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Properly timed exposure to central ANG II prevents behavioral sensitization and changes in angiotensin receptor expression

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Santollo J, Whalen PE, Speth RC, Clark SD, Daniels D. Properly timed exposure to central ANG II prevents behavioral sensitization and changes in angiotensin receptor expression. Am J Physiol Regul Integr Comp Physiol 307: R1396–R1404, 2014. First published October 29, 2014; doi:10.1152/ajpregu.00373.2014.—Previous studies show that the angiotensin type 1 receptor (AT1R) is susceptible to rapid desensitization, but that more chronic treatments that stimulate ANG II lead to sensitization of several responses. It is unclear, however, if the processes of desensitization and sensitization interact. To test for differences in AT1R expression associated with single or repeated injections of ANG II, we measured AT1R mRNA in nuclei that control fluid intake of rats given ANG II either in a single injection or divided into three injections spaced 20 min apart. Rats given a single injection of ANG II had more AT1R mRNA in the subfornical organ (SFO) and the periventricular tissue surrounding the anteroventral third ventricle (AV3V) than did controls. The effect was not observed, however, when the same cumulative dose of ANG II was divided into multiple injections. Behavioral tests found that single daily injections of ANG II sensitized the dipsogenic response to ANG II; however, this effect was absent in rats treated for 5 days with four daily ANG II injections. Taken together, these data suggest that a desensitizing treatment regimen prevents behavior- and receptor-level effects of repeated daily ANG II.

AT1R: water intake; drinking microstructure; fluid balance

REGULATION OF BODY FLUID HOMEOSTASIS requires constant coor-

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Animals and housing. Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 225–250 g upon arrival at the facility were used in all experiments. Rats were individually housed in
stainless-steel wire-hanging cages with access to tap water and rat chow (Teklad 2018; Harlan Laboratories) in a temperature- and humidity-controlled colony room on a 12:12-h light-dark cycle (lights on 0700). All behavioral experiments occurred during the early hours of the light phase. All experimental protocols were approved by the Animal Care and Use Committee at the University of Buffalo, and the handling and care of the animals were in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Surgery. All animals underwent stereotaxic surgery to implant a chronic cannula aimed at the right lateral ventricle following standard laboratory procedures. Briefly, rats were anesthetized with an injection of a ketamine (70 mg/kg im; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (5 mg/kg im; Akron, Decatur, IL). The rat’s head was shaved and secured into a stereotaxic frame. A small incision was made on top of the skull, a small hole was drilled, and a 26-gauge guide cannula was implanted using the following coordinates: 0.9 mm posterior and 1.4 mm lateral to bregma, and 2.8 mm ventral to the skull. The cannula was fixed to the skull with bone screws and dental cement. All rats received a single injection of carprofen (5 mg/kg sc; Pfizer Animal Health, New York, NY) after surgery to minimize pain. One week later and five days prior to experiments, accurate cannula placement was verified by measuring the drinking response to an injection of 10 ng ANG II. Only rats that drank at least 6 ml in 30 min after ANG II treatment were included in the study.

cDNA synthesis and RT-PCR. Real-time PCR was used to quantify AT1R mRNA levels in samples from the periventricular tissue surrounding the anteroventral third ventricle (AV3V), subfornical organ (SFO), and paraventricular nucleus of the hypothalamus (PVN). DNA-free total RNA was purified using the E.Z.N.A. MicroElute Total RNA kit (Omega Bio-Tek, Norcross, GA), including a deoxyribonuclease step. Reverse transcription was performed with 500 ng of RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was carried out using SYBR Green master mix (Bio-Rad), according to the manufacturer’s instructions. GAPDH was used as an internal control for quantification of mRNA. The primer sequences used were AT1R sense, 5′-CGGCCCTTGGATTAACATGA, antisense, 5′-CCTGCCATCCTACCCCTCAAAACCA, GAPDH sense, 5′-AACCCGACCTTCCTTAGAC, and antisense, 5′-TACACGCTGATATATGAC. The primer sequence was selected on the basis of a previous report (16) and is specific for the type 1A AT1R.

