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DIFFERENTIAL EFFECTS OF MINERALOCORTICOID AND ANGIOTENSIN II ON INCENTIVE AND MESOLIMBIC ACTIVITY

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Abbreviated title: Aldo and AngII-induced motivation for sodium

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ABSTRACT

The controls of thirst and sodium appetite are mediated in part by the hormones aldosterone and angiotensin II (AngII). The present study examined the behavioral and neural mechanisms of altered effort-value in animals treated with systemic mineralocorticoids, intracerebroventricular AngII, or both. First, rats treated with mineralocorticoid and AngII were tested in the progressive ratio operant task. The willingness to work for sodium versus water depended on hormonal treatment. In particular, rats treated with both mineralocorticoid and AngII preferentially worked for access to sodium versus water compared with rats given only one of these hormones. Second, components of the mesolimbic dopamine pathway were examined for modulation by mineralocorticoids and AngII. Based on cFos immunohistochemistry, AngII treatment activated neurons in the ventral tegmental area and nucleus accumbens, with no enhancement by mineralocorticoid pretreatment. In contrast, western blot analysis revealed that combined hormone treatment increased levels of phospho-tyrosine hydroxylase in the ventral tegmental area. Thus, mineralocorticoid and AngII treatments differentially engaged the mesolimbic pathway based on tyrosine hydroxylase levels versus cFos activation.

Key words: Aldosterone; Angiotensin; Dopamine; Motivation; Nucleus Accumbens; Sodium Appetite; Thirst; Ventral Tegmental Area

Introduction

Fluid depletion can be life threatening, and animals must carefully titrate their intake of water and sodium to restore and maintain osmotic and volemic balance. Sodium replacement requires the goal-directed behavior known as sodium appetite (Andersson, 1977), a behavior that can be prompted by mineralocorticoids, such as aldosterone, and angiotensin II (AngII) (Johnson and Thunhorst, 1997). AngII acts in the brain to elicit water intake and sodium intake (Epstein et al., 1969). During fluid depletion, suppression of either central AngII or aldosterone action does not eliminate sodium appetite, but blocking the central actions of both hormones abolishes the behavior (Buggy and Jonklaas, 1984; Sakai et al., 1986). Conversely, when both aldosterone and AngII are given exogenously, sodium appetite is potentiated (Fluharty and Epstein, 1983). The behavioral and neural basis for the combined effect of aldosterone and AngII on sodium appetite remains undefined.

The behavioral effects aldosterone and AngII to promote sodium ingestion may involve parallel behavioral mechanisms. For example, sodium ingestion could be enhanced by a change in the hedonic strength of the sodium tastant. Indeed, in rats placed on a sodium deficient diet, which increases aldosterone and AngII levels, sodium ingestion is preferred to moderately reinforcing brain stimulation, which suggests sodium appetite involves the modulation of the pleasurable properties of sodium intake (Conover et al., 1994). In parallel to altered taste value, sodium appetite may involve a recalibration of incentive-based effort. In this regard, rats treated with both aldosterone and AngII run faster on a runway to gain access to sodium, compared with rats treated with either

hormone alone (Zhang et al., 1984), which suggests that the combined hormone treatment increases the incentive value of sodium. The progressive ratio task is a quantitative assay for incentive-based effort, but the effects of combined mineralocorticoids and AngII on this behavioral test have not been reported.

The mesolimbic dopamine system has been widely implicated in effort-related behaviors (Barbano and Cador, 2006; Floresco et al., 2008; Kelley et al., 2005; Phillips et al., 2007; Salamone et al., 2009). Dopamine neurons in the ventral tegmental area (VTA) project to the accumbens, which in turn projects to brain regions such as the ventral pallidum to generate goal directed movement (Carelli, 2002). Previous work has implicated this brain system in sodium appetite. For example, the accumbens receives multisynaptic input from aldosterone-sensing and sodium-sensing neurons in the hindbrain (Miller and Loewy, 2014; Shekhtman et al., 2007). Sodium depletion alters the level of dopamine transporters and opioid peptides in the accumbens (Grondin et al., 2011; Lucas et al., 2003; Roitman et al., 1999). In addition, sodium depletion modifies the dendritic arbor of ventral striatum neurons (Roitman et al., 2002). Although these studies have suggested a role for mesolimbic activity in sodium appetite, the separate effects of aldosterone and AngII on mesolimbic activation have not been studied.

