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Associative learning contributes to the increased water intake observed after daily injections of angiotensin II

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Abstract

Daily injections of angiotensin II (AngII) cause a progressive increase of water intake that resembles a classically ascribed non-associative sensitization. Consistent with the presumption that the observed increase in intake was sensitization, we hypothesized that it resulted from a pharmacological interaction between AngII and its receptor. To test this hypothesis, and remove the influence of drinking itself, we implemented a delay in water access after injection of AngII (icv) on four consecutive ‘induction days,’ and then measured intake on the next day (‘test day’) when rats were allowed to drink immediately after AngII. The delay in water access effectively reduced water intake on the four induction days, and rats with longer delays in access drank less on the test day than did rats allowed to drink immediately after AngII on the induction days. Additional experiments ruled out a role for a conditioned drinking response to the injection alone, and demonstrated a lack of conditioned appetite after pairing injections of AngII with water given by intragastric catheter. Taken together, these findings suggest that the increased drinking observed after daily injections of AngII is a conditioned appetite after repeated pairings of AngII and water intake. We further conclude that repeated stimulation of the AngII receptor alone is not sufficient to drive appetite.

Keywords

angiotensin; sensitization; associative learning; appetite; drinking; thirst

1. Introduction

Peripheral or central injections of angiotensin II (AngII) reliably elicit a drinking response, largely through actions at the angiotensin type 1 receptor (AT₁R) [22, 30]. Repeated injections of AngII have bivalent effects that depend on the timing of the injections. Injections given within a relatively short period decrease the response to subsequent AngII [29]; however, daily injections of AngII increase water intake [14, 23]. More specifically, the

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drinking response to AngII by the fifth day of testing was significantly greater than the intake after AngII on the first day [14, 23]. The mechanism underlying the increased intake remains unclear, but the pattern of intake resembles a classical non-associative sensitization. Analysis of receptor binding produced results that may be inconsistent with this hypothesis. Specifically, a reduction in AT₁R binding was observed in the caudal AV3V and dMnPO, but not in other areas known to be targets of AngII for the control of drinking behavior, after daily exposure to AngII [23]. This could be consistent with sensitization if the receptor or its downstream signaling somehow becomes more efficient, but it also could indicate that the changes in intake require changes beyond a simple receptor-level sensitization, and may instead involve a form of associative learning.

Associative learning has been implicated in the development and maintenance of ingestive behaviors, such as flavor preference conditioning [8, 16], conditioned taste aversion [10, 17, 26], and drug intake [1, 7, 12]. Studies using weanling rats show that rats need prior experience in order to engage in appetitive behaviors towards a water source in response to subsequent dehydration [15], indicating that the pairing of the need state with relief of that state is an important learning signal. Moreover, although presumed to be sensitization, the increased sodium intake occurring after several bouts of sodium appetite induction is prevented by NMDA receptor antagonists [11] that are known to prevent other types of associative learning. Accordingly, our previous conclusion that a receptor-level sensitization underlies the changes in intake observed after repeated injections of AngII requires further investigation.

The present experiments were designed to test for the sufficiency of a receptor-level sensitization in the progressive increases in intake observed after daily AngII. To this end, we took advantage of previous studies showing that a delay in water access decreases intake after AngII [9], and used this strategy to isolate the AngII-receptor interactions from the drinking response on four days, followed by a test day during which rats were allowed to drink immediately after AngII. We also tested if the injection itself could condition a drinking response, and if water delivered directly into the stomach was sufficient to facilitate a conditioned increase in water intake after AngII.

2. Materials and Methods

2.1 Subjects and housing

Male Sprague Dawley rats (200–250 g) were ordered from Envigo Laboratories (Indianapolis, IN). All rats were individually housed in hanging stainless-steel, wire mesh cages (Unifab, Kalamazoo, MI) in a temperature and humidity controlled room on a fixed 12-h light, 12-h dark cycle. Experimental injections started during the first 2–4 h of the light portion of the cycle. Food (Teklad 2018; Envigo Laboratories) and tap water were accessible *ad libitum*, unless otherwise noted. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo.

