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Endogenous angiotensin II-induced p44/42 MAPK activation mediates sodium appetite but not thirst or neurohypophysial secretion in male rats

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ABSTRACT

The renin-angiotensin-aldosterone system makes a critical contribution to body fluid homeostasis, and abnormalities in this endocrine system have been implicated in certain forms of hypertension. The peptide hormone angiotensin II (AngII) regulates hydromineral homeostasis and blood pressure by acting on both peripheral and brain targets. In the brain, AngII binds to the angiotensin type 1 receptor (AT1R) to stimulate thirst, sodium appetite and both arginine vasopressin (AVP) and oxytocin (OT) secretion. The present work used an experimental model of endogenous AngII to examine the role of p44/42 mitogen-activated protein kinase (MAPK) as a signalling mechanism to mediate these responses. Animals were given a combined treatment of furosemide and a low dose of captopril (furo/cap), a diuretic and an angiotensin converting enzyme inhibitor, respectively, to elevate endogenous AngII levels in the brain. Furo/cap induced p44/42 MAPK activation in key brain areas that express AT1R, and this effect was reduced with either a centrally administered AT1R antagonist (irbesartan) or a p44/42 MAPK inhibitor (U0126). Additionally, furo/cap treatment elicited water and sodium intake, and irbesartan markedly reduced both of these behaviors. Central injection of U0126 markedly attenuated furo/cap-induced sodium intake but not water intake. Furthermore, p44/42 MAPK signalling was not necessary for either furo/cap- or **exogenous** AngII-induced AVP or OT release. Taken together, these results indicate that p44/42 MAPK is required for AngII-induced sodium appetite, but not thirst or neurohypophysial secretion. This result may allow for discovery of more specific downstream targets of p44/42 MAPK to curb sodium appetite, known to exacerbate hypertension, while leaving thirst and neurohypophysial hormone secretion undisturbed.

Introduction

The renin-angiotensin-aldosterone system promotes body fluid homeostasis by coordinating physiological and behavioural responses to correct fluid perturbations (1, 2). Hypotensive and hypovolemic events prompt the release of the enzyme renin from the kidneys, which initiates a catalytic cascade that ultimately elevates blood levels of the hormone angiotensin II (AngII) (3). Peripherally, AngII constricts blood vessels to support blood pressure. C**irculating** AngII binds to brain receptors in the subfornical organ (SFO), organum vasculosum of the lateral terminalis (OVLT), brain regions with an incomplete blood-brain barrier. **In addition, AngII produced locally by the brain renin-angiotensin system acts** on relays in the median pre-optic nucleus (MNPO), paraventricular nucleus of the hypothalamus (PVN) and supraoptic nucleus (SON) (4). **Acting upon these central nuclei, AngII** prompts thirst, sodium appetite (5, 6), and both arginine vasopressin (AVP) and oxytocin (OT) secretion (7). While the physiological actions of AngII are well known, our understanding of its cellular actions is incomplete.

The central actions of AngII are mediated by the angiotensin type 1 receptor (AT1R), which belongs to the super family of G protein coupled receptors and associates with G_q to trigger inositol 1,4,5-triphosphate (IP3) production and Ca^{2+} mobilization (8, 9). *In vitro* research uncovered a parallel signal transduction pathway that phosphorylates MAPK Kinase (MEK), which activates mitogen-activated protein kinase (MAPK) (10) , including the p42 and p44 isoforms, also referred to as extracellular signal-regulated kinase 1 and 2. In turn, p44/42 MAPK provokes a variety of downstream changes in cellular activity, such as changes in gene expression (11-13). Although the majority of studies examining AT1R-induced p44/42 MAPK activation were performed *in vitro*, p44/42 MAPK activation also occurs in the SFO and PVN after a systemic AngII injection (14). As **these** AT1R signalling molecules were detected in the brain, researchers have begun to investigate their role in the neural actions of AngII.

A previous study employed signalling selective AT1R ligands and inhibitors to reveal that the inositol triphosphate (IP_3) branch of AT1R signal transduction mediates thirst, whereas the p44/42 MAPK signalling pathway mediates sodium appetite (15, 16). This finding was novel and intriguing; however, it remained unclear whether the result reflected a normal physiological process or a pharmacological phenomenon based on exogenously administered AngII. Therefore, to establish the physiological significance of AngII-induced p44/42 MAPK on sodium appetite, we exploited a preparation for endogenous AngII-induced sodium appetite (17). More specifically, a combined treatment of furosemide and a low dose of captopril (furo/cap), a loop diuretic and an inhibitor of angiotensin converting enzyme (ACE) inhibitor, respectively, elevates brain levels of AngII. The low dose of captopril blocks the conversion of diuresis-provoked angiotensin I to AngII in the periphery, but still allows conversion in circumventricular organs, which contain very high levels of ACE in comparison to the periphery (18). The high level of endogenous AngII production in the brain elicits rapid and robust thirst and sodium ingestion (19). We used this model to test the hypothesis that p44/42 MAPK phosphorylation mediates sodium, but not water, ingestion induced by endogenous AngII production.

