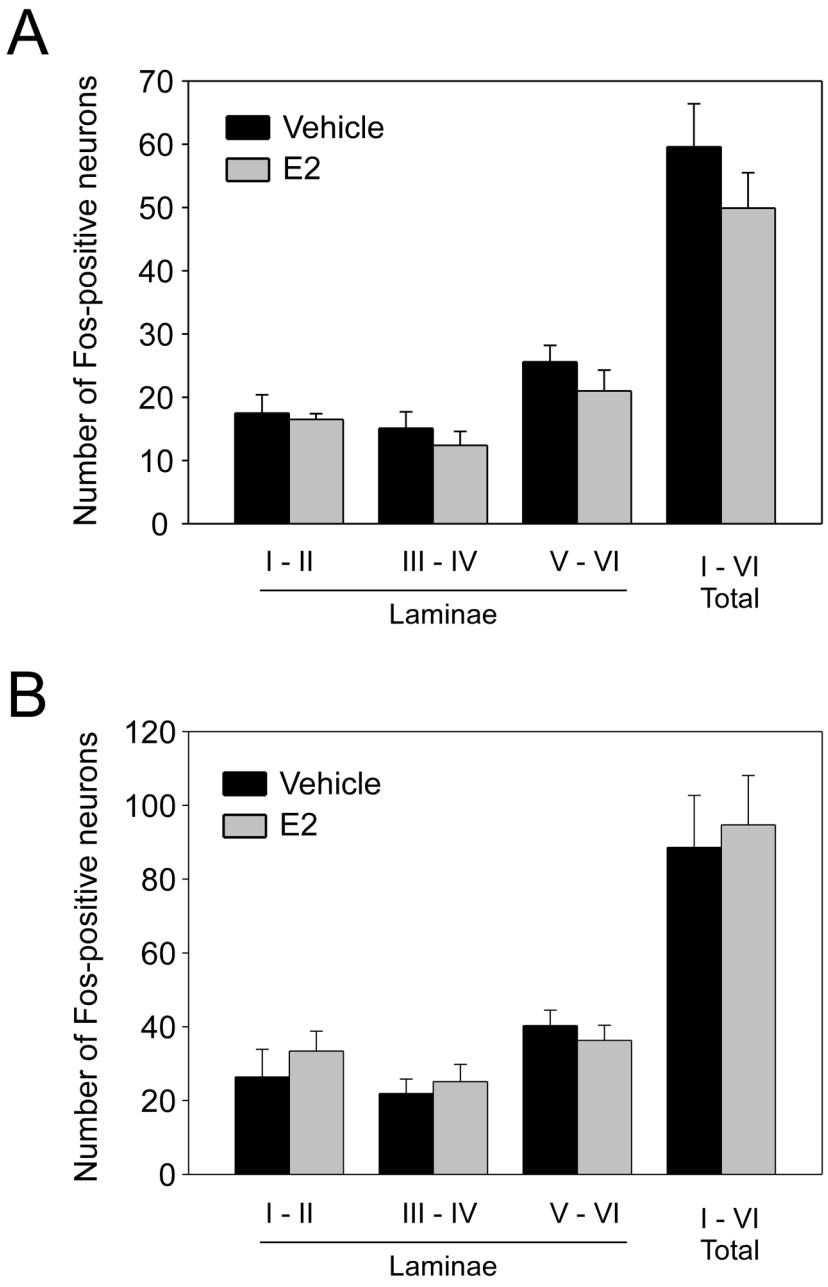

Figure 3.

Effect of E2 on (**A**)1.25% or (**B**) 5% formalin-evoked Fos immunohistochemistry in the ipsilateral spinal dorsal horn. Data represent the mean ± SEM. No significant differences were detected by unpaired Student's t-test; n = 8 for 1.25% formalin; for 5% formalin Veh n = 5, E2 n = 6.