2.2 Surgery

Rats were anesthetized using a mixture of ketamine (70 mg/kg im; Zoetis, Kalamazoo, MI) and xylazine (5 mg/kg im; Akorn Inc, Decatur, IL). Isoflurane was used as an anesthetic supplement when necessary. A subset of rats (Experiment 3) were implanted with an intragastric (IG) catheter and all rats were implanted with a cannula aimed at the lateral ventricle (LV). For rats receiving an IG catheter, a dorsal midline incision between the shoulder blades and a ventral abdominal midline incision were made. Another incision was made on the abdominal wall and a catheter was attached to the stomach through a puncture opening. Stomach and abdominal wall incisions were closed using 4-0 silk sutures. The exposed tubing of the catheter was guided through the subcutaneous tissue and released at the dorsal midline incision site. A back-mounted cannula (Plastics One, Roanoke, VA) was attached to the end of the tubing and the ventral and dorsal incisions were closed with staples. All rats were secured in a stereotaxic frame and a 26-ga guide cannula (Plastics One, Roanoke, VA) was inserted through a small hole drilled in the skull using the following coordinates for LV placement: 0.9 mm posterior and 1.4 mm lateral to bregma, and 2.8 mm ventral to the surface of the skull. The cannula was secured to the skull with screws and dental cement (Reliance Dental Mfg Co., Alsip, IL). Septocaine with articaine HCl and epinephrine (Septodont, Lancaster, PA) was topically applied to the skull prior to drilling, and rats received an injection of carprofen (5 mg/kg sc; Zoetis Inc., Kalamazoo, MI) as an analgesic, and an injection of sterile saline (5 mL sc; Abbott Laboratories, Chicago, IL). In addition, rats in experiment 3 received an injection of Baytril (5 mg/kg sc; Bayer Healthcare LLC, Shawnee Mission, KS) pre-operatively and for 2 days post-surgery. Rats were allowed to recover for a minimum of 5 days before any behavioral testing was conducted. Accurate cannula placement was confirmed by a pre-experimental drinking response after injection of AngII [10 ng in 1 μ l Tris-buffered saline (TBS)].

2.3 Drug injections and intake measures

AngII (Bachem Bioscience Inc., King of Prussia, PA) was diluted in TBS and injected into the LV through a 31-ga internal cannula attached to water-filled PE-50 tubing and a Hamilton syringe (Hamilton Company, Reno, NV). Injectors were left in place for approximately 25 s after each injection.

Water intake was measured by computing the difference between bottle weights at the beginning and end of each experimental trial. An experimental trial began after icv injection of AngII and ended after each rat had access to their water bottle for 30 minutes. Food was removed for each experimental trial, and replaced 30 minutes after the water bottle was returned to the subject.

Lick data were collected using a custom-made contact lickometer (Psychology Electronics Shop, University of Pennsylvania, Philadelphia, PA). Data were processed using MATLAB (MathWorks, Natick, MA) and exported to Excel for final processing.

2.4 Experimental Designs

2.4.1. Injection nomenclature—The experiments described below included a series of injections of AngII or vehicle. Whether or not intake was measured, for the purpose of

clarity, ‘induction day’ will be used to refer to the first 4 days of injections, and ‘test day’ will refer to the fifth day of injections.

2.4.2 Experiment 1: effect of delayed water access on AngII-induced intake—

Rats (9–10 per group) were injected with 40 ng of AngII on each of 5 consecutive days. During the first four induction days, rats were divided into 4 groups that were either allowed to drink immediately after injection of AngII, or that were given water after a delay of 30 min, 1 h, or 3 h after AngII. On the test day (day 5), rats in all groups were given an injection of AngII and allowed to drink immediately after the injection. Injections contained 40 ng of AngII in 1 μ l of TBS.

2.4.3 Experiment 2: effect of conditioned injections on water intake—

Rats (4–5 per group) were injected with 40 ng of AngII or vehicle (1 μ l of TBS) with unlimited access to water on each of 4 consecutive induction days. On the test day, all rats received an injection of vehicle and subsequent water intake was measured.

