Activation of the Renin-Angiotensin System Specifically in the Subfornical Organ is Sufficient to Induce Fluid Intake

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Short title: SFO ANG induces fluid intake through PKC

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Abstract

Increased activity of the renin-angiotensin system within the brain elevates fluid intake, blood pressure, and resting metabolic rate. Renin and angiotensinogen are co-expressed within the same cells of the subfornical organ; and the production and action of angiotensin-II through the angiotensin-II type 1 receptor in the SFO is necessary for fluid intake due to increased activity of the brain renin-angiotensin system. We generated an inducible model of angiotensin-II production by breeding transgenic mice expressing human renin in neurons controlled by the synapsin promoter with transgenic mice containing a Cre-recombinase inducible human angiotensinogen construct. Adenoviral delivery of Cre-recombinase causes SFO-selective induction of human angiotensinogen expression. Selective production of angiotensin-II in the SFO results in increased water intake, but did not change blood pressure or resting metabolic rate. The increase in water intake was angiotensin-II type 1 receptor-dependent. When given a choice between water and 0.15M NaCl, these mice increased total fluid and sodium, but not water, because of an increased preference for NaCl. When provided a choice between water and 0.3M NaCl, the mice exhibited increased fluid, water and sodium intake, but no change in preference for NaCl. The increase in fluid intake was blocked by an inhibitor of protein kinase C, but not extracellular regulated kinases, and correlated with increased phosphorylated cyclic AMP response element binding protein in the subfornical organ. Thus increased production and action of angiotensin-II specifically in the subfornical organ is sufficient on its own to mediate an increase in drinking through protein kinase C.
Introduction

Tight regulation of fluid and electrolyte balance is crucial for cellular function and survival. The renin angiotensin system (RAS), one of the most important physiological regulators of fluid and electrolyte homeostasis, is expressed and regulated in a tissue specific manner (reviewed in (31)). In the brain, renin is expressed primarily in neurons while angiotensinogen, the substrate for angiotensin-II (ANG-II), is widely expressed in glial cells (2; 32; 40; 64; 68). Angiotensinogen is also expressed in neurons, but its expression in neurons is restricted to those nuclei important for cardiovascular regulation, such as the subfornical organ (SFO), paraventricular nucleus of the hypothalamus, and rostral ventrolateral medulla (20; 32; 39; 68). This expression profile suggests the potential for the de novo production of ANG-II in the brain. Indeed, studies in transgenic mice employing sensitive reporter genes controlled by the renin and angiotensinogen promoters has revealed areas of the brain where renin and angiotensinogen are expressed in adjacent cells (i.e. potential for local extracellular generation of angiotensin peptides), and regions where both renin and angiotensinogen are co-expressed in the same neuron (i.e. potential for intracellular generation of angiotensin peptides) (39; 40; 68).

The SFO has been implicated as an important nucleus for the control of fluid intake (reviewed in (62)). There is strong experimental support for the local action of ANG-II in the SFO. ANG-II AT$_1$ receptors (AT$_1$R) are expressed on SFO neurons, and on post-synaptic neurons in the paraventricular nucleus and supraoptic nuclei which receive projections from the SFO (9; 24; 28; 42; 47). Direct injection of ANG-II into the brain causes increased fluid intake in rats through an AT$_1$R-dependent pathway, which is abolished by lesions of the SFO (56-58). In mice, peripheral injection of ANG-II stimulates c-fos immunoreactivity in the SFO, as it does in rats, but in mice, the magnitude of the drinking response is diminished (54) or absent (36). Central injection of ANG-II in mice induces drinking (19; 36), and does so via an AT$_1$R-dependent pathway (17), and over-expression of either ANG-II (55) or AT$_1$R in neurons of mice induces
water intake (41). Genetic ablation of endogenous AT$_{1A}$R selectively in the SFO of mice carrying a conditional allele of the AT$_{1A}$R gene (AT$_{1A}$R$^{\text{flox}}$) attenuates the polydipsia and hypertension induced by DOCA-salt (33). This suggests that DOCA-salt-induced polydipsia and hypertension has an SFO AT$_{1R}$-dependent component. It should be noted however, that studies also implicate other nuclei located in the anteroventral third ventricle (AV3V), the organum vasculosum of the lamina terminalis (OVLT) for example, in mediating responses to ANG-II, because lesions of the SFO do not effectively ablate ANG-II-induced drinking when ANG-II is injected directly into the AV3V region (7; 8).

In addition to the local action of ANG-II, there is experimental support for the concept that ANG-II is locally generated within the SFO. Renin and angiotensinogen are transcriptionally co-expressed within the same cells in the SFO, and angiotensin peptide immunoreactivity is found within the soma and processes of SFO neurons (39; 44). Ferguson and colleagues have shown that ANG-II released from synaptic terminals of SFO neurons can activate neurons in the paraventricular nucleus, suggesting that ANG-II produced in the SFO can induce neuronal signaling in other nuclei that project from the SFO (reviewed in (23)).

