Prenatal exposure to ethanol causes partial diabetes insipidus in adult rats

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Knee, Daniel S., Aileen K. Sato, Catherine F. T. Uyehara, and John R. Claybaugh. Prenatal exposure to ethanol causes partial diabetes insipidus in adult rats. Am J Physiol Regul Integr Comp Physiol 287: R277–R283, 2004. First published May 13, 2004; 10.1152/ajpregu.00223.2003.—Chronic consumption of alcohol in adults is associated with impairment, and probably permanent damage, to the vasopressinergic systems in the brain, and brain damage is known to occur to a fetus exposed to alcohol; it is reasonable to hypothesize that damage could be done to the developing vasopressinergic systems in the brain.

Only two previous animal studies have been reported on water and electrolyte regulation and AVP responses in association with prenatal ethanol (PE) exposure. Dow-Edwards et al. (10) reported that basal levels of AVP were nearly sevenfold higher in prenatally alcohol exposed rats at 3 mo of age, and despite normal renal responses to 24 h of dehydration, this did not stimulate further release of AVP in these rats. McGivern et al. (22) reported that PE exposure produced permanent increased water drinking behavior in male rats but not female rats, and they observed no difference in the basal plasma levels of AVP at 80 days of age in rats regardless of PE exposure.

The present study was conducted to improve our understanding of AVP regulation in young adult rats after PE exposure. We hypothesized that osmoregulatory regulatory mechanisms would be impaired in the PE-exposed rats and the pituitary storage and hypothalamic synthesis of AVP would be reduced.

**MATERIALS AND METHODS**

**Experimental animals.** All experimental animals were acquired and used in compliance with federal, state, and local laws and institutional regulations. The experimental protocol was approved by Tripler Army Medical Center, Institutional Animal Care and Use Committee, and the program and institution are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Investigators complied with the policies as prescribed in the

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United States Department of Agriculture Animal Welfare Act and the National Research Council's Guide for the Care and Use of Laboratory Animals.

**PE and control diet procedures.** The pregnancies of nine Sprague-Dawley female rats (Taconic, Germantown, NY) were timed by pairing females with males on the day of proestrus, as determined by vaginal smears. The male rats remained with the female rats for up to 3 days, and pregnancy was determined by the presence of sperm in the vaginal smear, which was designated as day 1 of pregnancy.

On day 7 of pregnancy, four of nine pregnant rats were placed on a liquid diet containing 35% of the calories derived from ethanol (Bio-Serv, Frenchtown, NJ), and the remaining five pregnant rats were fed a similar liquid diet but with an equivalent caloric content of carbohydrate instead of ethanol. The diets were continued until gestational day 21 when the dams were returned to their normal rat chow. The pups were fed by their biological lactating mothers (20) and weaned at 21 days.

**Water intake measurements.** Ad libitum water intake was determined in two ways. Sixty- to 68-day-old rats from two PE litters and two litters not exposed to PE (NPE litters) were used in series 1 experiments. In this series, water intake was measured from the water bottles while the rats were housed in their normal cages. To verify that the increase in water drinking behavior was, in fact, accompanied by an increased urine flow, series 2 was conducted where 60- to 68-day-old rats were placed in metabolism cages that allowed for the collection of urine and water consumption. The hypertonic saline infusion experiment, described later, was completed using the rats from series 1 plus two groups of rats used in series 2 when they had reached 67–78 days old: one PE litter and one NPE litter. Sixty- to 68-day-old rats from one additional NPE litter and one PE litter were used in the urine collection determinations to provide adequate statistical power for urine volume comparisons.

**Surgical procedures.** At 67–75 days of age, the rats underwent surgical catheterization of the femoral artery and vein by procedures previously described (31). Ibuprofen, at 7.2 mg/2 ml, was administered for analgesia in a Jell-O jiggle before surgery and after surgery. Surgical anesthesia was achieved with isoflurane (5%) delivered with an anesthetic gas delivery system. Surgeries were performed aseptically. After the surgery the rats were given ibuprofen in their drinking water solution (0.6 mg/ml) for 24 h. The animals were used in an experiment 1 wk after surgery.