2.4.4 Experiment 3: effect of pairing gastric infusion of water and central injection of AngII on subsequent AngII-induced intake—

Rats were injected with 40 ng of AngII on each of four consecutive induction days and divided into two groups (6–7 rats per group) that were either allowed to drink immediately after injection (no-infusion), or that were given water infused through an IG catheter (infusion) at a rate and volume intended to approximate intake by AngII injected rats. Specifically, rats in the ‘infusion’ group were infused with 9.5 mL of water on day 1, 12.4 mL on day 2, 11.5 mL on day 3, and 11.2 mL on day 4 of the induction days. All infusions were given over 15–20 min. Water bottles were returned to rats in the infusion group 3 h after injection of AngII. The back-mounted cannulae of rats in both groups were attached to a connector assembly and plastic syringe. A Legato 100 syringe pump (KD Scientific Inc., Holliston, MA) was used to deliver fluid to rats in the infusion group, and was allowed to run while attached to empty syringes in the non-infused group, to control for the presence of pump noise. All rats received an injection of 40 ng of AngII on test day and were allowed to drink normally.

2.5 Data Analysis

Statistical testing was performed using Statistica software (version 9.0, Statsoft, Tulsa, OK). Results of analyses were considered statistically significant when $p < 0.05$, unless otherwise specified. For Experiment 1 a one-way ANOVA was used to compare water intake data between groups on testing day (day 5). Additionally, a paired t test was used to compare water intake on day 1 and day 5 within the no-delay group. A two-factor mixed design repeated measures ANOVA with day as the repeated measure was conducted to examine the effect of day (days 1–4) and delay group on water intake. Intake values on day 5 for rats that received no delay in access to water were compared with rats in the 3-h and 1-h delay conditions using Student’s t tests with a Bonferroni corrected critical value. Analyses of drinking microstructure were used to further probe differences in intake. For these analyses, a burst was defined as a series of two or more licks with interlick intervals no greater than 1 s, and burst size was a count of the number of licks in a given burst. A two-factor mixed design repeated measures ANOVA with day as the repeated measure was conducted to

compare burst number and burst size between the 3-h delay and no-delay groups on days 1 and 5. An unpaired *t* test was used to compare intake values of rats exposed to AngII treatment and those exposed to vehicle, and Student's *t* tests with a Bonferroni corrected value were used to analyze the effect of IG infusions on water intake. Student-Newman-Keuls post hoc test was used to explore statistically significant main effects and interactions.

3.1 Results

3.1.1 Experiment 1: effect of delayed water access on AngII-induced intake

We previously hypothesized that the increase in water intake is the result of a receptor-level sensitization that occurs independently of the behavioral response. Alternatively, the increased intake could be due to a learned association between the injection of AngII and the resultant water intake after several pairings of AngII and drinking [23]. To test these competing hypotheses, we added a delay period (30 min, 1 h, or 3 h) between AngII and drinking during 4 consecutive induction days to remove or reduce the behavioral response, and tested the effect of this delay on AngII-induced intake on a fifth day of treatment. Similar to previous findings [9], we found that delaying water access decreased the drinking after AngII. Specifically, a delay of 30 min, 1 h, and 3 h effectively attenuated the drinking response after administration of AngII ($F_{3,33} = 99.61$; $p < 0.01$; Figure 1a). Analysis of intake over all four induction days revealed a main effect of delay on intake ($F_{3,33} = 194.295$; $p < 0.01$), but no main effect of day ($F_{3,99} = 2.42$; $p > 0.05$). A delay \times day interaction was detected ($F_{9,99} = 3.64$; $p < 0.01$) and post hoc tests found that this was largely driven by an increase in intake by rats in the no-delay group, whereas intake by rats in the other groups remained stable (Figure 1a). Additionally, the 3-h, 1-h, and 30-min delay groups drank less across all induction days when compared to intake of the no-delay group. Consistent with previous studies [14, 23], when no delay was imposed, rats drank more water on the fifth day of AngII than they drank on the first day of injections ($t(8) = -6.34$, $p < 0.01$; Figure 1b).