Given the myriad actions of AngII in the brain, it is important to consider the signalling pathways involved in other **AngII-induced effects, such as neuroendocrine responses**. In addition to its effects on water and sodium ingestion, central AngII is a potent stimulus for AVP and OT secretion (20). **While AVP promotes renal water reabsorption, OT increases renal sodium excretion and centrally inhibits sodium appetite** (21-23). AT1R-expressing neurones in the SFO send axonal projections to the neurosecretory PVN and SON, where AVP and OT neuronal activity is increased to hormone release from the neurohypophysis (24). We addressed the role of p44/42 MAPK signalling in AngII-induced AVP and OT by measuring plasma levels

of the neurohypophysial hormones after either furo/cap treatment or *icv* AngII in the presence of an AT1R antagonist or MEK inhibitor. Overall, our experiments better define the participation of p44/42 MAPK signalling in the regulation of fluid balance.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (n = 83) that weighed between 225-250 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. 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.

Drugs

Furosemide (Abbott Laboratories, N. Chicago, IL) and captopril (SQ-14225, Bristol-Meyers-Squibb Pharmaceutical Research Institute, Princeton, NJ) were administered subcutaneously (*sc)* at doses of 10 mg/kg and 5 mg/kg body weight, respectively, in 0.9% sterile saline. Equal volume 0.9% sterile saline was administered in control animals. The specific doses for each drug given intracerebroventricularly (*icv)* were as follows: 20 ng AngII (Bachem, King of Prussia, PA), 0.4 ug irbesartan (a generous gift from Dr. Sal Lucania, Bristol Myers Squibb), 2 nmol U0126 dissolved in **10%** DMSO (Promega, Madison, WI). Artificial cerebrospinal fluid

(aCSF; R&D Systems, Minneapolis, MN) **plus 10%** DMSO was administered *icv* as a vehicle control. All injections were administered *icv* in a volume of 2 ul.

Surgeries

Surgeries were performed under asceptic conditions. Animals were anaesthetised with a combination of ketamine and xylazine (70 and 5 mg/kg, respectively, intraperitoneally [*ip*]) before being fixed in a stereotaxic frame and implanted with 26-gauge guide cannulae (Plastics One, Roanoke, VA, USA) aimed at the lateral ventricle. The coordinates (0.48 mm caudal to bregma, 1.6 mm from mid-line and 4.2 mm ventral to dura mater) were chosen to allow an internal injection cannula to extend beyond the guide cannula into the ventricular space. The cannulae were fixed in place with dental cement and bone screws, and the animals were allowed at least five days to recover before verification procedures were performed. After surgery, animals were injected with yohimbine (0.11 mg/kg, Ben Venue Laboratories Bedford, OH) and orally administered Children's Tylenol (30 mg/ml, McNeil PPC Inc, Fort Washington, PA). Upon awakening, animals were singly housed.

Five days after surgery and prior to undergoing experimental treatments, animals were tested for correct cannula placement and patency. They were given an injection of 20 ng of AngII diluted in aCSF *icv* 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. Animals began testing three days after the *icv* test injections.

Enzyme Linked Immunosorbent Assays

Experiment 1. Animals were pretreated with either vehicle, irbesartan, or U0126 *icv*.

Fifteen minutes after the *icv* injection, they were given furosemide, *sc*, followed 10 minutes later by captopril, *sc*. Thirty minutes after the captopril injection, animals were rapidly decapitated **without anaesthesia** and the brains were flash frozen in hexane over dry ice. **Rats were not administered anaesthesia because it is known to increase phosphorylated p44/42 MAPK levels, which would compromise our results** (25). Micropunches (1 mm diameter) of the brain regions of interest (OVLT, SFO, PVN, SON, and primary somatosensory cortex) were obtained from 300-µm sections cut on a cryostat. OVLT/SFO and PVN/SON punches were combined from each rat to ensure detectable levels for analysis. The micropunched tissue samples were immersed in the lysis buffer provided in the Pathscan p44/42 MAPK Sandwich ELISA kit (Cell Signalling Technology, Danvers, MA) plus 1 mM phenylmethanesulfonylfluoride (Sigma, St Louis, MO). The brain tissue samples were sonicated three times for one second (incubated on ice between bursts) followed by centrifugation at 14,000 rpm at 4°C for 10 minutes. Supernatant was collected and a protein assay was performed on five microliters of each sample. Based on the protein levels detected by Bicinchoninic Acid Assay, appropriate amounts of lysis buffer, sample, and sample diluent were mixed for each sample. This mixture (100 µl) was loaded into corresponding wells in both the Total and Phospho p44/42 MAPK Sandwich ELISA kits. The ELISA was performed according to the protocol provided by each kit.

