Effects of 17β-Estradiol on Responses of Viscerosomatic Convergent Thalamic Neurons in the Ovariectomized Female Rat

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Reed WR, Chadha HK, Hubscher CH. Effects of 17β-estradiol on responses of viscerosomatic convergent thalamic neurons in the ovariectomized female rat. J Neurophysiol 102: 1062–1074, 2009. First published June 24, 2009; doi:10.1152/jn.00165.2009. Ovarian hormones have been shown to exert multiple effects on CNS function and viscerosomatic convergent activity. Ovariectomized (OVX) female rats were used in the present study to examine the long-term effects of estrogen levels of 17β-estradiol (EB) delivered by a 60-day time-released subcutaneous pellet on the response properties of viscerosomatic convergent thalamic neurons. In addition, avoidance thresholds to mechanical stimulation for one of the convergent somatic territories, the trunk, was assessed using an electro–von Frey anesthesiometer before and at the end of the 6-wk post-OVX/implant period prior to the terminal electrophysiological experiments, which were done under urethane anesthesia. Rats implanted with an EB-containing pellet, relative to placebo controls, demonstrated 1) altered thalamic response frequencies and thresholds for cervix and vaginal but not colon stimulation; 2) some response variations for just the lateral group of thalamic subnuclei; and 3) altered thalamic response frequencies and thresholds for trunk stimulation. Thalamic response thresholds for trunk pressure in EB versus placebo rats were consistent with the avoidance thresholds obtained from the same groups. In addition, EB replacement affected visceral and somatic thresholds in opposite ways (i.e., reproductive-related structures were less sensitive to pressure, whereas somatic regions showed increased sensitivity). These results have obvious reproductive advantages (i.e., decreased reproductive organ sensitivity for copulation and increased trunk sensitivity for lordosis posturing), as well as possible clinical implications in women suffering from chronic pelvic pain syndromes and/or neuropathic pain.

INTRODUCTION

There are considerable sex- and gender-related factors contributing to the expression and magnitude of pain perception and analgesia (Craft 2003a,b; Fillingim and Gear 2004; Fillingim et al. 1997; Greenspan et al. 2007). The ovarian steroid hormones have separately and synergistically been shown to influence pain sensitivity; however, the results have often proven inconsistent due in part to the lack of understanding of how these hormones modulate the response and physiology of the CNS to noxious input (Atkinson et al. 1965; Craft et al. 2008; Dawson-Basoa and Gintzler 1993; Frye and Duncan 1994; Frye et al. 1992; Gintzler and Bohan 1990; Mannino et al. 2007; Martinez-Gomez et al. 1994; Meyerson 1967; Ren et al. 2000; Selye 1941). Although estrus-cycle–related changes to nociceptive stimuli can be partially attributable to peripheral mechanisms, central changes and integration likely play important roles (Bradshaw and Berkley 2003). Correlations have been shown to exist between estrus stage and certain central neuronal properties (Bradshaw and Berkley 2000, 2003; Chadha and Hubscher 2008; Haskins and Moss 1983; Smith 1995, 1998) and also to the presence and fluctuation of CNS ovarian steroid receptor densities (Bereiter et al. 2005; Monks et al. 2001). The activation effects of ovarian hormones have been attributed to mechanisms such as, for example, the modulation of opioid systems, cytokine release, vasodilatory effects, and/or neurotransmitter release (Coughlan et al. 2005; D’Amico et al. 1991; Danzbrink et al. 1995; Dawson-Basoa and Gintzler 1997; Dawson-Basoa MB and Gintzler 1993; Dawson-Basoa ME and Gintzler 1996; Gupta et al. 2007; Martin and Behbehani 2006a,b; McEwen 2002; Melzack 1999; Smith et al. 2006).

Clinical and experimental studies investigating responses to noxious somatic or visceral stimuli indicate that pain thresholds and/or brain activation patterns are directly related to the actions of sex hormones and can vary during the menstrual/estrus cycle (Aloisi and Bonifazi 2006; Choi et al. 2006; Cimino et al. 2000; Craft et al. 2004; de Leeuw et al. 2006; Drury and Gold 1978; Fillingim and Ness 2000; Fillingim et al. 1997; Frye et al. 1992,1993; Giambardino et al. 1997a,b; Hellstrom and Anderberg 2003; Kayser et al. 1996; Riley 3rd et al. 1998, 1999; Sapsed-Byrne et al. 1996; Smith et al. 2006; Stoffel et al. 2003). For example, significant bilateral cerebral activation levels were reported in the insula, thalamus, and cingulate gyrus of women in response to a painful unilateral (left) thermal facial stimuli during both low- and high-estrogen serum phases of their menstrual cycle, as well as cerebral activations unique to either low- or high-estrogen serum levels (de Leeuw et al. 2006). In addition, regulatory effects of low and high levels of estradiol on functional responses of μ-opioid receptor-mediated endogenous opioid neurotransmission in response to a sustained pain challenge in women have been demonstrated in the medial thalamus, anterior hypothalamus/medial nucleus accumbens, and amygdala (Smith et al. 2006). Central activation and response differences attributed to serum estradiol levels suggest ovarian steroids project a multifaceted influence over signal interpretation, integration, and response to noxious stimuli.