**Experimental design.** Osmotic stimulation experiments were conducted on 17 PE rats (10 males and 7 females) from three litters with 5, 6, and 6 animals from each litter contributing to the group. Twenty rats not exposed to PE (NPE-exposed rats; 10 males and 10 females) from four litters with a distribution of 7, 5, 5, and 3 were used in this group. The time-control subgroups were infused with normal saline instead of hypertonic saline and consisted of five rats (2 males and 3 females) in the PE group from two litters and seven rats (3 males and 4 females) in the NPE group from two litters. On three separate days, before experimentation, the rats were placed in plastic Braintree experimental chambers (Braintree, MA) for periods of 30, 60, and 90 min, respectively, to allow them to become accustomed to the restrained environment. On the day of the experiment the rats were weighed and placed in the plastic chambers. The arterial catheter was used to record blood pressure and heart rate using MacLab/8 recording instrument (ADInstruments, Castle Hill, NSW, Australia) and for obtaining blood samples. The venous line was connected to an infusion pump (Harvard Apparatus, model 22, Holliston, MA) for the infusion of 5% NaCl. After an acclimation period of 60–90 min, baseline cardiovascular measures were obtained. After this, a 1.2-ml blood sample was obtained from the arterial line while 1.2 ml of 0.9% NaCl was simultaneously infused using the venous line to help maintain volume homeostasis. At this point in the venous infusion of 5% NaCl at a rate of 0.05 ml-kg⁻¹-min⁻¹ was started in those animals receiving the hypertonic saline infusion, or else 0.9% NaCl was infused at the same rate in the control series. Three additional blood samples of similar volume were taken at 40-min intervals during the infusion. From each blood sample two micropipette tubes were collected for hematoctrit determination, and the remainder was transferred to a 3-ml vacutainer with sodium heparin (BD Vacutainer, Franklin Lakes, NJ) and centrifuged at 1,000 g at 4°C for 15 min. The plasma from the vacutainer was separated from the red blood cells and further separated into two samples. To a 400-μl sample, 40 μl of 1 N HCl was added for subsequent AVP analysis, and the sample was frozen until assayed. The remaining plasma was used to measure osmolality by freezing point depression (Advanced Micro Osmometer, 3M, Norwood, MA). At each sampling time, the replacement fluid contained the red blood cells of the previous sample suspended in a saline solution to a volume of 1.2 ml. The replacement solution was in three increasing degrees of hypertonic saline: 297 mosmol/kgH₂O for sample 2, 304 mosmol/kgH₂O for sample 3, and 311 mosmol/kgH₂O for sample 4. In the control series, the replacement fluid remained as normal saline with a measured osmolality of 290 mosmol/kgH₂O.

**Tissue harvesting.** After the experiment, the animals were euthanized with at least 100 mg/kg of pentobarbital sodium given via the venous or arterial catheters. The brain was removed from the cranium, the neurohypophysis was separated from the adenohypophysis, and the former was frozen until assayed for AVP content. A block of the brain including the supraoptic, paraventricular, and suprachiasmatic nuclei was then excised, placed in RNA later (Ambion, Austin, TX), and frozen for later analysis of AVP mRNA. All samples were stored at −70°C until further assayed.

**Assay procedures.** Vasopressin was extracted from 0.4-ml plasma samples by passage through octadeclisilane cartridges (SepPak C₁₈, Waters Associates, Milford, MA) by methods and assay specificity previously described (30). The eluate was dried under vacuum (SpeedVac Concentrator, Savant, Farmingdale, NY), taken up in 0.4-ml assay buffer, and assayed as a single dose.

The between-assay coefficient of variability for the assay was 8.2%, and the average recovery of unlabeled AVP in the extraction procedure was 88%. The sensitivity of the assay was 0.625 μU/ml plasma. The pituitary content of AVP was determined on dilutions of extracts of the neurohypophysis, which was extracted according to the method described by Crofton et al. (8).