Behavioural Study

For behavioural experiments, rats were removed from their home cages approximately one hour before the onset of the dark phase. The rats were weighed and then placed in individual wire mesh-bottomed cage equipped with a funnel and calibrated tube for the collection of urine, and with two 25-ml bottles available for drinking tap water and 1.5% saline, each marked with 0.2 ml

graduations. The rats were allowed to acclimate for one hour before the experiment began. After the last drug treatment was given, water, saline, and urine levels were measured at 15 min intervals for the first hour and then at 30 min intervals for the remaining time.

Experiment 2A. The goal of the first behavioural experiment was to examine the role of p44/42 MAPK activation in mediating the effects of endogenous AngII on water and sodium intake. The animals were assigned to one of four treatments in a crossover design, which allowed **each rat to receive all treatment conditions over the course of the study without all the animals receiving the drug combinations in the same order**. The control condition was two vehicle injections (0.9% saline, *sc*) with a 10-min interval between them. All other treatments included injections of furosemide and, 10 min later, captopril. *Icv* injections were given 15 minutes before the furosemide injection. **Fluid ingestion was measured for three hours when animals were given furo/cap or saline. Based on this initial study, it was noted that the induced ingestion mainly occurred within the first 90 minutes; therefore, subsequent studies used this time course.** To assess the role of AT1R, animals were treated with irbesartan *icv*, and to assess the role of p44/42 MAPK, they were injected with U0126 *icv*. Water and 1.5% saline intakes **as well as urine output,** were measured for 90 minutes.

Experiment 2B. The goal of the second behavioural experiment was to examine the role of p44/42 MAPK activation on water intake in the absence of sodium ingestion. The animals were treated with either vehicle or U0126 *icv* followed 15 minutes later by furosemide, and 10 minutes later by captopril in a crossover design. Rather than being presented with two bottles, as in the first experiment, animals were presented with a single bottle containing tap water. Water intake was monitored for the next 90 minutes.

AVP and OT Secretion

Experiment 3A. Rats were pretreated centrally with either vehicle, irbesartan, or U0126, followed by either vehicle or furo/cap, *sc* as described above. Thirty minutes after the captopril injection rats were rapidly decapitated and trunk blood was collected into 10 ml BD Vacutainer blood collection heparinised glass tubes on ice (Franklin Lakes, NJ). The thirty-minute time point was chosen based on the emergence of diuresis and drinking behaviour in our initial furo/cap studies. Additionally, the ELISA study showed that U0126 effectively inhibited p44/42 MAPK activation at this time point. Previous research examined a ninety-minute time point after furo/cap treatment, but did not observe increases in oxytocin (19). The tubes then were centrifuged at 4^{o}C at 1300 relative centrifugal force (rcf) for 10 min. The plasma supernatant was extracted with acetone and petroleum ether. Plasma levels of AVP and OT were measured with a specific radioimmunoassay performed by the laboratory of Dr. Joseph G. Verbalis, as described previously (26) . The standard curve for each peptide was linear between 0.5 and 10.0 pg per tube with the use of a synthetic AVP and between 0.25 and 5 µU per tube with the OT standard (Bachem Americas, Inc, Torrance, California). The minimum detectable concentration of AVP or OT in extracted plasma was 0.5 pg/ml and 0.25 µU/ml, respectively. The AVP antiserum (R-4) displayed < 1% cross-reactivity with OT, and the OT antiserum exhibited < 1% cross-reactivity with AVP.

Experiment 3B. Rats were assigned to one of the following pretreatment groups: vehicle or U0126 (1mM) followed 15 minutes later by AngII (20 ng) *icv*. Two or fifteen minutes after the last *icv* injection, rats were rapidly decapitated and their trunk blood was collected. The two- and fifteen-minute time points were based on the emergence of drinking behaviour in previous exogenous AngII studies and selected to establish the range of neurohypophysial hormone levels in the plasma after the treatments. Previous studies used a 90-second time point and observed

increases in plasma vasopressin (27) . The protocol described in Experiment 3A was then followed to measure AVP and OT levels.