The present studies tested the overall hypothesis that mineralocorticoids and AngII recalibrate the willingness to work for sodium versus water. In particular, the willingness to work was measured with the progressive ratio task. In addition, the activity of the mesolimbic dopamine pathway was assessed with immunohistochemistry for cFos and western blot analysis for tyrosine hydroxylase. Although it is recognized that sodium depletion is a complex physiological state that is imperfectly mimicked with

mineralocorticoid and central AngII treatments, this preparation has yielded useful insights into the neuroendocrine actions that influence motivated behavior.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (weight between 225 and 250 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were pair-housed in plastic tubs with standard bedding and with food and water available *ad libitum*, except during experimental procedures. The temperature in the colony was maintained at 22°C with a 12:12 h reversed light/dark cycle. Behavioral testing, described below, was conducted during the lights-out phase. Animals were allowed at least one week to acclimate to the colony before any procedures were performed. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures with animals.

Surgery

Surgeries were performed in aseptic conditions. Animals were anesthetized with inducted and maintained with isofluorane anesthesia for stereotaxic surgery. A 26-gauge guide cannula (Plastics One, Roanoke, VA, USA) was aimed at the lateral ventricle using these coordinates: 0.48 mm caudal to bregma, 1.6 mm from mid-line and 4.2 mm ventral to dura mater. The cannulae were fixed in place with dental cement and bone screws. Upon completion of surgery procedures, animals were injected with yohimbine (0.11

mg/kg, ip, Ben Venue Laboratories Bedford, OH), and upon awakening animals were returned to the housing facility and singly housed. The animals were allowed at least five days to recover before verification procedures were performed.

Prior to undergoing experimental treatments, animals were tested for correct lateral ventricle cannula placement and patency. They were given an *icv* injection of 20.0 ng of AngII diluted in artificial cerebrospinal fluid (aCSF) via a Hamilton syringe connected with PE-10 tubing to an injector that terminated 1 mm beyond the guide cannula. Animals were excluded from the experiment if they failed to demonstrate a drinking response in less than 30 seconds, consuming at least 3 ml of water, in two separate AngII challenges. Experiments began three days after these *icv* test injections.

Experimental Design

In all experiments, animals were assigned to one of four treatments in a 2 x 2 design, with a crossover in behavioral experiments. Animals were first pre-treated twice daily (10 hours apart) for three days with a subcutaneous injection of sesame oil or an aldosterone analog, deoxycorticosterone acetate (DOC; 0.25 mg/0.2 ml sesame oil; Sigma, St Louis, MO). DOC penetrates the blood brain barrier more easily than aldosterone due to its low capacity for hydrogen bond formation (Kraulis et al., 1975). Animals then were injected *icv* with either artificial cerebrospinal fluid (aCSF; R&D Systems, Minneapolis, MN) or 20.0 ng AngII in a volume of 2.0 ul (Bachem, King of Prussia, PA). The treatment groups will be referred to as follows: Veh/Veh, DOC/Veh, Veh/AngII, DOC/AngII. Using this experimental design and identical doses, the

DOC/AngII treatment has been shown to elicit a greater than additive effect on sodium intake, but not water intake (Grafe et al., 2014).

Experiment 1. Progressive Ratio Task

Rats were acclimated to wire mesh cages for one hour with two 25-ml bottles containing tap water and 3% saline, each marked with 0.2 ml graduations. These bottles were then removed, and rats were water restricted for 23 hours per day for the next six days. During these six days, rats underwent operant lever pressing training in conditioning boxes for 30 minutes per day (Med Associates; MDPC IV Software, St. Albans, Vermont). The conditioning boxes contained levers for both water (right lever) and 3% saline (left lever), both simultaneously present. A lever press lowered a syringe pump, which delivered a 0.1 ml drop of the appropriate liquid into a cup available to the rat. The saline and water each had their own syringe pump and their own cup. During the first two training days, to facilitate learning, an aliquot was dispensed every 300 sec that elapsed without bar pressing. In addition, animals could earn a 0.1 ml of water or saline for each bar press, depending on which of the two levers was pressed. During the subsequent two training days, the animals earned a 0.1 ml water or saline for each lever press, followed by two training days during which three lever presses were required for each aliquot of water or saline. Animals were considered to have learned the lever-fluid contingencies when they had made at least 10 lever presses for water during the 30-min session. Once this occurred, rats were given ad libitum access to water again. Rats were then assigned to treatment groups, as described above, and given no further operant

training while they received their three days of pretreatment injections (vehicle or DOC). After pretreatment was complete, 24 hours after the last DOC treatment, rats were administered their assigned *icv* injection (vehicle or 20 ng AngII), and immediately given a test with a progressive ratio (PR) schedule. Thus, animals were water replete at the beginning of the PR test. The response requirement of the PR schedule increased progressively for both saline and water, as previously described (Davis et al., 2011). The breakpoint for each animal was defined as the final reinforced bar pressing set that preceded a 10-minute period without earning a reinforcment, with a two-hour limit total. Food was not available during this task.

