Mechanisms of brain renin angiotensin system-induced drinking and blood pressure: importance of the subfornical organ

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Coble JP, Grobe JL, Johnson AK, Sigmund CD. Mechanisms of brain renin angiotensin system-induced drinking and blood pressure: importance of the subfornical organ. Am J Physiol Regul Integr Comp Physiol 308: R238–R249, 2015. First published December 17, 2014; doi:10.1152/ajpregu.00486.2014.—It is critical for cells to maintain a homeostatic balance of water and electrolytes because disturbances can disrupt cellular function, which can lead to profound effects on the physiology of an organism. Dehydration can be classified as either intra- or extracellular, and different mechanisms have developed to restore homeostasis in response to each. Whereas the renin-angiotensin system (RAS) is important for restoring homeostasis after dehydration, the pathways mediating the responses to intra- and extracellular dehydration may differ. Thirst responses mediated through the angiotensin type 1 receptor (AT1R) and angiotensin type 2 receptors (AT2R) respond to extracellular dehydration and intracellular dehydration, respectively. Intracellular signaling factors, such as protein kinase C (PKC), reactive oxygen species (ROS), and the mitogen-activated protein (MAP) kinase pathway, mediate the effects of central angiotensin II (ANG II). Experimental evidence also demonstrates the importance of the subfornical organ (SFO) in mediating some of the fluid intake effects of central ANG II. The purpose of this review is to highlight the importance of the SFO in mediating fluid intake responses to dehydration and ANG II.

angiotensin; blood pressure; barin; fluid; renin

FLUID BALANCE is crucial for the homeostasis and survival of organisms because they have to properly maintain fluid and electrolyte concentrations for the normal function of all cells. Fluid balance can go awry in pathological states such as in chronic kidney disease and diabetes. Among its many roles in maintaining homeostasis, the renin-angiotensin system (RAS) is an important regulator of fluid balance. The RAS achieves this through the actions of its main effector peptide, angiotensin II (ANG II) through the ANG II type 1 (AT1R) and type 2 (AT2R) receptors. Activation of AT1R by blood-borne ANG II in target tissues causes increases in sodium and water retention, arginine vasopressin (AVP), and aldosterone release, vasoconstriction, increased sympathetic nervous system activity, and increased fluid intake. ANG II is produced by the consecutive enzymatic action of renin on its substrate angiotensinogen (AGT) and by angiotensin-converting enzyme (ACE) on its substrate angiotensin I (ANG I). The renin-AGT cleavage step is generally the rate-limiting step in the production of ANG II, and this is certainly true in the brain where the amount of renin is limiting. The RAS exists in two forms: a circulatory form, where local production of ANG II acts in an autocrine or paracrine manner to induce AT1R signaling on ANG II producing or nearby cells (75). Intracrine, or intracellular forms of the RAS, have also been described (46, 109). Although the generation and action of other angiotensin peptides in the brain (such as ANG[1–7] by ACE2) has garnered considerable attention (147), this review will focus primarily on ANG II.

Localization of RAS Components Required for ANG II Production

In 1961, Bickerton and Buckley (5) demonstrated that angiotensin injections into the brain increased blood pressure. In 1968, Booth (7) demonstrated that injection of angiotensin amide into the hypothalamus caused a robust and specific drinking response. Finally in 1971, Ganten and colleagues (40) demonstrated that renin was expressed within the brain of nephrectomized dogs. Subsequent studies of primary neuronal and glial cultures, and from whole mouse and rat brains, identified that renin is mainly expressed in neurons, though it can also be found in glial cells (39, 50, 53). Several genetic strategies have also been useful to identify the cell types expressing renin in the brain. In the first, enhanced green fluorescent protein (eGFP) was expressed in transgenic mice (Ren-1-GFP) using a region of the mouse Ren-1 promoter shown to accurately target renin expressing cells (65, 72, 73). In the second, knock-in mice expressing Cre-recombinase inserted into the Ren-1 locus were bred with mice that express β-galactosidase in response to Cre-recombinase (145). Another
study took a similar strategy but employed the human renin promoter to drive expression of Cre-recombinase in mice also carrying a Cre-activatable reporter (1). The latter two approaches identify cells which at any time in their developmental history expressed renin. Renin was expressed mainly in neurons of nuclei important for cardiovascular regulation, such as the subfornical organ (SFO), parabrachial nucleus (PB), rostral ventrolateral medulla (RVLM), and paraventricular nucleus of the hypothalamus (PVN). Renin was also possibly found to be expressed in oligodendrocytes of the RVLM and hippocampus, but not in astrocytes.

