Enhanced central response to dehydration in mice lacking angiotensin AT$_{1a}$ receptors

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Received 26 May 2000; accepted in final form 1 November 2000

Morris, Mariana, Shelia Means, Michael I. Oliverio, and Thomas M. Coffman. Enhanced central response to dehydration in mice lacking angiotensin AT$_{1a}$ receptors. Am J Physiol Regulatory Integrative Comp Physiol 280: R1177–R1184, 2001.—The objective was to determine the central nervous system (CNS) responses to dehydration (c-Fos and vasopressin mRNA) in mice lacking the ANG AT$_{1a}$ receptor [ANG AT$_{1a}$ knockout (KO)]. Control and AT$_{1a}$ KO mice were dehydrated for 24 or 48 h. Baseline plasma vasopressin (VP) was not different between the groups; however, the response to dehydration was attenuated in AT$_{1a}$ KO mice (24 ± 11 vs. 10.6 ± 2.7 pg/ml). Dehydration produced similar increases in plasma osmolality and depletion of posterior pituitary VP content. Neuronal activation was observed as increases in c-Fos protein and VP mRNA. The supraoptic responses were not different between groups. In the paraventricular nucleus (PVN), c-Fos-positive neurons (57.4 ± 10.7 vs. 98.4 ± 7.4 c-Fos cells/PVN, control vs. AT$_{1a}$ KO) and VP mRNA levels (1.0 ± 0.1 vs. 1.4 ± 0.1 μCi, control vs. AT$_{1a}$ KO) were increased with greater responses in AT$_{1a}$ KO. A comparison of 1- to 2-day water deprivation showed that plasma VP, brain c-Fos, and VP mRNA returned toward control on day 2, although plasma osmolality remained high. Data demonstrate that AT$_{1a}$ KO mice show a dichotomous response to dehydration, reduced for plasma VP and enhanced for PVN c-Fos protein and VP mRNA. The results illustrate the importance of ANG AT$_{1a}$ receptors in the regulation of osmotic and endocrine balance.

central nervous system; hypothalamus; water balance; blood pressure; molecular genetics; knockout models; paraventricular nucleus

There are strong links between the central ANG II and vasopressin (VP) systems. ANG II activates VP neurons, as seen by neuronal firing, c-Fos and VP mRNA expression, and VP secretion (6, 8, 23, 24, 26, 35). ANG peptides and their receptors are present in the neurosecretory, paraventricular (PVN), and supraoptic nuclei (SON), and in brain regions that are osmosensitive (13, 15). A recent study found a similar distribution pattern for ANG-(1–7), VP, and colocalization in a subpopulation of PVN neurons (15). Alterations in fluid balance produced by dehydration or salt loading result in increases in central nervous system (CNS) ANG receptors, ANG AT$_{1a}$ receptor mRNA, and VP mRNA (2, 3, 20, 27, 37, 39, 40).

ANG receptors are differentiated into AT$_1$ and AT$_2$ subtypes with the ANG AT$_{1a}$ receptor clearly associated with CNS, vascular, and endocrine effects. Pharmacological antagonists to the AT$_1$ receptor are used clinically for the treatment of hypertension and cardiac failure and as tools for the study of pharmacological action. There is evidence that the AT$_1$ antagonists block the pressor, endocrine, and cellular responses to ANG II stimulation (7). However, when ANG AT$_{1a}$ antagonists were tested against physiological stimulation, certain questions and controversies emerged. For example, losartan, an AT$_1$ antagonist, was much less effective than ANG AT$_{2}$ antagonists in blocking the drinking response to dehydration (17, 38). Studies in animals lacking vasopressin suggested that neither AT$_1$ nor AT$_2$ receptors were involved in the response to dehydration (44). Additionally, losartan was not effective in blocking the plasma VP response to hemorrhage (33). Questions have been raised concerning the specificity of losartan and the possibility of other central ANG receptor subtypes (9, 29, 46).

