CENTRAL VS PERIPHERAL ESTROGEN EFFECTS IN ENHANCED BEHAVIORAL RESPONSES TO A SYSTEMIC NACL LOAD

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CHAPTER I

INTRODUCTION

Body fluid balance is critical for survival and is tightly regulated despite changing internal and external environmental conditions. Body fluid homeostasis involves osmotic, volume, and pressure factors, all of which are maintained within very narrow limits, called 'set points'. Deviation from the set point for osmotic regulation causes compensatory behavioral, hormonal, and neural responses that restore body fluid concentration to proper levels. The central nervous system (CNS) is essential in coordinating and integrating compensatory responses to osmotic challenges. For example, changes in plasma osmolality are detected by osmoreceptors located in areas of the brain with an incomplete blood-brain barrier (BBB), called circumventricular organs (CVOs). CVOs, in turn, project to other areas of the CNS that are involved in body fluid homeostasis and, thus, function in the regulation of fluid and electrolyte balance.

Estrogen is a steroid hormone that is critical for reproductive behavior in females and also has been implicated in the control of body fluid balance. Circulating estrogen levels change across the reproductive

cycle and, in many cases, exert effects by binding to receptors in peripheral tissues. In addition, however, estrogen receptors have been localized to central areas, including many that are involved in body fluid regulation. These observations suggest that estrogen influences body fluid balance. In fact, estrogen has been shown to affect vasopressin release from neurosecretory neurons in the hypothalamus in response to increased plasma osmolality. Estrogen also influences compensatory behavioral responses to body fluid challenges. For example, estrogen decreases water intake stimulated by extracellular dehydration via interactions with the Renin-Angiotensin-Aldosterone System (RAAS).

On the other hand, the evidence for the involvement of estrogen in water intake stimulated by intracellular dehydration has been less clear. However, recent work from our laboratory showed that estrogen treatment decreased the latency to begin drinking in ovariectomized rats given a slow, hypertonic saline infusion, but did not significantly affect the duration of the first drinking bout, water intake during the first bout, or the total volume of water consumed. The decreased latency to begin drinking in estrogen-treated rats could be due to a difference in renal function as a result of estrogen treatment, or to a difference in the ability of the CNS to detect changes in plasma osmolality as a result of estrogen treatment. Accordingly, the goal of these studies is to determine whether the decreased latency to begin drinking is due to the effects of estrogen at the

level of the kidney or on the detection of increased osmolality at the level of the CNS.

CHAPTER II

REVIEW OF LITERATURE

Survival during constantly changing internal and external environmental conditions requires adequate perfusion of all body tissues to provide sufficient fuels for cellular metabolism. The fluid required to supply tissues must be delivered in appropriate volumes and pressures to facilitate exchange of metabolic fuels and gases, and it must contain electrolytes in appropriate concentrations for proper enzymatic and neural function. Thus, body fluid regulation involves osmotic, volume, and pressure regulation, all of which are maintained within very narrow limits, called 'set points'.

Under normal conditions, plasma osmolality ranges from 289-295 mOsmol/kg water in rats and 285-290 mOsmol/kg water in humans[11]. An increase or decrease of as little as 1-2%[17] from the set point for osmotic regulation results in compensatory behavioral, hormonal, and neural responses that restore body fluid concentration to proper levels. For example, environmental conditions of extreme heat lead to excessive fluid loss in sweat, and thereby elevate plasma Na^+, the primary electrolyte in body fluids. In response, vasopressin (VP) is released from the posterior

pituitary, leading to urinary water retention. At the same time, increased release of oxytocin (OT) from the posterior pituitary and atrial natriuretic peptide (ANP) from the heart promote renal excretion of Na^+. Together, these responses reduce or dilute plasma Na^+, as does absorption of water ingested in response to activation of peripheral and central osmoreceptors. Further increases in plasma Na^+ are prevented by changes in activation of central neural pathways that mediate salt intake, as well as by suppression of aldosterone and angiotensin II (AngII), both of which act at central sites to stimulate salt intake[28].

The central nervous system and osmotic regulation: detection of changes in plasma osmolality.

The central nervous system (CNS) is critical in coordinating and integrating compensatory responses to osmotic challenges. Clearly, to accomplish this, the CNS must be able to detect changes in plasma osmolality. The brain is normally buffered from potentially harmful constituents circulating in the blood by the blood-brain barrier (BBB), an organization of non-fenestrated capillaries that, by virtue of a continuous epithelium with a dense arrangement of glial processes on the brain side of the capillary basilar membrane, prevents the movement of many organic compounds and ions. Passage across the BBB is biochemically and locally selective[3]. Lipophilic messenger molecules may diffuse through the lipid bilayers of the endothelial cell walls, whereas some polar

compounds are transported across the BBB endothelia by specific molecule carriers. In general, however, transfer of most large molecules is possible only in specific small structures with an incomplete or "leaky" BBB due to fenestrated capillaries. These structures are the circumventricular organs (CVOs), specialized central areas that serve as the functional interface between the brain and the periphery[28].

The primary CVOs include the hindbrain area postrema (AP), the hypothalamic subfornical organ (SFO), and the organum vasculosum of the lamina terminalis (OVLT). The arrangement of capillaries and glia in CVOs suggest that these structures receive peripheral signals from molecules such as hormones involved in body fluid regulation, and from electrolytes that circulate throughout the bloodstream. In other words, sensory CVOs serve as targets on which circulating agents act to influence brain function. Consistent with this role, the OVLT, which lines the recessus supraopticus, is a site of osmoreception; the SFO, which is located dorsal and caudal to the anterior commissure, is an important target for circulating angiotensin II; the AP, which is located at the dorsal surface of the medulla oblongata and bordered ventrolaterally by the nucleus of the solitary tract (NTS), monitors circulating hormones, particularly vasopressin. In addition to receiving circulating hormonal and electrolyte signals, CVOs share reciprocal neural connections with other brain areas. That is, CVOs not only relay information to other central areas, but also receive input from other central areas. In short, CVOs

receive peripheral input, process these signals via local (synaptic and/or humoral) interactions, and eventually send their integrated output into other areas within the brain.