Analysis of intake on the test day (day 5) revealed group differences in intake ($F_{3,33} = 8.04$, $p < 0.01$; Figure 1c). Post hoc tests showed that rats in the 1-h and 3-h delay groups drank less water on day 5 relative to rats in the no delay groups ($p < 0.05$). Intake by rats in the no-delay and 30-min delay groups did not differ ($p > 0.05$). Additionally, intake by rats in the 1-h and 30-min groups did not differ ($p > 0.05$). Planned comparisons using *t* tests with a Bonferroni corrected critical value did not detect a difference in intake between rats in the no-delay group on day 1 and rats in the 3-h delay group ($t(17) = 1.06$, $p = 0.30$) or 1-h delay group ($t(16) = -1.34$, $p = 0.20$; Figure 1d). Collectively, these results indicate that the imposed delay effectively reduced intake on induction days, and that this disassociation of drinking behavior from AngII was sufficient to prevent the expected increase in water intake.

In order to more fully explore the differences in drinking response between groups, analysis of drinking microstructure was performed comparing day 1 and day 5 of the no-delay group, and day 5 of the 3-h delay and no-delay group. Due to technical issues, lick data were not collected from two subjects that were, therefore, excluded from the analysis. A main effect of delay ($F_{1,15} = 6.27$, $p < 0.05$), day ($F_{1,15} = 4.85$, $p < 0.05$), and a delay \times day interaction were detected ($F_{1,15} = 7.82$, $p < 0.05$) for burst number. Post hoc tests revealed that this was

due to a reduced burst number in the 3-h delay group on the first induction day ($p < 0.05$; Figure 2a). There was no effect of burst number between day 1 and day 5 of the no-delay group ($p = 0.68$), or between day 5 of the 3-h delay and day 1 of the no-delay group ($p = 0.68$; Figure 2a). There was no main effect of delay ($F_{1,15} = 0.72$, $p = 0.41$) and no day \times delay interaction was observed ($F_{1,15} = 1.30$, $p = 0.27$) for burst size. A main effect of day was detected ($F_{1,15} = 7.52$, $p < 0.05$), and post hoc tests revealed that this resulted from an increase in burst size from day 1 to day 5 in the no-delay group ($p < 0.05$; Figure 2b). No differences in burst size between day 5 of the 3-h delay group and no-delay group ($p = 0.20$), as well as day 1 of the no-delay ($p = 0.50$) and 3-h delay ($p = 0.51$) groups, were detected (Figure 2b).

3.1.2 Experiment 2: effect of conditioned injections on water intake

To test the possibility that the increased water intake on the fifth day of AngII was, at least partly, the result of a conditioned drinking response to the injection itself, rats (4–5 per group) were injected with either AngII or vehicle on four consecutive induction days. All rats were given vehicle on day 5 (test day) and allowed to drink. We found no group differences in intake ($t(7) = 1.45$, $p = 0.19$; Figure 3), indicating that an injection without AngII was not sufficient to produce a conditioned drinking response.

3.1.3 Experiment 3: effect of pairing gastric infusion of water and central injection of AngII on subsequent AngII-induced intake

Fluid intake includes the act of drinking, as well as delivery of fluid into the gut. The previous experiments show that rats consume more after repeated bouts of AngII, and that water intake during the induction days is required for this response. This leaves open the question of which components of the behavioral response are needed to generate that increased intake. To test if pairing AngII with delivery of fluid into the gut is sufficient to increase the drinking response to subsequent AngII, we used rats with IG catheters to pair AngII and gastric infusions of water on 4 consecutive induction days, and measured drinking in response to AngII on the subsequent test day. Consistent with the results from Experiment 1, rats in the no-infusion group that received their water by drinking, drank more on day 5 than they drank on day 1 ($t(6) = 8.17$, $p < 0.01$; data not shown). A comparison of drinking on day 5 showed that rats in the infusion group drank less than rats in the no-infusion group ($t(11) = 3.87$, $p < 0.01$; Figure 4), suggesting that delivery of fluid into the gut was not sufficient to cause the increased intake observed here and elsewhere [14, 23].