Fluid intake measures. Total intake was calculated by weighing the water bottles before and after each test period. Licking behavior was measured using a contact lickometer (designed and constructed by the Psychology Electronics Shop, University of Pennsylvania, Philadelphia, PA) that recorded individual licks to allow for analysis of drinking microstructure. The lickometer interfaced with a computer using an integrated USB digital I/O device (National Instruments, Austin, TX). Home cages were affixed with an electrically insulated metal plate with a 3.175-mm-wide opening, through which the rat found access to the drinking spout. The drinking response to an injection of 10 ng ANG II. Only rats that drank at least 6 ml in 30 min after ANG II treatment were included in the study.

AT1R autoradiography. Brains were sectioned on a cryostat at a thickness of 20 μm from the level of the AVG3 (+.6 mm bregma) through the PVN (~2.15 mm bregma), with the first three of every six sections being mounted on gelatin-coated slides, stored with desiccant at 4°C for 2 h, and then stored at ~20°C for no more than 5 days. On the day of processing, slides were thawed at RT for 45 min. Slides were then preincubated at RT in buffer (150 mM NaCl, 5 mM EDTA, and 50 mM NaPO4 at pH 7.1–7.20) for 30 min. Slides were then incubated at RT for 2 h in either 500 mM125I-Sar1ANG II (22, 25), and the AT1AR is the dominant subtype expressed in forebrain (15).

Materials. 125I-Sar’1ANG II was prepared using the chloramine T procedure (8) with HPLC purification to theoretical specific activity (2175 Ci/mmol), as described previously (26). ANG II was obtained from Phoenix Pharmaceuticals ( Burlingame, CA) or American Peptides (Sunnyvale, CA); PD123319 from Tocris (Bristol, UK); EDTA, and NaCl were obtained from WVR International (Radnor, PA).

Data presentation and analysis. All data are presented as means ± SE. Images showing autoradiography were generated using screen captures and were organized into panels using Adobe Photoshop and Illustrator. No contrast adjustments were made. Images of Nissl-stained sections were captured using a Nikon Eclipse 80i microscope with an attached Nikon DS-Fi1 camera. Images were acquired using NIS-Elements software and were contrast adjusted (applied to the whole image, not to specific parts) using Adobe Photoshop, before being assembled into panels in Adobe Illustrator. Drinking microstructure analysis was processed in a MatLab (MathWorks, Natick, MA) software environment before being ported to Excel (Microsoft, Redmond, WA) for final analysis. A burst was defined as at least two licks with an interlick interval of no more than 1 s. Burst size was defined as the average number of licks within a burst. RT-PCR values were calculated using the ΔΔCT quantification method with GAPDH as the normalizing housekeeping gene. Statistical analyses were performed using Statistica (StatSoft, Tulsa, OK). Changes in AT1R mRNA were analyzed using one-way ANOVAs for each brain region. Water intake and drinking microstructures on days 1 and 5 were analyzed using two-factor mixed design ANOVAs (group × day). AT1R binding was analyzed using one-way ANOVAs for each brain region. Fisher’s post hoc tests were used throughout to determine individual group differences after significant main or interaction ANOVA effects.

Experimental designs: experiment 1: does a desensitizing treatment regimen affect AT1R mRNA levels? This experiment was performed to evaluate changes in AT1R mRNA caused by doses and administration paradigms used previously to show desensitization (32–35) and to compare these effects to administration of the same amount of ANG II given in a single bolus. To this end, 24 rats were divided into one of three groups. Rats in group 1 (n = 8) received three intracerebroventricular injections of 1 μl TBS vehicle spaced 20 min apart. Rats in group 2 (n = 8) received three intracerebroventricular injections, spaced 20 min apart, of the following: 1 μl TBS vehicle, 900 ng ANG II dissolved in 1 μl TBS vehicle, and 1 μl TBS vehicle. Rats in group 3 (n = 8) received three intracerebroventricular injections of 300 ng ANG II dissolved in 1 μl TBS vehicle spaced 20 min apart. Food and water were removed before the first injection. Four hours and 20 min after the final injection, rats were anesthetized by a 90-s exposure to isoflurane and then decapitated. This time point was chosen on the basis of previous reports demonstrating that AT1R mRNA can change as early as 3 h after manipulation (11). Brains were immediately removed from the skull, flash frozen with 2-methyl-butane (Sigma-Aldrich, St. Louis, MO) and stored at ~80°C. The AV3V, SFO, and PVN regions of the brain were obtained by sectioning 300-μm coronal sections on a cryostat and then taking four 1-mm punches from each brain region. Tissue punches were stored at ~80°C until processing for AT1R mRNA content by RT-PCR.