Experiment 2: Hormone-Induced cFos expression

To observe brain activation after DOC and AngII treatments, rats were assigned to treatment groups, as explained above. Sixty minutes after the last *icv* injection, each rat was anesthetized with 50 mg/kg ketamine and 20 mg/kg xylazine, intraperitoneally. As discussed below, there were group differences in the effort for sodium versus water during the first few minutes of the progressive ratio task, making 60 minutes post-treatment a reasonable time to expect differences in cFos levels. Rats were perfused transcardially with 100 mL of heparinized saline followed by 200 ml 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). The brains were isolated, post-fixed in paraformaldehyde overnight at 4°C, and then submerged in 20% sucrose in 0.1 M phosphate buffer for three days. Coronal sections were cut on a freezing microtome into three serial sets of 40-um-thick sections. These sectons encompassed the VTA and

the shell and core of the accumbens. One set of sections from each animal underwent immunohistochemical staining and analysis; the other two sets of sections were preserved in cryoprotectant as reserve material.

Sections were washed in Tris-buffered saline (TBS; pH 7.4) and then incubated with a cFos antibody (1:500, sc-52, rabbit; Santa Cruz Biotechnology, Santa Cruz, CA) in TBS with 0.2% TritonX-100 and 3% normal donkey serum (Jackson Immunoresearch; West Grove, PA) overnight at 4°C. After several washes, sections were incubated with a Biotin-SP-conjugated AffiniPure Donkey Anti-rabbit IgG (1:100, Jackson Immunoresearch) in TBS with 0.2% TritonX-100 and 3% normal donkey serum for 2 hours at room temperature. After several washes, sections were incubated with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for one hour. This was followed by another set of washes before staining with 3'3'-diaminobenzidine (Sigma-Aldrich) for 10 minutes. After a final set of washes, sections were mounted on slides, air-dried, and cover slipped with DPX mounting media (Electron Microscopy Sciences: Fort Washington, PA).

Photomicrographs were acquired with a digital camera (Diagnostic Instruments, Sterling Heights, MI, model RTKE), maintaining the same microscope and camera settings to ensure the same level of light and exposure for all images. Background was subtracted from images using Photoshop, and images were set to the same threshold level (200). Images were further analyzed in NIH Image J using standardized boxes for each brain region (based on Paxinos & Watson Rat Brain Atlas, shown in **Figure 1A**), using the analyze particles function, which counts objects within specific size and shape parameters. Pixel size minimum was 15 and circularity was set to 0.35.

Experiment 3: Hormone Regulation of Tyrosine Hydroxylase

Animals were assigned to four treatment groups, as described above. Five minutes after the last injections, animals were rapidly decapitated and brains were flash frozen in hexane over dry ice. The five-minute time point was selected based on its correlation with a significant increase in lever presses for sodium in the progressive ratio experiment. Using a cryostat, 1-mm punches of the brain regions of interest (VTA, accumbens shell and core, shown in **Figure 1B**) were collected from 300-µm sections. Punches were immersed in lysis buffer containing 25 mM Tris-HCl (pH 8), protease inhibitors (pepstatin, leupeptin, aprotinin), and phosphatase inhibitors (sodium pyrophosphate, sodium fluoride, sodium molybdate, phenylarsin oxide, and sodium orthovanadate). The VTA and accumbens (shell and core) punches were immersed in 50 and 100 uL of lysis buffer, respectively. Brain punches were sonicated for three seconds followed by centrifugation at 14,000 rpm in 4°C for 15 minutes. Supernatant was collected and a Bicinchoninic acid (BCA) protein assay was performed on five microliters of each sample. Based on the protein levels detected by the BCA assay, appropriate amounts of sample and sample buffer were loaded into wells of a 10% sodium dodecyl sulfate polyacrylamide gel. Western blots for phospho- and total TH were performed using the LiCor Odyssey System. The following antibodies were used: monoclonal anti-tyrosine hydroxylase antibody T2928 at 1:8000 (Sigma Aldrich); tyrosine hydroxylase pS31 rabbit polyclonal antibody #36-9900 at 1:400 (Life Technologies, Carlsbad, CA); IRDye 800CW Goat anti-Mouse IgG (H + L) 926-32210 at 1:2000 (Li-Cor Biosciences, Lincoln, NE); IRDye 680LT Goat anti-Rabbit IgG (H + L) 926-68021 at 1:4000 (Li-Cor

Biosciences). Phosphorylation sites on tyrosine hydroxylase generally increase catecholamine production. Serine 31, the residue attended to in the present study, is phosphorylated by depolarization and extracellular receptor-activated kinases 1 and 2 (Tekin et al., 2014).