AGT is expressed at much higher levels than renin in the brain and is easily detectable by immunohistochemistry and in situ hybridization. Both methods show that AGT is widely expressed in astrocytes (32, 131), and in those cells, was localized in the nucleus (122). Genetic deletion of glial-specific AGT lowers arterial pressure in double transgenic mice expressing both human renin and human AGT (121), and antisense inhibition of glial AGT in rats results is altered baroreflex regulation and increased exercise tolerance (43, 119). AGT is also expressed in neurons, specifically within nuclei of the brain important for cardiovascular (CV) regulation, such as the SFO, RVLM, PB, and PVN (58, 136, 150). ACE is widely expressed in areas important for CV regulation; e.g., the SFO, organum vasculosum of the lamina terminalis (OVLT), PVN, and supraoptic nucleus of the hypothalamus (SON) (14, 91, 110).

Using a dual transgenic reporter system, we showed that renin and AGT are coexpressed within cells of the SFO, PB, CA1–3 of the hippocampus, and the central nucleus of the amygdala (CeA) (72). The dual reporter model also revealed that renin and AGT are expressed in adjacent cells within the inferior olivary nucleus, reticular formation, ventromedial nucleus of the hypothalamus, CA1–3 of the hippocampus, RVLM, PVN, SCN, SFO, and the CeA. Consistent with a concept for coexpression of renin and AGT, angiotensin peptides were identified within cell bodies of the magnocellular parts of the SON and PVN, the cell bodies and fibers of the SFO, and the cell bodies of the BNST, CeA, and nucleus tractus solitarius (NTS) (81). The presence of intracellular angiotensin peptides is also consistent with molecular and functional evidence for the expression of a novel intracellular form of renin in the brain (74, 125), although genetic analysis revealed that the intracellular form of renin cannot compensate for a loss of renal-derived secreted renin (146). These studies support the hypothesis for local activation of the RAS within the brain and data supporting the concept that ANG II is synthesized within the SFO and acts in the PVN as a neurotransmitter (35, 78).

Increased expression of both the human renin and AGT genes in the brain results in increased blood pressure and water intake (96, 97) and depending on the cellular sites of production (glia vs. neurons) can differentially modulate baroreflex function (118). Moreover, increased fluid intake and blood pressure caused by increased production of ANG II in the brain is attenuated when the source of the ANG II substrate, AGT, is selectively deleted from the SFO (117, 126), and mice selectively expressing ANG II only in the SFO exhibit increased fluid intake (20). These studies convincingly show that ANG II production in the SFO plays an important role in both blood pressure and fluid balance. It is notable that unlike rats, mice appear to be resistant to the dipsogenic effects of peripheral ANG II, but will increase water intake after ANG II is directly injected into the brain, albeit with lower sensitivity (31, 114).

Localization of RAS Components Required for ANG II Action: Focus on ANG II Receptors

Mice and rats express two isoforms of AT1R termed AT1A and AT1B. AT1A and AT1B are differentially expressed (12), and in the brain are differentially regulated by dehydration and dietary sodium (18, 19). A comparison of AT1AR-deficient and AT1BR-deficient mice revealed that the pressor response to central ANG II infusion is mediated by AT1AR (24). On the contrary, AT1BR appears to be important for central ANG II-induced fluid intake since the polydipsia that occurs with intracerebroventricular ANG II is attenuated in AT1BR knockout mice (24). Mice deficient in AT1R exhibited an enhanced pressor response to intracerebroventricular ANG II, and the polydipsia caused by central ANG II infusion was abrogated in mice deficient in both AT1AR and AT2R (79). The difference between the effects of AT1AR and AT1BR, in particular, highlight a molecular divergence of pathways that mediate central ANG II phenotypes; i.e., fluid intake can be separated from blood pressure, at least in mice. Humans express only a single isoform of the AT1R, which is typically but incorrectly attributed only to the AT1AR. Studies on the role of these receptors in the dipsogenic response to ANG II will be discussed below.