Experiment 2: does a desensitizing treatment regimen affect water intake sensitization? Thirty four rats were housed in custom-designed stainless-steel hanging wire cages, which allowed for continuous measurement of drinking behavior. Rats received one of four treat-
ment regimens: 1) a single daily intracerebroventricular injection of 1 μl TBS vehicle (n = 5); 2) a single daily intracerebroventricular injection of 10 ng ANG II (dissolved in 1 μl TBS; n = 9); 3) a single daily intracerebroventricular injection of 40 ng ANG II (n = 10); and 4) four daily intracerebroventricular injections of 10 ng separated by 20 min each (n = 10). The treatment paradigm used was chosen to mimic the timing of injections used previously to study desensitization (32–35), but using a lower dose of ANG II to more closely resemble the dose used in earlier studies showing sensitization (19). This occurred daily for five consecutive days. Food and water were removed prior to the start of the experiment. Immediately after the final injection of the day, water was returned, and intake was monitored for 30 min. Food was not available during the 30-min water intake test.

Experiment 3: does a desensitizing treatment regimen influence AT1R binding? To test the hypothesis that changes in AT1R binding are associated with the behavioral effects shown by experiment 2, we performed radioligand autoradiography in rats given the same treatments as described in experiment 2. To this end, 14 rats were divided into one of three groups: 1) a single daily intracerebroventricular injection of vehicle (1 μl TBS; n = 4); 2) single daily intracerebroventricular injection of 40 ng ANG II (n = 5); and 3) four daily intracerebroventricular injections of 10 ng separated by 20 min each (n = 5). This treatment regimen was repeated daily for five consecutive days. On the morning of day 6 (24 h after the previous injection), rats were anesthetized by 90-s exposure to isoflurane and then decapitated. Brains were immediately removed from the skull, flash frozen with 2-methyl-butane, and stored at −80°C until processing.

RESULTS

Experiment 1: does a desensitizing treatment regimen affect AT1R mRNA levels? To evaluate changes in AT1R mRNA as a result of any ANG II treatment (Fig. 1). In the AV3V, rats given a 900-ng ANG II injection in a single bolus (n = 5) had greater levels of AT1R mRNA than was found in the brains of vehicle-treated rats (n = 6). Brains of rats given 900 ng of ANG II delivered in three 300-ng injections over 40 min (n = 7), however, had levels of AT1R mRNA that were not different from the controls (F2,15 = 4.23; P < 0.05; Fig. 1A). Similarly, in the SFO, treatment with 900 ng ANG II (n = 8) was associated with greater levels of AT1R mRNA than was observed in vehicle-treated rats (n = 8), but this effect of ANG II was absent when the ANG II was delivered in three 300-ng injections (n = 8; F2,21 = 3.55; P < 0.05; Fig. 1B). Finally, in the PVN, there was no change in AT1R mRNA as a result of any ANG II treatment (n = 6–8/group; F2,19 = 0.07; P = 0.93; Fig. 1C). A small number of samples were excluded from the analysis because of organic contamination, and the sample sizes provided above reflect this exclusion.

Experiment 2: does a desensitization treatment regimen affect water intake sensitization? Repeated injections of ANG II in close temporal proximity desensitize the drinking response to ANG II (32–34), but single daily injections appear to cause sensitization (19). To this end, we gave rats daily injections of vehicle, 10 ng ANG II, 40 ng ANG II, or a total of 40 ng ANG II/day in four 10-ng injections with 20 min between each. Daily ANG II treatment significantly influenced drinking behavior across the testing period (Fig. 2). We found a main effect of group (F3,30 = 25.12; P < 0.05), a main effect of day (F1,30 = 17.67; P < 0.05), and a group × day interaction (F3,30 = 4.87; P < 0.05). Post hoc tests showed that rats that received a single daily