Statistical Analysis

Data are presented as the mean ± the standard deviation of the mean. For all experiments, comparisons were made between treatment variables with a two-way ANOVA. When warranted, Bonferonni-corrected t-tests were performed. Effect sizes were calculated using η^2 and Cohen's d. All hypothesis tests used α=0.05 as the criterion level of significance. Statistical analyses were conducted using Prism 2.0 software (La Jolla, CA).

Results

The hormone regimen used in the present study was previously demonstrated to increase sodium ingestion in the Veh/AngII and DOC/AngII groups and to significantly water intake in the Veh/AngII condition (Grafe et al., 2014). Here, the progressive ratio operant task was used to compare the effort rats are willing to exert for sodium ingestion after these hormone treatments. Rats were pretreated with oil or DOC, followed by *icv* treatments of vehicle or AngII and allowed access to two levers: one for access to 3% saline and the other for access to water (0.1 ml per reinforcement). **Figure 2A** illustrates

the cumulative number of lever presses across the duration of testing for 3% saline and water. DOC/AngII treated rats bar-pressed for a longer duration for 3% saline compared with the other treatment groups.

We conducted separate two-way ANOVA tests to examine effects of these hormones on bar presses for sodium and water. The two-way ANOVA supported a main effect for AngII ($F(1,29) = 13.9$, $p = 0.0008$, $\eta^2 = 0.31$) but not DOC ($F(1,29) = 0.1$, $p = 0.752$, $\eta^2 > 0.01$) on 3% saline lever presses. Bonferroni-corrected post-hoc t tests revealed that the DOC/AngII group pressed significantly more on the saline lever compared with the Veh/Veh and DOC/Veh groups (Veh/Veh: 10.9 presses (SD2.4), DOC/Veh: 6.4 presses (SD1.7), Veh/AngII: 19.1 presses (SD6.4), DOC/AngII: 26.0 presses (SD2.8); $p = 0.001$, $p = 0.0001$; $d = 1.50$). A two-way ANOVA established a main effect for AngII ($F(1,29) = 5.7$, $p = 0.023$, $\eta^2 = 0.13$) but not DOC ($F(1,29) = 1.7$, $p = 0.203$, $\eta^2 = 0.04$) on water lever presses, and a significant interaction between the two hormones ($F(1,29) = 8.4$, $p = 0.007$, $\eta^2 = 0.19$). Post-hoc analysis revealed that rats treated with Veh/AngII pressed more for water than any other treatment group (Veh/Veh: 16.9 presses (SD5.2), DOC/Vehicle: 29.5 presses (SD4.9), Vehicle/AngII: 58.9 presses (SD10.7), DOC/AngII: 25.5 presses (SD8.9); $p = 0.003$, $p = 0.020$, $p = 0.030$; $d = 1.47$).

With a progressive ratio schedule, the response requirement to attain rewards increases according to the same rule throughout the test session until the rat stops responding (Sclafani and Ackroff, 2003). The highest set of bar presses completed is referred to as the break point and provides a measure of incentive. In the present experiment, rats had simultaneous access to work for sodium and water. Treatments for Veh/Veh, DOC/Veh and Veh/AngII elicited more work for water compared with sodium,

whereas the DOC/AngII-treated animals exhibited the opposite pattern. A two-way ANOVA established an interaction between AngII and DOC ($F(1,29) = 4.6$, $p = 0.046$; $F(1,29) = 8.6$, $p = 0.006$; $\eta^2 = 0.18$). DOC/AngII treated rats had significantly higher ratio of the breakpoint for sodium versus breakpoint for water than all other treatment groups.