AVP mRNA and β-actin mRNA were quantified by analysis of real-time PCR of single-strand cDNA (iCycler, Bio-Rad Laboratories, Hercules, CA). Total RNA was extracted from the blocks of brain by the use of TRIzol Reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed by the use of Superscript First Strand Synthesis System (Invitrogen), and PCR was performed using Platinum qPCR, SuperMix-UDG (Invitrogen). The forward and reverse primers used for the PCR of the AVP cDNA were 5'-tacctctgctgctc-3′ and 5'-cgcagggagactgt-3′, respectively, which produced an amplicon of 87 bp that spanned between the first and second exons. The Taqman probe used for the AVP cDNA PCR analysis had the following structure: 5'-6-fluorescein (FAM)-ccacatcagcatgaaggaggagctag-Black Hole Quencher (BHQ)-1-3′. The forward and reverse primers for β-actin cDNA PCR were 5'-caataggcgtggtg-3′ and 5'-ccacagcagctgaggtg-3′, which produced an amplicon of 148 bp that spanned between the third and fourth exons. The sequence of the β-actin probe was 5'-6-FAM-tccgtgagctgacagagtt-BHQ-1-3′. All primers and probes were synthesized by Biosearch Technologies, Navato, CA.

A standard curve was performed with each assay using a stock of cDNA from a rat hypothalamus. The data are expressed as a ratio of relative copies of AVP mRNA to β-actin mRNA.

**Statistics.** Basal differences between the PE group of rats and the NPE group were performed with a two-way ANOVA, with “group” being one factor and “sex” being the other factor. All rats were included regardless of the experiment that was conducted, i.e., hypertonic saline infusion or normal saline infusion. If there was no effect of sex, only the effects of groups were reported. If sex was significant,
then a post hoc test was performed to determine which means were different from each other, a Tukey-Kramer honestly significantly different test.

The evaluation of the relationship between plasma osmolality and plasma AVP was assessed by determination of the individual slopes and intercepts for each rat by least squares linear regression. Then the osmolality differences between the two lines were determined at a middle range of the values with the AVP values set at 2.5 μU/ml and at 3.0 μU/ml. That is, for each rat the plasma AVP values were set once at 2.5 and once at 3.0 μU/ml, and the corresponding plasma osmolality was calculated from the equation of the line. The means of these calculated values were then compared with an unpaired t-test. Significance was set at an α-error of 0.05. All statistics were performed with JMP version 4 software. (SAS Institute, Cary, NC).

RESULTS

The female rats exposed to ethanol consumed an average of 271 kcal·kg⁻¹·day⁻¹ while on the diet and the control fed rats consumed 273 kcal·kg⁻¹·day⁻¹, based on the manufacturer’s caloric value of the liquid diet at 1 kcal/ml. Despite this similarity in caloric intake, the ethanol-fed dams gained an average of 62 g from day 7 to day 21 of pregnancy compared with a weight gain of 115 g during the same period in the control fed dams (P = 0.0006). The average body weight of the PE-exposed and NPE-exposed rats on day 7 of life was similar, 13.2 and 13.5 g, respectively. At the time of the experiments the average body weight for the female rats was 237 ± 5 and 232 ± 3 g for NPE- and PE-exposed groups, respectively (P = 0.46). The corresponding male weights were 376 ± 9 and 367 ± 7 g (P = 0.63).

PE-exposed rats from two litters consumed more water than NPE-exposed rats, also from two litters, when daily ad libitum water consumption was monitored while the rats were in their normal cages (Fig. 1, series 1). Furthermore, these female rats were observed to consume more water per kilogram body weight than male rats. To confirm that the water drinking behavior was reflected in increased urine output, some of the rats from two additional litters of PE-exposed rats and three additional litters of NPE-exposed rats were placed in metabolism cages for 48 h (Fig. 1, series 2). Unfortunately, the animals consumed significantly less amounts of water in the metabolism cages. Therefore, the water-drinking data could not be pooled with the series 1 data. Furthermore, the collected urine samples appeared variably contaminated by fine food particles, and we felt that accurate measurements of osmolality or electrolytes could not be made and were therefore not attempted. Despite these apparent problems with acclimation, urine output was observed to be significantly greater in the PE-exposed rats than the NPE-exposed rats. There was a similar trend toward PE-exposed animals having a greater water intake than NPE-exposed animals (17–20% increase), but this did not reach statistical significance. In both series the females had higher water intake than did the male counterparts.