Since the thalamus receives and processes all nociceptive information destined to reach the cortex, it may play a key role in the pathophysiology leading to a higher female prevalence of chronic and/or neuropathic pain. Widespread convergence to various types of somatic and/or visceral stimuli into assorted thalamic subnuclei has been reported (Berkley et al. 1993, 1995; Bruggemann et al. 1998; Hubscher and Johnson 2003; Kawakita et al. 1997; Moncouttet et al. 2003; Yang et al. 1998, 1999). Pain processing by both lateral and medial systems, the
lateral thalamus and somatosensory cortices for localization (Andersson et al. 1997; Gingold et al. 1991; Ploner et al. 1999; Talbot et al. 1991), and the medial thalamus and limbic structures for unpleasantness (Rainville 2002; Rainville et al. 1997; Sikes and Vogt 1992; Vogt and Sikes 2000) is widely recognized yet the underlying integrative network and ovarian hormone modulation of these two components for pain perception remains poorly understood. It has been shown—for example, in female rats—that individual neurons in and around the ventrobasal (VB) complex of the thalamus respond to stimulation (noxious and nonnoxious) of one or more reproductive-related structures (i.e., uterus, cervix, or vagina) in addition to small cutaneous regions, whereas intralaminar neurons exhibit responses to widespread cutaneous pinch and nocioceptive-related stimuli (Berkley et al. 1993, 1995). The integration of convergent activity among thalamic subnuclei in response to episodic or persistent noxious viscerosomatic stimulation remains unclear. However, one group (Sanoja and Cervero 2005) reports that ovariectomized (OVX)-induced hyperalgesia requires a long time course (4 wk) to develop as well as the finding of OVX hyperalgesia prevention using time-released estrogen pellet implantation in mice. Aware of potential time-delay requirements for the development of visceral and/or somatic hyperalgesia, we have chosen to use a long-term estradiol replacement paradigm in OVX female rats. At 6 wk, the impact of EB treatment on thalamic response thresholds and degree of convergent activity was investigated using single-unit electrophysiological techniques. Direct nerve stimulation, low- and high-threshold mechanical (touch/stroke, pinch), and visceral (vaginal, cervix, colon) stimulation were used. Avoidance-response thresholds in the same rats were also tested while not under the influence of anesthesia for one somatic territory a few days prior to the terminal experiments. Comparisons with both the thalamic response thresholds and the pre-OVX/implant values are presented. Opposing effects are reported between visceral (reproductive-related) and somatic thalamic response thresholds with EB treatment.

METHODS

In all, 17 adult female Wistar (Harlan, Indianapolis, IN) rats (175–200 g/53–63 days) were used. All animal procedures were reviewed and approved by the Institutional Animal Use and Care Committee at the University of Louisville. Animals were housed individually and exposed to a 12-h light/dark cycle.

The estrus cycle was monitored with daily vaginal smears for 2 wk using traditional stage assignments (Becker et al. 2005; Feder 1981; Hubscher et al. 2005; Young et al. 1941). Each rat was then anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg, administered intraperitoneally [ip]) for a bilateral OVX via small lumbar incisions (Hubscher 2006; Olson and Bruce 1986). Immediately after the completion of the OVX procedure, subcutaneous implantation was performed of either a 60-day time-released 17β-estradiol pellet (EB, 0.25 mg; n = 7) or a placebo pellet (P); similar pellet without active compound; n = 10; Innovative Research of America, Sarasota, FL) just medial to the scapula. The 60-day time-released pellet was selected to ensure an adequate time course for investigating chronic (5 to 6 wk) effects of hormone replacement on thalamic response thresholds. The presence of hormonal supplement was verified with vaginal smears taken weekly throughout the duration of the experiment (Hubscher et al. 2005).

At the end of the electrophysiological experiment, a cardiac blood sample was collected for determination of EB serum concentrations. Serum radioimmunoassays were performed by the Ligand Assay and Analysis Core Laboratory at the Center for Research in Reproduction, University of Virginia Health Science Center/National Institutes of Health cooperative agreement U54 HD-28934 as part of the Specialized Cooperative Centers Program in Reproductive Research. All pellets required for the study were purchased on a single order with EB concentration levels selected to meet or exceed normal proestrus levels (Nechin et al. 1979). Our previous study in the rostral medulla indicates EB neuronal response properties during the proestrus stage of the cycle only (Hubscher 2006).

Three days prior to OVX/pellet implantation, lateral trunk pressure baseline measurements were taken for all the rats while awake using an electro–von Frey meter with a rigid tip (2390 series, IITC), measuring the amount of pressure required to elicit an avoidance response (freezing, grabbing, or escape). Head orientation and vocalization were not considered a higher-order avoidance response (Woolf 1984). Lateral trunk electro–von Frey testing was again performed at 38 days post-OVX/pellet implantation for all rats just prior to the terminal electrophysiological experiment at 6 wk. The trunk was chosen to serve as a baseline for ongoing studies on hormone-induced changes in trunk sensitivity in spinal-cord–injured rats (Hubscher and Johnson 1999, 2006). Testing for avoidance threshold was done every 10 times per rat, with an interstimulus interval of 2 min, alternating between the left and right sides (five times each side). The stimulus location on the lateral trunk, midway between the hindlimb and forelimb, was varied slightly from test to test to reduce any potential for sensitization and/or habituation.

For terminal electrophysiological recordings, all animals were anesthetized with an ip injection of 50% urethane (1.2 g/kg) and maintained with supplements (5% urethane, administered intravenously [iv]) as needed (Hubscher and Johnson 1996, 2003). Reflexes involving the reproductive organs in female rats are not affected by urethane anesthesia (McKenna et al. 1991). Note that no differences in thalamic response properties and characteristics have been found between urethane and a variety of other anesthetics (Detsch et al. 1999; Dostrovsky and Guillaud 1988; Kawakita et al. 1993; Vahle-Hinz and Gottschaldt 1983; Vahle-Hinz and Hicks 2003). The jugular vein and trachea were intubated for the purposes of iv infusion route and tracheal bifurcating. Body temperature was maintained at 37°C through an esophageal thermometer and circulating-water heating pad. The pelvic nerve (PN) and dorsal nerve of the clitoris (DNC, the sensory branch of the pudendal nerve) were exposed bilaterally as previously described (Chadhia and Hubscher 2008; Hubscher 2006). The somatic motor branch running with the PN was cut and removed (leaving only the viscerocutaneous branch intact). Specially fabricated bipolar siliccone-cuff microelectrodes were then placed around both DNCs and viscerocutaneous branches of the PN (Hubscher and Johnson 1996, 2003) (Fig. 1A). For stimulation of the abdominal branches of the vagus nerve (8-mA stimulus intensity; 0.5 train/s, 2-ms duration), a bipolar ring electrode was inserted just caudal to the esophageal hiatus (Hubscher et al. 2004; Kaddumi and Hubscher 2006, 2007b; Khasar et al. 1998).