4. Discussion

Sensitization is a pervasive form of non-associative learning observed in many systems, such as gill and siphon withdrawal in *Aplysia californica* [4] and drug effects in rats and humans [3, 28]. Previous studies found what appeared to be sensitization of fluid intake after repeated exposure to AngII [14, 23], after repeated bouts of sodium deprivation [11, 21], or after repeated bouts of dehydration with partial rehydration [19]. Sensitization under at least some of these conditions can generate cross-sensitization of other responses, including responses to amphetamine [20]. Although there is ample evidence for what can correctly be considered sensitization in these cases, the present experiments provide evidence that what

appeared to be a non-associative sensitization of water intake after repeated AngII [14, 23] more likely involves an associative component that increases appetite signals [24], thereby increasing intake after AngII. This conclusion is supported by the findings that, 1) imposing a delay between AngII and water intake prevented the increased intake otherwise observed after repeated AngII, 2) that a conditioned drinking response to the injection itself was not responsible for the increased intake, and 3) that water delivered directly to the gut after AngII was not sufficient to produce increased intake after a subsequent injection of AngII. These data are consistent with earlier studies showing that ‘thirst’ provides a negative-valence teaching signal [2], and suggests that repeated exposures to this teaching signal, and the negative reinforcement that arises from the resultant water intake, can strengthen the reinforcing value of the water consumed. Taken with the present findings, this collectively provides evidence that increased water intake under these conditions is the result of associative learning, rather than a more traditionally ascribed non-associative sensitization.

We initially hypothesized that the sensitization of water intake was a result of changes at the level of the angiotensin type 1 receptor (AT₁R) [5, 23]. The present studies tested the hypothesis that receptor activation by AngII, without concomitant drinking behavior, would still produce the previously observed increase in water intake, thereby supporting the presumption that the increased drinking behavior was caused by a receptor-level sensitization. To this end, we took advantage of previous studies showing that delaying access to water after icv administration of AngII attenuated or prevented the drinking response [9]. Using this strategy to reduce or eliminate AngII-induced intake, while keeping the administration of AngII intact, we confirmed that delaying access to water after injection of AngII effectively reduced water intake, and found that the reduced water intake on days 1–4 (induction days) was associated with a lack of increased intake on the fifth and final day of testing (test day), when rats had immediate access to water after AngII. This finding, which is inconsistent with our initial hypothesis, provided support for our revised working model that the increased intake observed previously (and here) requires more than a simple ligand-receptor mediated sensitization. Accordingly, the present findings suggest that the drinking response itself is a necessary component in what we previously referred to as sensitization.

The experimental design used in the present study involves several facets that could be at the root of the conditioned appetite suggested by the results. In an attempt to identify these facets, we performed additional experiments and analyses to improve our understanding of which factors may be involved in the difference in intake observed. Specifically, we examined drinking microstructure to test for differences that could provide information about the nature of the enhanced drinking response after daily AngII. Moreover, we used gastric infusions to test if the water entering the system was sufficient to condition a strengthened drinking response, and we tested the possibility that the injection itself conditioned a drinking response.

Analysis of drinking microstructure can be helpful in understanding the nature of any observed changes in intake [6, 25]. In our analyses of drinking microstructure, we found that burst size, but not burst number, was larger in no-delay group rats on the test day when compared to the same no-delay group rats on the first induction day. In other words, the

greater intake observed on test day, compared with the drinking on the first induction day, was a function of greater burst size, rather than of greater burst number. This is consistent with earlier studies in our laboratory [23], and, based on previous work on drinking microstructure [6, 25], suggests that the difference in intake was a result of altered orosensory feedback (perhaps increased hedonic value) of the water consumed. Although intake was different on day 5 (test day) between the rats with a 3 h delay and rats with no delay on the induction days (days 1–4), this was not accompanied by statistically significant differences in either burst size or burst number. Accordingly, the analyses were helpful in that they provided potentially useful information about the nature of the changes in intake in the positive control rats (no-delay group), but were less helpful in improving our understanding of the intake differences between the treatment groups on the test day. As expected, a 3-h delay in water access reduced intake, and our analysis of lick patterns found that this was a function of reduced burst number, without any change in burst size. Although this requires a more complete set of experiments, these findings suggest that a delay in water access reduces AngII effects by altering postingestive feedback, rather than orosensory properties of the consumed water.