Double transgenic mice (termed sRA) expressing human renin in all neurons via the synapsin promoter (sR mice) and human angiotensinogen (A mice) driven by its endogenous promoter exhibit robust angiotensinogen and angiotensin peptide expression in the SFO and exhibit markedly increased fluid intake (30; 55). AT$_{1R}$ blockade blunts the increase in drinking in this model, as does selectively eliminating the expression of human angiotensinogen in the SFO in double transgenic mice carrying a conditional allele of human angiotensinogen (sRA$^{\text{flox}}$) (55; 59). Thus, production and action of ANG-II in the SFO is necessary to increase fluid intake. What remains unknown is whether selective ANG-II production only in the SFO is sufficient on its own to increase fluid intake and BP. We tested this directly by generating and analyzing a unique
mouse model where production of ANG-II can be genetically induced in any region of the brain. Herein we used an adenovirus encoding Cre-recombinase to specifically target ANG-II production within the SFO.
**Methods**

*Generation of the A\textsuperscript{Red} construct.* The A\textsuperscript{Red} construct contains: 1) the ubiquitous CAG promoter consisting of the cytomegalovirus (CMV) early enhancer element and the chicken β-actin gene promoter, first exon and intron, the rabbit β-globin gene splice acceptor, 2) a transgene encoding the dsRed fluorescent protein and a transcriptional and translational STOP signal surrounded by loxP sites, and 3) a human angiotensinogen transgene (Figure 1A). In response to Cre-recombinase, the dsRed gene and STOP signal is eliminated, and expression of human angiotensinogen is induced wherever Cre-recombinase is expressed. In practice, dsRed was easily detectable in cultured cells whereas it was not detectable in the mouse brain.

The A\textsuperscript{Red} construct was created by first subcloning the CAG promoter from pDRIVE-CAG into PCR-Blunt-II-TOPO to yield pTOPO-CAG. It was then subcloned from pTOPO-CAG into pSTEC1 to yield pSTEC1-CAG (63). The human AGT gene was removed from pTOPO-hAGT and cloned into pSTEC1-CAG to yield pSTEC1-CAG-hAGT. The loxP-DsRed-STOP-loxP was removed from the Cre-Stoplight and cloned into pSTEC1-CAG-hAGT, which yielded A\textsuperscript{Red} (69). The A\textsuperscript{Red} construct was transfected into Cos-7 cells treated with AdCRE. DsRed fluorescence and hAGT expression were assayed. The final construct was isolated after cutting with *Bam*H1 and injected into the pronuclei of C57BL/6J X SJL/J 1-cell fertilized mouse embryos. The mice were first backcross bred to C57BL/6 and then to sR mice to generate the sRA\textsuperscript{Red} double transgenic strain. The sR strain of transgenic mice carries the neuron-specific synapsin promoter driving a secreted form of active human renin due to replacement of the prorenin converting enzyme cleavage site with a furin cleavage site (50).

*Detection of tdTomato:* tdTomato reporter mice (ROSA) mice were injected with AdCRE ICV alongside each cohort of sRA\textsuperscript{Red} experimental mice that was injected. Three to six weeks after
injection they were anesthetized with ketamine/xylazine and were intracardially perfused with 4% paraformaldehyde in phosphate buffered saline. The fixed brains were then removed and sectioned at 40 μm on a vibratome for visualization of tdTomato fluorescence.

Quantitative RT-qPCR: The median preoptic nucleus (MnPO), SFO, and bilateral PVN were collected from individual sRA\textsuperscript{Red} and littermate control mice (33). Three to six weeks after ICV injection of AdCRE the mice were killed via CO\textsubscript{2}, their brains were removed, sunk and frozen in optimal cutting tool (OCT; Sakura). The brain was sectioned on a cryostat, and the MnPO, SFO, and bilateral PVN were identified according to “The Mouse Brain Atlas in Stereotaxic Coordinates” (Paxinos and Franklin, Academic Press)(53). These brain areas were punched with a 0.5 mm punch (Stoeling, Co.). RNA was isolated from the punches and converted into cDNA for RT-qPCR. hAGT-FAM and mouse actin-VIC labeled TaqMan probes (Applied Biosystems, Inc.) were used, so that hAGT and actin could be measured within the same reaction. Each sample was run with a no-Reverse Transcriptase control. If the CT value of the Reverse Transcriptase sample was greater than or equal to the no-Reverse Transcriptase control CT, it was considered to not be expressed. Expression of hAGT was quantified via a standard curve from 30 to 3x10\textsuperscript{5} copies of recombinant hAGT cDNA (OriGene Technologies, Inc.), and was normalized to actin.

Animals, Surgery, and Pharmacology: Male and female mice 12-20 weeks of age were used in this study. All procedures were approved by the University of Iowa Animal Care and Use Committee in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Double transgenic sRA\textsuperscript{Red} mice consist of transgenic mice expressing human renin selectively in all neurons via the synapsin promoter (sR mice) bred with A\textsuperscript{Red} transgenic mice (50; 55). There were no differences in survival comparing sRA\textsuperscript{Red} mice with
single transgenic or non-transgenic littermates before or after AdCRE. Experimental mice were sRARed whereas control mice consisted of either single transgenic sR, single transgenic ARed mice, or non-transgenic littermates. There was no increase in drinking when these control mice were given AdCRE ICV. Cre-recombinase reporter mice (B6.Cg-Gt(ROSA)26Sor tm14(CAG-tdTomato)Hze/J) mice were obtained from the Jackson laboratory (stock number 007914).