**Fig. 1.** Timing of ANG II-associated changes in angiotensin type 1 receptor (AT1R) gene expression. In the SFO and AV3V, a single injection of 900 ng ANG II increased AT1R gene expression. When ANG II was delivered in three 300-ng injections, spaced 20 min apart, there was no change in AT1R gene expression (A and B). There was no change in AT1R gene expression in the PVN, regardless of ANG II treatment (C). *Significantly greater than vehicle and repeated ANG II, P < 0.05.
from the burst analysis. There was no effect of group, errors, data from three rats were lost and, therefore, excluded injections of 10 and 40 ng of ANG II. Because of technical from rats in the groups that were treated with single daily intake, burst analysis was conducted on the drinking patterns with vehicle, regardless of day.

**Experiment 3:** does a desensitization regimen influence AT$_{1}$R binding? Given the acute changes in mRNA found in experiment 1, we hypothesized that differences in AT$_{1}$R binding were responsible for the behavioral effects of repeated injections of ANG II. To test this hypothesis, rats were given single daily injections of vehicle, of 40 ng of ANG II, or a daily treatment regimen of four 10-ng injections given 20 min apart. After 5 days of this treatment, brains were removed, and AT$_{1}$R binding was evaluated using autoradiography. AT$_{1}$R binding differed as a function of the treatment in distinct brain regions (Figs. 4−7). We did not find a change in AT$_{1}$R binding across the entire AV3V ($F_{2,11} = 1.63; P = 0.348$; Fig. 4A). Because the AV3V is composed of multiple nuclei, it is possible that specific regions within the AV3V respond differently to ANG II treatment. In support of this, when only the caudal sections of the AV3V were analyzed, a planned comparison determined that the group treated with a single daily injection of 40 ng of ANG II had significantly less binding than that found in the vehicle group or the group treated with four daily injections of 10 ng ANG II ($F_{2,11} = 3.42; P = 0.069$; Fig. 4A). In the dorsal portion of the MnPO (dmnPO), there was less AT$_{1}$R binding in the group treated with a single daily injection of 40 ng of ANG II than there was in the other groups ($F_{2,11} = 4.34; P < 0.05$; Fig. 5A). There were no treatment effects in AT$_{1}$R binding in the SFO ($F_{2,11} = 0.14; P = 0.864$; Fig. 6A) or PVN ($F_{2,9} = 0.21; P = 0.816$; Fig. 7A). The analysis of the PVN excluded two subjects because of tissue damage in this region.

**DISCUSSION**

The present results show, for the first time, that behavioral and molecular changes caused by single daily injections with ANG II do not occur when the same cumulative amount of ANG II is given in multiple daily injections. Previous studies show that repeated treatment of ANG II desensitizes the AT$_{1}$R injection of 10 or 40 ng of ANG II increased their water intake from day 1 to day 5 ($P < 0.05$); however, this increase was absent in rats treated with four daily injections of 10 ng ANG II ($P > 0.05$). Minimal drinking was observed in rats treated with vehicle, regardless of day.

To further investigate the nature of the sensitization of water intake, burst analysis was conducted on the drinking patterns from rats in the groups that were treated with single daily injections of 10 and 40 ng of ANG II. Because of technical errors, data from three rats were lost and, therefore, excluded from the burst analysis. There was no effect of group, $F_{1,14} = 0.06$, day, $F_{1,14} = 1.35$, or an interaction between group and day, $F_{1,14} = 0.06$, all $P$ values $>0.263$ (Fig. 3A) on the number of bursts within the 30-min test period. A main effect of day, $F_{1,14} = 10.88; P < 0.05$, on burst size during the 30-min test period was detected (Fig. 3B). Burst size was significantly greater on day 5 compared with day 1. There was no effect of group, $F_{1,14} = 0.59$ or an interaction of group and day, $F_{1,14} = 1.27$, on burst size, all $P$ values $>0.277$.