A new method that is useful for the study of functionality is gene deletion or knockout models. The premise is that the removal of an essential receptor protein and subsequent evaluation of phenotypic expression and physiological responses will, by inference, provide information on function. An advantage of gene deletion models is that the absence of the specific protein means that the blockade resulting from the receptor loss should be complete. This is in contrast to protocols that rely on pharmacological antagonism, in which drug concentrations and tissue levels are constantly changing and specificity may be a question. The drawback to genetic knockout models is that the protein of interest is removed from conception onward. Thus development proceeds in its absence, which may produce secondary changes or compensatory effects.

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However, at least in the AT\textsubscript{1a} KO, there is no evidence for changes (upregulation) of other ANG receptor subtypes (27). Published reports suggest that the AT\textsubscript{1a} KO model is useful for studying physiological genomics, because the basic phenotypic characteristics are compatible with the known functions of the AT\textsubscript{1a} receptor: reduced blood pressure, increased drinking, and reduced urine osmolality (12, 31, 41). However, investigations have just begun to tap the utility of the model for studying the role of ANG AT\textsubscript{1a} receptors in basic neuronal and endocrine physiology.

The AT\textsubscript{1a} KO strain provides a unique model for the study of angiotensinergic control of the VP axis. Investigation of the neurosecretory system under basal and osmotically stimulated conditions will provide clues as to the role of the peptide and its receptor in endocrine and osmotic balance. We hypothesize that if ANG AT\textsubscript{1a} receptors are essential to VP neuronal function and osmotic responsiveness, that these will be attenuated in the absence of the receptor. Dehydration is used as the stimulus with evaluation of VP secretion and storage and activation of central osmoregulatory regions, PVN, SON, subfornical organ (SFO), and median preoptic (MnPO) using c-Fos and VP mRNA.

**METHODS**

**Animals.** Male mice lacking ANG AT\textsubscript{1a} receptors were bred and maintained in the animal center of the Durham Veterans Affairs Medical Center. The mice were F\textsubscript{2} progeny derived from crosses of (129\times C57Bl/6) F\textsubscript{1} AT\textsubscript{3} and AT\textsubscript{1a} KO parents. This F\textsubscript{2} generation of AT\textsuperscript{1a+/-} and AT\textsuperscript{1a-/-} possesses similar random assortment of background genes making it an appropriate match for study. ANG AT\textsubscript{1a} genotypes were determined by Southern blot analysis of DNA isolated from tail biopsies (12). The animals were housed singly with free access to water and food.

**Protocol.** Six groups of animals were studied: AT\textsubscript{1a} KO and their controls with water ad libitum or after 24 or 48 h of water deprivation. The average body weights for the groups were 33.5 ± 1.7 vs. 35.1 ± 1.3 g (control vs. AT\textsubscript{1a} KO, respectively). The mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and rapidly decapitated. Brains were collected in fixative (Bouin’s solution); posterior pituitaries were frozen on dry ice, and trunk blood was collected on ice in heparinized tubes.

**RIA and osmolality.** Plasma was separated for measurement of osmolality by freezing point depression (20 μl) and of VP by RIA. For RIA, plasma samples (100–200 μl) were extracted with acetonitrile and petroleum ether. The lyophilized extract was resuspended in assay buffer, and VP concentrations were measured using a specific and sensitive assay. The ED\textsubscript{50} for the VP assay is 4–5 pg/tube. Preliminary studies showed that baseline plasma levels were similar in conscious or pentobarbital sodium-anesthetized animals. Acid extracts (0.1 N HCl) of posterior pituitaries were diluted, and VP was assayed by RIA and osmolality. The labeled c-Fos antisera was provided by P.J. Larson and J. Mikkelsen (Panum Institute, Denmark) and used at a dilution of 1:50,000. It was made against the NH\textsubscript{2}-terminal region of synthetic human c-Fos protein (residues 4–17) and cross-reacts with both mouse and rat c-Fos (16). The rabbit VP antisem was made against VP-neuropehysin and used at a dilution of 1:100,000 (NP 818, A. Robinson, University of California at Los Angeles, Los Angeles, CA). For quantifying, we used an image analysis system that combines captured video images (Optronics-DEI 750 D, charge-coupled device camera, Optronics Engineering, Goleta, CA) with image analysis software (MetaMorph Imaging System, West Chester, PA). The numbers of c-Fos-positive neurons within the areas of interest, PVN, SON, MnPO, and rostral forebrain were measured. For the SON and PVN, c-Fos was measured in magnocellular neurons located in the midregion of the nuclei.