Experiment 2: Hormone-Induced cFos expression

The observed unique effect of DOC/AngII treatment on willingness to work for sodium versus water suggested an underlying difference in mesolimbic activity. As shown in **Figure 3**, the number of cFos labeled cells was quantified in the VTA and the accumbens after Veh/Veh, DOC/Veh, Veh/AngII, and DOC/AngII treatment. A two-way ANOVA supported a main effect for AngII ($F(1,13) = 39.94$, $p < 0.0001$, $\eta^2 = 0.64$), but not for DOC or an interaction between the two hormones in the VTA. Post hoc tests indicated that Veh/AngII and DOC/AngII treatments increased cFos expression compared with Veh/Veh ($p = 0.0001$, $p = 0.0003$, respectively; $d = 3.77$). In the nucleus accumbens core, a two-way ANOVA revealed a main effect for AngII ($F(1,13) = 12.82$, $p = 0.0034$, $\eta^2 = 0.44$), but no effect for DOC or their interaction on cFos expression. Post-hoc tests indicated that Veh/AngII and DOC/AngII treatments increased cFos levels compared to Veh/Veh ($p = 0.0004$, $p = 0.0046$, respectively; $d = 2.12$). Lastly, a two-way ANOVA for the nucleus accumbens shell revealed a main effect for AngII ($F(1,13) = 18.6$, $p = 0.0008$, $\eta^2 = 0.52$), but no effect for DOC or the two-hormone interaction on cFos expression. Post-hoc t tests indicated that each AngII treatment group induced significant cFos expression compared to the Veh/Veh treatment (Veh/AngII $p = 0.0012$; DOC/AngII $p =$

290 0.002; d = 2.48). To summarize, AngII treatment activated cFos expression in the VTA
291 and nucleus accumbens, but DOC pretreatment did not enhance this activation.

292

293 *Experiment 3: Hormone Regulation of Tyrosine Hydroxylase*

294 The AngII-induced expression of cFos of the VTA observed in Experiment 2
295 suggested a possible increase in dopamine neurotransmission. The level of tyrosine
296 hydroxylase, the rate-limiting enzyme for dopamine production, and its phosphorylation,
297 were measured by western blot analysis. Rats were pretreated with vehicle or DOC
298 followed by *icv* injections of vehicle or AngII, and tissue punches were collected five
299 minutes after the last injection. In the VTA, where dopaminergic neurons reside, a two-
300 way ANOVA supported a main effect for both DOC and AngII on tyrosine hydroxylase
301 levels in the VTA ($F(1,8) = 16.6$, $p = 0.004$, $\eta^2 = 0.51$; $F(1,8) = 6.0$, $p = 0.04$, $\eta^2 = 0.19$),
302 as illustrated in **Figure 4A**. Post hoc tests indicated each treatment group significantly
303 enhanced tyrosine hydroxylase levels compared with vehicle ($p = 0.003$, $p = 0.04$, $p =$
304 0.02 for DOC/Veh, Veh/AngII, and DOC/AngII, respectively; $d = 1.99$). However, DOC
305 pretreatment did not further enhance the effects of AngII on tyrosine hydroxylase levels.
306 For phospho-tyrosine hydroxylase, the value for each animal, as determined by western
307 blot analysis, was divided by the value for total tyrosine hydroxylase (p-TH/TH). In the
308 VTA, a two-way ANOVA supported a main effect for DOC ($F(1,8) = 10.37$, $p = 0.012$, η^2
309 = 0.52), but not AngII treatment, as shown in **Figure 4B**. Post-hoc analysis indicated that
310 in the VTA DOC/AngII groups displayed increased p-TH/TH levels compared with
311 Veh/Veh ($p = 0.02$; $d = 0.78$).

In the nucleus accumbens core, neither DOC nor AngII had a significant main effect on tyrosine hydroxylase levels. In the shell of the nucleus accumbens, DOC, but not AngII, had a main effect on levels of tyrosine hydroxylase ($F(1,8) = 9.3$, $p = 0.02$, $\eta^2 = 0.40$). Post-hoc analysis revealed DOC/Veh increased tyrosine hydroxylase levels compared with Veh/Veh ($p = 0.04$; $d = 2.17$). In both the core and shell DOC and AngII treatments significantly increased p-TH/TH compared with vehicle (Core: Veh/Veh: 0.02 (SD0.2), DOC/Veh: 0.42 (SD0.2), Veh/AngII: 0.37 (SD0.14), DOC/AngII: 0.38 (SD0.01), $p = 0.011$, $p = 0.020$, $p = 0.018$; $d = 0.72$); (Shell: Veh/Veh: 0.07 (SD0.05), DOC/Veh: 0.46 (SD0.02), Veh/AngII: 0.38 (SD0.06), DOC/AngII: 0.39 (SD0.04); $p = 0.0008$, $p = 0.0028$, $p = 0.0022$, respectively; $d = 0.51$). In summary, in both regions of the nucleus accumbens, DOC and AngII pretreatments increased phosphorylation levels of tyrosine hydroxylase, usually without detectably increasing total tyrosine hydroxylase levels.

