Osmotic Regulation of Estrogen Receptor-β Expression in Magnocellular Vasopressin Neurons

Requires the Lamina Terminalis

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Running title: AV3V lesions and ER-β expression in vasopressin neurons

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Abstract

Estrogen receptor-β (ER-β) expression in rat magnocellular vasopressin (VP) neurons of the supraoptic (SON) and paraventricular nuclei (PVN) becomes undetectable following 72h of 2% NaCl consumption. To test the hypothesis that osmosensitive mechanisms originating in the region of the organum vasculosum lamina terminalis (OVLT) control ER-β expression in SON and PVN, animals were water deprived following electrolytic lesions of the area anterior and ventral to the 3rd ventricle (AV3V). Such lesions prevent osmotic stimulation of VP release. 4 wks post-surgery, male rats [lesioned (n=16) or sham (n=14)] were water deprived for 48h or allowed water ad libitum. Water deprivation eliminated ER-β-immunoreactivity (-ir) in SON and magnocellular PVN of sham-lesioned animals. Fos-ir was evident in these neurons, and plasma osmolality (pOsm) and hematocrit (Ht) were significantly elevated compared to the sham-hydrated rats (pOsm: 304±1 vs 318±2 mOsm/kg H2O, p<0.001; Ht: 49.6±0.6 vs 55.0±0.9%, p<0.001). ER-β expression was comparable in sham-hydrated, AV3V-hydrated, and in 6 of 8 AV3V-dehydrated rats despite significant increases in pOsm in both groups (AV3V-hydrated: 312±2; AV3V-dehydrated: 380±10; p<0.001). OVLT was not ablated in the AV3V-dehydrated rats in which ER-β was depleted. Fos-ir was low or undetectable in SON in the AV3V-hydrated animals in spite of the elevated pOsm. In AV3V-dehydrated rats, it was significantly less than in sham-dehydrated animals, but was significantly increased compared to the sham-hydrated group. This could reflect activation by non-osmotic parameters that do not inhibit ER-β expression. These data support the hypothesis that inhibition of ER-β expression in SON by osmotic stimulation is mediated by osmoreceptive neurons in the lamina terminalis.
Key words: anteroventral third ventricle
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I. Introduction

The control of body fluid homeostasis is achieved through a series of coordinated behavioral, autonomic and endocrine responses. Vasopressin (VP), a hormone synthesized by the hypothalamic magnocellular neurosecretory cells (MNCs) in the supraoptic (SON) and paraventricular (PVN) nuclei and secreted from the neurohypophysis, regulates water reabsorption by the kidney and is an important component of the endocrine homeostatic response.

Although a variety of stimuli activate the MNCs and hence initiate VP release from the neurohypophysis, an increase in plasma osmolality is the most potent physiological stimulus for VP release. Osmotic stimulation occurs as a result of interactions between the intrinsic osmosensitive properties of the MNCs and synaptic inputs from afferent osmosensitive pathways arising from rostral forebrain structures anterior to the third ventricle. Electrolytic lesions of the tissue anterior to the ventral 3rd ventricle (AV3V lesion) disrupt osmotic regulation of VP secretion. These lesions typically destroy the organum vasculosum of the lamina terminalis (OVLT), the ventral median preoptic nucleus (vMnPO), and fibers of passage from the subfornical organ (SFO). Both OVLT and SFO contain osmoreceptive neurons and both project directly to the SON and PVN as well as indirectly via the MnPO (5,14,23).

The MNC VP neurons express estrogen receptor β (ER-β) (1,12,19,28,29). While the precise role of ER-β in MNCs remains to be established, evidence from this laboratory suggests
an inhibitory role that modulates osmotic responses. Physiological concentrations of estradiol (E2) inhibit both osmotically-stimulated and NMDA-induced VP release from explants of the hypothalamo-neurohypophyseal system (31,34). Genistein, an ER-β agonist, mimics the effect of E2 on NMDA-induced VP release while a specific ER-β antagonist blocks E2 inhibition (31). We have also shown that ER-β expression is inversely correlated with the osmotic state of the animal. Thus, ER-β is depleted in MNCs following 72h of 2% saline drinking and is upregulated by chronic hyponatremia (32). This inverse correlation with plasma osmolality supports an inhibitory role for ER-β that is removed during osmotic stimulation of VP release.

In the present study we used AV3V lesioned rats to evaluate the role of osmosensitive mechanisms originating in the lamina terminalis in osmotically-stimulated inhibition of ER-β expression in SON and PVN. Water deprivation (48h) was used to induce a chronic increase in plasma osmolality.