The localization of AT1R expression in the brain and elsewhere has been reported in studies too numerous to cite here (reviewed in Ref. 2). Moreover, immunocytochemical detection of these receptors has been hampered by the recent recognition that antiser for specifically detect either AT1R (51) and AT2R (48) actually exhibit very poor specificity. Consequently, other approaches are required. Gonzalez et al. (41) used the NZ44 transgenic mouse (on the C57BL/6 genetic background) generated by the “Gene Expression Net- silgent System Atlas” (also known as GENSAT) where egFP was placed under the control of the mouse AT1AR promoter in a large bacterial artificial chromosome containing the AT1AR locus. They reported that AT1AR is expressed in a variety of nuclei throughout the forebrain, midbrain, and hindbrain. Of importance for CV regulation, AT1AR was highly expressed in neurons of the SFO, OVLT, SFO, SON, PVN, CeA, RVLM, and NTS. In the SFO, both the perikarya and dendrites were intensely labeled. This was particularly true in the dorsolateral outer shell and ventromedial core of the SFO, which, as will be described below, appears to be important for blood pressure regulation. In the PVN, AT1AR-positive cells were densely packed together, were found in both perikarya and dendrites, but did not coexpress AVP. Astrocytes in the PVN also expressed AT1AR. There was light labeling for AT1AR in the caudal portion of the SON where AVP-producing cells are generally found. In the NTS, many cells were labeled in the medial and commissural subdivisions, and AT1AR was also expressed within astrocytes. Most of the cells in the RVLM expressed AT1AR on both their perikarya and dendrites, and these cells coexpressed tyrosine hydroxylase implying that they are serotonergic and part of the sympathetic nervous system.

Overexpressing AT1AR in all neurons of mice increases sympathetic tone, water and saline intake, and the pressor...
Table 1. Afferent and efferent connections of the SFO

<table>
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<tr>
<th>Afferent</th>
<th>Efferent</th>
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<tbody>
<tr>
<td>MnPO</td>
<td>MnPO (****)</td>
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<tr>
<td>SON</td>
<td>OVLT (****)</td>
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<tr>
<td>PVN (thalamus and hypothalamus)</td>
<td>SON (*)</td>
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<tr>
<td>MnPO</td>
<td>Lateral preoptic area</td>
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<tr>
<td>Anterior hypothalamic nucleus</td>
<td>Lateral hypothalamus</td>
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<tr>
<td>Substantia innominata</td>
<td>PVN</td>
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<tr>
<td>BNST</td>
<td>SCN</td>
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<tr>
<td>Rostral parts of the zona incerta</td>
<td>Medial habenular nucleus</td>
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<td>NTS</td>
<td>Infrafimbic area of the prefrontal cortex</td>
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SFO, subfornical organ; OVLT, organum vasculosum of the lamina terminalis; SON, supraoptic nucleus; PVN, paraventricular nucleus of the hypothalamus; MnPO, median preoptic nucleus; BNST, basal nucleus of the stria terminalis; SCN, suprachiasmatic nucleus; NTS, nucleus tractus solitarius. The number of * indicates the relative density of efferent fibers from the SFO.

response to ANG II, responses that are AT1R dependent (76, 77). The first genetic studies probing the function of AT1AR in specific nuclei of the brain were performed using RNA interference (17). More recently, the importance of AT1AR in the SFO has been examined by genetic manipulation in mice carrying a conditional allele of the AT1AR (AT1ARFlox) gene. Intracerebroventricular injection of an adenovirus encoding Cre-recombinase (AdCRE) (127, 128) in AT1ARFlox mice results in an SFO-specific decrease in the expression of AT1AR mRNA, which attenuated the water and saline intake and blood pressure response to deoxycorticosterone acetate (DOCA) salt (44). Coupled with the discussion above, these studies highlight the importance of both ANG II production and action in the SFO in regulating blood pressure and fluid homeostasis. Details of the cellular mechanisms mediating these pathways in the SFO will be discussed later in this review.