Discussion

During sodium deficiency, aldosterone and AngII act in concert to produce an avid consumption of sodium (Fluharty and Sakai, 1995), and combined treatment with these hormones is a useful experimental model for investigating the biological basis of this striking example of motivated behavior. The current experiments tested the overall hypothesis that DOC and AngII treatments uniquely affect the willingness to work for access to sodium, potentially by modulating neural activation and neurochemistry in the mesolimbic dopamine pathway. DOC/AngII-treated rats steadily exerted effort for access to 3% sodium solution while decreasing their effort for access to water compared with the

Veh/AngII-treated rats. DOC and AngII had differential effects on cFos activation and phospho-tyrosine hydroxylase levels in the VTA. Additive effects of DOC and AngII were not observed when these treatments were combined, although it is possible that cFos immunohistochemistry and tyrosine hydroxylase immunoblots were limited by a ceiling effect. Instead, the co-treatment of DOC and AngII was associated with a unique combination of enhanced dopamine synthetic capacity and neural activation. **Figure 5** summarizes these results according to hormone treatment, brain region and behavior.

Several caveats are well known for the interpretation of cFos expression. First, neuronal activation may occur without cFos induction. Thus, the lack of cFos activation after DOC treatment may not reflect the recent activity of these neurons. In addition, the neural consequences of cFos activation are not fully understood, making cFos merely a marker of recent activity. Thirdly, without knowing the phenotype of the cFos labeled neurons in this study, the increased activity may occur in excitatory or inhibitory neurons, which would have quite different physiological consequences. With those points notwithstanding, cFos expression in the VTA and ventral striatum has been a valuable a proxy for measuring neuronal activation in many studies (Kovács, 2008).

Behavioral mechanisms

Healthy rats normally avoid concentrated sodium solutions (Berridge et al., 1984); however when levels of both mineralocorticoids and AngII are elevated, a robust sodium appetite emerges (Fluharty and Epstein, 1983; Shade et al., 2002). A component of sodium appetite is altered gustatory hedonic processing, and subsequent increased

pleasure, of passively received oral sodium. In the taste reactivity test, sodium appetite is associated with reduced gaping and increased pre-swallowing behaviors in response to orally presented sodium solutions (Berridge et al., 1984; Clark and Bernstein, 2006). Further evidence that sodium depletion increases the “liking” of concentrated sodium solutions is the depletion-specific response of neurons in the ventral pallidum, a brain region thought to encode pleasure, in response to sodium ingestion (Tindell et al., 2006). The ventral forebrain may contribute to the hormonal modulation of sodium as a tastant (Garcia et al., 2008; Geerling and Loewy, 2006; Lundy, 2008).

In addition to enhanced palatability, sodium appetite includes a willingness to work for sodium. For example, rats run faster along a runway to gain access to sodium after co-administration of aldosterone and AngII (Zhang et al., 1984). In the progressive ratio test, the highest set of bar presses completed, referred to as the break point, provides a quantification of goal value (Hodos, 1961). Progressive ratio schedules model foraging in a natural setting; as resources are consumed in a given area, more effort is required to obtain them. This method has been used to quantify the motivation for sodium after body fluid depletion (Clark and Bernstein, 2006; Starr and Rowland, 2006). The present experiment used a two-bottle progressive ratio task to document the effects of mineralocorticoids and AngII on the relative effort-value of 3% sodium solution versus water. This preparation revealed a shift in effort-value, with AngII alone biasing the animals to exert more effort for water than sodium, whereas DOC/AngII treatment drove them to work more for 3% saline than water. Unlike previous studies of sodium appetite that focused on the quantity of sodium ingested, the two-bottle progressive ratio task quantifies the competing drives for sodium and water without changing fluid balance.

Differences in effort-value led us to investigate effects of mineralocorticoids and AngII on mesolimbic regions, a brain system that regulates effort-related behaviors (Barbano and Cador, 2006; Floresco et al., 2008; Kelley et al., 2005; Phillips et al., 2007; Salamone et al., 2009).

In the present study, no simple correlation exists between the level of cFos activation in a specific brain region and behavior. Rather, similar cFos levels were observed in the VTA and nucleus accumbens when animals would have been working for water versus sodium. One interpretation of this finding is that cFos activation in the mesolimbic dopamine pathway represents a non-specific increase in willingness work. However, it remains uncertain whether the similar numbers of cFos labeled cells were of the same cell type. Furthermore, despite the similarities in cFos levels despite during these different motivated states, it should be noted that regulation of tyrosine hydroxylase varies, suggesting differential dopamine transmission. Thus, further studies are needed to understand the details of motivation-specific regulation of mesolimbic dopamine.