The in situ hybridization method for VP mRNA used an oligonucleotide probe as previously described (37). The 30-base pair oligonucleotide is complementary to exon C of vasopressin prohormone mRNA (GIBCO BRL Custom Primers, Rockville, MD). The sequence was GGTGAGGCG-GAAAAACCGTCGTTGGCACTC. The probe was labeled with [\textsuperscript{35}S]dATP using 3-terminal deoxynucleotidyl transferase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and purified using a Nensorb column (DuPont, Boston, MA). The tissue sections were mounted on coated slides (UltraStick, Fisher, Pittsburgh, PA) and air-dried. After being washed in PBS (0.1 M, pH 7.2) and 2× SSC, the labeled probe (~0.4 × 10\textsuperscript{6} cpm/100 μl) was added directly to the tissues. The slides were incubated overnight at 37°C in a humidified chamber and washed sequentially in 1× SSC (1 h at room temperature), 0.5× SSC (1 h at room temperature) and 0.5× SSC (1 h at 50°C). For quantification of the hybridization signal, we used a direct radioactive capture method (Fuji FLA-200, Fuji, Stamford, CT). After air drying, the slides and [\textsuperscript{14}C] standards (ARC, St. Louis, MO) were placed in a Fuji cassette (BAS III). After the appropriate exposure time (7 days for this study), the imaging plate was scanned and the digitized image measured. The amount of radioactivity is proportional to photostimulated luminescence (PSL), which can be quantified with the [\textsuperscript{14}C] standards. The intensity of PSL within the midregion of the PVN or SON provides an index of the labeling and mRNA levels. The method is superior to film densitometry that has a more limited range. Figure 1 shows an example of the digitized brain images and the [\textsuperscript{14}C] standards. There is clear resolution of the label within the PVN and SON. The PSL within thebrain regions is translated into microcuries using the [\textsuperscript{14}C] standard curve (R\textsuperscript{2} = 0.989).

For visual evaluation, the slides were coated with photographic emulsion (Kodak NTB-2). They were stored at 4°C for 7 days. After development and a light cresyl violet counterstain, sections were examined using dark-field and bright-field microscopy. Images were taken using a CCD video camera (Optronics-DEI 750 D).

**Statistical analysis.** The data are presented as the means ± SE. ANOVA for multiple groups was used to determine significance (P < 0.05) followed by a Newman-Keuls post hoc comparison.

**RESULTS**

Dehydration produced time-dependent changes in plasma VP, with the highest levels seen after 1 day of water deprivation (Fig. 2). The plasma VP response was markedly attenuated in the AT\textsubscript{1a} KO group, even
though osmolality was increased more than 30 mosmol/kgH₂O (Fig. 2 and Table 1). However, a comparison of the groups showed that there was no significant change in the responses for osmolality or loss in body weight (Table 1). Posterior pituitary VP content was decreased after dehydration with no differences noted between the groups (Fig. 3). Levels were depleted 61 and 59% (control vs. AT1a KO) with no further decrease noted after 2 days of dehydration.

The neuronal response to osmotic stimulation was accentuated in AT1a KO mice as seen with both c-Fos and VP mRNA levels. The difference was specific for the PVN region and not seen in the SON or MnPO. There was almost a twofold increase in the PVN c-Fos response in the AT1a KO (Fig. 4). Figure 5 shows c-Fos immunoreactive staining in PVN in the various groups. There was no staining in AT1a KO mice consuming water (Fig. 5A), but a marked increase after 1 day of dehydration in control (Fig. 5B) and AT1a KO (Fig. 5C) animals. Expression was lower after 2 days of deprivation (Fig. 5D). Neurons in the SFO, MnPO, and paraventricular thalamic nucleus (PVA) were also activated after dehydration (Figs. 4 and 6 and Table 2), although there was no difference between the pattern of activation between the groups.