The rat’s head was clamped in a stereotoxic device (David Kopf Instruments, Tujunga, CA) with the skull positioned flat. An opening was made over the cortex on the left side by first drilling a small hole into the skull and then expanding the opening with bone rongeurs. Thalamic exposure coordinates were 1.5 through 4.5 mm caudal to bregma and 3.5 mm from midline laterally (Hubscher and Johnson 2003). The hindquarters were pivoted with hip pins and the tail was tied in an upward direction to allow for cervix, vaginal, colon, and perineum surface stimulation.

Single neurons were recorded extracellularly with DiI (1,1’-dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate; Invitrogen, Carlsbad, CA) coated tungsten microelectrodes, 6- to 8-MΩ impedance (FHC, catalog no. UEWMGSMXNG; http://www.fh-co.com) attached to a hydraulic probe (controlled by a motorized drive unit; FHC) as previously described in other protocols (Chadhia and Hub-
Frey.

colon; gentle brush: hindquarters, rest of body; trunk pressure: electro–von Frey.
cervix pressure-glass probe; distention (balloon) of vaginal canal, distal stimulation left/right PN; left/right DNC; abdominal branches of the vagus; bilateral dorsal nerve of the clitoris (DNC) stimulation; ear pinch; (bilateral DNC/PN and/or ear pinch): bilateral pelvic nerve (PN) stimulation; trigeminal nerve stimulation (S) and recordings (R) from the thalamus. Sequence of testing once a neuron is found that responds to one of the search stimuli was kept (track location and depth). All search-stimuli–responsive neurons were tested further for responses to abdominal branches of the vagus nerve (Hubscher et al. 2004).

Single identified responsive neurons (somatodendritic) were recorded extracellularly and neural activity was monitored on an oscilloscope, with a spike-triggered analog delay module as previously described (Hubscher and Johnson 1996), and stored on videotape for off-line analysis using Data-wave SciWorks (data acquisition and analysis program; http://www.dwavetech.com). Response latency for bilateral electrical stimulation of PN, DNC, and vagus nerve was calculated from the beginning of the stimulus artifact in the record to the beginning of the neuronal response. Visceral pressure readings (cervix, vaginal, and colon distention) were taken with a pressure transducer (WPI, Sarasota, FL) and responses recorded on videotape. A response was noted if the number of spikes exhibited was at least twice (excitation) or half (inhibition) of background levels (immediately prior to onset of stimulus). For units that did not have a spontaneous discharge, a minimum of three spikes was required to consider them excitatory (Chadha and Hubscher 2008; Hubscher 2006).

Repetitive electrical stimuli were at times required to activate a neuron from a given nerve or receptive field (“wind-up”) (Johnson and Hubscher 1998). Spontaneous discharge rates were measured from oscilloscope records using established protocols (Hubscher and Johnson 2003; Hubscher et al. 2004). Only single discriminated potentials isolated from other background activity were counted in the sample. Careful records of the stereotaxic location of each neuron were kept (track location and depth). All search-stimuli–responsive neurons were tested further for responses to abdominal branches of the vagus stimulation (Hubscher et al. 2004).

Whole body cutaneous receptive fields were tested and mapped using handheld probes to determine whether convergent inputs from outside the DNC and PN territories were present. Gentle stimuli, generating a low-threshold (LT) neuronal response included touch (camel’s hair brush), probing (visceral), and gentle pressure (visceral/somatic). Noxious somatic stimuli, generating high-threshold (HT) neuronal responses included pinch (gentle, moderate, and strong) with small serrated forceps (Hubscher and Johnson 1999, 2002) and pressure measurements (electro–von Frey meter/rigid tip) applied to the rostral, mid-, and lower trunk bilaterally (results reported as mean values for right/left sides). As with behavioral testing, the location of lateral trunk mechanical stimuli sites was varied slightly from test to test to reduce any potential long-term effects (sensitization, habituation) on the territory and thus neuronal activity. Visceral stimulation (cervix pressure and vaginal and colonic balloon distention) was conducted as previously described (Chadha and Hubscher 2008; Hubscher 2006). In a few instances, testing of a given neuron was incomplete for all parameters of interest, so those data were not included in the statistical analysis.

At the end of the electrophysiological experiment, a cardiac blood sample was collected immediately before the animal was perfused transcardially with 0.9% saline followed by 4% paraformaldehyde.

FIG. 1. A: diagram illustrating the experimental setup for bilateral electrical nerve stimulation (S) and recordings (R) from the thalamus. Sequence of testing once a neuron is found that responds to one of the search stimuli (bilateral DNC/PN and/or ear pinch): bilateral pelvic nerve (PN) stimulation; bilateral dorsal nerve of the clitoris (DNC) stimulation; ear pinch; stimulation left/right PN; left/right DNC; abdominal branches of the vagus; cervix pressure-glass probe; distention (balloon) of vaginal canal, distal colon; gentle brush: hindquarters, rest of body; trunk pressure: electro–von Frey. B: example of a single row of DiI-coated electrode tracks through the thalamus (×40). CL, centrolateral; CM, centromedial; LDVL, lateral dorsal ventrolateral; Rt, reticular; Sub, submedius; TH, thalamus; VL, ventrolateral; VM, ventromedial; VPL, ventroposterolateral; VPM, ventroposteromedial.
The thalamic tissue containing the recording sites was removed and stored overnight in a 30% sucrose/10% formalin solution. Microelectrode track locations were visualized in 30-μm sections using the Nikon Eclipse E400 with a DFL-EPI fluorescence attachment connected to the EXFO X-CITE 120 fluorescence illumination system using a tetramethylrhodamine filter with 555-nm absorption wavelength and emission at 576 nm. Postmortem histological reconstructions were made as previously described (Hubsch and Hubsch 1998; Kaddumi and Hubsch 2006, 2007a) (Fig. 1B).