II. Methods

**Animals.** Adult male Sprague-Dawley rats (Crl:CD(SD)BR; 250-300g; Charles Rivers Laboratories, Wilmington, MA) were singly housed and maintained on a 12h light:dark cycle with lights on at 0600h, ambient temperature at 21±1°C. Male rats were used in this study, because ER-β is expressed in males as well as females, and testosterone can be metabolized to E2 as well as to other steroids that represent potential ligands for ER-β. In addition, variation in E2
secretion across the female estrus cycle was avoided. Standard rat chow was available *ad libitum* throughout the entire experiment. Body weight and fluid intake from each rat were determined daily between 0700-1000h. All protocols used were performed in accordance with the NIH guideline for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Colorado Health Science Center.

**AV3V lesion and pre- and post-surgery drinking regimen.** Rats were allowed 4 days to acclimate to their new surrounding before surgery. They had *ad libitum* access to tap water for the first day, followed by a 10% (w/v) sucrose solution on the second and third day, then back to tap water again the night before the surgery. The pre-surgery drinking regimen was to prime the rats to the sucrose solution that would be subsequently used to encourage drinking in rats with adipsia following the surgery, thus reducing the mortality rate. They were returned to tap water the night before the surgery to prevent association from the surgical trauma with the sucrose solution. AV3V lesions were performed as previously described (13). Specifically, the animals were anesthetized with Avertin (2,2,2 tribromoethanol, Sigma; 40mg/ml at 1ml/100g BW). A nichrome electrode was placed stereotaxically in the AV3V region (midline, 0.2mm posterior to bregma and 7.5mm below dura; the skull was leveled between bregma and lambda), and anodal direct current of 2.5 mA was passed through the electrode for 20s. For sham lesions, the electrode was lowered 6.5mm ventral to dura and no current was applied. All lesioned rats were functionally adipsic, (i.e. overnight fluid intake below 10ml) immediately following surgery. Adipsic animals were given *ad libitum* access to 10% sucrose solution to provide adequate hydration while sham-lesioned animals were continued on tap water. The rats were gradually
weaned from the sucrose solution to tap water, and the experiment began 4 wks after the surgery, at which point the lesioned and sham-operated animals consumed comparable amounts of tap water.

**Water deprivation.** Following the post-surgery weaning period, animals were either continued on *ad libitum* water (AV3V-hydrated or sham-hydrated, n=7 each) or were water-deprived (AV3V-dehydrated or sham-dehydrated, n=8 each) for 48h. All rats were anesthetized with Avertin and perfused transcardially with physiological saline, followed by 3.75% acrolein (EM Grade, Electron Microscopy Sciences, Fort Washington, PA) in phosphate-buffered 4% paraformaldehyde (pH 6.7; Sigma, St Louis, MO) between 0800-1300h. Brains were collected and processed for immunocytochemistry (see below). Blood was collected via cardiac puncture for hematocrit (microcapillary method), plasma osmolality (microvapour pressure osmometer; Wescor, INC, Logan, Utah), plasma sodium measurement (Flame photometry, Corning 435), plasma glucose (Accu-Chek, Roche Diagnostics Corporation, Indianapolis, IN), blood urea nitrogen (BUN; Beckman BUN analyzer 2, Beckman Instruments, Fullerton, CA), and free Testosterone (Active Free Testosterone Radioimmunoassay Kit, Diagnostic Systems Laboratories, Inc., Webster, Texas).