We also evaluated the pattern of hypothalamic VP immunoreactivity to determine whether removal of the AT1a gene had any effect. There was no change in the pattern of staining in PVN or SON (Fig. 7). Likewise, there was no difference in VP mRNA levels under basal conditions (Fig. 8). However, there was an increased mRNA response in AT1a KO animals, a change specific for the PVN (Fig. 5). The in situ hybridization reaction product was measured using a radioactive capture method that provides an accurate means of determining the signal over small brain regions (Fig. 1). A dark-field photomicrograph of emulsion-coated brain sections illustrates the pattern of changes in PVN (Fig. 9). There was a marked increase in hybridization signal in the AT1a KO after 1 day of dehydration (Fig. 9C) compared with AT1a KO mice consuming water (Fig. 9A), control dehydrated mice (Fig. 9B), or AT1a KO mice after 2 days of dehydration (Fig. 9D). There was no difference in the VP mRNA response in the SON (Table 3).

**DISCUSSION**

In gene deletion or knockout animal models, the consequence of loss of a gene product can provide clues as to functionality. In the case of the ANG AT1a receptor, the vascular sequel to gene deletion was as predicted. Removal of the ANG vascular pressor receptor produced hypotension and a lack of ANG II responsiveness (12, 41). The scenario for the VP system was more enigmatic, as seen in the present study that tested the effect of dehydration in AT1a KO mice. Although there is support for a critical role of AT1a receptors in the regulation of the VP axis, there were no alterations in hypothalamic VP anatomy or basal VP secretion in AT1a KO mice. The response was attenuated for
plasma VP and enhanced for the CNS, c-Fos, and VP mRNA in dehydrated mice. This Discussion tries to reconcile these findings and to support our conclusion that removal of the ANG AT_{1a} receptor produces a state of increased responsiveness to volume and osmotic changes.

The central organization of the ANG and VP systems shows a tight interrelationship. Within the PVN region there is a dense concentration of AT_{1} receptors, ANG peptides, and VP neurosecretory neurons (13, 15, 22, 27). The ANG receptors are not located directly on VP neurons, as was shown using double hybridization methods (19). However, there is evidence for ANG release within the PVN; for ANG effects on blood pressure, VP secretion, and neural activation; and for effects of ANG antagonists and ANG AT_{1} antisense oligonucleotides on PVN function (6, 8, 11, 20, 23, 24, 26, 34). In studies that have used ANG as a central stimulant, the consensus is that ANG is critical in VP neuronal responsiveness.

Gene deletion models provide a new means for the investigation of ANG-VP interactions. Strains have been developed that lack all components of the renin-angiotensin system (RAS), with the advantage that the proteins are absent in all tissues and at all times (4). Initial studies in the ANG AT_{1a} and angiotensinogen (Aogen) knockouts showed that fluid balance was altered after removal of the peptide or its receptor. Oliverio et al. (31) reported an increased urine volume and reduced osmolality under baseline conditions and a deficit in concentrating ability in AT_{1a} KO. They suggested that part of the problem was a structural abnormality in the renal papilla (31).

The Aogen knockout showed high urine excretion, low urine osmolality, and high urinary VP levels (14). The original assumption was that the changes in fluid balance were related to a deficit in VP neurosecretory function. However, our results showed that although hypothalamic ANG receptors were absent, the VP system was intact in AT_{1a} KO mice (27, 31). There were no changes in the hypothalamus, posterior pituitary or plasma compartments under baseline conditions. Likewise, the pattern of VP staining in the neurosecretory cells and regions was identical in the AT_{1a} KO and controls. However, when stimulated by 24 h of water deprivation, the AT_{1a} KO showed a reduced plasma VP response even though posterior pituitary levels were depleted to a similar extent. Oliverio et al. (31) reported no difference in dehydration-induced VP release. However, the high baseline levels of plasma VP (40–50 compared with 3–4 pg/ml) suggest that the VP axis was stimulated. Pharmacological blockade of the ANG AT_{1} receptors also produced alterations in hemorrhage-induced VP release. The results were different with acute vs. chronic blockade (18, 49). There was an attenuation of VP release with acute losartan treat-