In order to better understand the nature of the observed association, we tested if the strengthened drinking response observed on the test day was at least partly due to a response to the injection itself, conditioned by four days of AngII injections. To this end, we gave rats daily injections of AngII or vehicle during the induction days, and an injection of vehicle on test day. We found no difference in intake between vehicle-treated and AngII-treated groups on the test day. This suggests that the injection did not become a conditioned stimulus for drinking, and further shows that the injection itself was not crucial in driving the increased appetite observed after repeated injections of AngII.

In our continued attempt to understand the nature of the suspected conditioned appetite, we tested if pairing AngII with gastric delivery of water, thereby removing drinking from the training, was sufficient to strengthen the drinking response to subsequent AngII. In other words, could the pairing of AngII with stimulating water-receptive elements in the gut cause a conditioned appetite? The results of the experiment suggest that this is not the case. Specifically, pairing injections of AngII with gastric infusion of water was not sufficient to condition the increased appetite observed in rats after pairing AngII and normal access to water. Consistent both with earlier work showing intact drinking and satiation responses in a rat model of gastric bypass [13] and with the present finding that the altered drinking is a function of burst size differences, but not changes in burst number, the collective indication is that postingestive feedback alone is not sufficient to condition increased appetite to repeated AngII. This suggests that something about the orosensory experience of drinking or some other facet of the drinking experience is required for both intake termination and the increased appetite observed here. The critical element(s) of that experience is unknown at this time, and could involve the simple experience of water entering the mouth, or could involve an operant response that becomes associated with the restoration of fluid balance. In experiment 3, for instance, would the appetite effect have occurred if the rats were required to make an operant response to deliver water into the gut? Is the act of walking to and licking the spout required for the observed appetite effect? The present findings place

a strong emphasis on those open questions, and highlight the need to understand how orosensory, and possibly operant, events related to water intake are detected and integrated.

The present studies provide evidence supporting a role of a conditioned appetite in the observed response, but further studies are needed to confirm and support this conclusion. The present studies have not, for instance, ruled out the possibility that some kind of practice effect is responsible for the strengthened drinking response on the test day. It is possible that the repeated bouts of drinking, in response to the injections of AngII, provided training in licking behavior that allowed for increased licking on test day. Although we believe this possibility is unlikely, mostly because the rats had been licking a spout to get water since weaning, and therefore had abundant practice, it nevertheless provides a testable hypothesis for future studies. Moreover, although our presentation of these studies may give the sense that we see a dichotomy between a learned behavior and a pharmacological effect of AngII at its receptor, these studies do not rule out a role for altered AT₁R expression or signaling in the behavioral effects observed here. Indeed, our previous studies using daily injections of AngII, and our studies of the desensitizing effect of more acute repeated injections, found AngII-induced changes in receptor binding and expression [23, 27]. In addition, studies in mice have shown that AngII-sensitive SFO neurons fall into at least two categories of cells, glutamatergic or GABAergic, and that these cells can promote or inhibit drinking, respectively [18]. Although this may not occur similarly in rats, it opens the intriguing possibilities that repeated exposure to AngII either causes a shift in the relative proportion of these cell types, or shifts the receptor expression from one cell type to the other; a change that would not be detected by our previous studies testing for changes in receptor binding [23, 27]. These and other receptor-level changes may certainly be related to the appetite observed here. Accordingly, future studies are needed to parse the role of any receptor-level changes in the observed behavior.