Basal plasma AVP levels, obtained before the initiation of the infusion, were similar between the PE- and NPE-exposed rats (Fig. 2). This similarity of AVP levels continued throughout the infusion of 0.9% NaCl (Table 1). Although the AVP levels were similar between PE- and NPE-exposed rats, the plasma osmolality was ~2 mosmol/kg H₂O higher in the PE-than the NPE-exposed rats (Fig. 2). Plasma osmolality tended to decrease over time with the infusion of 0.9% NaCl in the time-control series but was only significant in the NPE-exposed rats (Table 1).

Mean arterial blood pressure and heart rate were not different between groups at any of the time periods during the hypertonic saline infusion (Table 2). The infusion of hypertonic saline caused a lowering of hematocrit by the third or fourth periods, most likely due to the osmotic gradient causing movement of fluids into the extracellular fluid space (Table 2).

The change in osmolality during the 5% NaCl infusion was similar for the PE- and NPE-exposed rats over the first three blood sampling periods (Fig. 3). However, at the fourth period, plasma osmolality was not further increased in the NPE group but was further increased in the PE group. The average slopes of the AVP vs. plasma osmolality (Fig. 3) derived from the slopes and intercepts of each rat were not different between the NPE- and PE-exposed rats. When values of AVP were set in the area of the relationship where measurements were obtained, that is, at 2.5 μU/ml (P = 0.058) and at 3.0 μU/ml (P = 0.045), the average extrapolated osmolality was higher in the PE-exposed rats. The data suggest that the AVP-plasma osmolality relationship was shifted to the right in the rats prenatally exposed to ethanol.

The pituitary AVP content was reduced by ~13% (P = 0.01) in the PE- compared with the NPE-exposed groups (Fig.
35% less in the PE-exposed rats than in the NPE-exposed rats.

Table 1. Effects of 0.9% NaCl infusion and blood sampling procedures on arterial BP, HR, and Hct

<table>
<thead>
<tr>
<th>Measurement/Group</th>
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<tr>
<td>NPE (0.9% NaCl)</td>
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<tr>
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<tr>
<td>NPE (5% NaCl)</td>
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<td>Hct, %PCV</td>
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<tr>
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<tr>
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<td>40.7±0.9</td>
<td>39.0±0.8</td>
<td>38.5±0.8</td>
<td>38.0±1.0*</td>
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Values are means ± SE. BP, blood pressure; HR, heart rate; Hct, hematocrit; PCV, packed cell volume. *P < 0.05 compared with blood sample 1.

Table 2. Effects of 0.9% and 5% NaCl infusion and blood sampling procedures on arterial BP, HR, and Hct

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In addition, the relative number of copies of AVP mRNA per copy of β-actin mRNA found in the hypothalamus was 35% less in the PE-exposed rats than in the NPE-exposed rats (P = 0.036).

**DISCUSSION**

Central diabetes insipidus (DI) is defined in Dorland’s Illustrated Medical Dictionary as “a metabolic disorder due to injury of the neurohypophyseal system, which results in a deficient quantity of antidiuretic hormone being released or produced, and thus in failure of tubular reabsorption of water in the kidney…” (9). The data of the present experiments suggest that PE exposure in rats can lead to a partial central DI evident in the young adult. This is supported by the observations that PE-exposed rats displayed reduced pituitary content of AVP and reduced hypothalamic AVP mRNA and had evidence for a shift to the right of the plasma osmolality-plasma AVP relationship compared with NPE-exposed rats. These observations are compatible with an overall suppression of the vasopressin system, which probably accounts for the increased urine volume in the PE-exposed rats. The increased water intake we observed would be necessary to maintain water balance. Last, the increased urine flow resulting from a decreased osmotic stimulation of AVP should be the result of increased free water excretion, which is the likely explanation for the tendency of plasma osmolality to be slightly increased in the PE-exposed rats, an observation also consistent with a decrease in the quantity of AVP produced, and thus in failure of tubular reabsorption of water in the kidney.
state of DI in these rats. The DI condition observed was only partial because evidence of circulating AVP is present, and it was increased by the infusion of hyperosmotic saline.