**Experiment 3:** does a desensitization regimen influence AT$_{1}$R binding? Given the acute changes in mRNA found in experiment 1, we hypothesized that differences in AT$_{1}$R binding were responsible for the behavioral effects of repeated injections of ANG II. To test this hypothesis, rats were given single daily injections of vehicle, of 40 ng of ANG II, or a daily treatment regimen of four 10-ng injections given 20 min apart. After 5 days of this treatment, brains were removed, and AT$_{1}$R binding was evaluated using autoradiography. AT$_{1}$R binding differed as a function of the treatment in distinct brain regions (Figs. 4−7). We did not find a change in AT$_{1}$R binding across the entire AV3V ($F_{2,11} = 1.63; P = 0.348$; Fig. 4A). Because the AV3V is composed of multiple nuclei, it is possible that specific regions within the AV3V respond differently to ANG II treatment. In support of this, when only the caudal sections of the AV3V were analyzed, a planned comparison determined that the group treated with a single daily injection of 40 ng of ANG II had significantly less binding than that found in the vehicle group or the group treated with four daily injections of 10 ng ANG II ($F_{2,11} = 3.42; P = 0.069$; Fig. 4A). In the dorsal portion of the MnPO (dmnPO), there was less AT$_{1}$R binding in the group treated with a single daily injection of 40 ng of ANG II than there was in the other groups ($F_{2,11} = 4.34; P < 0.05$; Fig. 5A). There were no treatment effects in AT$_{1}$R binding in the SFO ($F_{2,11} = 0.14; P = 0.864$; Fig. 6A) or PVN ($F_{2,9} = 0.21; P = 0.816$; Fig. 7A). The analysis of the PVN excluded two subjects because of tissue damage in this region.
injection of ANG II increased AT1R mRNA, but when the injections of ANG II (32–34) showed the expected sensitization of water intake (19), rats given 40 ng ANG II in four 10-ng injections each day showed no similar sensitization. Finally, because a change in receptor binding is one possible mechanism underlying these behavioral differences (12, 37), AT1R binding was analyzed in nuclei involved in controlling fluid intake. Paradoxically, we found that rats treated with a single injection of 40 ng for 5 days (a treatment that produced reliable sensitization of water intake) had an apparent decrease in AT1R binding in the caudal AV3V and dMnPO. Although the decreased binding was the opposite of what was predicted from the increased behavioral response after the same treatment, the results are consistent with the behavioral studies. Although not directly comparable because of the different doses used, the results from experiments 2 and 3 are consistent with the results of experiment 1 in that the observed effect was dependent on the timing of the injections. Accordingly, these studies provide convergent evidence suggesting that the changes in mRNA, behavior, and binding associated with sensitizing treatments are all prevented by repeated administration of ANG II within a short timeframe.

We first hypothesized that the timing of ANG II treatment would influence AT1R gene expression within nuclei involved in the control of fluid intake because previous studies show that the timing of ANG II treatment can influence drinking behavior. In vitro and in vivo studies show that AT1R is susceptible to rapid desensitization (7, 28, 29, 32–34) and AT1R is critical for the behavioral desensitization that is caused by repeated injections of ANG II (34). Accordingly, we tested the hypothesis that repeated ANG II treatment would influence AT1R gene expression in brain regions that are involved in fluid intake. Indeed, when rats received a single dose of ANG II, there was a significant increase in AT1R mRNA in both the AV3V and SFO, but not the PVN, and the effect was absent when ANG II treatment was delivered using the repeated-treatment regimen time frame. This suggests that the effect of ANG II on expression of its receptor is anatomically selective and is influenced by the timing of the exposure.

When considering the present experiments showing changes in mRNA, it is important to note that the expression of mRNA does not always correspond with the expression of the protein that it encodes. For example, estradiol can alter AT1R protein levels by posttranscriptional actions (14). Additionally, in the kidney, losartan increases AT1R mRNA, but decreases AT1R binding (36). Moreover, recent studies from our laboratory found that repeated injections of large doses of ANG II (the doses used in experiment 1 here) decreased radioligand binding (27), whereas the repeated injections here produced no difference in mRNA levels. As such, a change in mRNA should be interpreted with caution, and these examples highlight the importance of measuring levels of the relevant protein whenever possible.