**Immunocytochemistry for ER-β or Fos.** The immunocytochemistry procedure was performed as previously described (32). After fixation, brains were removed and allowed to sink in 30% aqueous sucrose for at least 72h. 30μM-thick cryostat brain slices (starting rostrally at the level
of OVLT and ending caudally at lateral hypothalamic area) were collected in a 1 in 4 series and placed in culture wells with cryoprotectant solution (30% sucrose, 30% ethylene glycol in 0.1M PBS) until processed. Each set of adjacent sections was used to localize either ER-β or Fos-immunoreactivity (-ir) in brain areas of interest. Cryoprotectant was removed from freely floating tissue sections with multiple rinses of 0.05M potassium phosphate-buffered saline (KPBS, pH 7.4). Tissues were then treated with 1% sodium borohydride for 20min, followed by several rinses with KPBS, and incubated with 0.2% Triton X-100 (2 times for 10min each) followed by 0.1M Glycine for 30min. All solutions were prepared in KPBS and multiple rinses with KPBS were used between each treatment. The sections were then incubated in blocking solution (20% normal donkey serum, 1% BSA and 1% H₂O₂ in KPBS) for 30min. All procedures were carried out at room temperature (RT). The tissues were subsequently incubated in primary antibody with an affinity purified rabbit polyclonal antiserum raised against amino acid 468-485 of ER-β (Z8P, Zymed Laboratories, San Francisco, CA) at 1:5,000 dilution made up in KPBS with 0.4% Triton X-100 / 1% normal donkey serum for 60min at RT, then 72h at 4°C. The sections were rinsed with KPBS (10 times for 6min each), incubated with a biotinylated donkey anti-rabbit serum (Jackson ImmunoResearch, West Grove, PA) at 1:600 in KPBS with 0.4% Triton X-100 for 1h 15min at RT. After several rinses, tissues were incubated in avidin-biotin complex solution for 1h 15min at RT (Vector Elite Kit, Vector Laboratories, Burlingame, CA; with 50µl of avidin and 50µl of biotin in 10ml of KPBS with 0.4% Triton X-100). Tissues were rinsed in KPBS (3 times for 5min each), followed by 3 rinses for 5min each in 0.175M sodium acetate. Primary antibody was localized using a conventional immunoperoxidase method with a 15min exposure to
nickel sulfate (25mg/ml) plus diaminobenzidine-HCl (DAB, 0.2mg/ml) in sodium acetate solution containing 0.83µl 3% H₂O₂. This yielded a blue-black reaction product in the nuclear compartment. The sections were rinsed in sodium acetate (3 times for 5min each), and then 3 rinses for 5min each in KPBS. Sections were mounted on poly-L-Lysine treated slides, air-dried overnight, dehydrated in a series of graded alcohols, cleared in Histoclear (National Diagnostics, Atlanta, GA) and cover-slipped with Protocol™ (Fisher Diagnostics, Middletown, VA).

For Fos-immunolabelling, all procedures were conducted in an identical manner to the ER-β-immunolabelling method, except that the ER-β primary antibody was replaced with a rabbit affinity-purified polyclonal antibody against c-Fos (sc-253; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:120,000 dilution in KPBS with 0.4% triton X-100 and 1% normal serum. Sections were incubated for 60min at RT and then at 4°C for 72h.

**Quantification of ER-β or Fos immunoreactivity.** Cells expressing nuclear blue-black immunoprecipitants after DAB-Nickel reaction were considered positive. ER-β and Fos expression were quantified in SON and PVN by counting the number of ER-β or Fos positive nuclei in one SON or PVN on one section from each rat (approximately bregma –1.3mm for SON and bregma –1.8mm for PVN) using image analysis software (NIH Image 1.55, available free online at [http://rsb.info.nih.gov/nih-image/](http://rsb.info.nih.gov/nih-image/)). Only intensely stained nuclei were counted to avoid interference of background staining (see later figures for examples), and the same threshold was applied to count all sections of ER-β-ir or Fos-ir. In PVN, counts were limited to the large,
circular, posterior magnocellular region of the nucleus in order to avoid contamination of the
counts with ER-β positive parvocellular neurons.

Statistical analysis. Student’s t-test, one or two-way ANOVA (or Kruskal-Wallis analysis on ranks) with post-hoc tests (Student-Newman-Keuls), Chi-square, and Fisher Exact test were used (SigmaStat software; SPSS, Chicago, IL) as appropriate to determine the statistical significance between groups. The alpha value was set at $p<0.05$. Results are expressed as the group means±sem.

Results

Characterization of AV3V lesion.

Post surgery adipsia provided an initial indication of lesion success. All AV3V-lesioned animals demonstrated post-surgical adipsia (defined as less than 10ml overnight). Decreased drinking was also observed in the sham-lesioned animals immediately post-surgery (pre-surgery: 43.1±3.1 ml; post-surgery: 25.2±2.2ml), but they resumed their pre-surgery drinking pattern within 24-48h.

Histological analysis of the lesion was performed on sections stained for ER-β and Fos. As shown in Figure 1, a typical lesion ablated the OVLT as well as the vMnPO, but left the SFO as well as SON and PVN intact. However, as discussed below, incomplete lesions were observed in 2 animals assigned to the AV3V-lesioned group.
Effect of AV3V lesion and water deprivation on body weight.

The initial weight and the rate of weight gain of the rats prior to surgery were comparable among all animals. Following the surgery, the body weight in sham-lesioned animals decreased slightly but then rapidly recovered and continued to increase throughout the recovery period. In contrast, the AV3V-lesioned animals had a dramatic drop in their body weight that persisted for several days before they began to gain weight again. The body weight of AV3V-lesioned animals remained significantly lower than the sham-lesioned animals throughout the experiment (Figure 2). Water deprivation for 48h induced a significant decrease in body weight in both AV3V- and sham-lesioned animals, compared to their respective water-replete controls (Figure 2).