The electrophysiological recordings were carried out by a single experimenter blind to pellet contents (EB or Pl) and different from the experimenter conducting the behavioral testing. The animals were identified numerically and a key to their pellet implant was kept separately and opened only at the end of the study when combining the data for analysis. Statistical analysis was performed with SigmaStat software (Jandel, San Rafael, CA). Standard t-tests with significance set at P < 0.05 were used for hormonal replacement and medial versus lateral thalamic subnuclei comparisons. Binomial proportion tests were used to determine frequency differences between groups (P < 0.05). All results are expressed as means ± SE.

RESULTS

General pellet implant effects

In all, 17 OVX Wistar rats were studied across two chronically implanted time-released pellet groups (EB, 7; Pl, 10). Vaginal smears were taken weekly from all rats throughout the 6-wk post-OVX/pellet implantation period. As anticipated, all EB pellet rats had smears resembling the proestrus-stage profile (Hubsch et al. 2005), whereas Pl pellet rats had smears resembling diestrus throughout the experiment duration. Mean plasma EB levels in OVX rats measured from serum taken immediately after terminal electrophysiological experiments at 6 wk were 121.5 ± 24.0 pg/ml (for the EB group) and 30.4 ± 7.2 pg/ml for the Pl animals (EB being significantly greater than Pl; P < 0.05). Although no difference in mean body weight between groups existed prior to OVX/pellet implantation (214.7 ± 8.7 g for EB; 217.5 ± 4.7 g for Pl; P > 0.05), a significant difference in mean body weight was found at the time of the terminal experiment (249.4 ± 5.5 g for EB and 356.6 ± 8.5 g for Pl; P < 0.001).

Trunk electro–von Frey behavioral findings

Significantly lower avoidance-response somatic trunk pressure thresholds were found in EB-treated rats at 38 days post-OVX/pellet implantation relative to the Pl group at 38 days (98.5 ± 3.7 vs. 114.3 ± 3.3 g/cm², respectively; t = -3.107, P = 0.002). No differences were found between the baseline group-response threshold data obtained pre-OVX/implant (120.3 ± 4.7 g/cm² for EB; 111.9 ± 3.3 g/cm² for Pl; P > 0.05). None of the rats was in the proestrus stage when the baseline testing was done (i.e., the stage when serum estradiol levels are elevated). Note that, as would be expected, the mean electro–von Frey trunk pressure required to elicit avoidance behavioral responses (i.e., freezing, grabbing) when awake was about 30% higher than the trunk pressure required to elicit thalamic responses under anesthesia (Pl, 114.3 vs. 87.8 g/cm²; EB, 98.5 vs. 76.6 g/cm²).

Terminal electrophysiological recordings in thalamus

A total of 291 (EB, 138; Pl, 153) neurons localized in either the medial or lateral thalamus responded to our search stimuli bilaterally. DNC/PN stimulation and/or ear pinch. The 204 electrode tracks containing these responsive neurons were located between -1.88 and -3.30 mm caudal to bregma (Figs. 27–33 in Paxinos and Watson 1998) and between 0.8 and 2.8 mm from midline. Thalamic subregions through which the electrodes passed during the search included the following: intralaminar (centromedial, centrolateral), lateral (dorsal, posterior), medial (dorsal, central, lateral, and medial), ventral (anterior, medial, lateral, posteromedial, posterolateral), posterior, submedius, and reticular nuclei. An example showing the responses of a single ventrolateral nucleus neuron to the search stimuli and other convergent territories is provided in Fig. 2.

Responsive neuron properties

Most of the 291 responsive neurons were excitatory (n = 286, 98%); 62% of these thalamic neurons had no resting discharges. EB-treated rats had, relative to Pl, significant increases (P < 0.05) in both the percentage of neurons exhibiting spontaneous resting discharges (45.6 vs. 34.6%, respectively) and rate of background activity (11.8 ± 1.3 vs. 7.7 ± 0.7 impulses/s, respectively). This EB-related increase in spontaneous resting discharge activity was found to occur in both lateral and medial thalamic nuclei. Of the neurons responding to peripheral nerve stimulation, 37/291 (12.7%) units required wind-up of four trains on average (given at a rate of one train/s; see METHODS) to elicit a thalamic neuronal response. Nearly half of neurons requiring wind-up (46%) as well as most of the neurons with inhibitory responses (4/5, 80%) were located within the ventrolateral (VL) and ventromedial (VM) thalamic subnuclei.

Thalamic responses to direct nerve stimulation

EB-treated rats had, relative to Pl, significantly fewer responses to bilateral PN stimulation (36.4 vs. 46.4%, respectively; P < 0.05) and significantly shorter (P < 0.05) mean response latencies (219 ± 19 vs. 265 ± 26 ms, respectively). Significantly fewer neurons in EB-treated OVX rats responded to bilateral DNC stimulation (35.6 vs. 59.5% for Pl), although no significant mean latency differences (P > 0.05) were found (195 ± 14 vs. 166 ± 15 ms, respectively). For the abdominal branches of the vagus, there were no significant differences between EB-treated and Pl groups both for the percentage of neurons responding (30.3 and 37.9% respectively) and for the response latency (235 ± 22 vs. 342 ± 40 ms, respectively).