Taken together, these findings support a critical role of associative learning in what previously appeared to be sensitization. The observed changes in behavior do not appear to be a consequence of postingestive feedback alone or a conditioned response to the injection procedure. Given the suggestion that what appeared to be non-associative sensitization more likely involves associative learning, in the form of a conditioned appetite, these findings may have implications for other studies on sensitization. Indeed, considering the role of associative learning in what is currently deemed non-associative sensitization could shape how treatment interventions are designed and, more broadly, how we understand the complex phenomenon of sensitization. More specifically, however, the present studies improve our understanding of the increased intake observed after repeated AngII injections, and open new questions about the processes involved in response to recurrent exposure to AngII.

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Highlights

- Delayed water access after AngII injections attenuated water intake
- Daily AngII without water intake did not progressively increase intake
- Increased intake was not a result of conditioning to injection procedure
- Gastric infusion of water did not substitute for normal drinking

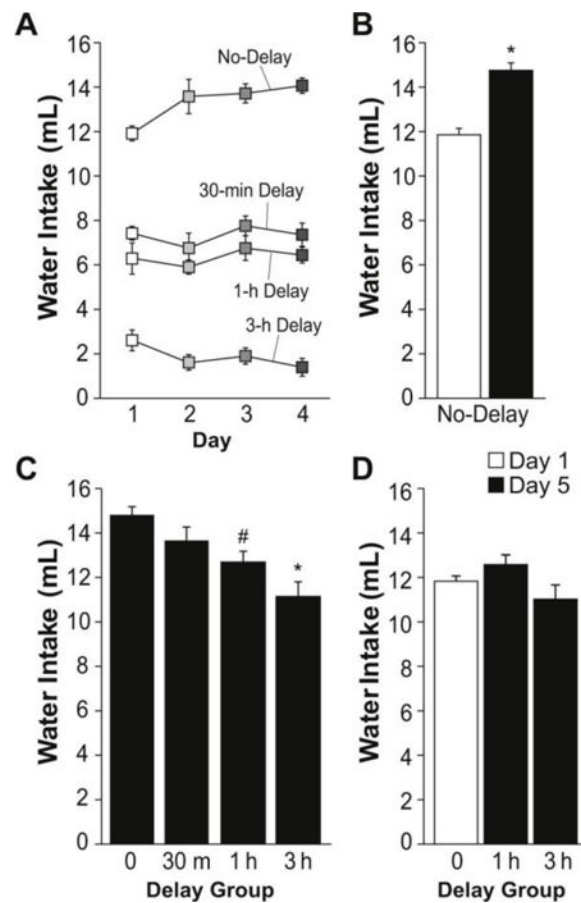


Figure 1.

Effect of dissociating AngII and water intake on subsequent AngII-induced intake. Data are shown as mean \pm SEM. Panel A, drinking after AngII on four induction days. A delay of 30 min, 1 h, or 3 h between the injection of AngII and access to water significantly attenuated the drinking response observed in the no-delay group. A main effect of delay group, and a delay \times day interaction were detected (see text of Results for details). Panel B, rats that received immediate access to water after AngII drank more on the test day (day 5) than they drank on the first induction day (day 1) * $p < 0.05$. Panel C, a comparison of intake on day 5 across all groups revealed a significant difference in intake in the 3-h and 1-h delay groups ($p < 0.05$). No differences were observed between the 30-min and no-delay groups. # different from no-delay control, * different from no-delay control and 30-min delay group. Panel D, the intake by rats in the 3-h and 1-h delay groups on test day was not different from the intake by rats that received immediate access to water on the first induction day ($p > 0.05$).

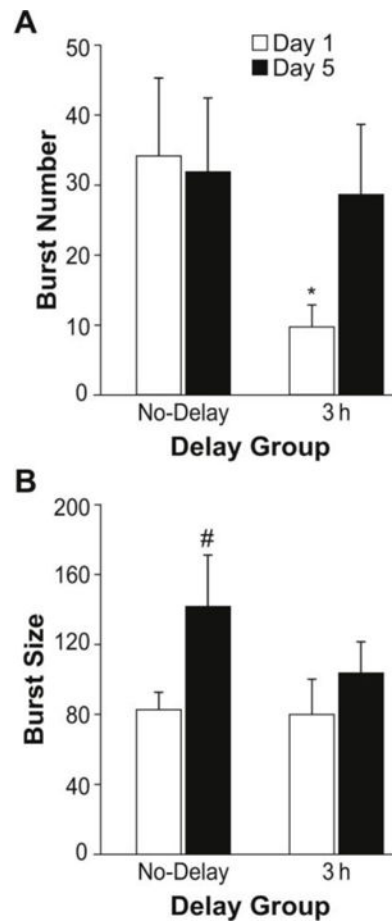


Figure 2.