Statistical Analysis

Data are presented as the mean ± the standard error of the mean. For the behavioural studies, comparisons were made between the treatment conditions and over time using repeated measure analysis of variance (two-way ANOVA; time point x treatment). For the p44/42 MAPK ELISA and AVP/OT radioimmunoassay results, comparisons were made between the treatment groups (one-way ANOVA). When warranted, planned comparison post-hoc t-tests were performed. 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

Furo/cap treatment activates p44/42 MAPK in the brain

In **Experiment 1**, rat brain punches were collected from coronal slices for several brain regions, namely, OVLT, SFO, PVN SON and primary somatosensory cortex as a control brain region (depicted in ***Figure 1***). As shown in ***Figure 2A***, total p44/42 MAPK levels between all of the treatment groups in the OVLT/SFO were not different (ANOVA $F(5,19) = 0.5$, $p>0.05$). However, p44/42 MAPK phosphorylation in the OVLT/SFO differed between treatment groups (ANOVA ($F(5,19) = 5.9$, $p<0.01$). More specifically, furo/cap treatment increased phospho p44/42 absorbance levels compared to control (1.6 ± 0.0 vs 1.1 ± 0.1, $p<0.001$). Furo/cap treatment paired with *icv* AT1R antagonist irbesartan or MEK inhibitor U0126 significantly reduced

absorbance levels compared to furo/cap with vehicle pretreatment (1.1 ± 0.1 vs 1.1 ± 0.0 vs 1.6 ± 0.0, respectively; $p<0.01$).

Likewise, ***Figure 2B*** illustrates that total p44/42 MAPK levels were equivalent for all treatment groups in the PVN/SON (ANOVA $F(5,19) = 0.2$, $p>0.05$). However, p44/42 MAPK phosphorylation in the PVN/SON of the hypothalamus differed between treatment groups (ANOVA $F(5,19) = 10.4$, $p<0.001$). In particular, furo/cap treatment augmented phospho p44/42 absorbance levels compared to control (1.7 ± 0.1 vs 1.1 ± 0.0, $p<0.001$). Pretreatment with either *icv* irbesartan or U0126 reduced absorbance levels of phospho p44/42 MAPK compared to furo/cap with vehicle pretreatment (1.2 ± 0.1 vs 1.0 ± 0.1 and 1.7 ± 0.1, respectively; $p<0.001$).

As shown in ***Figure 2C***, the control brain region S1 had similar levels of p44/42 total MAPK regardless of treatment group (ANOVA ($F(5,19) = 0.4$, $P>0.05$). Unlike OVLT/SFO and PVN/SON, S1 did not show an increase in p44/42 MAPK phosphorylation in response to furo/cap treatment (ANOVA $F(5,19) = 0.1$, $P>0.05$).

<u>The role of p44/42 MAPK in furo/cap-induced water and sodium ingestion</u>

Consistent with previous work (19), the furo/cap protocol produced a rapid diuresis and prompt water and sodium ingestion (as shown in ***Figure 3***). A two-way ANOVA established a strong main effect of furo/cap treatment on urine output ($F(1, 112) = 72.4$, $p<0.0001$). Animals that received furo/cap treatment excreted significantly more urine during the three hours after treatment than controls (13.6 ± 1.1 versus 2.9 ± 0.5 ml, $p<0.001$). This increase in urine output was first significant within 15 minutes of treatment ($p<0.01$). These animals also drank water robustly in response to the furo/cap treatment compared with controls ($F(1,112) = 23.5$, $p<0.001$; 7.8 ± 1.4 versus 0.8 ± 0.2 ml, $p<0.001$). In addition, furo/cap-treated rats consumed substantial

amounts of 1.5% NaCl solution compared with controls during this three hour time period ($F(1,112) = 9.0$, $p<0.01$; 5.7 ± 1.5 versus 0.7 ± 0.2 ml, $p<0.001$). The time point at which the furo/cap group intake became significantly elevated compared with the control group was 45 min for water intake ($p<0.01$) and 60 min for sodium intake ($p<0.05$) after the captopril injection.