Thalamic responsiveness to pelvic/visceral input

A significantly higher mean cervix pressure was required to elicit a thalamic response among EB-treated rats compared with placebos (Table 1), without differences in the percentage of neurons responding (Table 2). An example illustrating this difference in the response to cervix pressure for an ovariec-tomized EB-treated rat is provided in Fig. 3 (Pl in Fig. 2). Although a similar trend was found for thresholds to vaginal distention, significantly fewer neurons responded in EB-treated rats (Tables 1 and 2). Note that no overall differences between groups were found for distention of the distal colon (Tables 1 and 2). However, neurons in the VA and VL subnuclei did demonstrate a significant decrease in colonic distention-response thresholds with EB treatment (P < 0.05).
There were proportionately one third fewer neurons responding to cervix pressure (medial thalamus) and half as many responding to vaginal distention (medial and lateral thalamus) <40 mmHg in the EB-treated rats compared with placebo controls (Table 1). This shift away from the low end of the response-threshold range with EB treatment is likely responsible for the overall elevation of the mean values relative to placebos. In both EB- and Pl-treated rats, neurons responsive to very gentle levels of cervix pressure and vaginal distention were evenly distributed between medial and lateral thalamic subnuclei, with the majority being found within the ventral (VA or VL) thalamic subnuclei (Table 1). Note that both EB and Pl groups had some very high response thresholds (>200 mmHg), but these were relatively few in number (27/291; 9.3%). There were only five neurons (two EB, three Pl) responding to low-pressure levels of colonic distention, each of which was located within medial thalamic subnuclei. Neuronal responses to visceral stimulation were rarely time locked to the stimulus, with afterdischarges lasting from several seconds up to several minutes.

**TABLE 1. Visceral and somatic thalamic response thresholds**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cervix</th>
<th>Vaginal</th>
<th>Colon</th>
<th>Trunk</th>
</tr>
</thead>
<tbody>
<tr>
<td>All thalamic nuclei</td>
<td>59.3 ± 7.3</td>
<td>61.1 ± 3.1*</td>
<td>120.6 ± 4.4</td>
<td>87.8 ± 3.0</td>
</tr>
<tr>
<td>EB</td>
<td>93.3 ± 12.7*</td>
<td>93.3 ± 4.8*</td>
<td>114.8 ± 3.9</td>
<td>76.6 ± 2.9*</td>
</tr>
<tr>
<td>Medial nuclei</td>
<td>70.0 ± 11.8</td>
<td>65.7 ± 4.3</td>
<td>113.8 ± 5.7</td>
<td>86.4 ± 3.1</td>
</tr>
<tr>
<td>PL</td>
<td>91.0 ± 19.6</td>
<td>98.9 ± 8.0*</td>
<td>110.2 ± 6.4</td>
<td>79.8 ± 5.4</td>
</tr>
<tr>
<td>EB</td>
<td>10.0%</td>
<td>4.3%</td>
<td>2.0%</td>
<td>5.9%†</td>
</tr>
<tr>
<td>%LT</td>
<td>28.0%</td>
<td>9.8%</td>
<td>2.0%</td>
<td>5.9%†</td>
</tr>
<tr>
<td>Lateral nuclei</td>
<td>47.2 ± 8.0</td>
<td>55.7 ± 4.5</td>
<td>131.4 ± 6.6</td>
<td>84.9 ± 4.0</td>
</tr>
<tr>
<td>PL</td>
<td>93.5 ± 17.0*</td>
<td>89.0 ± 5.8*</td>
<td>120.3 ± 5.3</td>
<td>74.8 ± 3.0*</td>
</tr>
<tr>
<td>EB</td>
<td>29.4%</td>
<td>10.4%</td>
<td>0.0%</td>
<td>4.6%†</td>
</tr>
<tr>
<td>%LT</td>
<td>21.7%</td>
<td>5.8%</td>
<td>0.0%</td>
<td>7.2%†</td>
</tr>
</tbody>
</table>

Values are means ± SE or percentages. *EB-treated group is significantly different from the placebo (P < 0.05). Visceral pressure (mmHg), mean of right/left upper, mid-, and lower electro–von Frey somatic trunk pressure values (g/cm²); low threshold (LT) responses <40 mmHg; †percentage pertains to values taken at the mid-trunk level only. Medial nuclei include: VA, VM, MD, CM, CL, Sub; lateral nuclei include: VL, VPL, VPM, Po, LD, Rt.

**TABLE 2. Viscerosomatic mechanical stimuli responses**

<table>
<thead>
<tr>
<th>Response</th>
<th>PL</th>
<th>EB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral</td>
<td>83.7%</td>
<td>76.8%</td>
</tr>
<tr>
<td>Cervix pressure, % of total</td>
<td>69.9%</td>
<td>59.4%</td>
</tr>
<tr>
<td>Vaginal distention, % of total</td>
<td>58.2%</td>
<td>62.3%</td>
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</table>

Somatic

<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>EB</th>
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<tbody>
<tr>
<td>Ear pinch</td>
<td>98.7%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Face pinch</td>
<td>96.4%</td>
<td>98.5%</td>
</tr>
<tr>
<td>Hindpaw pinch</td>
<td>81.2%</td>
<td>87.4%</td>
</tr>
<tr>
<td>Trunk, % stroke</td>
<td>15.2%</td>
<td>13.7%</td>
</tr>
<tr>
<td>Trunk, % pinch</td>
<td>81.2%</td>
<td>92.7%*</td>
</tr>
</tbody>
</table>

*Significantly greater (P < 0.05) than other group.
**Thalamic responsiveness to somatic inputs**

A summary of neurons responding to somatic mechanical stimuli (innocuous and noxious) is provided in Tables 1 and 2. The EB-treated group demonstrated greater trunk sensitivity to noxious (pinch) trunk stimuli (Table 2; \( P < 0.05 \)). Neurons responding to innocuous stimuli were distributed throughout both medial and lateral thalamic subnuclei, with the majority being located within ventral (VA, VL) thalamic subnuclei. Responses to mechanical trunk pressure were measured bilaterally at the upper, mid, and lower trunk using an electro–von Frey with an attached rigid tip. The mean mechanical trunk pressure was significantly lower in the EB group (\( P < 0.01 \); Table 1), which is the opposite of the shift in responses for cervix and vaginal pressure (see values for the single VL neuron recording illustrated in Fig. 3).

