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# Estradiol selectively reduces central neural activation induced by hypertonic NaCl infusion in ovariectomized rats

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ABSTRACT

We recently reported that the latency to begin drinking water during slow, intravenous infusion of a concentrated NaCl solution was shorter in estradiol-treated ovariectomized rats compared to oil vehicle-treated rats, despite comparably elevated plasma osmolality. To test the hypothesis that the decreased latency to begin drinking is attributable to enhanced detection of increased plasma osmolality by osmoreceptors located in the CNS, the present study used immunocytochemical methods to label fos, a marker of neural activation. Increased plasma osmolality did not activate the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), or the nucleus of the solitary tract (NTS) in either oil vehicle-treated rats or estradiol-treated rats. In contrast, hyperosmolality increased fos labeling in the area postrema (AP), the paraventricular nucleus of the hypothalamus (PVN) and the rostral ventrolateral medulla (RVLM) in both groups; however, the increase was blunted in estradiol-treated rats. These results suggest that estradiol has selective effects on the sensitivity of a population of osmo-/$Na^+$-receptors located in the AP, which, in turn, alters activity in other central areas associated with responses to increased osmolality. In conjunction with previous reports that hyperosmolality increases blood pressure and that elevated blood pressure inhibits drinking, the current findings of reduced activation in AP, PVN, and RVLM—areas involved in sympathetic nerve activity—raise the possibility that estradiol blunts HS-induced blood pressure changes. Thus, estradiol may eliminate or reduce the initial inhibition of water intake that occurs during increased osmolality, and facilitate a more rapid behavioral response, as we observed in our recent study.



## 1. Introduction

Estrogen is a steroid hormone that is critical for reproductive behavior in females; however, effects of steroid hormones beyond those related to reproduction are increasingly being investigated. Reproductive hormones have been implicated in the control of body fluid balance in females of numerous species including humans and rodents [2,50,51]. More specifically, estrogens influence physiological processes related to body fluid balance, such as the regulation of blood pressure [27,28,37,57] and basal levels of the antidiuretic hormone, vasopressin (VP), as well as the release of VP stimulated by increased plasma osmolality (pOsm) [2,5,15,16,21]. Although behavioral responses to volume stimuli also are affected by estradiol [33–35], water intake stimulated by hyperosmolality was initially thought not to be influenced by estradiol, as ip or sc injection of hypertonic saline elicited drinking that appeared to be unaffected by estradiol ([33,34]; but see [58]). However, a recent study from our laboratory [30] showed that estradiol treatment of ovariectomized rats reduces the latency to begin drinking water during slow, intravenous (iv) infusion of a concentrated NaCl solution. The difference in the latency to begin drinking during hypertonic saline (HS) infusion is not secondary to a greater degree of hyperosmolality in estradiol-treated rats but, instead, appears to be due to enhanced sensitivity to more modest increases in pOsm. Thus, for water intake, as well as for VP release, estrogens may restore the sensitivity to hyperosmolality that was blunted by ovariectomy. Interestingly, estradiol effects on stimulated water intake are specific to increased pOsm, as prevention of osmotic dilution produced by water intake does not alter drinking [30].

Estradiol-mediated changes in behavior suggest changes in CNS activity. The idea that estrogens have central actions which influence compensatory responses to body fluid challenges also is supported by previous findings that estrogens alter hyperosmolality-induced increases in VP and oxytocin (OT) release from neuroendocrine neurons in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus. Clearly, to achieve the altered CNS output that underlies behavioral and physiological responses, perturbations of body fluid balance first must be detected by the CNS. This function is executed, in part, by circumventricular organs (CVOs), specialized central structures with an incomplete blood–brain-barrier that facilitates monitoring of peripheral osmolality. In fact, highly sensitive osmoreceptors have been localized to CVOs in the forebrain (subfornical organ, SFO; organum vasculosum of the lamina terminalis,

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OVLT) and hindbrain (area postrema, AP) [4,8,38,59]. In addition, neural afferents from visceral osmoreceptors terminate in the hindbrain nucleus of the solitary tract (NTS) [1,31,43]. Thus, hyperosmolality may be detected by any of these central nuclei, all of which are rich in estrogen receptors (ERs) [39,47,49] and project to CNS areas implicated in body fluid balance [9,11,45,46,55,56]. We, therefore, hypothesized that the decreased latency to begin drinking in response to iv HS observed in our previous study was attributable to estradiol-mediated alterations in CNS sensitivity to increased pOsm. Specifically, we hypothesized that estrogens enhance detection of increased pOsm by the CNS.

Accordingly, we assessed the effect of estradiol on neural activation in central areas that are associated with the detection of hyperosmolality. We used immunocytochemical methods to label for fos, the protein product of the immediate early gene *c-fos*, as a marker of neural activation in the SFO, OVLT, and AP, and in the NTS. Importantly, ERs are located in virtually all CNS areas implicated in body fluid balance [25,39,47–49,52]. Thus, the central effects of estradiol to alter physiological or behavioral responses to hyperosmolality may also—or instead—include actions at ERs on neurons in central areas 'downstream' from those associated with detecting changes in peripheral osmolality. Therefore, we also examined neural activation in central areas that receive input from the SFO, OVLT, AP, and NTS [9,11,45,46,55,56]. Given the elevated blood pressure and increased sympathetic nerve activity in response to hyperosmolality [3,22,53], along with the stimulation of VP and oxytocin release, this study focused on the PVN, the site of 'preautonomic neurons', the rostral ventrolateral medulla (RVLM), the hindbrain sympathoexcitatory area, the caudal ventrolateral medulla (CVLM), the hindbrain sympathoinhibitory area, and the SON, the site of neurosecretory VP and oxytocin neurons.