In terms of osmotic regulation, CVOs are able to "see" changes in plasma osmolality because of their incomplete BBB. More specifically, CVOs contain electrophysiologically defined osmoreceptive cells that function in the regulation of fluid and electrolyte balance[(see 3 for review)]. Although there is debate about the specific mechanisms for such osmoreceptivity, candidates include aquaporins, vanilloid receptors, and mechanical stretch-gated Na^+ channels. Regardless of the mechanism, though, the osmoreceptors that are more important for eliciting drinking are located in CVOs. More specifically, increases in water intake and VP release, typical responses to increased body fluid osmolality, are attenuated by lesions of the OVLT. Numerous other studies also have provided compelling evidence supporting the involvement OVLT in the osmotic regulation of neurohypophysial hormone release. For example, anatomical studies of male rats by Bourque[4] show that neurons within the OVLT send axonal projections into the supraoptic and paraventricular hypothalamic nuclei, areas containing the magnocellular neurosecretory cells (MNC) that release OT and VP. Moreover, electrophysiological studies have shown that an acute systemic hypertonic stimulus increases the firing rate of OVLT neurons that project to MNC, and also enchances production of the nuclear protein Fos by these OVLT neurons[14,15]. In

addition, injections of hypertonic saline directly into the OVLT *in vivo* cause an increase in the firing rate of MNC[14]. However, projections from OVLT neurons to MNC may be excitatory or inhibitory, as evidenced by reports that electrical stimulation of the OVLT can provoke both excitatory and inhibitory responses in MNC[14]. Taken together, these studies provide strong evidence that the OVLT is a critically important CVO in mediating central activation by and responses to osmotic challenges. At present, however, the specific role of the OVLT in osmotically stimulated water intake is not completely understood.

Estrogen and central estrogen receptors in osmotic regulation

Estrogen is a steroid hormone that is well established to be critical for reproductive behavior in females, and also has been implicated in the control of body fluid balance. Circulating estrogen levels fluctuate across the reproductive cycle and produce effects by binding to specific receptors in peripheral tissues. Estrogen also binds to receptors in the CNS and produces effects that are attributable to classic steroid actions: estrogen diffuses into the cell and forms a complex that is translocated to the nucleus where it binds to an estrogen response element, altering gene expression and transcription. The receptors involved in these effects are of two types: estrogen receptor α (ER- α) and estrogen receptor β (ER-β). It has recently become clear that estrogen not only produces slowly occurring genomic effects by binding to intranuclear receptors, but also

has more rapidly occurring nongenomic effects via actions at cell-surface receptors[13]. For example, estrogen alters neural responses to neurotransmitters by affecting intracellular protein kinase pathways. In addition, it has been reported by Lagrange and Mermelstein[2,5] that estrogen receptors are coupled to K^+ and Ca^{2+} channels.

Estrogen is a likely candidate to influence central control of body fluid balance, as evidenced by the localization of estrogen receptors to many central areas involved in body fluid regulation[25,26,29,30,31]. Receptors for estrogen have been localized to OVLT, as well as to NTS, ventrolateral medulla (VLM), and parabrachial nucleus (PBN), raising the possibility that estrogen affects detection of changes in hormones or electrolytes that signal changes in fluid balance or modulates activation in central pathways independent of changes in the initial detection. A recent study by Hartley[6] showed that estrogen increased neural activation, as indicated by immunolabeling for the *fos* protein, in the supraoptic nucleus (SON) of ovariectomized rats after an osmotic load. While these findings are an important link between estrogen enhancement of osmotically-stimulated VP release and central activation, they do not provide specific information about whether the effects are attributable to estrogen-mediated alterations in the detection of and/or sensitivity to the osmotic stimulus or to estrogen modulation of activation in central pathways, nor do they allow one to generalize to estrogen effects on osmotically stimulated water intake.

To date, studies examining the effect of estrogen on water intake in response to increased plasma osmolality produced by a systemic salt load have produced conflicting results. Kucharczyk[9] showed that water intake induced by 1M NaCl did not differ significantly during the various stages of the estrus cycle in rats. Krause et al[8] showed that there were no differences in the volume of water consumed by ovariectomized rats given a 2M NaCl injection, whether or not they were treated with estrogen. However, Thrasher and Fregly[10] showed that chronic treatment with synthetic estrogen attenuates the drinking response to an IP load of hypertonic saline (1 M NaCl, 1% of body weight). It seems likely that methodological differences contribute to the conflicting findings. For example, in the study by Krause et al, the salt load was administered subcutaneously, whereas Thrasher and Fregly delivered a different dose of hypertonic saline intraperitoneally. Moreover, Kucharczyk tested intact, cycling rats; Krause and colleagues tested ovariectomized rats that were given estrogen for two days and tested two days later; and Thrasher and Fregly gave intact female rats estrogen continuously via implantation with Silastic tubing containing estrogen for more than four months before testing. However, a more important complication in these previous studies centers around the route of administration and the volume of the hypertonic saline that was administered. Specifically, all of those studies employed rapid delivery of a large salt load that may have increased plasma osmolality well in excess of the threshold for water intake, and

thereby obscured subtle effects of estrogen on osmotically stimulated drinking.