**Viscerovisceral/viscerosomatic convergence**

Viscerovisceral convergence [cervix + vaginal + colon stimulation; 50.9% (148/291)] was widespread among thalamic neurons responding to our search stimuli, with 44.6% (66/148) of these viscerovisceral convergent neurons also responding to vagus stimulation. The medial lateral distribution of the 148 convergent neurons is presented in Table 3. An example showing the location of viscerovisceral convergent neurons at two anterior–posterior thalamic levels of the search region is provided in Fig. 4. Most viscerovisceral convergent neurons also exhibited responses to whole body somatic noxious stimulation (e.g., responses to pinching of the ears, trunk, forepaw, and hindpaw). Figure 2 shows an example of a thalamic neuron in VL with viscerovisceral/viscerosomatic convergent responses to both innocuous and noxious levels of stimulation. There were significant differences between EB- and PI-treated ovariectomized rats in terms of the response thresholds to reproductive organ stimulation for these viscerovisceral convergent neurons (Table 3).

**Medial versus lateral thalamic subnuclei**

Differences for the effects of EB replacement between the two major thalamic divisions, which have distinctive roles in noxious stimuli processing, were evaluated (see Table 1 legend for groupings). Cervix pressure and vaginal distention pressure in EB-treated rats had significant threshold increases in lateral thalamic subnuclei (Table 1). No colonic distention-response threshold differences were found overall or within either medial or lateral subnuclei (Table 1). For somatic electro–von Frey trunk thresholds, neurons within the lateral thalamic subnuclei made a greater contribution to the differences in trunk sensitivity between EB- and PI-treated rats (Table 1). A greater proportion of neurons (58 vs. 45%) receiving viscerovisceral convergent inputs were located medially than those located laterally (see Table 3).

**DISCUSSION**

The complex and multifaceted central effects of sex hormones in addition to the wide variety of methodologies and species used have slowed progress in understanding the mechanisms responsible for observed sex and gender differences in pain perception. Numerous experimental studies have investigated the effects of an OVX with or without hormone replacement on pain sensitivity (Fillingim and Gear 2004; Greenspan et al. 2007). In previous studies from our lab using cycling rats...
and/or acute hormone replacement therapy, neuronal response properties in the medullary reticular formation and forebrain regions were shown to vary with EB but not progesterone treatment (Chadha and Hubscher 2008; Hubscher 2006). Medullary reticular formation neuronal responses and thresholds, such as to cervix stimulation, for ovariectomized EB-treated rats were similar to nongonadectomized rats only when they were in the proestrus stage (elevated ovarian hormones) of their cycle (Hubscher 2006). In the present study, we used an estradiol depletion/long-term replacement paradigm to investigate whether there were hormone-dependent effects on viscerosomatic convergent responses in the thalamus. The results indicate an estradiol-dependent increase in thalamic pelvic/visceral response threshold as well as a decrease in thalamic somatic trunk response threshold, with some differences in responsiveness between medial and lateral groups of thalamic subnuclei.

**Estradiol levels**

EB levels produced by the implants were within the physiological range for proestrus at 6 wk postimplantation of the hormone replacement pellet (Bridges 1984; Nequin et al. 1979). By that time, there had been a 30% reduction in the body weight for only the EB-treated group of rats. Using a hormone depletion/long-term replacement paradigm similar to that in the current study, sustained weight loss at 5 wk post-OVX in pellet-supplemented rats was found with high but not regular EB serum levels (Dean et al. 2005). Inhibitory effects of EB on body-weight gain in animal models have been long recognized (Drewett 1973; Simpkins et al. 1988, 1989). EB-related inhibitory effects on body-weight gain are known to be mediated by multiple factors, including reduced food intake and increased metabolic expenditure and/or activity levels (Roesch 2006; Wade and Schneider 1992). There are conflicting reports of ovarian hormonal effects on activity levels (Beatty 1979, 1992; Brobeck et al. 1947; Hitt et al. 1968). One study, for example, reported that rats are significantly more active in the estrus phase than in the diestrus phase (Quadagno et al. 1972). We have preliminary data from other EB- and PI-treated rats from current studies in our laboratory (unpublished observations) that there is an overall increase in activity levels in EB-treated rats without a daily change in total food consumption.

Some of the differences found in the present study for thalamic response thresholds to stimulation of various visceral and somatic territories could result from indirect effects secondary to changes in fat content of the tissues stimulated. This possibility is not likely, since estradiol effects were seen in specific visceral and somatic regions. Potential relationships between nociceptive thresholds and changes in body mass over time are not well understood. Hargraves and Hentall (2005) previously showed that, in male mice, long-term caloric restriction (assumed less body fat) leads to significant somatic hypoalgesia when subjected to above-threshold pain of widely different durations (thermal nociceptive response). This is in contrast to our somatic threshold findings in which EB rats with a smaller body mass had greater somatic sensitivity to trunk pressure. However, one should note that somatic stimulation was not the same in these two studies.

**Behavioral effects of estradiol**

Trunk sensitivity was significantly greater with 6 wk of EB treatment in OVX rats, as evidenced by a decrease in avoidance pressure threshold. Although we used one concentration at proestrus levels to maximize any potential effects of EB, sustained serum EB concentrations have been shown to produce substantial effects on behavior in OVX rats at levels as low as 15 pg/ml (Albert et al. 1991). EB-related effects on trunk sensitivity may be of functional relevance during mating, since stimulation of these cutaneous regions when mounted by a male contributes to lordosis, a mating posture important for copulation and successful fertilization (Kow et al. 1976, 1979).
**Thalamic response properties**

EB treatment increases mean spontaneous resting discharge activity of thalamic neurons. EB has been shown to have profound effects on the discharge frequency of neurons in some other regions of the CNS following acute or extended exposure, including dopaminergic neurons in the substantia nigra, serotonergic neurons in the dorsal raphe, and cerebellar Purkinje neurons (Chiodo and Caggiula 1980; Robichaud and Debonnel 2005; Smith et al. 1988; Torres-Hernandez and Gonzalez-Vegas 2005), but no effects in other regions, including the nucleus reticularis gigantocellularis and gracile nucleus (Bradshaw and Berkley 2003; Hubscher 2006).