Experiment 2A verified that furo/cap-induced water and sodium ingestion could be attributed, at least in part, to AT1Rs. In particular, rats were pretreated centrally with the AT1R antagonist irbesartan (200 ng/ul) 15 min before furo/cap treatment (28) . A two-way ANOVA established a main effect of irbesartan on furo/cap induced water intake compared to furo/cap controls ($F(1,275) = 11.2$, $p<0.01$). A similar main effect occurred with irbesartan treatment and furo/cap-induced sodium intake ($F(1,270) = 9.2$, $p<0.01$). As shown in ***Figure 4A***, irbesartan pretreatment decreased furo/cap-induced water intake by 50% over 90 minutes (5.8 ± 0.6 versus 2.9 ± 0.5 ml, $p<0.001$), first becoming significant at 45 minutes ($p<0.001$). Sodium intake was reduced by 56% at 90 minutes (4.6 ± 0.6 versus 2.0 ± 0.5 ml, $p<0.001$), first becoming significantly attenuated at 45 minutes ($p<0.01$). This level of suppression of furo/cap-induced water and sodium intake by an AT1R antagonist is consistent with previous findings (19).

Next, we investigated whether furo/cap-induced water and sodium ingestion could be attributed, at least in part, to p44/42 MAPK signalling. In particular, rats were pretreated centrally with the MEK inhibitor U0126 15 min before the furo/cap treatment. The dose of U0126 was chosen based on previous efficacy (15), and was validated in our **Experiment 1**. A two-way ANOVA did not reveal a main effect of U0126 on furo/cap-induced water intake compared to controls ($F(1,275) = 2.5$, $p = 0.1$). However, there was a significant interaction between treatment and time ($p<0.01$). U0126 decreased furo/cap-induced water intake by 30% at 90 minutes (5.8 ± 0.6 versus 4.1 ± 0.5 ml, $p< 0.01$), as shown in ***Figure 4A***. A two-way ANOVA revealed a main

effect of U0126 on furo/cap-induced sodium intake ($F(1,280) = 13.0$, $p<0.001$). U0126 reduced sodium intake by 60% (4.5 ± 0.6 versus 1.8 ± 0.5 ml, $p < 0.001$), first becoming significant at 30 min ($p<0.05$). **Urine volume measurement confirmed that neither irbesartan or U0126 pretreatment the interfered with the diuretic effectiveness of the furo/cap treatment (12.3 ± 0.9 versus 12.4 ± 1.4 versus 14.0 ml, respectively; data not shown).**

Previous work that suggested that p44/42 MAPK signalling mediates AngII-induced sodium appetite but not thirst; however, we observed a reduction in water intake in the U0126-treated animals, albeit not until the 90-min time point. We reasoned that this delayed suppression of water intake was an artefact of the two-bottle test, given that consumption of hypertonic saline would provide an osmotic stimulus for water intake. Thus, as U0126 suppresses sodium ingestion, it may indirectly effect to reduce water intake. This interpretation of the data is supported by the fact that U0126 suppressed sodium intake at 30 min, and subsequently reduced water intake at 90 min. To test this hypothesis, the effect of U0126 was examined in a single-bottle test. In this case, a two-way ANOVA revealed that water intake was not significantly decreased with U0126 treatment ($F(1,105) = 1.2$, $p = 0.3$), compared with animals only treated with furo/cap. Furo/cap versus furo/cap + U0126 groups drank 4.3 ± 0.8 versus 5.0 ± 0.9 ml, respectively, at the 90-minute time point (***Figure 4B***).

The role of p44/42 MAPK in furo/cap- and AngII-induced AVP and OT release

Experiment 3A investigated whether or not p44/42 MAPK signalling mediated furo/cap-induced AVP and OT secretion. Rats were pretreated with vehicle, irbesartan, or U1026 *icv* paired with either vehicle or furo/cap *sc*. Thirty minutes after captopril treatment, plasma AVP levels differed between treatment groups (***Figure 5***; ANOVA ($F(5,19) = 6.2$, $P<0.01$). Post hoc analysis

revealed that furo/cap stimulated AVP compared to vehicle treatment (1.3 ± 0.2 vs 0.6 ± 0.1 pg/ml, $p<0.05$). However, when animals were pretreated centrally with irbesartan or U0126, furo/cap-induced AVP secretion was not significantly reduced compared with vehicle pretreatment (1.4 ± 0.1 vs 1.7 ± 0.3 vs 1.3 ± 0.2 pg/ml, $p>0.05$). Similarly, OT levels differed between groups thirty minutes after captopril treatment (ANOVA ($F(5,19) = 3.3$, $P<0.05$). Post hoc evaluation indicated that furo/cap stimulated OT compared to control (5.6 ± 1.0 vs 1.8 ± 0.5 pg/ml, $p<0.05$). However, furo/cap treatment paired with irbesartan or U0126 centrally did not reduce OT levels compared to furo/cap with vehicle pretreatment (6.0 ± 1.6 vs 4.7 ± 0.9 vs 5.6 ± 1.0 pg/ml, $p>0.05$).