Recent work from our laboratory showed that the latency to begin drinking during a slow hypertonic saline infusion was significantly less in estrogen-treated ovariectomized rats compared to ovariectomized rats without estrogen. There were no differences in the duration of the first drinking bout, in water consumed during the first bout, or in total volume of water consumed. The effect of estrogen to decrease the latency to osmotically-stimulated drinking may be due to alterations in renal function or to differences in the detection of changes in plasma osmolality by the CNS. In other words, the decreased latency to water intake could be attributable to differences in kidney function that lead to more rapid increases in plasma osmolality and/or to increases of a greater magnitude. Alternatively, estrogen may not affect renal function, but may alter the way the CNS "sees" the increase in plasma osmolality.

Goals and Objectives

The goal of this thesis is to determine which of these possibilities underlies the decreased latency to water intake in estrogen-treated rats after a systemic salt load. Therefore, I have conducted parallel experiments designed 1) to examine the effect of estrogen on plasma osmolality after a slow intravenous infusion of hypertonic saline and 2) to assess the effect of estrogen on neural activation in forebrain CVOs that

are associated with the detection of hyperosmolality after a slow intravenous infusion of hypertonic saline.

CHAPTER III

METHODOLOGY

General Methods

Animals

Adult female Sprague-Dawley rats weighing between 225-325 grams were used in these experiments. Animals were individually housed in plastic cages and given *ad libitum* access to rodent chow and water except as noted. Rats were kept in a temperature-controlled room (21-25^{o}C) on a 12:12 hour 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.

Ovariectomy

Under sodium pentobarbital anesthesia (50mg/kg body weight IP; Sigma-Aldrich), rats were bilaterally ovariectomized (OVX) using a ventral approach and given 7-10 days to recover.

Femoral Catheters

After OVX and recovery, rats were anesthetized with sodium pentobarbital (50mg/kg body weight) and chronic, indwelling femoral venous catheters consisting of PE-50 tubing fused to PE-10 tubing were inserted. Tubing was filled with heparinized 0.15ml NaCl (1000 U/ml) and the end of the catheter was tunneled subcutaneously to exit at the back of the neck. Rats were given one day to recover.

Estrogen Replacement

OVX rats with indwelling femoral venous catheters were given subcutaneous injections of 17-β-estradiol-3-benzoate (EB, Fisher Scientific; 10ug/0.1ml in sesame oil) or the oil vehicle (OIL, 0.1ml) 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).

Rats were weighed on both days of EB/OIL treatment and on the day of the test. The percent change in body weight from the first day of EB/OIL replacement (Day 1) to the test day (Day 4) was calculated for each rat as

100 * {[Day 4 weight (g) – Day 1 weight (g)] / Day 1 weight (g)}

These data are presented as group means +/- SE; differences in the percent change in body weight between EB- and OIL-treated rats were analyzed using independent two-tailed t-tests.

Experiment 1. Plasma Osmolality

Previous work from our laboratory showed that OVX rats treated with estrogen had a shorter latency to begin drinking water when given a hypertonic saline infusion than did OIL treated rats. As mentioned previously, one possible explanation for this observation is that estrogen affects renal function and leads to a larger or more rapid increase in plasma osmolality (pOsm). Therefore, Experiment 1 was designed to test the hypothesis that EB-treated OVX rats have higher pOSM than do OIL treated OVX rats after hypertonic saline infusion.

Procedures

1. NaCl Infusions. During the two days of EB/OIL injections, OVX rats were adapted to testing procedures 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 2.0M NaCl (HS) or 0.15M NaCl (ISO). Water was not available during the infusion. Based on the results of our previous water intake studies, rats were infused with HS (n=7 OIL/HS; n=6 EB/HS) or ISO (n=6 OIL/ISO; n=6 EB/ISO) at 35ul/min for 15 minutes. Immediately thereafter, rats were deeply anesthetized (sodium pentobarbital, 0.2ml, iv) and rapidly decapitated for collection of trunk blood.

2. Plasma osmolality, hematocrit, and plasma protein concentration. Aliquots of trunk blood were used for determination of

plasma protein concentration using a refractometer (Reichert, Depew, NY) and hematocrit reader (LW Scientific, Inc). The remaining blood was centrifuged and plasma was collected and stored at -20^{o}C prior to analysis of plasma osmolality (pOsm) using an osmometer (Vapor Pressure Osmometer, Wescor, Inc).

3. Statistics. Data are presented as group means +/- SE. Data were analyzed using Statistics (StatSoft, Tulsa, OK). Differences in plasma osmolality, hematocrit, and plasma protein concentration were assessed with a two-factor analysis of variance (ANOVA) with hormone treatement (EB or OIL) and test condition (ISO or HS) as the factors. Significant main effects or interactions ($P<0.05$) were analyzed using Student-Newman-Keuls tests.

Experiment 2. Fos-immunoreactivity

An alternative explanation for the decreased latency to begin water intake after HS infusion in EB-treated OVX rats is that estrogen enchances the detection of increased pOsm by the CNS. Therefore, Experiment 2 was designed to test the hypothesis that neural activation in the forebrain CVOs of EB-treated rats given HS is greater than that in OIL-treated animals given HS.

Procedures

1. NaCl Infusion. During the two days of EB/OIL injections, OVX rats were adapted to testing procedures 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 2.0M NaCl (HS) or 0.15M NaCl (ISO). Water was not available during the infusion. Again, based on the results of our previous water intake studies, rats were infused with HS or ISO at 35ul/min for 15 minutes.