ICV injection or placement of a chronic ICV cannula was performed as described previously (18). For acute ICV injection or placement of a chronic ICV cannula, anesthesia was induced with 5% isoflurane in O2 and maintained with 2 ± 1% isoflurane in O2 (33). Mice were placed in a stereotaxic apparatus, and the lateral ventricle was located at the following coordinates: AP: 0.3 mm; ML: 1.0 mm; DV: 3.0 mm. One μL of adenovirus was injected via a Hamilton syringe over 5-minutes. In order to selectively activate the ARed transgene in the SFO, 1 μL of Ad5CMV-Cre or Ad5-CMV-eGFP (1x10⁷ pfu/μL; University of Iowa Gene Transfer and Vector Core) was injected ICV via a Hamilton syringe over 5-minutes (60; 61). Mice recovered from anesthesia in their home cage on a hot pad for 24 hours after surgery. After ICV injection of adenovirus, mice were placed in a special room in the vivarium for 1 week as they were shedding virus after which time they were transferred to their normal room or were used for experiments. For ICV cannula placement, 26G guide cannulas were stereotaxically placed into the lateral ventricle, held in place with cranial screws and dental acrylic, and capped until injection with a 33G internal cannula (PlasticsOne, Inc.). ICV injections through the cannula were performed with a cannula injector attached to a gas-tight Hamilton syringe via tubing. Mineral oil was loaded into the tubing, and an air bubble was present between the drug and mineral oil to visualize movement.

Fluid intake was measured via a burette in either a home or metabolic cage (Nalgene). In some experiments, mice housed in metabolic cages were given a two-bottle comparator choice
between de-ionized water and different molarities of NaCl made in de-ionized water (30). For these experiments, mice were ICV injected with AdCRE. Eighteen days after injection they were placed into metabolic cages (Nalgene) and were given a two-bottle choice between water and 0.15M NaCl. After 2 days acclimation, 24 hour fluid and food, and body weight were measured and averaged for 2 days. The comparator drink was then changed to water and 0.3M NaCl, and they were given a day of acclimation after which time similar measurements were taken for 2 days; this was repeated for 0.5M NaCl.

Losartan (dose per figure legend; Sigma), a blocker of ANG-II AT1R, bisindolylmaleimide I (BIM, 4 μg in 2 μL of aCSF; Cell Signaling), a blocker of PKC, and FR180204 (4 μg in 2 μL of DMSO; Santa Cruz) a blocker of ERK1/2 were injected into sRARed and littermate control mice as described previously (18). The maximum change in water intake of Losartan from aCSF was calculated by 3-parameter, sigmoidal regression.

Blood Pressure and Metabolism: Tail-cuff (Visitech Systems BP-2000) and radiotelemeter (TA11PA-C10; Data Sciences International, DSI) measurements of blood pressure were performed as described previously (29). For tail cuff, mice were acclimated to the apparatus for 1 week. After acclimation, 2 weeks of baseline measurements were taken from the average of 30 measurements daily. Once baseline measurements were obtained, mice were injected with AdCRE ICV as described above, and then daily measurements resumed 1 week later and was continued for 62 days. At day 24/25 after ICV injection of AdCRE, water intake was measured from their home cage as described above. For radiotelemetry, mice were implanted with radiotelemeters (TA11PA-C10; Data Sciences International, DSI) into the common carotid artery while under ketamine/xylazine anesthesia (33). The mice underwent placement of a cannula ICV, and they recovered from the surgery for at least 5 days. After recovery, spontaneous physical activity, blood pressure, and heart rate was recorded every 10 seconds for 5 minutes.
using Dataquest software (DSI). Baseline measurements were taken for 1 week. The mice
then underwent ICV injection of AdCRE, recovered for 1 week, and daily measurements were
taken for 5 weeks. Any mouse with a poor radiotelemeter signal, pulse pressure below 15
mmHg, or a systolic BP above 200 mmHg (indicative of an occluded catheter) was excluded
from further analysis. To ensure transgene activation in the cohort of mice that underwent blood
pressure measurement, water intake was measured 24 days after AdCRE.

Mice were placed into temperature controlled, insulated chambers for estimation of heat
production by respirometry (10). First, the CO₂ (model CD-3A, AEI) and O₂ analyzers (model S-
3A/II, AEI) were calibrated to standardized air containing 5,000 ppm CO₂ and 20.50% O₂
(Praxair), respectively. Mice were then placed into water-jacket temperature controlled and air-
sealed chambers, and the change in effluent O₂ and CO₂ concentrations were recorded using a
PowerLab and Chart software (AD Instruments). Flow was determined by mass flow meters
(EM1, Sensiron) to STP-correct flow values. Heat production was estimated using the equation
based on Lusk (1928): Heat = VO₂ (1.232 RER + 3.815); where: VO₂ = [ΔO₂%] * [STP-corrected
flow] and RER = ΔCO₂% / ΔO₂%.

Serum Chemistry: mice were euthanized with CO₂ and truncal blood was collected into non-
heparinized tubes stored on ice. Whole blood from the non-heparinized tubes was taken into a
syringe and 95 μL was injected into a CHEM-8+ I-Stat cartridge (Abbott Point of Care). The
cartridges were run on a VetScan i-Stat1 (Abbott Point of Care).