Effect of AV3V lesion and water deprivation on plasma osmolality and hematocrit.

As shown in Table 1, plasma osmolality was increased by both the lesion (F=28.04, p<0.001) and dehydration (F=30.85, p<0.001). The AV3V lesion induced a rise of 8 mOsm/kg H$_2$O in plasma osmolality in non-dehydrated animals. Dehydration increased plasma osmolality by 14 mOsm/kg H$_2$O in sham-lesioned rats but further increased the osmolality by 76 mOsm/kg H$_2$O in AV3V-lesioned from the level in sham-hydrated controls (p<0.001). Plasma sodium (pNa), glucose, and BUN were analyzed to determine the relative contribution of these agents to the large increase in osmolality observed in the AV3V-dehydrated animals (Table 1). Similar to pOsm, pNa was increased by both the lesion (F=9.7, p<0.005) and dehydration (F=12.24, p<0.002). Interestingly, plasma glucose was significantly reduced by the AV3V lesion regardless of hydrational state (F=37.15, p<0.001). There was also a significant increase in BUN in the
AV3V-dehydrated group. However, the changes in plasma osmolality primarily reflected changes in these agents since pOsm predicted using the formula pOsm=2(pNa) + pGlucose/18 + BUN/2.8 (17) did not differ significantly from the measured pOsm in any of the groups (Table 1).

Hematocrit was significantly increased only in sham-dehydrated animals compared to other groups (F=13.102, p<0.001; Table 1). Thus, AV3V lesioned rats remained capable of regulating their blood volume, but failed to monitor changes in their plasma osmolality.

Expression of ER-β in SON and PVN magnocellular neurons

As previously reported (1,12,28,32), numerous intensely labelled ER-β-ir neurons were found throughout the rostrocaudal extent of SON (Figure 3A) and posterior magnocellular PVN neurons (Figure 4A) in sham-hydrated controls. Water deprivation for 48h virtually eliminated ER-β-ir from SON (Figure 3B) and magnocellular PVN neurons (Figure 4B) in sham-dehydrated rats. SON and magnocellular PVN were virtually devoid of ER-β positive nuclei in all 7 sham-lesioned dehydrated rats, although ER-β staining persisted in parvocellular regions of PVN. In contrast, the AV3V-hydrated animals continued to express ER-β in SON (Figure 3C) and magnocellular PVN (Figure 4C), despite an increased plasma osmolality that was comparable to the dehydrated sham-lesioned rats. All eight animals in this group retained prominent ER-β staining in MNCs. The majority of AV3V-dehydrated rats (6 out of 8 animals) exhibited prominent expression of ER-β in SON (Figure 3D). Again, this was despite an extreme increase in plasma osmolality (380±10 mOsm/kg, n=6) following water deprivation in these animals. ER-
β expression was absent in SON neurons in 2 of the AV3V-dehydrated rats, but histological examination demonstrated an incomplete ablation of OVLT in these animals (Figure 6). These 2 rats also had the lowest plasma osmolality (332 and 340 mOsm/kg) and highest hematocrit values (56 and 58%) among the rats in the AV3V-dehydrated group. Thus, based on histology, osmolality, and hematocrit, these animals likely had incomplete AV3V lesions. Nevertheless, Chi-square analysis for the presence or absence of ER-β staining in SON indicated a significant difference between the groups ($\chi^2 = 22.86$, $p<0.001$), and the Fischer Exact test for individual group comparisons demonstrated a significant difference between sham-dehydrated and AV3V dehydrated ($p=0.007$) even when the 2 animals with ER-β expression were included. Therefore, we conclude that the AV3V region is required for inhibition of ER-β expression in SON in response to water deprivation. As shown in Figure 5A, not only was ER-β expression not suppressed in SON in the AV3V-lesioned animals, the number of SON cells exhibiting intense ER-β-ir was significantly greater in the AV3V-hydrated as well as AV3V-dehydrated rats compared to the sham-hydrated rats ($p\leq 0.008$). This suggests that ER-β expression is partially inhibited in euhydrated, intact rats.