In **Experiment 3B**, rats were pretreated centrally with either vehicle or U0126 followed by AngII (20 ng). Two or fifteen minutes after treatment, plasma AVP differed between the groups (***Figure 6*****;** ANOVA ($F(2,38) = 5.6$, $p<0.01$). Given that there were no significant differences between the two and 15 min time points within each treatment group, these data were combined. Post-hoc analysis indicated that AngII treatment stimulated AVP secretion compared with vehicle treatment (2.5 ± 0.3 versus 1.5 ± 0.2 pg/ml, $p<0.01$). This level of AngII-induced AVP secretion is consistent with previous findings (29) . However, when animals were pretreated with U0126, the AngII-induced AVP secretion was not significantly different than when AngII was given alone (2.3 ± 0.2 versus 2.5 ± 0.3 pg/ml, $p = 0.7$). Likewise, plasma OT levels was increased 2 and 15 min after AngII treatment compared with vehicle (***Figure 6***; ANOVA $F(2,35) = 5.3$, $p<0.01$). Post hoc analyses revealed that AngII treatment stimulated OT secretion compared with vehicle treatment at levels congruous with previous findings (14.2 ± 2.8 versus 6.0 ± 1.3 pg/ml, $p<0.01$) (29) . However, AngII-induced OT secretion was not diminished by U0126 pretreatment (14.2 ± 2.8 versus 13.5 ± 2.4 pg/ml, $p = 0.9$).

Discussion

The goal of our experiments was to uncover the role of p44/42 MAPK in both behavioural and physiological actions of central AngII. **Experiment 1** demonstrated p44/42 MAPK activation in certain brain regions after endogenous AngII production, which was inhibited by an AT1R antagonist. **Experiment 2** established that endogenous AngII production promotes sodium appetite, but not thirst, via p44/42 MAPK activation. **Experiment 3** found that p44/42 MAPK activation does not mediate furo/cap- or AngII-induced AVP and OT secretion, underscoring the selective role of p44/42 MAPK in AngII-induced sodium appetite.

While previous findings have demonstrated the part played by p44/42 MAPK in AngII-induced sodium appetite, our studies confirm and extend these results (16). For example, although studies using exogenous AngII-induced sodium appetite demonstrated the stimulation of p44/42 MAPK activation by AngII, they did not show its reduction by MEK inhibitor U0126 in relevant brain regions (15, 16). The present studies demonstrate that furo/cap induces p44/42 MAPK activation in relevant forebrain areas, and that our doses of central irbesartan and U0126 restore phosphor p44/42 MAPK to control levels. The furo/cap treatment produces high levels of circulating renin and angiotensin I, and central AT1R appears to mediate a large proportion of the observed water and sodium ingestion (19, 30) . The present results further support the notion that AT1R-p44/42 MAPK activation is responsible for the increase in sodium appetite observed in our experiments, and that the reduction of the behaviour by U0126 is due to decreased p44/42 MAPK activation in relevant AT1R containing brain regions. Moreover, our studies provided an additional control to examine the unique role of p44/42 MAPK in sodium appetite through use of a single water bottle test.

Nevertheless, **our studies present their own limitations**. For example, AngII levels in

the brain may be unusually elevated by the peripheral blockade of angiotensin I conversion **using the furo/cap model**. However, in this protocol AngII production is constrained by physiological availability of the prohormone (19) . Another consideration is that furo/cap treatment may activate other interoceptive systems apart from the renin-angiotensin-aldosterone system. For example, low- and high-pressure baroreceptors would detect the diuresis-induced loss of blood volume and pressure, respectively, and relay this information to brainstem structures that may promote thirst, sodium appetite, or both (17) . Such baroreceptor-mediated signals may explain the partial effect of irbesartan pretreatment on water and sodium intake, and its ineffectiveness on AVP and OT secretion. **Another limitation of our studies is the combining of brain regions for the p44/42 MAPK ELISA. An immunohistochemical approach would allow for better localization of activated p44/42 MAPK. However, the objectivity, reproducibility, and semi-quantitative nature of ELISAs offer a clear advantage over the challenges of quantifying immunohistochemistry. Lastly, the *icv* injections used in our studies are not as spatially discrete as parenchymal injections. Future studies using local microinjections will be informative to pinpoint the contribution of parallel signalling pathways in specific brain regions.**