Immunohistochemistry: Mice were ICV injected with AdCRE. One month later they were
intracardially perfused with 4% paraformaldehyde and 0.1% gluteraldehyde in phosphate
buffered saline. The brains were removed, controls were marked for identification by a nick in
the cortex, sectioned frontally at 24 μm on a sliding microtome, collected into alternating wells
filled with phosphate buffered saline, and each well contained an experimental and control
group. Immunohistochemistry was performed for phosphorylated-CREB (1:400, Cell Signal Inc.)
using a biotinylated goat anti-rabbit IgG antibody (Vector Labs, Inc., #BA-1000) at a
concentration of 1:200. Biotin detection occurred by treating the sections with VECTASTAIN
ABC Elite kit (Vector Labs, Inc.), and then visualizing the immunoreactivity by 3,3’-
diaminobeznidine (DAB) plus hydrogen peroxide (SIGMAfast 3,3'-diaminobeznidine; Sigma
D4168). Sections were then rinsed in phosphate buffered saline, and cover slipped. The
number of immunopositive cell fragments was determined by counting cells greater in size than
a 3.0 μm diameter circle.

Statistics: We analyzed the data with one- or two-way ANOVA, with repeated measurements as
appropriate. Bonferroni multiple comparisons procedures were used to further explore
treatment effects. If equal variance or normality failed, we used non-parametric analysis of our
data, such as Mann-Whitney U or Wilcoxon tests. We considered significance at P<0.05, and
all data plotted are mean ± SEM.
Results

ICV AdCRE induces human angiotensinogen production in the SFO of sRARed mice

A construct with conditionally inducible human angiotensinogen expression was designed to test the hypothesis that production of ANG-II in the SFO is sufficient on its own to increase fluid intake. Upon Cre-mediated recombination, the gene encoding dsRED and a stop sequence are removed allowing the ubiquitous CAG promoter to drive expression of human angiotensinogen (A^Red construct; Figure 1A). The addition of Cre-recombinase to Cos-7 cells transfected with A^Red resulted in decreased dsRED fluorescence (Figure 1B), a 20-fold induction of human angiotensinogen mRNA, and an increase in hAGT protein (Figure 1C).

To test whether we can specifically induce human angiotensinogen expression in the SFO of A^Red mice, we used a method previously validated by us and others to selectively target the SFO (29; 59). As shown in CAG-LSL-tdTomato mice (Figure 1D), a reporter model for Cre-recombination, ICV injection of AdCRE induces robust expression of the tdTomato reporter in the SFO (Figure 1E). There was no induction of tdTomato expression in other regions of the brain including the median preoptic nucleus (MnPO), paraventricular nucleus, or rostral ventrolateral medulla in this experiment or in previously reported cohorts (13; 33). Consistent with the tdTomato reporter mice, ICV injection of AdCRE into A^Red transgenic mice significantly induced expression of human angiotensinogen mRNA selectively in the SFO (Figure 1F). There was no induction of human angiotensinogen mRNA when AdGFP was injected, and the induction in the paraventricular nucleus was not significantly different from that obtained with non-transgenic control mice.

Expression of the human RAS selectively in the SFO is insufficient to increase BP

We next bred mice expressing neuron-specific human renin (sR) with A^Red mice (double transgenic: sR^ARed) to allow Cre-mediated induction of ANG-II production in the SFO.
Unexpectedly, an analysis of multiple cohorts of sRA\textsuperscript{Red} mice revealed there was no significant difference in tail cuff systolic blood pressure (Figure 2A) or heart rate (Figure 2B) compared with control mice either before or after ICV injection of AdCRE. To ensure this was not due to a lack of sensitivity of the tail cuff measurement, we confirmed these results with radiotelemetry over a 5-week period after injection of AdCRE (Figure 2C-D). Five weeks is sufficient to mediate a blood pressure increase in the slow-pressor model of peripheral ANG-II administration (11; 21). Importantly, the same cohort of AdCRE-treated sRA\textsuperscript{Red} mice used for the tail cuff measurements of blood pressure exhibited increased water intake (at the end of the blood pressure experiment) compared with control mice (Figure 3A). This is important because it demonstrates that the lack of an increase in arterial pressure is not the result of a failed viral delivery or Cre-mediated recombination. The increase in water intake in AdCRE-treated sRA\textsuperscript{Red} mice was returned to normal by the AT\textsubscript{1}R blocker losartan (Figure 3B). The maximum change in water intake from vehicle to losartan was significantly greater in sRA\textsuperscript{Red} compared to control mice (Figure 3C). Thus, whereas increased expression of the human RAS specifically in the SFO of sRA\textsuperscript{Red} mice is sufficient to increase water intake, it is insufficient to raise blood pressure.

We previously showed that increased activity of the brain RAS increases resting metabolic rate (29; 30). We therefore sought to determine if an SFO-selective increase in human RAS activity increased metabolic rate. Like blood pressure, there was no difference in resting metabolic rate, oxygen consumption (VO\textsubscript{2}), and heat production estimated by respirometry compared with controls after injection of AdCRE (Table 1).

Expression of the human RAS selectively in the SFO is sufficient to increase fluid intake.