2. Fos-immunoreactivity. One hour and 15 minutes after the 15 minute infusion, rats were deeply anesthetized with sodium pentobarbital (0.2ml, iv). Blood was collected from the heart for analysis of plasma protein concentration, hematocrit, and pOsm. Animals were then perfused through the heart with ISO for exsanguination 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 at 40um and Fos immunoreactivity (Fos-ir) was demonstrated using the avidin-biotin-peroxidase technique as previously described[7]. Briefly, free-floating sections were rinsed in Tris-NaCl, soaked in 0.5%H_2O_2 for 30 minutes, soaked in 10% normal goat serum (NGS) for 60 minutes, and incubated with the primary fos antibody (Santa Cruz SC-52, rabbit anti c-fos, diluted 1:30,000), in 2% NGS at 4^{o}C overnight. Sections were rinsed and then

incubated in biotinylated goat anti-rabbit antibody for two hours. Sections were rinsed again with 2% NGS and Tris-NaCl and soaked in an avidin-biotin solution (Vectastain elite A+B kit) for 90 minutes. They were rinsed again with Tris-NaCl and then treated with hydrogen peroxide with diaminobenzidine (DAB) as the chromogen for 10-15 minutes.

3. Quantification of Fos-ir. For each brain, the sections were ordered, mounted on gelatinized slides, and allowed to dry overnight. The sections were then dehydrated in 70%, 95%, and 100% ethanol and xylenes before coverslipping. The sections were examined microscopically using a Nikon microscope equipped with a camera and NIS-Elements AR 2.30 software. Based on previous reports of the involvement of the OVLT in the detection of an increase in pOsm, I identified the OVLT as described in Paxinos and Watson[27]. The number of Fos-positive cells was counted in 2-4 matched, representative sections from each rat, and the average number of Fos-positive cells in the OVLT was calculated for each rat (n=3 EB/HS; n=3 OIL/HS). Another forebrain CVO, the subfornical organ (SFO), also was identified as described by Paxon and Watson[27], the number of Fos-positive cells was counted in 2-4 matched, representative sections from the SFO from each rat, and an average was calculated for each rat (n-3 EB/HS; n=OIL/HS).

4. Statistics. Data are presented as group means +/- SE. In this preliminary evaluation of neural activation in the OVLT and SFO, we limited our comparisons of Fos-ir in EB- and OIL- treated rats after HS

infusion. These data were analyzed using two-tailed independent students t-tests.

CHAPTER IV

FINDINGS

Body weights

Table 1 shows body weight during EB and OIL replacement. EB-treated rats lost body weight during the 4-day protocol, whereas OIL-treated rats gained body weight during that time. The percent change in body weight from Day 1 to Day 4 was significantly greater ($p<0.001$) in OIL-treated rats than in EB-treated rats. These results are consistent with our previous studies[e.g. 8] and indicate that estrogen treatment was effective.

	OIL				EB	
Day 1	Day 4	% change		Day 1	Day 4	% change
264.9	278.5	5.12		265.3	263.3	-0.72

Table 1. Percent change in body weight during EB and OIL replacement.

Plasma osmolality, hematrocrit, and plasma protein concentration after HS infusion

Figures 1, 2, and 3 summarize the results from Experiment 1, showing the effect of estrogen on pOsm, hematocrit, and plasma protein concentration after infusion of hypertonic saline (HS) or isotonic saline (ISO). Plasma osmolality (Figure 1) increased after HS infusion [$F(1,21) = 56.66$, $p<0.001$]; however, there

were no differences between EB and OIL-treated rats. Plasma protein concentration (Figure 2) decreased after HS infusion [$F(1,21) = 5.41$, $p<0.05$]; but again, there were no differences between EB and OIL-treated rats. Neither EB treatment nor HS infusion caused systematic differences in hematocrit (Figure 3).

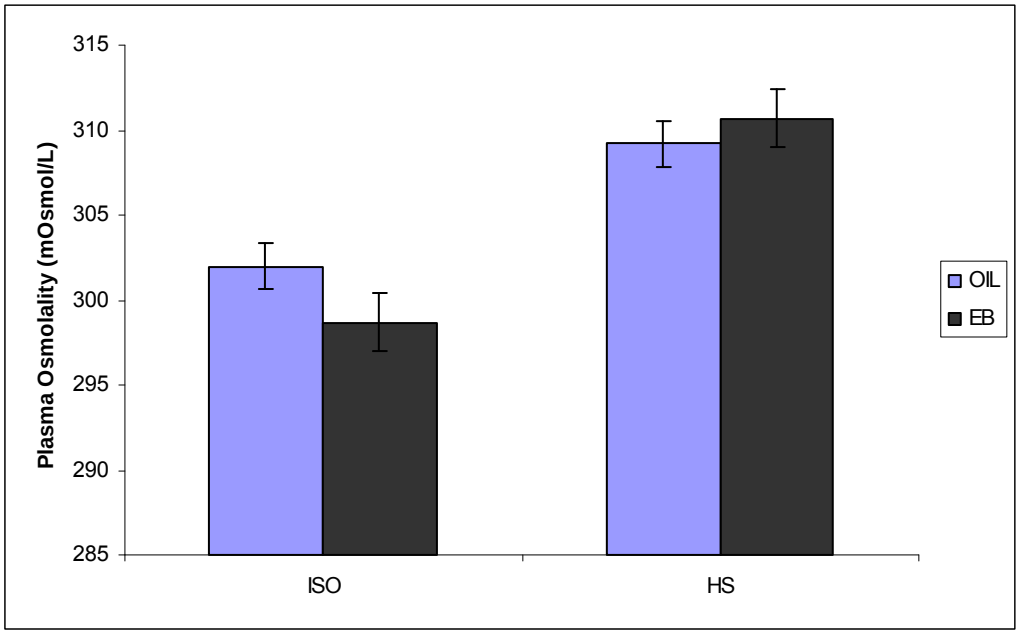

Figure 1. pOsm after IV infusion of HS in EB and OIL-treated rats.