Hormone-sensing brain regions

The initial brain targets of mineralocorticoids and AngII are known. Regarding mineralocorticoids, the nucleus of the solitary tract contains a unique population of neurons that co-express mineralocorticoid receptors and the enzyme necessary for mineralocorticoid-specific action, known as 11-β hydroxysteroid dehydrogenase 2 (Geerling et al., 2006; Geerling and Loewy, 2006). In contrast, humoral AngII acts initially on AngII type 1 (AT1) receptors in circumventricular organs, such as the subfornical organ

and the organ vasculosum of the lateral terminalis (Lind et al., 1985; McKinley et al., 1992b; Tanaka et al., 1986; Weiss and Hatton, 1990). Mineralocorticoids exert minor up-regulation of AT1 receptor levels and AT1 signaling (Grafe et al., 2014; Shelat et al., 1999a; Shelat et al., 1998; Shelat et al., 1999b). As a parallel mechanism, mineralocorticoids may exert a cooperative behavioral effect with AngII by acting elsewhere in a broader circuit. For example, mineralocorticoids appear to remove an inhibitory influence on sodium appetite, namely oxytocin activity in the PVN (Blackburn et al., 1992; Grafe et al., 2014; Roesch et al., 2001; Stricker and Verbalis, 1987).

A behaviorally relevant downstream connection of both mineralocorticoid- and AngII-sensing brain regions is the lateral hypothalamus (Camacho and Phillips, 1981), which is essential for the normal expression of sodium appetite (Dayawansa et al., 2011). The lateral hypothalamus, in turn, projects to the mesolimbic dopamine system via the medial forebrain bundle (Berk and Finkelstein, 1981; Saper et al., 1979). Relevant projections from the lateral hypothalamus to the VTA may include orexin neurons (Fadel and Deutch, 2002; Narita et al., 2006; Peyron et al., 1998), which promote incentive-based effort in the VTA (Borgland et al., 2009; Choi et al., 2010). Furthermore, stimulation of the lateral hypothalamus promotes sodium appetite (Liedtke et al., 2011). Likewise, AngII treatment enhances the release of striatal dopamine in freely moving rats within minutes, which then is associated with increased drinking behavior (Brown et al., 1996; Hoebel et al., 1994). Thus, a possible link between mineralocorticoid and AngII-sensing brain regions and midbrain dopamine neurons may be lateral hypothalamic orexin neurons. Future studies are needed to illuminate the details of this broader circuit.

Sodium appetite and motivation systems

The mesolimbic pathway is part of a motor loop that links the incentive properties of stimuli with the effort exerted to approach or avoid them (Berridge, 2007; Salamone et al., 2007). Neural adaptations in this brain region are associated with motivated behaviors (Gu et al., 2010). AngII-induced cFos expression in the mesolimbic area is likely the result of transynaptic activation because the VTA does not have AngII receptors (Song et al., 1992). Previous studies documented AngII-induced activation of the hypothalamus and taste nuclei (Han and Rowland, 1995; Houpt et al., 1998; McKinley et al., 1992a; Thunhorst et al., 1998; Vivas et al., 1995), but this is the first report of cFos activation in the VTA.

The VTA includes many dopaminergic neurons (Oades and Halliday, 1987). The AngII-induced increase in tyrosine hydroxylase levels supports the notion that dopamine neurons are activated by AngII treatment. A similarly rapid increase in tyrosine hydroxylase levels occurs during exposure to cocaine-paired contexts (Liang et al., 2012). AngII-induced cFos expression may contribute to the up-regulation of tyrosine hydroxylase (Gheea et al., 1998). DOC treatment markedly increased the phosphorylation of tyrosine hydroxylase, which augments catalytic activity of this rate-limiting enzyme (Dunkley et al., 2004). The combined actions of AngII and DOC on neuronal activation and tyrosine hydroxylase would be expected to produce a substantial increase in dopamine release at axonal targets. Future studies are needed to examine the consequences of these hormonal actions at downstream nodes in the motivation circuit.

Regarding the ventral striatum, neurons there exhibit neural plasticity during various motivational states, including depletion-induced sodium appetite (Roitman et al., 2002). As in the VTA, AngII treatment increased cFos levels without a detectable augmentation by DOC pretreatment. Unlike the VTA, all hormone treatments increased phosphorylation of tyrosine hydroxylase levels with detectable increases in total levels of this enzyme. It will be important for future studies to investigate the neurophysiological consequences and potential rewiring that may occur in the accumbens in response to AngII- versus DOC/AngII-induced changes in this dopamine pathway.