Because we observed region-specific differences in AT1R mRNA after acute bolus or timed ANG II injections, we next hypothesized that the timing of ANG II delivery would prevent the enhancement of water intake observed after single daily injections of ANG II. This intake sensitization has been studied using a variety of paradigms, including chronic central ANG II infusions, repeated bouts of deprivation with partial rehydra-

![Fig. 4. Effect of repeated injections of ANG II on AT1R binding in the anteroventral third ventricle (AV3V). Densitometric analysis of the entire AV3V region did not detect any treatment-associated changes in AT1R binding. A: when the caudal AV3V was analyzed separately, a decrease in AT1R binding was observed in rats treated daily with 40 ng of ANG II that was attenuated when rats were given the same cumulative dose of ANG II in four daily injections with 20 min between each injection. Representative autoradiograms of vehicle, single daily injections, and four daily injection treatments are shown in B–D, respectively. E: representative Nissl staining of a brain section from the vehicle treatment group is shown. 3V, third ventricle; LV, left ventricle; ac, anterior commissure. *Significantly less than Vehicle and 4 × 10 ng ANG II, P < 0.05.](image)
tion, and repeated injections of mineralocorticoids or diuretics, alone or in combination with ACE inhibitors (1, 3, 6, 9, 10, 20, 23, 24). Although the focus of these studies has been on the sensitization of sodium intake, both repeated daily injections and chronic exposure to ANG II sensitize the dipsogenic response (3, 19). Because our previous studies found that the desensitizing effect of acute repeated injections of ANG II was limited to water intake, without an effect on saline intake, we focused exclusively on a sensitizing treatment already known to enhance water intake (34). Indeed, whereas rats treated with a single daily injection of either 10 or 40 ng of ANG II increased their water intake across the 5-day testing period, rats given four daily injections of 10 ng of ANG II showed no sensitization of water intake. The inclusion of single injections of both 10 ng and 40 ng was important because each provides a direct respective comparison with the final injection of ANG II used in the desensitizing treatment regimen or with the cumulative total dose of ANG II provided. Given these comparisons, the results of this study demonstrate that properly timed administration of ANG II does not lead to behavioral sensitization of water intake and suggests this treatment regimen can prevent the behavioral sensitization of water intake that results from daily ANG II treatment.

To further understand the nature of the increase in water intake in this paradigm, we analyzed drinking microstructure. Analysis of drinking microstructure revealed that the sensitization of water intake, observed in the group treated with a single daily injection of 10 and 40 ng of ANG II, was mediated by a change in burst size. There were no significant changes in burst number. On the basis of previous studies (5), this suggests that the increased water intake is the result of changes in the orosensory value of the fluid and not the result of a change in postingestive feedback. To the best of our knowledge, this is the first examination of drinking microstructure across fluid sensitization. It is unclear whether the enhancement of intake in other sensitization paradigms is also the result of changes in orosensory properties vs. satiety signals. Therefore, future studies will be necessary to determine whether this is a general mechanism underlying sensitization of water intake or specific to daily repeated injections of ANG II. It is also important to recognize that the changes observed in the present study are occurring in a whole animal model, and, therefore, require extensive additional research to test for roles of confounding variables. Indeed, the treatments used here could have affected many things, including, but not limited to, vasopressin secretion, body temperature, and blood pressure. Because many of the results of this study demonstrate that properly timed administration of ANG II does not lead to behavioral sensitization of water intake and suggests this treatment regimen can prevent the behavioral sensitization of water intake that results from daily ANG II treatment.

Fig. 5. Effect of repeated injections of ANG II on AT1R radioligand binding in the dorsal median preoptic nucleus (dMnPO). A: Densitometry analysis of the dMnPO revealed a decrease in binding when rats were given daily injections of 40 ng ANG II, but this decrease was not observed when rats were given the same cumulative dose of ANG II spread across four daily injections of 10 ng. Representative autoradiograms of vehicle, once-daily injections, and four daily injection treatments are shown in B–D, respectively. E: representative Nissl staining of a brain section from the vehicle treatment group is shown. The dotted outline indicates the area included in the analysis. *Significantly less than vehicle and repeated ANG II, P < 0.05.