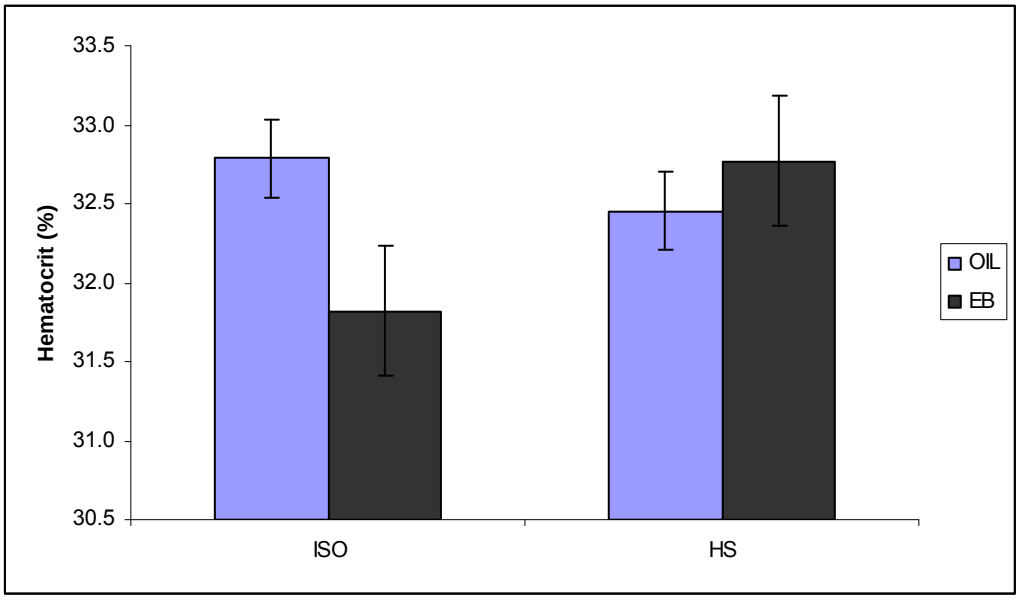

Figure 2. Hct after IV infusion of HS of EB and OIL-treated rats.

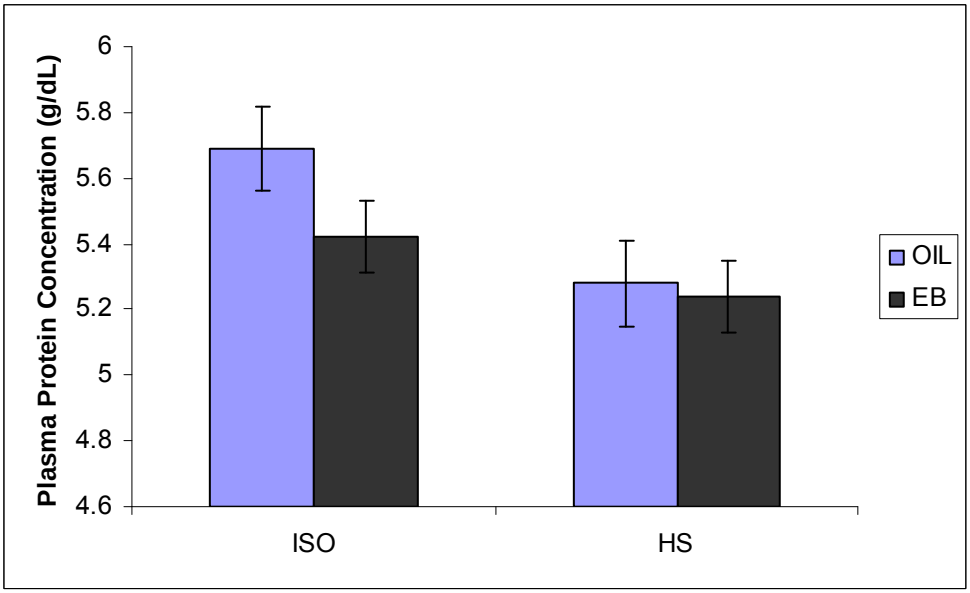

Figure 3. Plasma protein concentration after IV infusion of HS for EB and OIL-treated rats.

Fos-ir after HS infusion

Figures 4 and 5 summarize the results from Experiment 2, showing the number of Fos-positive cells in the OVLT and the SFO in EB and OIL-treated rats after HS infusion. There was no difference in the number of Fos-positive cells in the OVLT between EB and OIL-treated rats after HS infusion (Figure 4), nor did the number of Fos-positive cells in the SFO differ between EB- and OIL- treated rats after HS infusion (Figure 5).

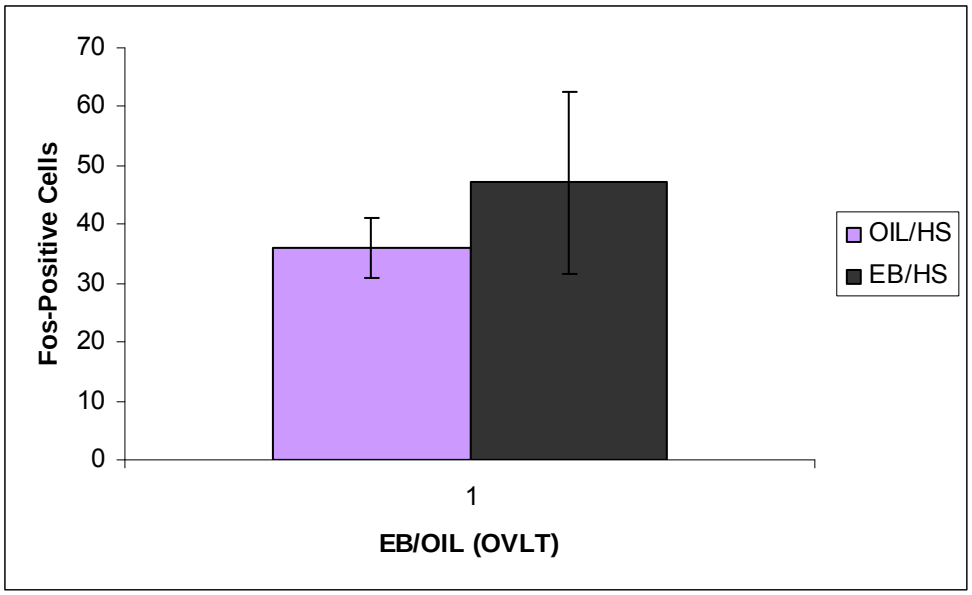

Figure 4. Fos-positive cells in the OVLT of EB and OIL-treated rats given a HS infusion.