The data above suggest that increased activity of the human RAS in the SFO induces water intake when water is the only fluid offered. Consequently, we next examined fluid intake in mice given a two-bottle choice between water and isotonic saline (0.15M NaCl). There was a
significant increase in 24 hour fluid intake in 2 separate cohorts of sRA\textsuperscript{red} mice compared to controls starting 20 days after ICV injection of AdCRE (Figure 4A). This reflected an increase in 0.15M NaCl (Figure 4B) and total sodium uptake (Figure 4C), but not an increase in water intake (Figure 4D). Thus, when given a two-bottle choice with 0.15M NaCl, the increase in fluid intake occurred due to an increased preference for saline (Figure 4E). There was no change in food intake (Figure 4F). Urine volume in AdCRE-treated sRA\textsuperscript{red} mice was unchanged at baseline and at day 14 when there was no increase in fluid intake, but increased 1.9-fold at day 21 when the maximal increase in fluid intake was reached. We confirmed there was an increase in the expression of human angiotensinogen mRNA in the SFO of one of these cohorts similar to data in Figure 1 (data not shown). There were no changes in serum chemistry or renal function (Table 2).

We next examined the intake and preferences for increased concentrations of saline. sRA\textsuperscript{red} and control mice were placed into metabolic cages with a two-bottle choice between water and a comparator drink (0.15M, 0.3M or 0.5M NaCl) 20 days after ICV injection of AdCRE (Figure 5). When presented with 0.15M NaCl as the comparator drink, total fluid intake in this cohort showed a trend toward being increased in sRA\textsuperscript{red} mice (P=0.056), and like the previous experiment and our previous studies in sRA\textsuperscript{flox} mice, was a reflection of increased NaCl, but not water (30). When presented with 0.3M NaCl as the comparator drink, total fluid, water and sodium was increased, but there was no increase in the preference for hypertonic saline. Urine sodium was also increased in AdCRE-treated sRA\textsuperscript{red} mice given 0.3M NaCl (Figure 5F). There was no increase in fluid, water, or NaCl intake when 0.5M NaCl was offered. Urine volume increased 2.13- and 2.26-fold in sRA\textsuperscript{red} mice given 0.15M and 0.3M NaCl, respectively, but were not different from controls at 0.5M NaCl.

*Increase fluid intake in AdCRE-treated sRA\textsuperscript{red} is mediated by PKC.*
In addition to the evidence for SFO-specific expression of human angiotensinogen mRNA obtained above, we sought to obtain direct evidence of increased human angiotensinogen protein or ANG-II in the SFO of AdCRE-treated sRA\textsuperscript{Red} mice. However, the level of both products was below the level of detection by immunohistochemistry perhaps explaining why there was no increase in blood pressure. We therefore sought to assay for surrogate markers downstream of AT\textsubscript{1}R activation. We have previously shown that elevated fluid intake due to hyperactivity of the brain RAS is mediated through PKC-\(\alpha\) (13). We injected the pan-active PKC inhibitor BIM into AdCRE-treated sRA\textsuperscript{Red}. PKC inhibition corrected the increased water intake in AdCRE-treated sRA\textsuperscript{Red} lowering it to the level observed in control mice (Figure 6A-B). There was no change in water intake when the ERK pathway was inhibited in AdCRE-treated sRA\textsuperscript{Red} mice with FR180204 (Figure 6C-D). Phosphorylation of CREB has been shown to be a downstream effector of ANG-II AT\textsubscript{1}R action in the rostral ventrolateral medulla and vascular smooth muscle cells (12; 67). We observed both an increase in the intensity (Figure 7) and number of phospho-CREB labeled cells (Figure 8) within the SFO and MnPO of sRA\textsuperscript{Red} compared to control mice. There was no increase in the number of phospho-CREB positive cells in the paraventricular nucleus, supraoptic nuclei, nucleus accumbens, or lateral hypothalamus, nor in the cortex and hippocampus (data not shown).
Discussion

There are several important findings in this study. First, the induction of human RAS expression selectively in the SFO of transgenic mice causes increased water intake that is mediated by an AT$_1$R- and PKC-dependent mechanism. Second, increased fluid intake, when given as a choice between 0.15M NaCl and water, is due to increased consumption of 0.15M NaCl but not water, suggestive of an increased preference for saline under non-aversive conditions. Third, when the mice are provided a choice between 0.3M NaCl and water, their increased fluid intake was largely due to a preference for water, although they exhibited increased sodium intake as well. There was no increase in fluid intake when 0.5M NaCl was provided suggesting it was aversive. From this we can conclude that localized production of ANG-II in the SFO in this model induces drinking, but does not alter sodium appetite, formally defined as increased preference for NaCl under aversive conditions. Finally, SFO-selective expression of the human RAS is insufficient to increase arterial pressure or resting metabolic rate, at least at the level of expression observed in the AdCRE-treated sRA$^{\text{Red}}$ model. Thus, it is possible that the neural pathways controlling drinking may be more sensitive to ANG-II, than the pathways controlling blood pressure or metabolism.