Fig. 6. Effect of repeated injections of ANG II on AT1R radioligand binding in the subfornical organ (SFO). Densitometric analysis of the SFO revealed no treatment effects on AT1R binding (A). Representative autoradiograms of vehicle, single daily injections, and four daily injection treatments are shown in B–D, respectively. E: representative Nissl staining of a brain section from the vehicle treatment group is shown.
these can directly or indirectly affect fluid intake, understanding the direct link to the observed changes requires further research.

Although the precise link between the treatment and the response remains unclear, the present studies revealed the striking finding that once-daily ANG II treatment increased water intake accompanied by a paradoxical decrease in AT1R binding in the caudal AV3V and dMnPO. Determining the precise anatomical location of these changes is difficult with the techniques employed. Indeed, the changes that we refer to as occurring in sections of the caudal AV3V and dMnPO may reflect a more generalized change in the MnPO because there is likely ventral MnPO in the sections considered caudal AV3V. Although the precise anatomical locus of the change is difficult to clarify from our data, it is clear that the differences in binding depended on the timing of the injections. Specifically, whereas a single daily injection of ANG II decreased AT1R binding in these brain areas, this did not occur when the same cumulative amount of ANG II was split into four injections. Moreover, the observed differences were not observed in the SFO or PVN. The changes in the more ventral regions of the lamina terminalis are consistent with previous studies showing that the AV3V is particularly important in the behavioral desensitization caused by acute repeated injections of ANG II (32). Although the direction of the change in behavior may have predicted an increase in binding, the decreased binding observed in the present report is consistent with other reports. Indeed, Moellenhoff et al. (19) reported a decrease in c-Fos in the MnPO after daily injections of ANG II that is consistent with the direction of the change in binding observed here. In that study, however, changes in c-Fos were also observed in the SFO, PVN, and SON (19), where we did not find changes in AT1R binding. Moreover, two studies using repeated mineralocorticoid treatment found increased AT1R binding that was associated with the sensitization of saline intake (12, 37), suggesting that the observed sensitization can occur by different underlying mechanisms. Nevertheless, and perhaps more important, the efficacy of the treatments to affect behavior corresponded to the observed changes in binding. Indeed, we consistently found differences in behavior, AT1R mRNA, and AT1R binding in the rats given a sensitizing course of treatment or single injection of ANG II, and these changes were each not present after the desensitizing treatment regimen, given either in a single day or across days. The present studies are limited, however, in that the doses used to study mRNA were different from those used to study behavior and receptor binding. Nevertheless, the changes in AT1R binding appear to be closely associated with, if not causing, the changes in behavior observed previously (32–35).

The most parsimonious explanation is that ANG II binding to the AT1R directly causes the changes in radioligand binding observed here and that this change in receptor availability or affinity makes the animal less responsive to the dipsogenic effects of ANG II. Other less direct mechanisms are possible and could be suggested on the basis of previous studies on the mechanism(s) underlying sensitization of water and saline intakes after different means to produce the sensitization. For instance, the requirement for altered circulating hormones in the intake sensitization after repeated bouts of sodium depletion were ruled out by demonstrating that there are no lasting changes in circulating ANG II or aldosterone (24). Although our studies suggest a role for AT1R that is supported by other studies [e.g., AT1R blockade prevents furosemide/captopril-induced sensitization (20)], our studies do not address other systems that may be critical for the observed behavioral changes. Yet other studies have found that mineralocorticoid receptor (MR) and NMDA receptors are associated with the intake changes observed after sensitizing treatments (9, 23), making these important targets for future investigations of the neurochemical systems involved in the interactions between sensitizing and desensitizing treatments. With respect to the potential interactions involving mineralocorticoids, our finding that AT1R binding in the MnPO was affected by the treatments used here may relate to previous studies showing that DOCA causes increased spontaneous activity, increased firing rates, and prolongs the effect of ANG II on neurons in the MnPO (30). Studies of sodium appetite found that the sensitizing effect of repeated treatments with furosemide is prevented by pretreatment with an MR antagonist (23). Consideration of a contribution by NMDA receptors is also important. A recent study showed that an NMDA receptor antagonist prevented the sensitization of water and saline intake after repeated treat-

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**Fig. 7. Effect of repeated injections of ANG II on AT1R radioligand binding in the paraventricular nucleus (PVN).**

A: densitometric analysis of the SFO revealed no treatment effects on AT1R binding. Representative autoradiograms of vehicle, single-daily injections, and four daily injection treatments are shown in B–D, respectively. E: representative Nissl staining of a brain section from the vehicle treatment group is shown.
ments with furosemide and captopril (9). Accordingly, future studies testing whether these changes occur after daily ANG II treatment could provide important insight into understanding the mechanisms underlying the behavioral sensitization of water intake.