ER-β expression in magnocellular PVN was more variable than in SON in the AV3V-dehydrated group. As in SON, it was virtually absent in the 2 animals with incomplete AV3V lesions (Figure 6). However, it was also noticeably diminished in 4 out of the remaining 6 animals in the AV3V-dehydrated group in contrast to the strong expression of ER-β in SON in the same animals (Figure 7). ER-β expression was comparable to sham-hydrated rats in the
remaining 2 AV3V-dehydrated animals (Figure 4). As shown in Figure 5B, cell counts in magnocellular PVN corroborated the partial decrease in ER-β expression in the AV3V lesioned animals. The number of ER-β positive cells was significantly reduced in the AV3V-dehydrated group in comparison to the sham-dehydrated group, but it was significantly greater than in the sham-dehydrated group.

The decrease in ER-β-ir in the sham-dehydrated groups is not due to receptor regulation by circulating gonadal steroids, because the free plasma testosterone concentration was not significantly different among the groups (Table 1), and thus, there was no correlation between ER-β and free testosterone.

**Expression of Fos in magnocellular neurons**

Fos-ir was increased in SON (Figure 3F) and magnocellular PVN (Figure 4F) neurons following water deprivation in sham-dehydrated rats, compared to sham-hydrated controls (Figures 3E and 4E). Thus, as observed following chronic saline consumption (32), an inverse correlation exists between Fos and ER-β expression in MNCs in the sham-lesioned groups. As reported previously (24,35), it was low or undetectable in SON (Figure 3G) and magnocellular PVN (Figure 4G) neurons in the AV3V-hydrated animals in spite of the elevated plasma osmolality. In AV3V-dehydrated rats, however, Fos expression was variable. In both SON and magnocellular PVN, the number of Fos positive cells in the AV3V-dehydrated group was significantly increased compared to the sham-hydrated group, but was significantly less than in the sham-dehydrated group (excluding the 2 animals with incomplete lesions; 2-way ANOVA
F=53.00, p<0.001 for SON and F=22.07, p<0.001 for PVN). In PVN, Fos expression was inversely correlated with ER-β staining (Figure 7) suggesting that the lesions caused variable destruction of osmotic afferents to PVN. In SON, the continued expression of ER-β in the presence of Fos activation suggests that in AV3V-lesioned animals water deprivation activated neurons by pathways that were ineffective in inhibiting ER-β expression (Figure 7).

**Discussion**

In this study we examined the contribution of neural input from AV3V region in regulating the expression of ER-β in MNCs during water deprivation. Although VP and OT neurons themselves are osmoreceptive and respond directly to changes in the osmotic pressure of their extracellular environment, they require excitatory synaptic input to fire in response to increases in osmolality (21,26). Osmosensitive neurons also reside in the OVLT and communicate osmotic information to the MNCs via glutamatergic synapses (26). Thus, interaction between the intrinsic properties of the MNCs and osmosensitive synaptic inputs dictate the magnitude of the hormonal responses. AV3V lesions destroy the osmosensitive neurons in the OVLT and disrupt afferents to the MNCs from the SFO and MnPO. This results in impaired release of VP (and oxytocin) from the neurohypophysis following osmotic stimulation in animals with AV3V lesions (20,25). The current study demonstrates that AV3V lesions also disrupt inhibition of ER-β expression in SON MNCs following water deprivation. This supports the hypothesis that osmotically-sensitive afferents from the AV3V region are
responsible for inhibition of ER-β expression during chronic dehydration, and demonstrates that the intrinsic osmosensitive properties of the MNCs are not sufficient to inhibit ER-β expression.