There were also significant differences in the response latency of thalamic neurons to bilateral PN nerve stimulation with EB treatment, which was not seen in our previous study of neurons in the medullary reticular formation (Hubscher 2006). Different methodologies in the amount and/or duration of the EB treatment (i.e., chronic vs. acute) may account for the effect on latency in the current study. There was no response latency difference for pudendal nerve stimulation (DNC branch) in either the present study or the study on medullary reticular formation neurons (Hubscher 2006).

Proposed mechanisms behind the increase in spontaneous discharge frequency with prolonged EB treatment include (but are not limited to) interactions with γ-aminobutyric acid type A (GABA_A) receptor binding sites, as demonstrated in such regions as the substantia nigra, striatum, globus pallidus, and nucleus accumbens (Bosse and DiPaolo 1996; Maggi and Perez 1984), or effects on glutamate receptors, as seen in the striatum, nucleus accumbens, and frontal cortex of the female rat CNS (Cyr et al. 2000). Although the underlying mechanisms are not well understood, ovarian hormones can modulate neurotransmission by regulating the expression of neurotransmitters synthesizing enzymes or their receptors. Estradiol has been shown to significantly increase protein expression of the NR1 subunit of the N-methyl-d-aspartate (NMDA) receptor in the lumbosacral spinal cord compared with OVX rats. Spinal NMDA receptors have been shown to contribute to somatic and visceral nociceptive processing (Cairns et al. 2003; Ji and Traub 2001; Woolf and Thompson 1991). In hippocampal and cerebellar neurons, 17β-estradiol has been shown to enhance excitatory post synaptic potential amplitude by potentiating NMDA and non-NMDA glutamate receptor responses at concentrations as low as 100 pM (Foy et al. 1999; Gu and Moss 1996; Smith et al. 1987). Estradiol increases glutamic acid decarboxylase and tyrosine hydroxylase expression in the hippocampus, hypothalamus, and other areas of the rat brain (Joh et al. 2006; Nakamura et al. 2004; Serova et al. 2004) as well as glutamate and GABA_A receptors in the rat hippocampus (Cyr et al. 2000). This suggests a possible estradiol mechanism of increased spinal processing of visceral nociception by increasing NMDA or non-NMDA receptor function and/or expression.

Inhibitory interactions mediated by GABA are known to occur throughout the somatosensory thalamic nuclei to shape the temporal but not spatial characteristics of neuronal responses in the ventrobasal complex (Hicks et al. 1986; Salt 1989). It is possible that estradiol alters the sensitivity of neurons to neurotransmitters either by modifying membrane ionic conductance (Gu and Moss 1996) or by affecting receptor expression (Rosas-Arellano et al. 1999). Estrogens have also been shown to have widespread effects throughout the CNS such as modulating enkephalinerergic neurons in the spinal cord, catecholaminergic neurons in the brain stem and midbrain, midbrain serotonergic pathways, and the basal forebrain cholinergic neurons (Amandusson et al. 1999; McEwen et al. 1997). Estradiol has also been associated with increased activation of the endogenous mu-opioid system during exposure to a noxious stimulus (Smith et al. 2006).

**Reproductive versus nonreproductive pelvic/visceral responses**

Cervix and vaginal distention pressure required to elicit thalamic responses was significantly greater (organ hypersensitivity) in EB-treated rats. A shift away from the low end of the response-threshold range with EB treatment is likely responsible for the overall elevation of the mean values relative to placebos. Support for a supraspinal EB-dependent shift in neuronal responsiveness is provided not only by similar response-threshold increases to cervix stimulation found in the medullary reticular formation (Hubscher 2006), but also by increasing (150%) vocalization thresholds to foot shock following mechanical cervix stimulation (Crowley et al. 1976). Additionally, vaginal pressures vary as a function of estrus stage with the proestrus and estrus phases requiring the greatest amount of pressure to elicit an escape response (thereby indicating less sensitivity of the vaginal wall at higher EB serum levels) (Bradshaw et al. 1999). EB pellet supplementation does not prevent the marked reduction of uterus/vagina weight following OVX (Sanoja and Cervero 2005); therefore the functional changes in visceral responsiveness observed in the present study are likely not due to gross anatomical alterations caused by hormone implantation.

The EB-dependent decrease in central neuronal responsiveness was found for colon distention thresholds in only a few thalamic subregions. Also, no effects were seen in studies of the forebrain or rostral or caudal medulla (Bradshaw and Berkley 2000, 2003; Chadha and Hubscher 2008; Hubscher 2006). Intracolonic reflexes to colon distention, however, have been reported to vary with the rat’s estrus cycle, with the greatest sensitivity being exhibited during the proestrus phase when plasma EB is at its peak (Sapsed-Byrne et al. 1996). Although peripheral variations for nerves supplying the reproductive organs across the estrus cycle have also been shown (Robbins et al. 1992; Zoubina et al. 1998), the hormone effects in the present study on only a few specific thalamic subregions for the colon argue against a peripheral effect.

Further investigation involving additional reproductive and nonreproductive visceral organs/structures coupled with central recordings will be required to clarify where in the nervous system EB is exerting its effect and what mechanisms lead to altered pelvic/visceral response thresholds from some structures but not from others. Visceral pain is one of the most common complaints for those seeking medical attention, with a higher prevalence among women (Berkley 1997; Cervero and Laird 1999; Mayer et al. 2004). Estrogen receptors have been reported on ganglion and spinal sensory and brain stem neurons, indicating that both primary afferents and central neurons are potential targets for ovarian hormone modulation of sensory input (Amandusson et al. 1995; Bereiter et al. 2005; Flores et al. 2006; Nakamura et al. 2004; Serova et al. 2004).
Mechanical thresholds of female rats in proestrus and estrus treatments were found to be lower (increased sensitivity) in EB-treated rats. This finding—coupled with the hypersensitivity of thalamic neurons to trunk stimulation with EB treatment—is consistent with our previously reported results in the medullary reticular formation (Hubscher 2006). The increases in the electro–von Frey pressure thresholds in nonanesthetized versus anesthetized rats can be explained by the additional delay required to process and generate a voluntary behavioral avoidance response (i.e., freezing, grabbing) to the sensory stimulus.