It has long been known that direct administration of ANG-II to the SFO induces drinking, and that the induction of drinking in response to centrally or peripherally administered ANG-II can be blocked by lesions of the SFO (49; 57; 58). We previously showed that over-expression of the human renin and angiotensinogen genes throughout the brain (the sRA$^{\text{Flox}}$ model) results in a marked elevation of both water intake and blood pressure that is dependent upon expression of the ANG-II substrate in the SFO (59). In a recent study, we showed that the increase in ANG-II-mediated drinking is due to activation of PKC$\alpha$ in the SFO (13). In the current study, we generated a new model to address if SFO-selective induction of ANG-II production is sufficient on its own to mediate increased drinking and blood pressure. Unlike the sRA$^{\text{Flox}}$ model where
Cre-recombinase causes an ablation of human angiotensinogen expression, in the sRA\textsuperscript{Red} model, angiotensinogen expression and consequently ANG-II production is induced by Cre-recombinase. The strength of this model is that the A\textsuperscript{Red} construct allows for temporal and spatial control over the production of ANG-II dictated by the site of Cre-recombinase expression in the presence of human renin.

We showed that ICV injection of AdCRE into A\textsuperscript{Red} mice significantly induces the production of human angiotensinogen mRNA in the SFO, but not the MnPO. While the increase in human angiotensinogen mRNA in the paraventricular nucleus was not significant, there was a trend for its induction which may be due to inter-animal variability. There is substantial evidence supporting an angiotensinergic neural connection between the SFO and the paraventricular nucleus which utilizes ANG-II as the neurotransmitter (reviewed in (24)). Moreover, ANG-II directly injected into the paraventricular nucleus increases blood pressure (3). Thus, we cannot rule out that in some mice, increased ANG-II production in the paraventricular nucleus may have contributed to the phenotypes we observed. We also cannot rule out the possibility of increased production of human angiotensinogen in other relevant nuclei. This may be important as nuclei in the anteroventral third ventricle (AV3V) region, which have extensive interconnectivity with the SFO, have been implicated in mediating drinking responses to ANG-II (7). For example, although there was no evidence for induction of human angiotensinogen in the MnPO, and no evidence that AdCRE caused recombination in the MnPO, we found elevated phosphorylated CREB in the MnPO of AdCRE-treated sRA\textsuperscript{Red} mice. The increase in phosphorylated CREB could be an indicator of Ang-II-mediated neural activity in the MnPO, and would be consistent with extensive angiotensinergic interconnectivity between the SFO and MnPO, and the role of the MnPO in drinking (14; 25; 35; 43).

There were several surprising results of our study. First, the increase in fluid intake in AdCRE-
treated sRAT treated mice occurred over a period of 3 weeks. This is surprising because direct
injection of ANG-II centrally or directly into the SFO elicits a rapid increase in drinking behavior
(56; 58). The increase in water intake we observed in sRAFlo mice was quite robust, but
because those transgenes were not inducible and expressed life-long, there was no way to
determine when the dipsogenic response started. We know that the production of human
angiotensinogen mRNA in AdCRE-treated sRAT infected mice was an order of magnitude lower than
observed in sRAFlo mice, and human angiotensinogen protein and angiotensin peptides were
both undetectable in sRAT infected mice. Thus the delayed drinking response may result from an slow
accumulation of ANG-II over time. We are certain that the increased drinking is ANG-II-
dependent because single transgenic sR or AAT infected mice treated with AdCRE had no response,
and is AT1R-dependent because it was normalized with losartan. The delayed response may
also be due to the observation from some studies that compared with rats, higher doses of
ANG-II have been reported to be required to mediate drinking responses in mice (54). Other
studies reported similar drinking responses to central ANG-II between mice and rats (19; 36).

The second surprising finding was the lack of an increase in baseline blood pressure. Lesions
of the SFO block the pressor action of peripheral ANG-II, AT1R in the SFO are thought to
mediate responses to some psychological stressors, and hypertension can be induced by
electrically stimulating the SFO (34; 38; 48; 56). The SFO richly expresses AT1R, which are
required to mediate changes in blood pressure in response to central sodium and DOCA-salt
(33; 65). On the contrary, lesions of the SFO did not attenuate experimental renal hypertension
nor the hypertension caused by short term ANG-II infusion, and in fact augmented blood
pressure in the latter (4; 37). We have to consider the possibility that whereas the level of
expression of human renin and angiotensinogen may have been sufficient to stimulate the
neural pathways involved in drinking, it may have been insufficient to stimulate the pathways
required to mediate an increase in blood pressure. Additional studies would be needed to test
the hypothesis that there is a differential sensitivity to the dipsogenic and pressor actions of
ANG-II in the SFO. Lesion studies suggest that other brain regions in addition to the SFO
mediate blood pressure increases to ANG-II in a model of Ang-II/high salt hypertension (52).
Differential effects of ANG-II on blood pressure and drinking were also observed in Sprague-
Dawley in response to AT$_2$R inhibition (66).

AdCRE-treated sRA$^{\text{Red}}$ mice increase their water intake when water is the only fluid offered.
This is likely due to the direct effects of ANG-II within the brain because it is blocked by ICV
administration of either losartan or an inhibitor of PKC, and because there is no evidence for an
alteration in renal function and serum chemistry. We recently showed that drinking in the
sRA$^{\text{Flo}}$ model is due to PKC$\alpha$ activation in the SFO (13). In rats, ANG-II stimulates central
PKC$\alpha$ activity and PKC blockers prevent the dipsogenic response to ICV ANG-II (27). On the
contrary, there was no effect on ANG-II-stimulated water intake when the ERK pathway was
blocked. This is consistent with studies showing that ANG-II-induced water and salt intake are
mediated by divergent intracellular signaling cascades downstream of the AT$_1$R, PKC for water
intake and MAP kinase for NaCl intake (15; 16; 22). This divergence in AT$_1$R signaling
pathways controlling water and salt intake was recently supported by the identification of a novel
small peptide encoded by the AT$_1$R gene which selectively blocks the ANG-II-ERK1/2 pathway
both in vitro and in vivo (45). As we did not measure the effect of ERK inhibition when mice
were provided saline we are unable to determine if the ERK pathway is selectively activated in
response to SFO ANG-II under those conditions.