While demonstrating that AT1R-MAPK mediates sodium appetite, our studies indicate that water intake and the secretion of AVP and OT do not require p44/42 MAPK activation. Circulating AngII accesses AT1R located on neurones in the circumventricular organs, such as the SFO and the OVLT (4) , whose neurones project to the magnocellular neurones in the PVN and SON, which also express AT1R (14, 24) . Centrally administered AngII induces c-Fos expression in the PVN and SON and increases the firing rate of AVP and OT neurones (31, 32) , resulting in plasma levels of these hormones being elevated within minutes after AngII injection (33) . **These**

hormones contribute to fluid balance, with AVP prompting water retention and OT stimulating natriuresis (21, 23). In furo/cap-treated animals, the AT1R antagonist irbesartan does not reduce AVP and OT secretion, which suggests that other mechanisms, such as baroreceptors, trigger neurohypophysial release in this circumstance. **However, it is possible that a role for AT1R and m44/42 MAPK in furo/cap-induced AVP and OT release could be found at different time points**.

The present study focused on a subset of the MAPK family, namely p44/42. The role of other MAP kinases in the central effects of AngII remains unclear. It is possible that p38 MAPK and/or JNK play a role in mediating the central actions of AngII not blocked by the p44/42 MAPK inhibitor. Additionally, as shown in previous studies, it is possible for some MAP kinases, but not others, to be involved in mediating central AngII effects, such as AT1R upregulation (14). It would be important for future studies to investigate the role of other MAP kinases in drinking behaviour and neurohypophysial release.

Studying the signalling molecules involved in the central actions of AngII is the first step to understanding how central AngII exerts its physiological effects, but much remains to be uncovered about the cellular mechanisms by which they mediate these actions. For example, there are several plausible mechanisms by which p44/42 MAPK may mediate sodium appetite in the brain. One possibility is that AngII-MAPK signalling elicits sodium appetite by changing neuronal excitability in circumventricular neurones or forebrain integration sites that project to motor generators involved in salt ingestive behaviour (4, 34) . This rapid effect on synaptic transmission could explain the relatively fast time course of furo/cap-induced sodium appetite (35) . Alternatively, p44/42 MAPK may be involved in long-term gene transcription and protein synthesis for sodium and potassium channel subunits or AT1R. For example, MAPK up-regulates

AT1R in the SFO and PVN, and increased levels of AngII binding to its receptor may explain the robust sodium appetite, although not the lack of p44/42 MAPK-mediated water ingestion (14). Other downstream consequences of p44/42 MAPK activation include rearrangements of the cytoskeleton, which could include structural neural plasticity (36, 37). This mechanism is highlighted in a study by Swank and Sweatt, which found that p44/42 MAPK activation is important for plasticity in taste learning (38). Other research has demonstrated that sodium appetite is associated with dendritic growth and the development of dendritic spines in the nucleus accumbens (39). p44/42 MAPK activation may underlie strengthened connections within this brain region associated with incentive sensitization, which would explain the sudden avidity for sodium. It will be imperative for future studies to discern what downstream consequences of p44/42 MAPK signalling contribute to AngII-induced sodium appetite, keeping in mind there may be several parallel mechanisms.

Extending beyond drinking behaviours and neurohypophysial secretion, central AngII has a multitude of actions in the brain. For example, AngII causes sympathetic stimulation, hypothalamic-pituitary-adrenal activation, increased sympathetic tone, and ultimately an increase in mean arterial pressure (1). Other investigators have begun to uncover the AT1R signalling mediating these actions. Specifically, Wei et al have shown that p44/42 MAPK mediates the effect of central AngII to increase sympathetic nerve activity **in heart failure, another syndrome adversely affected by salt intake** (40). An important avenue for future investigation is to determine the locus within the network of AngII-modulated neural circuitry wherein sodium appetite and sympathetic activity share a common dependence on p44/42 MAPK activation. Another key question to address is the effect of elevated p44/42 MAPK activity in specific brain regions on the many central actions of AngII.

In summary, these studies provide new evidence that the diverse effects of central AngII rely on divergent signalling mechanisms. More specifically, we found that sodium appetite requires p44/42 MAPK activation, whereas thirst and neurohypophysial hormone secretion do not. It will be important for future studies to assess the role of both p44/42 MAPK and other MAPK kinases in other central AT1R actions that remain undefined, such as hypothalamic-pituitary-adrenal activation. Moreover, it will be critical to find the downstream targets of MAPK and determine the cellular mechanism of AngII in exerting these central effects.