The increased water consumption of PE-exposed males and females agrees with the findings of others (10, 22), who also reported an increase of about 20–40% in water consumption after PE exposure. McGivern et al. (22) found the increased water intake in male rats only. In those studies the PE exposure was only from day 14 through parturition. The decreased PE exposure in those studies may account for the lack of significant response in the female rats. Dow-Edwards et al. (10), using an alcohol exposure similar to the present study, reported an ~40% increase in daily water consumption of male Long-Evans rats exposed prenatally to ethanol, but they did not study female rats.

We observed a greater urine output in PE-exposed rats than in NPE-exposed rats. This difference appeared greater in the male rats than in the female rats, but statistical analysis could not confirm this. The observed increase in urine output in PE-exposed rats is consistent with findings in humans. Assadi (3) found that among six children who met clinical criteria for fetal alcohol syndrome, none were able to concentrate their urine after 12 h of water deprivation to a normal range that was established in age- and sex-matched control subjects [560 ± 107 compared with 965 ± 77 mosmol/kgH2O in control subjects (P < 0.001)]. However, their data suggest that a large component of the defect may be of renal origin. They found that when vasopressin was administered to the children, they were only able to further increase their urine osmolality to 578 ± 101 mosmol/kgH2O. Plasma AVP was not measured in those studies, and the source or demonstration of vasopressin potency was not presented. This aspect of a renal defect merits further study.

We also found that female rats drank more water than the males, in agreement with others (22). Our inability to demonstrate a similar difference in urine excretion may be possibly explained by the general decrease in drinking behavior while the rats were in the metabolism cages. This resulted in very small urine collections, especially in the female rats because of their smaller size. The increased water drinking in female rats would, however, suggest a greater water turnover rate when calculated on a weight basis. These observations are consistent with results from human studies indicating an increased excretion of a modest water load in female subjects compared with males (6). This sexual dimorphism may be related to the renal responsiveness to AVP. Wang et al. (32) found that male rats exhibit a threefold increase in antidiuretic activity in response to AVP compared with females. This increase in AVP sensitivity was associated with a twofold increase in V2 receptors in papillary collecting ducts of males compared with females. The increased sensitivity to AVP would explain the significantly different water consumptions while having similar AVP plasma levels.

The plasma AVP values for PE- and NPE-exposed rats were similar, as reported by others (22). However, Dow-Edwards et al. (10) found a highly variable, sevenfold increase in AVP in PE-exposed rats. A difference in the method of blood sampling may explain why this difference is present. In our study we used indwelling central catheters for blood draws, whereas Dow-Edwards et al. (10) and McGivern et al. (22) collected trunk blood after decapitation. The use of decapitation may involve varying degrees of handling of the rats and may cause an elevation in AVP levels. This was shown by Husain et al. (15), who reported that holding a rat for 3 min resulted in a 25-fold increase in AVP.

Although starting plasma AVP levels observed in both NPE- and PE-exposed rats were similar, the plasma osmolality was greater in the PE-exposed rats. A similar tendency in mean plasma osmolalities was reported between PE and NPE in the euhydrated and dehydrated states by Dow-Edwards et al. (10), but these differences did not reach statistical significance in their studies. This increased plasma osmolality would suggest a different osmotic threshold for the release of AVP. The present studies reveal that the slope of the plot of plasma AVP as a function of plasma osmolality in both PE- and NPE-exposed rats was similar, and both were significant. That is, increased osmolality was associated with increased AVP. In the study by Dow-Edwards et al. (10), dehydration did not further increase plasma AVP levels in the PE-exposed rats, but the euhydrated levels were already sevenfold higher than in the NPE-exposed rats. Thus the sensitive osmotic stimulation of AVP may have been overwhelmed by other stimuli to the AVP-releasing neurons. In the present study the mean calculated plasma osmolality was significantly greater in the PE-exposed rats at an AVP level of 3 μU/ml, indicating a rightward shift of the relationship within the response limits of the experiment. Taken together, the data suggest that the PE-exposed rats have an increased osmotic threshold for AVP release.