Conclusions

In summary, the DOC/AngII-induced sodium appetite involves a shift in the effort value of sodium versus water consumption, noted in the break point for lever pressing. DOC and AngII affect the mesolimbic dopamine pathway differently, with AngII treatment increasing cFos activation in the VTA and nucleus accumbens, and DOC increasing phosphorylation of tyrosine hydroxylase. Thus, these two hormones may act in parallel to enhance mesolimbic dopamine transmission through complementary mechanisms.

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Figure Legends

Figure 1. Drawings of rat brain coronal sections depicting the regions of interest for both cFos immunohistochemistry (**Panel A**) and tyrosine hydroxylase activation assays (**Panel B**). **Panel A.** Boxes surrounding each brain region represent the areas analyzed for cFos cell counts. **Panel B.** Circles within each brain region represent 0.75-mm micropunches collected for the tyrosine hydroxylase western blots. The brain regions examined include both the core and shell of the accumbens, as well as the VTA. Abbreviations: AcbC = Core of the accumbens, AcbS = Shell of the accumbens, CPu= caudate putamen, ml = medial lemniscus, LS = lateral septal nucleus, PAG = periaqueductal gray, VTA= ventral tegmental area.

Figure 2. DOC/AngII treatment increases relative motivation for sodium (n = 5-6/group). **Panel A.** Line graphs illustrating cumulative presses over time for 3% Saline and Water after Vehicle, DOC, AngII, or DOC plus AngII treatments. **Panel B**. Bar graphs illustrating total lever presses for 3% saline and water after Vehicle, DOC, AngII, or DOC plus AngII treatments. AngII-only treatment increased bar presses for water and sodium, whereas DOC plus AngII treatment mainly increased presses for sodium. **Panel C.** Bar graphs illustrating the breakpoint ratio of sodium to water after Vehicle, DOC, AngII, or DOC plus AngII. DOC plus AngII causes the highest sodium to water breakpoint ratio. Abbreviations: AngII= Angiotensin II, DOC = deoxycorticosterone acetate, Veh = vehicle.

Figure 3. DOC/AngII treatment increases cFos expression in the ventral tegmental area and the accumbens (n = 3-6 per group). Bar graphs illustrate the cFos cell counts in the ventral tegmental area and accumbens after either vehicle or DOC pretreatment followed by *icv* vehicle or AngII. Representative images of the VTA and accumbens (coronal plane, 10x) in each treatment condition are shown above the bar graphs. AngII and DOC/AngII treatment increased cFos expression in the VTA, with DOC/AngII inducing the most immunostaining. In the core and shell of the nucleus accumbens, each hormone treatment induced cFos immunostaining compared with vehicle; DOC/AngII induced the highest amount of cFos immunostaining. Asterisks indicate a significant increase compared with the Veh/Veh group. Abbreviations: AngII = Angiotensin II, DOC = deoxycorticosterone acetate, NuAcc= Accumbens, Veh = Vehicle, VTA= Ventral Tegmental Area

Figure 4. DOC treatment increases tyrosine hydroxylase activation in the VTA and the accumbens (n = 3/group). **Panel A.** Bar graphs illustrate tyrosine hydroxylase levels in the VTA and the accumbens (core and shell) after oil versus DOC pretreatment and *icv* saline versus AngII. Representative western blot images of tyrosine hydroxylase are shown above each quantified bar. Each treatment increased tyrosine hydroxylase expression in the VTA. **Panel B.** Bar graphs illustrate phosphorylated tyrosine hydroxylase levels in the VTA and the accumbens (core and shell) after either oil or DOC pretreatment followed by *icv* treatments with AngII. Only DOC pretreatment significantly increased phosphorylated tyrosine hydroxylase expression compared to vehicle in the VTA and accumbens core. However, each treatment increased phosphorylated tyrosine

hydroxylase expression in the accumbens shell. Panel C. Representative western blot images labeled with corresponding treatment groups and brain regions assayed for tyrosine hydroxylase (left) and phosphorylated tyrosine hydroxylase (right). Abbreviations: AngII = Angiotensin II, Acc= Accumbens, DOC = deoxycorticosterone acetate phospho-TH = phosphorylated tyrosine hydroxylase, TH = tyrosine hydroxylase, Veh = Vehicle, VTA= ventral tegmental area. Asterisks indicate $p<0.05$ compared with the Veh/Veh group.

Figure 5. Diagram summarizing the effects of DOC and AngII on the mesolimbic system and behavior. Abbreviations: AngII = Angiotensin II, NuAcc= Nucleus Accumbens, DOC = deoxycorticosterone acetate, p-TH = phosphorylated tyrosine hydroxylase, TH = tyrosine hydroxylase, VTA= ventral tegmental area.