Subfornical Organ: Anatomy, Connections, and Physiological Functions

The SFO can be anatomically subdivided into a core and peripheral portion. The core of the SFO is highly vascularized, and the local vasculature lacks a blood-brain barrier (BBB). Ultrastructural examination of the SFO has demonstrated that the capillaries have tongue-like projections and pinocytic vesicles in the endothelial cell layer. These projections and vesicles provide an anatomic basis for the SFO to be exposed to factors in the blood (28). Fluorescence was observed in the core portion of the SFO when a small-molecular-weight fluorescent marker was injected in the heart of mice (98). Thus the core of the SFO is anatomically situated to be permeated by blood-borne, low-molecular-weight molecules, such as ANG II, aldosterone, and AVP. The peripheral portion of the SFO is not as highly vascularized as the core, but it is located to respond to factors in the cerebrospinal fluid (CSF), such as ANG II and sodium (28).

The SFO protrudes ventrally from the fornix into the third ventricle just caudal to the foramen of Monroe at the confluence of the lateral to third ventricles. The lateral ventricles produce CSF and contain cilia to aid in the circulation of CSF through the ventricular system. The CSF has to converge and pass by the SFO located near the foramen of Monroe. Consequently, CSF bastes the SFO as it flows through the ventricular system. Ultrastructural examination of the SFO demonstrates that some SFO neurons project into the ventricles (29, 86). These projections have vesicles that indicate either release into or uptake of CSF from the ventricles. Ependymal cells located along the ventricular wall also form channels into the SFO. This may be another means for the SFO to secrete into or extract factors from the CSF. Furthermore, tanycytes lining the ventricles around the SFO project from the ventricles to neurons within the SFO. These tanycytes contain vesicles that could sample factors from the CSF. Thus the core portion of the SFO is anatomically situated to respond to blood-borne factors while the peripheral portion of the SFO is positioned to respond to factors in the CSF, making the SFO a unique sensory organ.

The SFO is also a distinctive nucleus in the brain in that its afferent and efferent projections are well placed to respond to and integrate both blood-borne and central nervous system (CNS) signals (Table 1). The rostral portion of the SFO mainly contains efferent axons while the caudal portion contains cell bodies (30). The SFO sends efferent axonal projections to the MnPO, OVLT, SON, the lateral preoptic area, the lateral hypothalamus adjacent to the SON, PVN, suprachiasmatic nucleus (SCN), and the medial habenular nucleus (82, 93, 94, 134) (Fig. 1). The connection to the MnPO appears to be...
especially dense since it has high terminal labeling (33, 82). After the MnPO, the OVLT followed by the SON appear to be the next nuclei, respectively, receiving the highest number of efferent projections from the SFO (94). In the SON, it is primarily the magnocellular (i.e., vasopressinergic) neurons that receive SFO input (13, 93, 133); and injection of a retrograde tracer into the SON demonstrated that it is mainly the peripheral portion of the SFO that projects to the SON (64). The connection of the SFO to the magnocellular PVN (mPVN) has also been demonstrated by an injection of the neuronal tracer, biotinylated dextran amine (BDA), into the peripheral portion of the SFO (68). In addition, injecting two different retrograde tracers into the MnPO and PVN revealed that a small proportion of SFO neurons in the periphery have collateral projections to both the MnPO and PVN that likely affect the vasopressin system (33). Neurons in the core portion of the SFO also project to the parvocellular PVN (pPVN), which synthesizes corticotropin-releasing hormone, and to the BNST (133). The anatomic specificity for SFO efferent projections was confirmed by injecting BDA into the mPVN or pPVN and observing the tracer within the peripheral and core portions of the SFO, respectively (68). Thus the SFO is anatomically structured for a divergence of physiological effects; the peripheral portion being connected to areas important for fluid balance (i.e., the vasopressin system) while the core is connected to areas controlling blood pressure (i.e., the sympathetic system; Fig. 1). A detailed review of how the connection between the SFO and PVN is angiotensinergic and mediates an elevation of blood pressure can be found by Ferguson and colleagues (36).

In addition to sending projections elsewhere in the brain, the SFO receives projections from the MnPO, SON, PVN of the hypothalamus and thalamus, medial preoptic, anterior hypothalamic, substantia innominata, BNST, rostral parts of the zona incerta, NTS, and the infralimbic area of the prefrontal cortex (13, 33, 66–68, 133). Retrograde tracing from the SFO and anterograde tracing from the MnPO and PVN revealed that the MnPO sends the greatest number of projections to the SFO. Retrograde tracing also further highlights the anatomical segregation of the SFO since the substantia innominata, rostral parts of the zona incerta, and the infralimbic area of the prefrontal cortex project to the peripheral portion of the SFO while the BNST projects to the core of the SFO.