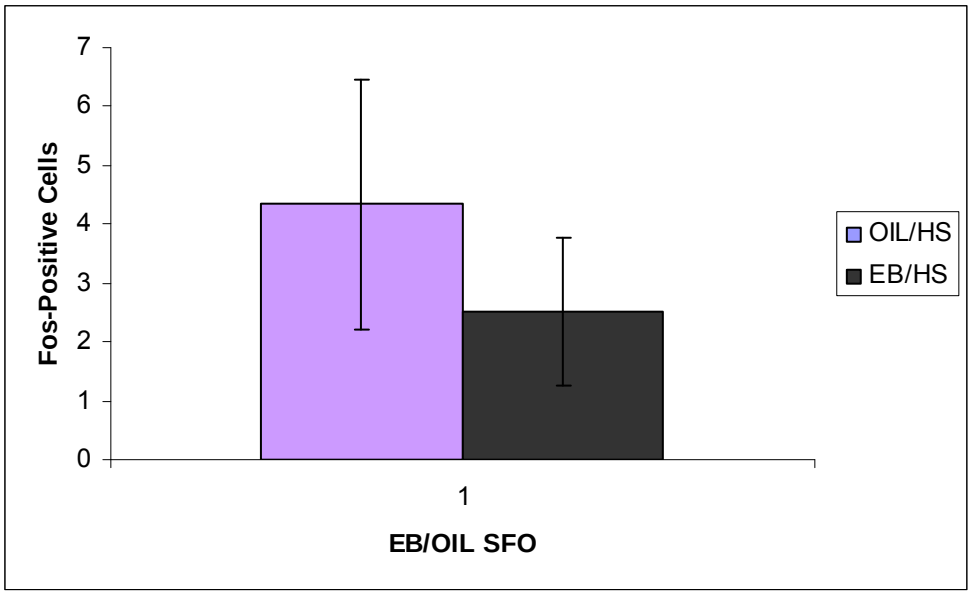

Figure 5. Fos-positive cells in the SFO of EB and OIL-treated rats given a HS infusion.

In these initial evaluations of neural activation, the numbers of rats infused with ISO were insufficient to legitimately include the data in statistical comparisons; therefore, we limited our analyses to EB- and OIL-treated rats infused with HS. However, several points are worth noting. First, after ISO infusion, the number of Fos-positive cells did not appear to differ between EB- and OIL- treated rats in either the OVLT (46 vs 51, respectively) or the SFO (3 vs 3, respectively). Second, comparison of these numbers with the data shown in Figures 5 and 6 suggest that HS infusion did not increase the number of Fos-positive cells in the SFO or in the OVLT in either group.

Figure 6, which shows representative sections from the OVLT of rats infused with ISO and HS, illustrates the lack of effect of HS infusion on Fos-ir in the OVLT. In contrast, Fos-ir increased substantially in the SON after HS, as illustrated by the representative sections from the SON of rats infused with ISO

and HS shown in Figure 7. There was no effect of estrogen treatment on pOsm after HS infusion (OIL: 287.5 +/- 1.8 mOsm/L, EB: 286.3 +/- 2.0 mOsm/L), nor were hct or pPro affected by estrogen.

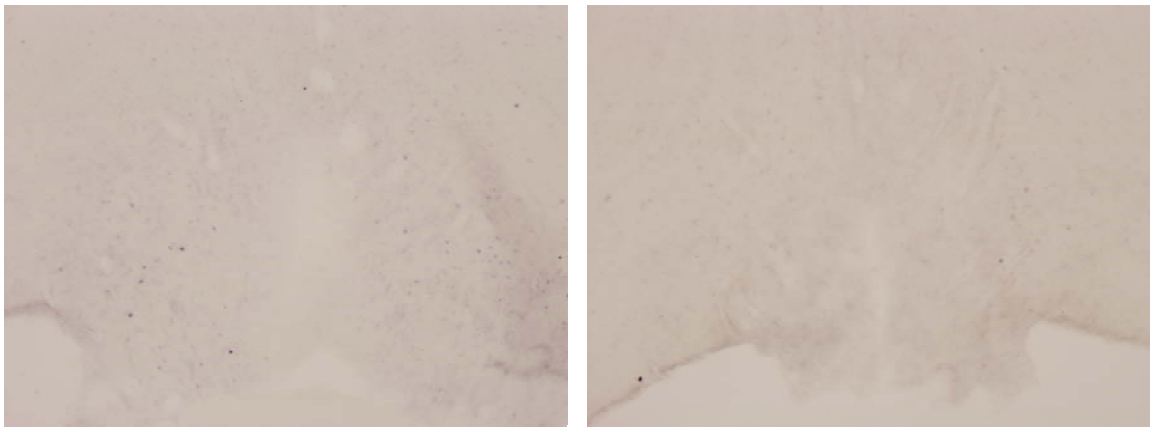

Figure 6. Sections from OVLT of rats infused with ISO(left) and HS(right)