## 2. General methods

### 2.1. Animals

Adult female Sprague–Dawley rats weighing 225–325 g were used in these experiments. Animals were individually housed in plastic cages and given *ad libitum* access to Harlan rodent diet (no. 2018) and deionized water except as noted. Rats were maintained in a temperature-controlled room (21–25 °C) on a 12:12 light/dark cycle with lights on at 7:00 AM. All procedures were approved by the Oklahoma State University Center for Health Sciences Animal Care and Use Committee.

### 2.2. Ovariectomy and femoral catheters

Under sodium pentobarbital anesthesia (50 mg/kg body weight IP; Sigma-Aldrich), rats were bilaterally ovariectomized (OVX) using a ventral approach. Seven to ten days later, OVX rats again were anesthetized with sodium pentobarbital and chronic, indwelling catheters consisting of PE-50 tubing fused to PE-10 tubing were inserted into the femoral vein. Tubing was filled with 0.15 mL of heparinized 0.15 M NaCl (1000 U/mL) and plugged. The end of the catheter was tunneled subcutaneously to exit at the back of the neck.

### 2.3. Estrogen replacement

Twenty four hours after implantation of femoral venous catheters, OVX rats were given subcutaneous injections of 17-B-estradiol-3-benzoate (EB, Fisher Scientific; 10 μg/0.1 mL sesame oil) or the vehicle (OIL; 0.1 mL sesame oil) on a schedule that mimics patterns of estrogen fluctuations during the estrous cycle. Specifically, rats were given EB or OIL daily for two consecutive days and were tested two days after the second injection (i.e. on day 4). Previous work [62] showed that this estradiol replacement protocol produces circulating plasma levels of estradiol at the time of testing that are comparable to those at estrus; moreover, we and others have used this approach with reliable and replicable results [13,18,20,34,35] including in our study of EB effects on water intake stimulated by iv HS infusion [30]. Rats were weighed on both days of EB/OIL treatment and on the day of the test.

### 2.4. Intravenous (iv) infusions

During both of the two days of EB/OIL injections, OVX rats were adapted to testing procedures (approximately 60 min/day) by connecting the catheters to tubing attached to an infusion pump. On day 4, catheters were connected to the infusion pump and rats were infused intravenously with 2.0 M NaCl (HS) or 0.15 M NaCl (ISO). Based on the results of our previous study in which EB-treated OVX rats began to drink water after 15 min [30], rats were infused with HS or ISO at 35 μL/min for 15 min. Neither food nor water was available during the infusion.

### 2.5. Perfusion, blood and brain collection

Seventy-five minutes after the 15 min infusion, rats were deeply anesthetized with sodium pentobarbital (0.2 mL) through the femoral venous catheter. Blood was collected from the heart and retained on ice until centrifugation for plasma protein concentration (using a refractometer; Reichert) and plasma sodium concentration (using ISE; Easylyte). Separate aliquots were drawn into microcapillary tubes and centrifuged for determination of hematocrit. Animals then were perfused through the heart with 0.15 M NaCl followed by 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde overnight and then placed in a 30% sucrose solution for cryoprotection. Brains were sectioned coronally on a cryostat (Leica) at 40 μm in a 1:3 series and stored in cryoprotectant [61] at −20 °C until processed.

### 2.6. Immunolabeling

#### 2.6.1. fos

fos immunoreactivity was demonstrated using the avidin-biotin-peroxidase technique. Briefly, one series of free-floating sections from each rat was rinsed in 0.05 M Tris–NaCl, soaked in 0.5% $H_2O_2$ for 30 min, rinsed again, and then soaked in 10% normal goat serum (NGS) for 60 min before being incubated in the fos primary antibody (Santa Cruz SC-52, rabbit anti c-fos, diluted 1:30,000 in 2% NGS) at 4 °C overnight. Sections then were rinsed and incubated in a biotinylated goat anti-rabbit antibody (Vector Laboratories BA 1000, diluted 1:300 in 2% NGS) for 2 h at room temperature. Sections were rinsed again with 2% NGS and Tris–NaCl, and then soaked in an avidin–biotin solution (Vectastain Elite ABC kit) for 90 min. They were rinsed again with Tris–NaCl and then treated with $H_2O_2$ with nickel-intensified diaminobenzidine (Peroxidase substrate kit, SK-4100; Vector Laboratories) as the chromogen for 10–15 min. This reaction was terminated with multiple rinses in 0.05 M Tris–NaCl.

#### 2.6.2. fos + DBH

As an initial effort to identify the phenotype of activated neurons in the RVLM, we focused on norepinephrine (NE). A subset of hindbrain sections was double immunolabeled for fos and dopamine-β-hydroxylase (DBH), an enzyme involved in NE biosynthesis, as a marker for NE-containing neurons, as described [10,13]. Briefly, after processing for fos immunolabeling, sections were incubated in the DBH primary antibody (mouse anti-DBH; Chemicon; diluted 1:1000 in 2% NGS) for 48 h at 4 °C. DBH immunolabeling was visualized by incubating in a Cy2-conjugated anti-mouse secondary antibody (Jackson Immunoresearch; diluted 1:300 in 2% NGS) for 6 h at room