The continued expression of ER-β in SON of AV3V-dehydrated rats also suggests that afferent pathways other than those from lamina terminalis to the MNCs are not effective in inhibiting ER-β expression under these conditions. This is based on the presence of Fos-ir in the SON of AV3V-dehydrated rats indicating that these neurons are being activated by excitatory afferents. The excitatory input is probably eliciting VP release, because AV3V-lesioned animals excrete urine that is comparably concentrated to that of sham-lesioned rats following 3 days of water deprivation (11). Specific candidates for stimulation of VP release during water deprivation in AV3V-lesioned animals are pathways carrying information from peripheral osmoreceptors (or sodium receptors), and pathways carrying information about blood volume. Extensive evidence for regulation of VP secretion by visceral osmo- or sodium receptors exists (14). Specifically, water and sodium are more effective in eliciting diuresis and antidiuresis, respectively, when administered into the portal vein than when given IV (10), and hyperosmotic solutions administered intragastrically or into the portal vein increase plasma VP without altering systemic osmolality (2,7,8,33). However, information from these receptors is then transmitted to the MnPO in the hypothalamus via multisynaptic pathways where it is integrated with information from the central osmoreceptors (15). Thus, AV3V lesions not only destroy central osmoreceptive mechanisms, but also interrupt the pathways carrying information from peripheral osmoreceptors. Therefore, the presence of Fos-ir in MNCs of the AV3V-dehydrated rats cannot be due to peripheral osmoreceptors.
Blood volume is another important regulator of VP secretion during dehydration in intact rats. Information about blood volume is monitored by cardiopulmonary receptors, transmitted to the brainstem via the vagus, and to the hypothalamic MNCs via pathways that do not involve the AV3V region (30). Thus, it has been demonstrated that hypovolemic stimulation of VP release is maintained in AV3V-lesioned rats (4,9). The current experiment arose from our previous observation that chronic hypertonicity induced by 2% NaCl drinking eliminated ER-β expression in MNCs (32). However, in the current study we used water deprivation rather than 2% NaCl drinking to elicit hypertonicity, because AV3V-lesioned rats do not consume 2% NaCl as readily as intact rats (25). Thus, any effect on ER-β expression that might have been detected with 2% NaCl drinking paradigm following AV3V lesion could, arguably, be due to the difference in the amount of their fluid intake. Therefore, water deprivation provided a uniform manipulation in the sham- and AV3V-lesioned groups. However, water deprivation not only causes hypertonicity but it also induces hypovolemia, both of which potently activate MNCs. Therefore, if ER-β had been eliminated in the AV3V lesioned rats it might have reflected activation of the MNCs by hypovolemia rather than a sustained effect of hypertonicity. However, ER-β was not depleted in the animals with complete AV3V lesions suggesting that hypovolemia did not contribute to the regulation of ER-β expression in MNCs in these animals. This could indicate that the brainstem afferents carrying cardiovascular information do not regulate ER-β expression in MNCs, or it could indicate that the hypovolemia in AV3V-lesioned animals was too small to suppress ER-β expression. The latter possibility is supported by the lack of evidence for hypovolemia in the AV3V-dehydrated rats. Hematocrit was used as an index
of blood volume, and although it was significantly elevated in the sham-dehydrated rats indicating water deprivation-induced hypovolemia, it was not significantly elevated in the AV3V-lesioned rats. However, a prior study did report a significant decrease in both blood and plasma volume without a detectable increase in hematocrit in AV3V-lesioned rats (4). Thus, there may have been a decrease in blood volume that was not detected as a change in hematocrit. Nevertheless, during water deprivation, the AV3V-lesioned rats conserved blood volume better than the sham-lesioned animals, and therefore stimulation of VP release by hypovolemia may have been inadequate to suppress ER-β expression. The conservation of blood volume may be due to decreased dehydration-induced natriuresis in the AV3V-lesioned animals. In sheep, lesions of the OVLT/MnPO attenuate dehydration-induced natriuresis resulting in extreme hypernatremia as was observed here (22).

The SON also receives numerous afferent projections from other brainstem, limbic, and hypothalamic regions (30) that could contribute to the Fos response in AV3V-dehydrated animals. In addition, although the endogenous osmoreceptivity of MNCs is not normally sufficient to elicit VP release without excitatory input from the AV3V region (21,26), given the extreme increase in plasma osmolality induced by water deprivation in the AV3V-dehydrated rats, it is possible that direct osmotic activation of the MNCs occurred in these animals which could have contributed to Fos expression without inhibiting ER-β expression. Regardless of the source of stimulation of the SON MNCs in the AV3V-dehydrated rats, there clearly was a disruption in the previously observed inverse correlation between Fos and ER-β in SON (32).
The conservation of ER-β expression in Fos expressing neurons supports the hypothesis that ER-β expression is selectively regulated by specific afferents to SON.

The partial depletion of ER-β in magnocellular PVN among the AV3V-dehydrated animals as well as the presence of strong Fos expression in these animals suggests that the AV3V lesion did not completely eliminate osmoreceptive afferents to the PVN. A differential effect of AV3V lesions on the response to water deprivation in SON and PVN was observed in early ultrastructural studies (6). Although axonal degeneration occurred in both SON and PVN following an AV3V lesion, by 5 weeks post lesion, PVN neurons of water deprived AV3V-lesioned rats exhibited fine structural changes characteristic of increased secretory activity (e.g. cellular hypertrophy, increased number of Golgi stacks, and increased neurosecretory granules). In contrast, SON neurons appeared smaller and contain fewer neurosecretory granules than control animals (6). Similarly and comparable to our results, Xu and Herbert (36) reported that 24hr water deprivation-induced Fos expression was less completely suppressed in PVN than in SON by AV3V lesions. The likely explanation for this difference is that some PVN afferents from the SFO exit the lamina terminalis above the lesion site while SFO afferents to SON continue to descend in the lamina terminalis and therefore are completely disrupted by the lesion (6,36). Therefore, afferents to SON are more completely destroyed than those to PVN.