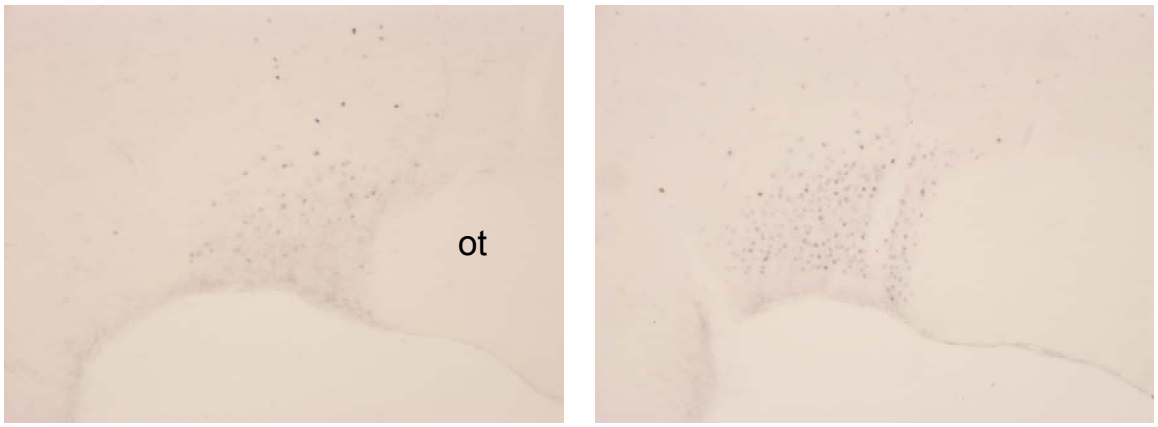

Figure 7. Sections of SON from rats infused with ISO (left) and HS (right).
OT= optic tract

CHAPTER V

CONCLUSION

Previous studies from our laboratory showed that OVX rats treated with estrogen had a shorter latency to begin drinking water when given a hypertonic saline infusion compared to OIL-treated rats. These findings parallel the results of studies showing that estrogen increases osmotically-stimulated VP release and suggest the involvement of estrogen actions in the CNS in mediating these effects. However, the decreased latency to begin drinking in EB- treated rats could instead be due to differences in the function of the kidney. In these experiments, I sought to determine whether the decreased latency to water intake in EB treated rats after a systemic salt load is attributable to differences in renal function that lead to larger and/or more rapid increases in plasma osmolality or to alterations in the detection of the salt load by the CNS.

Experiment 1 tested the hypothesis that EB-treated OVX rats have higher pOsm than do OIL-treated OVX rats after HS infusion. The results showed no differences in pOsm between EB-treated OVX rats and OIL-treated OVX rats. Moreover, there were no estrogen effects on plasma volume, as indicated by

hematocrit and plasma protein concentration, that could indirectly affect water intake. These findings suggest that even though the kidneys express both the classic ER-α and the more recently discovered ER-β[18], renal function in response to an intravenous infusion of hypertonic saline is not affected by estrogen. In other words, estrogen enhances the behavioral effects of hyperosmolality without differentially affecting plasma osmolality. The possibility cannot be ruled out that our methods were insufficiently sensitive to detect differences in plasma osmolality that, while slight, may have contributed to the difference in the latency to begin drinking. Nonetheless, we did not observe statistically significant differences in plasma osmolality between estrogen and OIL-treated rats. Thus, it is feasible that estrogen-induced differences in the behavioral response to an osmotic stimulus are attributable to estrogen effects on receptors within the CNS[25,26,29,30,31] that alter the detection of hyperosmolality.

Changes in plasma osmolality are detected by osmoreceptors in forebrain CVOs and Simon's[3] work indicates that the osmoreceptors important for eliciting drinking are located in the OVLT. Moreover, estrogen receptors are located in CVOs, including the OVLT[25,26,29,30,31]. Accordingly, Experiment 2 tested the hypothesis that neural activation in the forebrain CVOs, and in particular the OVLT, of EB- treated rats given hypertonic saline is greater than in OIL-treated rats. The results showed no differences, as indicated by Fos-ir, in the OVLT or in the SFO between EB- and OIL- treated OVX rats after HS infusion. These findings suggest that the enhanced behavioral effects are not attributable to estrogen-mediated alterations in the detection of osmotic stimuli by forebrain

CVOs. In other words, estrogen does not increase the sensitivity of forebrain CVOs to increased plasma osmolality. Importantly, HS infusion increased neural activation in other CNS areas such as the SON. Thus, the absence of a clear effect of HS infusion on fos-ir in SFO and OVLT was not because the stimulus was insufficient to increase neural activity.

The findings from the present study using Fos-ir as a marker of neural activation are surprising. There is some evidence that the SFO is activated by osmotic stimuli[(e.g.15)], which clearly was not the case in the present study; however, most evidence points to the OVLT in the central detection of hyperosmolality[(e.g.15)]. Nonetheless, despite those findings, and despite findings by Simon and others[3] of osmoreceptors for drinking in the OVLT, we observed no differences in Fos-ir in the OVLT between EB- and OIL-treated rats. It is possible that HS infusion differentially inhibits neural activation within a specific subpopulation of neurons. If that is the case, given that fos expression does not increase with neural inhibition, use of the fos technique would not allow us to detect group differences in cells in the OVLT that are inhibited by an osmotic stimulus. However, although Bourque[14] showed inhibitory responses in central areas that *receive* projections from OVLT neurons after electrical stimulation of the OVLT, to my knowledge, there are no reports that OVLT neurons are *themselves* inhibited by increased plasma osmolality. In contrast, there are many reports of increased neural activation in the OVLT in response to hyperosmolality[(e.g.15)]. Thus, it seems unlikely that the lack of effect of estrogen

on neural activity in the OVLT (or the SFO) is attributable to a methodological limitation that precludes the detection of differences in neural inhibition.