It is well established that central ANG-II causes increased intake of dilute saline solutions (5; 6;
22) and reviewed in (26). Consistent with this, when given a choice between 0.15M NaCl and
water, sRA$^{\text{Red}}$ mice prefer saline over water. On the contrary, when offered a choice between
0.3M NaCl or water, the preference is for water; and thus sRA$^{\text{Red}}$ mice do not exhibit an increase in salt appetite despite the preference for palatable saline. Increased sodium appetite has been reported in sodium-depleted rats caused by co-administration of furosemide and captopril, which was attenuated by lesion of the SFO (46; 51). Rats given peripheral injections of either renin or ANG-II, central injections of ANG-II, or DOCA-salt also exhibit a sodium appetite (1; 5). Sodium appetite is also increased in central ANG-II-treated transgenic mice over-expressing AT$_1$R receptors selectively in neurons (41). It is possible that we did not observe an increase in sodium appetite in sRA$^{\text{Red}}$ mice because the mice were sodium replete on a standard rather than low-sodium diet, or that the low level of ANG-II produced in this model did not gain access to the MnPO, although this is not consistent with our data showing increased phosphorylated CREB in the MnPO (25). It should be noted that the mice used to measure phosphorylated CREB were not provided saline. It is notable that compared with rats, mice are much more resistant to ANG-II-induced drinking despite a similar increase in c-fos staining in the SFO (54). Also, sRA$^{\text{Flox}}$ mice exhibit the same pattern of increased sodium intake when palatable, but not when aversive, and therefore exhibit increased “intake” but not “appetite” (30). Thus our data using ICV AdCRE-treated sRA$^{\text{Red}}$ mice suggest that SFO-specific ANG-II hyperactivity is sufficient to recapitulate the fluid intake patterns that are present with whole-brain RAS hyperactivity in mice.

Perspectives and Significance

We show that production of ANG-II selectively in the SFO is sufficient on its own to increase sodium and fluid intakes via a preference for palatable saline, though there is no increase in sodium appetite or change in blood pressure. This suggests that the mechanisms controlling drinking and blood pressure in response to local production and action of ANG-II in the SFO may differ, either in terms of its dose response, or more likely the cellular specificity of where ANG-II acts. Whether ANG-II acts on neurons projecting to nuclei controlling water and
electrolyte homeostasis or to nuclei controlling the preganglionic neurons of the sympathetic nervous system likely dictates the response.
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Disclosures: None.
References


25. Fitts DA, Freece JA, Van Bebber JE, Zierath DK and Bassett JE. Effects of forebrain circumventricular organ ablation on drinking or salt appetite after


Table 1: Resting metabolism as measured by indirect calorimetry in littermate control (n=6) and sRAd mice (n=6) 68-days after ICV injection of AdCRE.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>sRAd</th>
</tr>
</thead>
<tbody>
<tr>
<td>VO₂ (mL/min)</td>
<td>0.80 ± 0.02</td>
<td>0.71 ± 0.03</td>
</tr>
<tr>
<td>VO₂ (mL/100 g/min)</td>
<td>3.26 ± 0.17</td>
<td>2.95 ± 0.22</td>
</tr>
<tr>
<td>RQ</td>
<td>0.93 ± 0.01</td>
<td>1.02 ± 0.10</td>
</tr>
<tr>
<td>Heat (kcal/hr)</td>
<td>0.24 ± 0.01</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Heat (kcal/kg/hr)</td>
<td>9.70 ± 0.52</td>
<td>8.90 ± 0.53</td>
</tr>
<tr>
<td>TCO₂</td>
<td>24.00 ± 0.91</td>
<td>24.33 ± 0.49</td>
</tr>
</tbody>
</table>
Table 2: Serum chemistry of littermate control (n=5) and sRA<sup>Red</sup> (n=6) 32-days after ICV injection of AdCRE (B).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>sRA&lt;sup&gt;Red&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt; (mmol/L)</td>
<td>148.50 ±1.04</td>
<td>148.00 ±0.97</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt; (mmol/L)</td>
<td>6.98 ±0.39</td>
<td>6.87 ±0.18</td>
</tr>
<tr>
<td>Cl&lt;sup&gt;-&lt;/sup&gt; (mmol/L)</td>
<td>116.25 ±0.63</td>
<td>116.50 ±0.89</td>
</tr>
<tr>
<td>iCa&lt;sup&gt;2+&lt;/sup&gt; (mmol/L)</td>
<td>1.20 ±0.03</td>
<td>1.20 ±0.05</td>
</tr>
<tr>
<td>AnGap (mmol/L)</td>
<td>15.75 ±0.48</td>
<td>16.20 ±0.89</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>21.00 ±3.29</td>
<td>23.67 ±2.04</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.23 ±0.03</td>
<td>0.20 ±0.00</td>
</tr>
<tr>
<td>Hct (%PCV)</td>
<td>46.50 ±1.04</td>
<td>46.00 ±0.58</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1: Inducible expression of human angiotensinogen.