Table 1. Effect of dehydration on plasma osmolality and change in body weight

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<tr>
<th>Group</th>
<th>Water</th>
<th>Dehydration, 1 Day</th>
<th>Dehydration, 2 Days</th>
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<tr>
<td>Control</td>
<td>328 ± 6.9</td>
<td>340 ± 6.6</td>
<td>-3.5 ± 0.5</td>
</tr>
<tr>
<td>AT_{1a} KO</td>
<td>321 ± 4.8</td>
<td>352 ± 6.0</td>
<td>-3.9 ± 0.4</td>
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</table>

Values are means ± SE. Plasma osmolality (mosmol/kg H_{2}O) and decrease in body wt (g); ANOVA showed P < 0.001 for osmolality (treatment); effects for group or interaction not significant (P > 0.16 and 0.15, respectively); effects for body wt changes not significant (P > 0.14, 0.07 and 0.72 for treatment, group and interaction, respectively); n = 5 or 6/group.

![Fig. 3](image-url) Fig. 3. Effect of dehydration on posterior pituitary VP content. ANOVA shows effect of treatment (P < 0.001). **P < 0.01 water vs. dehydration; n = 5 or 6 per group.

![Fig. 4](image-url) Fig. 4. Effect of dehydration on c-Fos-immunoreactive cells in paraventricular nucleus (PVN) and median preoptic region (MnPO). ANOVA shows effect of group (P < 0.02), treatment (P < 0.06), and interaction (P < 0.04) in PVN. No significant changes noted in MnPO. *P < 0.05 Control vs. AT_{1a} KO; n = 6 per group.
ment as seen with our study, whereas chronic blockade caused an accentuated response.

Further proof that the AT\textsubscript{1a} KO animals are responsive to the dehydration stimulus is seen in the hypothalamic c-Fos and VP mRNA data. For the VP system, c-Fos and mRNA levels are often used as indexes of secretory activity. c-Fos is a part of the immediate early gene cascade, which occurs with neuronal activation, showing both anatomical and temporal specificity (10). In VP neurons, c-Fos is expressed after dehydration, salt loading, hypovolemia, ANG, and other stimuli (6, 10). Likewise, VP mRNA levels are correlated with cellular activity and increased under similar conditions as c-Fos (2, 23, 28, 37, 40). For c-Fos there was activation in the osmosensitive rostral forebrain regions, SFO and MnPO, in both AT\textsubscript{1a} KO and controls. This indicates that removal of the AT\textsubscript{1a} receptor does not abolish cellular activation as would be predicted on the basis of pharmacological studies (7). In the hypothalamus, both c-Fos and VP mRNA levels were increased after dehydration. The response was accentuated in the AT\textsubscript{1a} KO, specifically within the PVN. Recent studies bear relevance to our results, showing that treatment with converting enzyme inhibitors or AT\textsubscript{1} antagonists causes activation of c-Fos in PVN neurons (5, 32). Likewise, chronic central blockade of AT\textsubscript{1} receptors produced an increase in the plasma VP response to hemorrhage, suggesting an activation under these conditions (49).

Dehydration results in both volume and osmolality changes, making it difficult to determine whether the enhancement in the AT\textsubscript{1a} KO is related to hypovolemia, hypertonicity, or a combination. Because renal concentrating ability is reduced in the knockout (31), this might result in an accentuated effect of water deprivation. Although there was no significant difference in body weight loss or plasma osmolality between the groups, there was a trend for an increase in osmolality. Even so, the AT\textsubscript{1a} KO mice showed a reduced plasma VP response, which suggests a deficit in the

<table>
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<th>Group</th>
<th>Dehydration, 1 Day</th>
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<tr>
<td></td>
<td>SON</td>
<td>SFO</td>
</tr>
<tr>
<td>Control</td>
<td>50.7±13.0</td>
<td>31.3±10.8</td>
</tr>
<tr>
<td>AT\textsubscript{1a} KO</td>
<td>74.3±8.9</td>
<td>33.3±8.8</td>
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Values are means ± SE. Number of immunoreactive c-Fos positive cells in supraoptic nucleus (SON) and subfornical organ (SFO). There was no detectable c-Fos staining under control conditions. ANOVA for days 1 and 2 of dehydration was not significant in SON and \( P < 0.052 \) (treatment) in SFO.
secretory cascade. This is in contrast to the CNS changes, which were accentuated in the dehydrated AT1a KO mice. Thus, the lack of ANG AT1 receptors may produce a state of dissociation between the sensory component, particularly that which is connected via the PVN, and the final common pathway.