Perspectives

Elements of the renin-angiotensin-aldosterone system are frequently implicated in the pathogenesis of hypertension, although the cause of hypertension is often unknown (7). Hypertension is a common and serious health concern (41). One in three adults have chronically elevated blood pressure, which shortens a person's life span by three to five years (42) . The identification of signalling-selective subsets of AngII action in the brain presents an opportunity to more precisely target central pathways that regulate blood pressure (43, 44) .

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Figure Legends

Figure 1. Drawing of a coronal hemi section of rat brain depicting the brain regions of interest for p44/42 MAPK activation assays. Circles within each brain region represent 1 mm micropunches collected for the p44/42 MAPK ELISAs. The brain regions examined include circumventricular organs OVLT and SFO, as well as the PVN and SON of the hypothalamus. Additionally, the S1 was collected as a control brain region to assay P44/42 MAPK activation.

Abbreviations: S1 = primary somatosensory cortex, CPu = caudate putamen, MS = medial septal nucleus, OVLT = organum vasculosum of the lateral terminalis, SFO = subfornical organ, fi = fimbria of the hippocampus, ic = internal capsule, PVN = paraventricular nucleus of the hypothalamus, SON = supraoptic nucleus of the hypothalamus.

Figure 2. Bar graphs illustrating the levels of total p44/42 MAPK and phospho p44/42 MAPK in the OVLT/SFO (panel A), PVN/SON (panel B), and S1 (panel C) after either vehicle or furo/cap treatment *sc* and either vehicle, irbesartan, or U0126 administered *icv* (n = 3-5/group). Total p44/42 MAPK levels do not change, whereas furo/cap treatment increases phosphorylated p44/42 MAPK in the OVLT/SFO and PVN/SON, but not in S1. Both irbesartan and U0126 prevent this furo/cap induced increase in p44/42 MAPK phosphorylation. Abbreviations: OVLT = organum vasculosum of the lateral terminalis, SFO = subfornical organ, PVN = paraventricular nucleus of the hypothalamus, SON = supraoptic nucleus of the hypothalamus, S1 = primary somatosensory cortex.

Figure 3. Line graphs illustrating the effect of furo/cap treatment on urine volume (panel A) and

water and sodium intake during a two-bottle test (panel B). On the x axis, zero minutes represents the time of captopril injection. Compared with vehicle treatment, rats given furo/cap treatment excreted significantly more urine within 15 minutes (n=16). Furo/Cap also induced a robust intake of water and 1.5% sodium within 45 minutes and 60 minutes of treatment, respectively. Asterisks indicate that $p<0.01$ when comparing Furo/Cap to vehicle treatment.

Figure 4. Line graphs illustrating the effect of blocking AT1R receptors and MAPK activation after furo/cap treatment in a two bottle test (panel A) and in a one bottle test (panel B). On the x axis, zero minutes represents the time of captopril injection. In the two bottle test, compared with Furo/Cap + Vehicle treatment, rats given the Furo/Cap + AT1R antagonist irbesartan treatment consumed significantly less water within 45 minutes after treatment (n=28). Irbesartan also reduced the intake of 1.5% saline within 45 minutes of treatment. Compared with Furo/Cap + vehicle treatment, rats given Furo/Cap + the MEK inhibitor U0126 treatment consumed significantly less water within 90 minutes after treatment and significantly less saline within 30 minutes of treatment (n=28). In the one bottle test, compared with vehicle treated, rats given the MEK inhibitor U0126 treatment consumed similar amounts of water (n=12). Asterisks indicate that $p<0.05$ when comparing to Furo/Cap + Veh treatment. Stats for Furo/Cap + Veh compared to Veh are shown in **Figure 3.**

Figure 5. Bar graphs illustrating the role of p44/42 MAPK activation on Furo/Cap-induced neurohypophysial secretion. Plasma AVP (Panel A) and OT (Panel B) after vehicle or Furo/Cap treatment combined with either vehicle, irbesartan, or U0126 administered *icv* (n= 3-5/group). Furo/Cap-induced AVP and OT secretion was not diminished with either the AT1R antagonist

irbesartan or the MEK inhibitor U0126. Abbreviations: AVP = arginine vasopressin, OT = oxytocin.

Fig 6. Bar graphs illustrating the effect of inhibiting p44/42 MAPK activation on AngII-induced neurohypophysial hormone secretion. Plasma AVP (Panel A) and OT (Panel B) levels two and fifteen minutes after *icv* Veh, AngII, or U0126 plus AngII (n = 10-16/group). AngII-induced AVP and OT levels were not inhibited with the MEK inhibitor U0126. Abbreviations: AVP = arginine vasopressin, OT = oxytocin.