During our control arm of the experiment, normal saline was infused rather than a hyperosmotic solution. The control arm used replacement fluids (1.2 ml/sample) consisting of red blood cells from the previous blood draw and normal saline. The results of the control arm demonstrate no significant difference in AVP values. The similar AVP values indicate that the methods used to draw blood samples did not influence the results.

The mechanism for the decreased osmotic stimulation of AVP in rats that were prenatally exposed to ethanol was initially hypothesized to result because of alterations in brain morphology due to either the direct or indirect effects of alcohol exposure that had been previously reported. For instance, chronic alcohol exposure has been shown to damage AVP-producing cells in adult animals and humans (19, 13), and also morphological alterations had been reported in AVP-producing cells of the hypothalamus as a result of PE exposure (26). An alternative explanation may, however, involve the influence of the hypothalamic-pituitary-adrenal (HPA) axis. Although the basal levels of corticosterone (16) or ACTH (18) do not appear to be affected by PE exposure, recent evidence demonstrates an augmented response of the HPA axis (11, 12, 18) to stressors. It is well established that increased corticosterone, or cortisol, can interfere with the stimulated release of AVP (25). Therefore, it is tempting to speculate that the decreased response to an osmotic stimulation observed in the present studies could have been due to simultaneous stress-induced stimulation of corticosterone, possibly due to the 2 h of restraint in the plastic experimental chambers or other causes, but corticosterone was not measured in these studies. It is noteworthy that inhibition of the osmotically stimulated release of AVP by glucocorticoids has been demonstrated in vitro hypothalamic-neurohypophysial explants, but no changes.
in the hypothalamic mRNA were detected (24). Those authors concluded, therefore, that corticosterone interferes with AVP release by nongenomic mechanisms. It is possible that stress-induced increases in corticosterone could have contributed to the impaired osmotic stimulation of AVP, but the decreased mRNA and pituitary stores of AVP suggest that other mechanisms may be involved.

Last, it is important to consider the relevance of the dose of alcohol used in the current studies with human alcohol consumption. First, we selected this dose because a large portion of the previous work has used this dose and this period of administration, and specifically those that had studied potential disturbances of AVP and water balance (17, 22). While on the ethanol diet, the rats consumed ~18.3 ml (14.4 g) ethanol/kg each day in the current study, similar to ~1.3 l/day in a 70-kg human from a volume comparison. In the rat, this diet produces blood alcohol levels of ~130 mg/dl 1 h into the dark cycle (17). When 5.1 g of ethanol/kg were administered as a bolus, peak blood alcohol levels reached ~300 mg/dl (20). These levels of blood alcohol are commonly achieved in humans (National Institute on Alcohol Abuse and Alcoholism, http://www.niaaa.nih.gov/extramural/impasp.htm).

Perspectives

The reduction in pituitary AVP levels and hypothalamic AVP mRNA in the PE-exposed rats would indicate that the DI resulting from PE exposure has a central component. The fact that the adult rats are affected from prenatal exposure would indicate that the effect is permanent. We are not able to address the issue of renal DI with our current data. However, Assadi (3) noted that the patients with fetal alcohol syndrome had renal defects, including a reduced ability to concentrate their urine and to excrete an acidified urine, and fractional potassium excretion was lower than normal. Further investigation of AVP receptor mRNA in the kidneys needs to be assessed in the PE- and NPE-exposed rats to evaluate possibilities for nephrogenic DI.

Last, in view of the extensive literature regarding the involvement of AVP in learning and memory and other behavioral processes (for review, see Ref. 4), it is possible that a component of the mental retardation that can accompany fetal alcohol syndrome may be due to depressed vasopressinergic brain systems.

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