The CNS and plasma responses showed highest levels after 1 day of dehydration. The parameters returned toward baseline on day 2, although the stimulus (high osmolality) was present. These results differ from those in rats and other species, in which there is long-lasting activation of the neurosecretory neurons. Peptide secretion is maintained, as is the neural activity, after prolonged dehydration or salt loading (37, 43, 45). Likewise, mRNA levels were elevated in rats long after rehydration, when osmolality levels had returned to control (50). The results suggest that the balance between transcriptional activation and secretion is different in mice from other rodents. Perhaps the levels of mRNA are sufficient to maintain secretion, or there is an active negative feedback between the plasma and hypothalamic systems.

The question that arises from this data is how to reconcile the apparent increased osmotic response in AT1a KO mice with reports suggesting that ANG receptors are required for CNS signaling. The strongest case for a requisite role for ANG receptors in neuronal responsiveness comes from studies that test the effect of ANG II. AT1 antagonists normally block the neurophysiological, endocrine, intake, and pressor responses to central ANG II. However, in situations in which fluid balance is altered, the AT1 antagonists are not uniformly effective. For example, the AT2 antagonist completely blocked the response to dehydration while losartan was much less effective (17). ANG antagonists did not alter hemorrhage-induced VP secretion and only partially reduced dehydration-stimulated release (33, 48). However, centrally administered losartan reduced the drinking response to dehydration (36) as well as the drinking, VP, and pressor responses to hypertonic saline injection (25). Neurophysiological studies demonstrate that ANG II has both excitatory and inhibitory effects on PVN neurons (1, 21). The situation is complicated with neurons receiving input from different brain regions that have a variety of peptides and neurotransmitters. One must also consider that in the gene deletion models there may be compensation by other receptor subtypes, such as the AT1b or AT2. Oliverio et al. (30) showed that AT1a KO mice were responsive to ANG II stimulation after blockade with losartan. They suggested that the pressor response was mediated by AT1b receptors. Studies in our laboratory show that AT1b mRNA is present in osmosensitive brain regions in mice but is unresponsive to dehydration (3). However, using autoradiographic binding methods, we found no evidence for the presence of AT1b receptors in the AT1a KO mice and no indication of an upregulation after dehydration (27). A role for AT2 receptors in volume regulation is suggested by studies in which treatment with AT2 antagonists modified the response to salt and ANG stimulation (11, 17, 38, 44). Whether, AT2 or AT1b receptors are involved in the accentuated central responses in the AT1a KO remains to be determined.

Our results suggest that the cascade for ANG-VP interactions is more complicated than previously envisioned. In the whole animal, it is probably not a simple afferent/effector connection with ANG activating VP neurons and eliciting peptide secretion. The use of a gene deletion model has unmasked an interesting facet

![Fig. 8. Effect of dehydration on VP mRNA levels in PVN. ANOVA shows effect of group ($P < 0.05$) and treatment ($P < 0.0001$). *$P < 0.01$ water vs. dehydration; +$P < 0.05$ control vs. AT1a KO; $n = 5$ or 6 per group.](image1)

![Fig. 9. In situ hybridization for VP mRNA in PVN. A: AT1a KO, consuming water; B: control, 1-day dehydration; C: AT1a KO, 1-day dehydration; D: AT1a KO, 2-day dehydration.](image2)

<table>
<thead>
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<th>Table 3. Effect of dehydration on SON VP mRNA</th>
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<td><strong>Group</strong></td>
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<tr>
<td>Control</td>
</tr>
<tr>
<td>AT1a KO</td>
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Values are means ± SE. Vasopressin (VP) mRNA ($\mu$Ci/mm$^2$). *$P < 0.004$ effect of treatment (ANOVA). Basal data (water consumption) are not available because of insignificant number of readable samples.
REFERENCES