Mechanical thresholds of female rats in proestrus and estrus are lower than those in metestrus and diestrus for both tail and hindpaw stimulation (Kayser et al. 1996). Thresholds for these regions were not tested in the present study. Several other reports have also noted that rats became more sensitive to hindquarter somatic stimulation during proestrus than at other estrus cycle stages (Adler et al. 1977; Bradshaw and Berkley 2000; Drury and Gold 1978; Frye et al. 1992; Kayser et al. 1996). All of these findings are consistent with early reports that reported to occur in a variety of thalamic subnuclei in the male (Hubscher and Johnson 2003) and female (Berkley et al. 1993, 1995) rat. Note that fewer DNC/PN-responsive neurons were found in VPL than in other thalamic regions within our search area. As seen in the present study, nociceptive neurons and wide dynamic range ventrobasal complex neurons are often located in the periphery or region surrounding the nucleus (Hubscher and Johnson 2003; Nishikawa et al. 1999). Thalamic neuronal activity in a visceral pain model using c-fos mRNA expression showed that the paraventricular and mediodorsal thalamic nuclei had marked c-fos expression at 1 and 2 h following cyclophosphamide administration to the bladder (Nishii et al. 2008). Only weak expression was found in the ventrobasal complex. Whether this weak ventrobasal response applies only to noxious stimulation of the bladder is a possibility. However, a similar study looking at c-fos expression following proximal colonic phasic distension also failed to show strong ventrobasal labeling in the thalamus (Martinez et al. 2006).

Other supraspinal regions in the rat demonstrating similar widespread viscerosomatic and viscerovisceral convergence include the nucleus reticularis gigantocellularis and surrounding subnuclei, the lateral reticular nucleus, the gracile nucleus, the preoptic area of the hypothalamus, and the bed nucleus of the stria terminalis (Bradshaw and Berkley 2003; Chadha and Hubscher 2008; Hubscher 2006; Hubscher and Johnson 1996; Hubscher et al. 2004; Kaddumi and Hubscher 2006; Robbins et al. 2005). For example, convergence of noxious visceral sensory inputs from both the urinary bladder and the colon with widespread somatic regions occurs throughout many reticular nuclei across the medulla (Kaddumi and Hubscher 2006; Robbins et al. 2005). Therefore higher centers such as the thalamus may exhibit viscerovisceral convergence as a result of input not only from secondary spinal neurons but also from medullary centers because all of these regions are interconnected by both direct projections as well as indirectly via multiple neurons.

Widespread viscerosomatic input in the thalamic subnuclei of rodents is consistent with data reported from other species, such as the squirrel monkey whose thalamus has been shown to receive extensive overlap from both lemniscal and spinothalamic inputs (Ma et al. 1986). The finding that EB treatment markedly decreases the percentage of both medial and lateral neurons responding to reproductive organ stimulation at the low end of the range of response thresholds (<40 mmHg) is not that surprising since within the rat, spinothalamic tract neurons have projecting branches in both medial and lateral thalamic nuclei (Kevetter and Willis 1983). The medial thalamus receives ascending projections from the spinal cord via the rat spinothalamic tract (Cliffer et al. 1991; Giesler Jr et al. 1981a,b) and has been shown to contain neurons responsive to cutaneous, deep somatic, and visceral noxious stimuli, with the majority of nociceptive-specific neurons having large bilateral receptive fields (Berkley et al. 1995; Dostrovsky and Guilbaud 1990; Kawakita et al. 1993). Within the brain stem, many of the spinothalamic tract fibers destined for the thalamus give off collaterals to sites such as the medullary reticular formation and periaqueductal gray matter of the midbrain (Kevetter and Willis 1983; Willis and Coggshall 2004; Yezierski et al. 1987;
Zhang et al. 1990), which may account for thalamic activation latency differences.

Summary and implications

In summary, the role of sex hormones (particularly estradiol) in modifying pain remains an area of interest, particularly when women report higher incidences of recurrent pain compared with men (reviewed by Unruh 1996). A meta-analytic review of pain perception across the menstrual cycle of healthy females indicated that for pressure-induced pain, the cold pressor tasks, thermal heat stimulation, or ischemic muscle pain, there was less pain sensitivity during the follicular phase (when estrogen levels are high), with inconsistencies across other phases (Riley 3rd et al. 1999). A number of animal studies have also demonstrated response-threshold changes to somatic and visceral nociceptive stimuli at different stages of the estrus cycle in rats (Bradshaw et al. 1999; Fillingim and Ness 2000; Giamberardino et al. 1997a; Kayser et al. 1996; Martinez-Gomez et al. 1994; Sapsed-Byrne et al. 1996). We report opposing effects in thalamic thresholds to noxious visceral and somatic stimuli due to EB treatment, in addition to demonstrating widespread viscerosomatic convergence among both medial and lateral subthalamic subnuclei. Lower somatic trunk pressures and higher reproductive-related visceral pressure suggest that EB likely mediates the responsiveness (either directly or indirectly) on neuronal circuitries related to mating and/or nociception. Although the widespread thalamic viscerosomatic convergence demonstrated provides the potential of increased awareness to peripheral stimuli, sustained EB treatment may have a potential clinical implication in subpopulations of individuals suffering from chronic pelvic pain/disfunction, end-stage visceral disease (i.e., cervical cancer), and/or neuropathic pain, particularly given the current widespread use and availability of estrogen therapy.

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