The observation that ER-β-ir is actually increased in SON of the AV3V-lesioned rats suggests that ER-β is partially suppressed in normally hydrated rats. This is consistent with our previous observation that ER-β mRNA is increased by chronic hyponatremia (32). It is also
consistent with the early ultrastructural observation that the MNCs in AV3V-lesioned rats appear quiescent and contain fewer neurosecretory granules than control animals (6).

In conclusion, we have demonstrated for the first time that ER-β expression in rat MNCs is depleted by 48hr of water deprivation. Since this response is lost in SON and attenuated in PVN by lesions of the AV3V region, it occurs in response to osmoreceptive elements and/or fibers of passage in the AV3V region.

Perspectives

The physiological significance of ER-β expression in the MNCs remains an important question. Although the evidence to date supports an inhibitory role for ER-β during osmotic stimulation of VP release, it is evident that euhydration adult animals retain the ability to release VP in response to increases in plasma osmolality in spite of exposure to gonadal steroids. Possible explanations for this are: 1) In the whole animal the gonadal steroids may have multiple, offsetting actions such that the overall responsiveness of the neurohypophyseal system to osmotic control is retained. This possibility is supported by the fact that the results of numerous studies evaluating the impact of gender and gonadal steroid hormone production on VP secretion have been inconsistent (see refs (10,32,34) for review of the literature). 2) Gonadally produced steroid hormone may not be the primary endogenous ligand for the receptor. There is evidence for de novo neural production of steroids (3), and several enzymes required for steroidogenesis are present in hypothalamus including steroidogenic acute regulatory protein and P450 aromatase (16,27). Furthermore, in addition to estrogen, an androgenic metabolite, 5α-
androstan-3α, 17-β-diol, has affinity for ER-β (18). 3) ER-β may inhibit transcription of genes that are important for responses to chronic or intense stimulation rather than those involved in responses to small fluctuations in water balance. Thus, complete understanding of the physiological role of ER-β in the hypothalamo-neurohypophyseal system awaits elucidation of the endogenous ligand for the receptor as well as identification of the genes regulated by ER-β.
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Figure Legends.

Figure 1. Micrographs of OVLT (A,B), vMnPO (C,D), SFO (E,F), SON (G,H), and PVN (I,J) from sham- (A,C,E,G,I) and AV3V-lesioned (B,D,F,H,J) rats. In the AV3V-lesioned sections, note tissue damage to OVLT (B) and vMnPO (D), but not to SFO (F), SON (H), or PVN (J). These sections were stained for ER-β. ER-β positive cell nuclei are present in the anterior ventral periventricular region (C), SON (G,H) and PVN (I,J), but not in OVLT (A), vMnPO (C), nor SFO (E,F). Note: The dark material in B and D is debris remaining from tissue damage associated with the lesion, and not ER-β staining. AC-anterior commissure; mpv-medial parvocellular ventral zone of PVN; pm-posterior magnocellular region of PVN; oc-optic chiasm; 3V-third ventricle. Scale bars: 1000um – C,D; 500um - A,B,E,F; 200um – I,J; and 100um – G,H.

Figure 2. Effect of AV3V lesions and water deprivation on body weight. Closed circles – sham-hydrated animals; open circles – sham-dehydrated animals; closed triangle – AV3V lesioned-hydrated; open triangle – AV3V-dehydrated animals.

Figure 3. ER-β (A-D) and Fos (E-H) immunostaining in SON of sham-hydrated (A and E); sham-dehydrated (B and F); AV3V-hydrated (C and G); and AV3V-dehydrated (D and H). Note the disappearance of ER-β staining in the sham-dehydrated animal (B), but not in the AV3V-dehydrated rat (D). Note the prominent Fos expression in the sham-dehydrated animal (F), with only scattered Fos positive cells in SON of the AV3V-dehydrated rat (H) and fewer in the AV3V-hydrated animal (G) despite a significant increase in pOsm. Note the increase in the
number of ER-β intensely stained cells in the AV3V-lesioned animals (C and D) compared to the sham-hydrated (A). Scale bars: 100um.