The SFO is also highly interconnected with the anteroverentral third ventricle region (AV3V), which includes the OVLT, periventricular preoptic nucleus, and nucleus medianus. Electrolytic ablation of the AV3V initially makes rats adipsic to spontaneous water intake, blood-borne and CSF ANG II (9, 11, 62). During this acute postlesion period, the abolished fluid intake is specific for water and not generalized to all fluids or permanent. Rats will drink sugar-water while initially adipsic, and over time their spontaneous water intake eventually returns. Once spontaneous water intake returns, ANG II-induced and hypertonic saline-induced water intake is diminished, though hypovolemia-induced water intake is less affected. Obstructing ventricular flow with a plug around the AV3V blocks central ANG II-induced polydipsia but enhances peripheral ANG II-induced polydipsia (10). If the efferent projections from the SFO are severed, rats do not drink to peripheral ANG II, though they still increase fluid intake to intracerebroventricular injection of ANG II (80, 94). Electrolytic ablation of the SFO permanently abolishes fluid intake to sodium depletion and systemically administered ANG II (124, 129, 139). Overall the body of experimental evidence indicates that the dipsogenic effect of systemically administered ANG II is abolished by SFO lesions in the rat, although the involvement of structures located downstream of the SFO are likely to be involved. Ablation of ANG II-sensitive structures along the ventral part of the lamina terminalis (i.e., the MePO and OVLT) abolishes drinking to both systemically and centrally administered ANG II (61). So clearly, the SFO is a key sensory circumventricular organ involved in receiving systemically generated signals, integrating this information and relaying it to other areas involved in the generation of fluid intake (63).

**Stimulators of the Brain RAS and Physiological Effects of SFO Activation**

Dehydration can be classified as either extracellular (i.e., volumetric) or intracellular (i.e., osmotic). Extracellular dehydration induces drinking and an increase in salt preference and sodium appetite (137). Furosemide treatment, intraperitoneal ip polyethylene glycol (PEG), fluid restriction, or hemorrhage can all induce extracellular dehydration. Extracellular dehydration induced by fluid restriction or furosemide treatment increases the expression of AT1AR, activates ERK1/2, and increases the expression of AGT within the SFO (4, 19, 54). Similarly, elevating blood-borne ANG II either exogenously or indirectly by decreasing blood pressure via isoproterenol increases neuronal activity, the expression of AT1R within the SFO, and water intake (57, 69, 115). Fluid restriction also increases the expression of c-Fos (i.e., an indirect indicator of cellular activity) within the SFO, which remains elevated immediately after rehydration, whereas in other nuclei, c-Fos expression returns to normal (25). Table 2 summarizes stimuli that influence fluid intake and involve the SFO.

**AGT-deficient mice (AGT−/−)** exhibit an impaired dipsogenic response to acute extracellular dehydration suggesting a requirement for de novo synthesis of ANG II (90). In contrast, intracellular dehydration (discussed in more detail below) induced water intake in AGT−/− mice. This difference between extra- and intracellular dehydration was also seen when AT1R was knocked down in the SFO by antisense RNA (69). Thus production of ANG II by extracellular dehydration appears to increase fluid intake by acting through an AT1R-dependent mechanism located in the SFO.

Hypertonic saline is commonly used to increase osmolality thereby inducing intracellular dehydration. Intracellular dehydration increases fluid intake, decreases salt preference, and increases vasopressin (100, 137). Rats injected with intravenous hypertonic saline increase immunoreactivity for c-Fos within the SFO, OVLT, mPVN, SON, MnPO, NTS, RVLM, and lateral PB (56, 130). c-Fos immunoreactivity is selectively decreased in the MnPO, mPVN and SON, if the SFO, the ventral portion of the AV3V or these combined areas are lesioned (56). If the entire AV3V is lesioned, drinking to systemic hypertonic saline is abolished (9, 10). In sheep, lesion of the SFO alone does not alter water intake in response to intravenous hypertonic saline, whereas lesioning the OVLT or MnPO attenuates water intake and ablation of all three nuclei abolishes water intake in response to cellular dehydration (89).
Extracellular dehydration and sodium deficiency can be induced by the combined actions of furosemide (a diuretic/natriuretic agent). Rats with water and sodium deficit do not increase their water and sodium intakes if the SFO is lesioned (99, 129). If the cell bodies within, but not the fibers passing by the MnPO (a main efferent target of the SFO) are ablated, the increase in saline intake is reduced but water intake is not altered (26). Thus the SFO may also act as a sensor for intracellular dehydration by signaling to other nuclei, such as the OVLT and/or MnPO in the process of generating a sodium appetite.