From a logical perspective, the critical question raised by the present experiments is how an increased behavioral response to ANG II is associated with a decrease in AT1R binding? We propose four nonmutually exclusive answers to this perplexing question. First, it is possible that the observed sensitization of water intake is independent of the changes in AT1R, but it is more directly linked to a learning effect. Specifically, during the days of the treatments, the rats may strengthen the association between the act of drinking water and the alleviation of the ANG II-induced drive to drink. Accordingly, this stronger association leads to increased intake. Indeed, previous studies described above showing a role for NMDA receptors in a different type of drinking sensitization is consistent with a role of learning (9). Second, it is possible that the intake sensitization is secondary to more primary changes in blood pressure. ANG II treatment induces a pressor response and increased blood pressure inhibits fluid intake (13, 31). If repeated ANG II treatment has a primary effect that desensitizes the pressor response (consistent with the decreased binding), this could inhibit the effect of increased blood pressure on fluid intake. Previous studies using different models of sensitization are, however, inconsistent with this explanation. Specifically, 8 wk of DOCA treatment caused a sensitized drinking and pressor response to either central or peripheral ANG II (37); however, this study also showed an increase in AT1R binding, suggesting that the responsible mechanisms are quite different. Third, the decreased binding that was associated with increased behavior in the present studies may reflect an enhanced efficiency of the receptor. This could be a result of neural plasticity [consistent with the above-mentioned requirement for NMDA receptors (9)] that perhaps involves changes in specific signal-molecules downstream from the AT1R. Fourth, although the focus of the present and of previous studies has been on the apparent sensitization of behavior, which might predict an increase in a critical receptor, it is possible that this perspective is flawed and that the increased behavior is not because of sensitization of the behavior itself, but is instead due to a developed tolerance to the satiating effect of the consumed fluid. In this case, the reduction in receptor binding would be in the same direction as the reduced satiating potency of the consumed fluid. This possibility, however, requires that the AT1R be reconsidered as a critical part of drinking termination, rather than an integral part of the onset of drinking. This seems unlikely, and the present drinking microstructure analysis suggests that a change in the orosensory properties of the fluid, not a change in satiety, was involved in the increased drinking. Nevertheless, these possibilities offer reasonable starting points that can be used to direct future studies.

Perspectives and Significance

The results here provide the first studies suggesting that properly timed application of ANG II can prevent the sensitizing effect normally observed after daily injections of ANG II. Whether or not these results apply to chronically elevated ANG II or any of the sensitization that may underlie fluid balance disorders, such as hypertension remains an avenue for future research. Nevertheless, the studies open the door to the exciting, although perhaps remote, possibility that a paradigm shift in the treatment of hypertension is worth some consideration. Indeed, current strategies that target the renin-angiotensin system to control hypertension all seek to decrease ANG II activity, and an increase in ANG II would be contraindicated. Our studies show that the timing of ANG II produces marked differences in the response to the treatment, suggesting that proper timing of ANG II may prevent some of the consequences of elevations of ANG II. Accordingly, it is tempting to speculate that future views of the anti-ANG II strategy could be viewed similarly as the current perspective on the treatment of congestive cardiomyopathy. Indeed, several decades ago, treatment of chronic heart failure focused on maintaining or increasing adrenergic tone, but now the opposite approach, using cardio-selective β-blocking agents, is preferred and viewed as far more effective (2). In this respect, a new approach, which was the exact opposite of the previous approach and which was formerly contraindicated, was found to be far more effective. Perhaps fanciful, but it is possible that these studies provide the groundwork for a similar reversal in the treatment of hypertension by drugs targeting the renin-angiotensin system.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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