A) Map of the A^RED construct. Cre-mediated deletion of the dsREN and STOP signal will allow transcription of human angiotensinogen. B) DsRED fluorescence in A^RED transfected COS cells infected with either AdGFP or AdCRE. C) Expression of human angiotensinogen protein in response to AdCRE in A^RED transfected COS cells. D) Map of the Cre-reporter construct. Cre-mediated deletion of the STOP signal will allow transcription of tdTomato. E) One-month after ICV injection of AdCRE in ROSA-TdTomato mice, fluorescence was found exclusively in the SFO (A; SFO at arrow; 3V=third ventricle; * indicates corpus callosum). F) A^Red construct induces the expression of human angiotensinogen cDNA after Cre-mediated recombination. Expression of human angiotensinogen (copies normalized to actin) in the MnPO, SFO, and paraventricular nucleus of A^Red mice (AdGFP, n=10; AdCRE, n=20) compared to control mice (AdGFP, n=8; AdCRE, n=8) after ICV injection of AdCRE. White bar is 100 μm. *P<0.01, Bonferroni post-hoc comparison.

Figure 2: Blood pressure in sRA^Red mice.

Tail-cuff was used to measure systolic BP (A) and heart rate (B) in control (n=6) and sRA^Red mice (n=6) before and up to 9-weeks after ICV injection of AdCRE. Radiotelemeters were used to measure 24 hour mean arterial pressure (MAP; C) and heart rate (D) in control (n=3-5) and sRA^Red mice (n=4-5) before and 5-weeks after ICV injection of AdCRE.

Figure 3: Water intake in sRA^Red mice.

A) Water intake in one of the cohorts of control and sRA^Red mice used for the tail cuff measurements in Figure 1 was taken at the end of the blood pressure experiment. B) Twenty-four hour water intake after either aCSF or increasing doses losartan ICV in littermate control (n=4) or sRA^Red (n=5) mice previously injected with AdCRE ICV. C) 3-parameter logarithmic
regression was used to determine the max change in water intake from aCSF to losartan ICV.

*P<0.05, †P=0.056, Bonferroni post-hoc comparison.

Figure 4: Fluid intake in 2-bottle choice test.
Intakes of total fluid (A), 0.15M NaCl (B), total sodium (C), water (D), sodium preference (E), and food (F) in control (n=6-13) and sRA\textsuperscript{Red} (n=6-12) mice before and up to 24 days after ICV injection of AdCRE. Mice were given a two-bottle comparator drink between 0.15M NaCl and water. There are statistically significant interactions between genotype and period for total intakes of fluid and sodium. *P<0.05, Bonferroni post-hoc comparison.

Figure 5: Fluid intake in 2-bottle choice test with different comparator drinks.
Total fluid (A), water (B), saline (C), and sodium (E) intakes in littermate control (n=9) and sRA\textsuperscript{Red} (n=6) mice after ICV injection of AdCRE and given sequential two-bottle comparator drink tests of water versus NaCl (0.15M, 0.3M, or 0.5M). Preference for drinking NaCl (D) and excretion of sodium in the urine (D) of control and sRA\textsuperscript{Red} mice. There are statistically significant interactions between genotype and molarity for total fluid intake and urine sodium.

*P<0.05, †P=0.056, Bonferroni post-hoc comparison.

Figure 6: Water intake in response to PKC and ERK inhibition.
Water intake (A) and the change in water intake (B) of control (n=8) and sRA\textsuperscript{Red} (n=11) mice previously injected ICV with AdCRE and treated with either aCSF or BIM. Total fluid intake (C) and the change in total fluid intake (D) of littermate control (n=5) and sRA\textsuperscript{Red} mice (n=7) previously injected ICV with AdCRE, given a two-bottle comparator drink of water versus 0.3M NaCl, and treated with either aCSF or FR180204 ICV. *P<0.05, Bonferroni post-hoc comparison, and †P<0.05, independent t-test.
Figure 7: Expression of P-CREB.

Representative images of phosphorylated-CREB within the SFO and MnPO of littermate control and sRA\textsuperscript{Red} mice previously injected ICV with AdCRE; Scale is 100 μm for the SFO and 200 μm for the MnPO. cc indicates corpus callosum; 3V indicates third ventricle; ac indicates anterior comissure.

Figure 8: Quantification of P-CREB.

The number of immuno-positive cells within the SFO, MnPO, paraventricular nucleus (PVN), and supraoptic nuclei (SON) of littermate control (n=5) or sRA\textsuperscript{Red} (n=5-8) mice previously injected with AdCRE ICV (C). *P<0.05 vs control.
Figure 1

A. Schematic representation of the gene expression system with AdGFP and AdCRE.

B. Fluorescence images showing AdGFP and AdCRE infection.

C. Western blot analysis of hAGT expression with AdGFP and AdCRE.

D. Schematic representation of the gene expression system with AdGFP and AdCRE.

E. Immunofluorescence images showing tdTomato expression in MnPO, SFO, and PVN.

F. qPCR analysis of hAGT expression levels in MnPO, SFO, and PVN with AdGFP, AdCRE, and AdGFP, ARED.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8