Figure 4. ER-β (A-D) and Fos (E-H) immunostaining in PVN of sham-hydrated (A and E); sham-dehydrated (B and F); AV3V-hydrated (C and G); and AV3V-dehydrated (D and H). Note the loss of ER-β staining in the MNCs, but not the parvocellular neurons in the sham-dehydrated animal (B), and the prominent Fos expression (F). ER-β and Fos are comparable in the AV3V-hydrated (C and G respectively) to that of sham-hydrated (A and E) in spite of the elevated pOsm. ER-β persists in the AV3V-dehydrated rats (D). Scattered Fos expression is present in PVN MNCs in the AV3V-dehydrated (H). Scale bars: 200um.

Figure 5. Cell counts of ER-β (solid bars) and Fos (gray bars) immunopositive cell nuclei in SON (A.) and magnocellular PVN (B.). ER-β staining was virtually absent in the sham-dehydrated group in both SON and PVN (*p≤0.008 versus sham-hydrated). In contrast, Fos is significantly increased by dehydration in the shams (*p≤0.02). In the AV3V-lesioned rats, the number of positive ER-β nuclei is greater in SON, but reduced in PVN compared to the sham-hydrated group (* p≤0.008). However, in PVN ER-β is still greater in the AV3V-lesioned groups than in the sham-dehydrated group (#p<0.02). In both SON and PVN, Fos is lower in the AV3V lesioned groups compared to the sham-dehydrated group (#p≤0.02) in spite of the elevation in pOsm. However, it is greater in the AV3V-dehydrated group compared to the AV3V-hydrated
group ($p \leq 0.003$) in both SON and PVN. *=vs. sham-hydrated; #=vs.sham-dehydrated; $=$vs. AV3V-hydrated.

Figure 6. Histology in the 2 rats with incomplete lesions from the AV3V-dehydrated group. Pictures A-E are from rat A and Pictures F-J are from rat B. Note the intact OVLT in both rats (A and F), the virtual absence of ER-β staining in SON (C and H) and PVN MNCs (E and J) and the prominent Fos staining (SON: B and G; PVN MNCs: D and I) in both rats. Scale bars: A,F: 500 um; B,C,G,H: 100 um; D,E,I,J: 200 um.

Figure 7. Examples of variable effect of AV3V lesions on ER-β and Fos immunoreactivity in PVN and SON. The first group of pictures (A-D) is from a rat with prominent ER–β expression in SON (B), but depleted ER-β in PVN (D; compare to figure 4B and C). Note the prominent Fos expression in both SON and PVN (A and C, respectively). The second group of pictures (E-H) is from a rat with again prominent ER–β expression in SON (F), but decreased expression in PVN (H). However, this rat shows differential Fos expression in SON and PVN (e.g. only scattered versus prominent in SON and PVN respectively). Thus, the first rat is an example of an animal with sustained ER-β expression in the presence of Fos activation in SON while the second rat is an example where the inverse relationship between ER-β and Fos is present in both SON and PVN, but PVN is stimulated to a greater degree than SON. Scale bars: A,B,E,F: 100um; C,D,G,H: 200 um.
Table 1. Plasma osmolality, constituents contributing to osmolality, hematocrit values, and free testosterone.

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<td>Sham-hydrat.</td>
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<td>AV3V-hydrat.</td>
<td>AV3V-dehydrat.</td>
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<td>pOsm (mOsm/kg H₂O)</td>
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<td>pNa (mEq/liter)</td>
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<td>pGlucose (mg/100 ml)</td>
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<td>BUN (mg/100 ml)</td>
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<td>Predicted pOsm</td>
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^Only values from animals with all data available to calculate predicted pOsm are included except for Hematocrit and Testosterone where all values are included and n is indicated in ()

*p<0.001 vs all other groups
‡p<0.01 vs sham-hydrated by independent Student’s t-test
†p<0.001 sham vs lesioned
§p<0.05 vs all other groups
Fig 2

*Surgery vs Sham, p < 0.005,

*Water deprivation vs Sham, p < 0.05

Day

Body weight (g)

240
260
280
300
320
340
360
380
400
420

* p < 0.005, # p < 0.05 between Sham vs AV3V surgery
Fig 4

Sham-hydrated  Sham-dehydrated  AV3V-hydrated  AV3V-dehydrated

A  B  C  D

E  F  G  H
Fig. 5

A

SON

No. of cells

Sham-Hyd. Sham-Dehyd. AV3V-Hyd. AV3V-Dehyd.

B

PVN

No. of cells

Sham-Hyd. Sham-Dehyd. AV3V-Hyd. AV3V-Dehyd.
Fig 6
Fig 7

Fos-ir

ER-β-ir

A

B

OC

C

D

3V

E

F

OC

G

H

3V