Analysis of drinking microstructure. Data are shown as mean \pm SEM. Panel A, the number of drinking bursts was not different between day 1 and day 5 in rats that received immediate access to water (no-delay), but burst number was lower in rats in the 3-h delay group on day 1 ($p < 0.05$, *different from all other groups). Panel B, burst size was significantly increased in no-delay rats on day 5 when compared to drinking patterns by the same rats on day 1 ($\# p < 0.05$). No significant difference in burst size was observed between rats in the 3-h and no-delay groups when licking on day 5 was analyzed ($p > 0.05$).

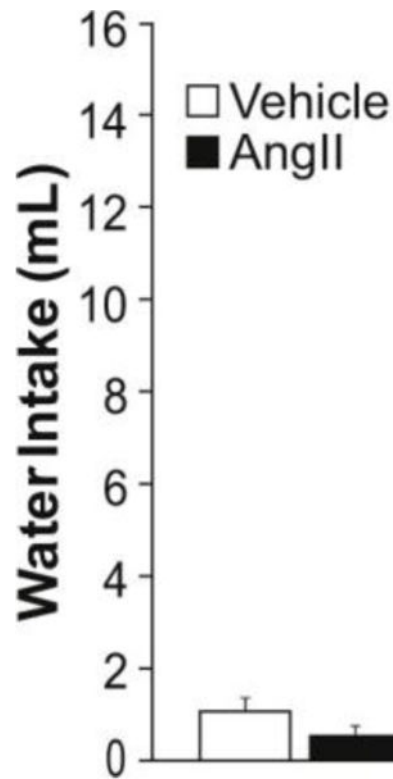


Figure 3. Response to a vehicle injection after four days of AngII injections. Four days of AngII injections without a delay in water access after each injection failed to condition a drinking response to the injection in the absence of AngII ($p > 0.05$). Data are shown as mean \pm SEM.

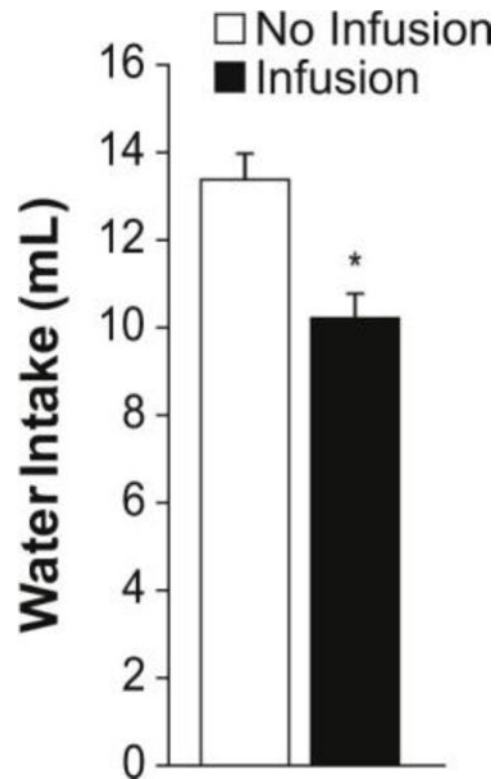


Figure 4.

Effect of pairing AngII and gastric infusions of water on subsequent drinking after AngII. Rats receiving gastric infusion of water after AngII during four induction days drank less in response to AngII than did rats allowed to drink normally after AngII ($*p < 0.05$). Intake on the test day (day 5) is shown, when all rats were injected with AngII and were allowed to drink normally. Data are shown as mean \pm SEM.