Although the results from the present studies do not provide a conclusive explanation for the enhanced behavioral effects of estrogen, they do provide directions for further investigations. For example, it is possible that estrogen enhances detection of increased osmolality by osmoreceptors in a CVO in the hindbrain, the area postrema. The area postrema is known to be important in osmoregulation, as area postrema lesions in adult male rats produce a spontaneous intake of NaCl up to as much as 108ml/day[24]. Moreover, Simonian and Herbison found that the rat area postrema contain immunoreactive estrogen receptors (ER-ir)[25]. In fact, Scott and colleagues found that the area postrema in sheep contain the largest and most densely stained population of ER-ir cells in the brainstem[26]. Accordingly, additional studies of Fos-ir may be conducted to determine whether estrogen affects osmotically-stimulated neural activation in the area postrema.

An alternative possibility is that estrogen enhances behavioral responses by affecting osmoreceptors located in the periphery and, more specifically, in the hepatic portal venous system. In fact, Adachi et al[16] demonstrated that the hepatic vagus involves two different types of osmosensitive afferent fibers using electrophysiological and behavioral methods. Thus, one strategy to address the question of whether estrogen influences peripheral osmoreceptors is to record electrophysiological activity from their neural afferents to the CNS in EB- and OIL- treated OVX rats during HS infusion.

A different approach involves examination of the afferent terminal sites of the peripheral osmoreceptors within the CNS. These osmoreceptors send neural input to the NTS[19] which, in turn, projects to other CNS areas, including vasopressin neurons in the hypothalamus[32]. In fact, Kahrilas and Rogers[19] showed that the NTS is responsive to changes of 1% or less in portal blood Na^+ concentration. Thus, activity of neurons in vagal terminal sites in the NTS may reflect estrogen-induced changes in the sensitivity of peripheral osmoreceptors. Accordingly, an additional study might examine differences in responsiveness of the NTS to increased plasma osmolality after estrogen treatment using Fos-ir.

Finally, it is possible that estrogen effects could be due to modulation of activation in central pathways independent of changes in the initial detection by osmoreceptors in CVOs or the periphery. Studies with *c-fos* and other immediate early genes[7] have provided evidence that intracerebroventricular (ICV) injections of carbachol induce activity in central structures associated with water intake, including the OVLT and hypothalamus. In fact, there is considerable evidence to suggest a cholinergic link in the neural control of vasopressin release. Intracarotid administration of acetylcholine (Ach) increases the electrical firing rate of MNC in the SON, as well as the output of vasopressin in hydrated rats[22]. Moreover, Iitake et al[21] demonstrated that acetylcholine and its agonists can act centrally to increase the activity of vasopressinergic MNC, likely via cholinergic receptors on MNC in the SON and PVN[22]. Finally, Gosbee and Lederis[23] demonstrated increased vasopressin release in rats after direct application of 5-20ng of carbachol to the neural lobe of the pituitary.

In addition, numerous studies of water intake by male rats have used intracerbroventricular (ICV) injections of carbachol[33], a procedure commonly thought to mimic activation of central pathways involved in osmotically-stimulated drinking[28,33]. Given estrogen effects on vasopressin release in response to increased osmolality[6,11], estrogen-induced changes in central cholinergic pathways seem a likely candidate for the reduced latency to water intake by EB-treated rats in response to HS infusion. Observations by Kisley et al[20] that water intake induced by ICV injection of carbachol was not affected by estrogen treatment would seem to argue against this idea. However, the aim of those investigators was to elicit water intake comparable to that produced by ICV administration of angiotensin II, which stimulates less water intake by estrogen-treated rats. Consequently, they used only a single, large dose (365.2 ng) of carbachol, which may have been well above the threshold for water intake and therefore produced maximal drinking regardless of the presence of estrogen. Accordingly, an additional study using a dose-response analysis of water intake with ICV carbachol might reveal effects of estrogen. For example, findings that carbachol produces water intake at lower doses in EB-treated rats would support the idea that estrogen modulates activity in central pathways involved in osmotically-stimulated drinking.

In summary, these experiments attempted to determine the mechanism that underlies the decreased latency to water intake in estrogen-treated rats after a systemic salt load. The results of Experiment 1 suggest that the decreased latency to water intake is not attributable to differences in renal function that lead

to more rapid increases in plasma osmolality and/or to increases of a greater magnitude. This finding led to the alternative hypothesis that estrogen alters the detection of the increase in plasma osmolality by the CNS, in particular, by forebrain CVOs. The results of Experiment 2 rule out estrogen-mediated changes in detection of the salt load by the SFO. Moreover, it seems unlikely that estrogen alters neural activation in the OVLT in response to increased osmolality, though it will be necessary to increase the number of animals in each group to make this determination conclusively.

Nonetheless, these results, in conjunction with previous studies, do allow conclusions to be drawn. Specifically, estrogen reduces the latency to drinking in response to a systemic salt load, but this effect does not appear to be the result of changes in renal function or of estrogen actions of forebrain CVOs. Rather, estrogen may influence osmotically-stimulated water intake by altering the sensitivity of hepatic osmoreceptors or of osmoreceptors in the hindbrain CVO, the AP. An alternative explanation is that estrogen changes neural activity in central pathways independent of changes in the detection of hyperosmolality. Clearly, additional studies will be necessary to determine which of these possibilities accounts for estrogen effects on water intake in response to increased plasma osmolality.

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Scope and Method of Study: To determine whether the effect of estrogen to decrease the latency to begin drinking during a hypertonic saline infusion is due to alterations in renal function that lead to a more rapid increase in plasma osmolality or is attributable to estrogen effects on receptors within the central nervous system that detect hyperosmolality.

Findings and Conclusions: There were neither differences in plasma osmolality between estrogen and OIL-treated rats nor differences in the number of Fos-positive cells in the forebrain circumventricular organs (CVOs). The findings from Experiment 1 suggest that the decreased latency to water intake in estrogen treated rats is not attributable to differences in renal function that lead to a more rapid increase in plasma osmolality. The results of Experiment 2 make it seem unlikely that estrogen alters neural activation in the forebrain CVOs in response to increased plasma osmolality.

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