It was reported that in rats, fluid intake induced by subcutaneous hypertonic saline is attenuated by a high dose of PD123319 (administered centrally), an AT2R antagonist, but not by an AT1R antagonist (112). Similarly, intracerebroventricular injection of an AT2R antagonist attenuated increased

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Route</th>
<th>Molecular Effect</th>
<th>Abolition</th>
<th>Ref No.</th>
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</thead>
<tbody>
<tr>
<td>Furosemide and captopril</td>
<td>iv</td>
<td>Increased p-p44/42 MAPK in OVLT/ SFO and SON/PVN</td>
<td>SFO lesion (water and Na⁺ intake)</td>
<td>34, 83, 99, 106, 140</td>
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<td></td>
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<td>Increased ANG II in the SFO, OVLT, MnPO, PVN</td>
<td>MnPO lesion (Na⁺ intake)</td>
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<td></td>
<td>Increased c-Fos in the SFO, OVLT, MnPO, mPVN, PB, AP, NTS</td>
<td>AT1R inhibition (iv or ip)</td>
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<td>Polyethylene glycol</td>
<td>ip</td>
<td>Increased c-Fos in SFO, OVLT, SON</td>
<td>AT2R inhibition</td>
<td>4, 19, 25, 54, 100, 112</td>
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<tr>
<td>Fluid restriction</td>
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<td>Increased SFO expression ATG, ANG II, AT1AR, ERK1/2</td>
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<td>Increased SFO neuronal activity</td>
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<td>Increased expression of c-Fos OVLT, MnPO, SFO, SON</td>
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<td>Increased PRA</td>
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<td>Isoproterenol</td>
<td>iv</td>
<td>Increased SFO expression AT1R and neuronal activity</td>
<td>AT2R inhibition</td>
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<td>SFO AT1R</td>
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<td>SFO and PVN AT1R</td>
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<td>Increased plasma, hypothalamus, and hippocampus Aldo</td>
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<td>Hypertonic saline</td>
<td>iv, sc</td>
<td>Increased c-Fos in the SFO, OVLT, MnPO, mPVN, SON, NTS, RVL, lateral PB</td>
<td>AV3V or lamina terminalis lesion</td>
<td>9, 37, 56, 88–90, 112, 130</td>
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<td></td>
<td></td>
<td>Increased AT1R in MnPO, SFO, PVN, NTS, AP</td>
<td>SFO/OVLT lesion together or each separate</td>
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<tr>
<td>Deoxycorticosterone acetate salt</td>
<td>sc</td>
<td>Increased c-Fos OVLT, BNST, MnPO, PVN, SON, amygdala, preoptic area</td>
<td>AV3V lesion</td>
<td>8, 44, 47, 52, 60, 102</td>
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<td>Increased RAS-gene expression</td>
<td>AT1AR removal from SFO (polydipsia and hypertension)</td>
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<td>Increased c-Fos OVLT, BNST, MnPO, PVN, SON, amygdala, preoptic area</td>
<td>icv Benzamil</td>
<td>148, 149</td>
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<tr>
<td>Aldosterone</td>
<td>iv, sc</td>
<td>Increased c-Fos OVLT, BNST, MnPO, PVN, SON, amygdala, preoptic area</td>
<td>icv Benzamil</td>
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<td>Sodium depletion</td>
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<td>Increased c-fos in SFO, MnPO, OVLT, PVN, SON</td>
<td>sc Captopril</td>
<td>84, 129</td>
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<td>Increased ANG III and Aldo in plasma and forebrain</td>
<td>SFO lesion</td>
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<td>Decreased ACE and AT1R in PVN, SON, and OVLT</td>
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<td>Renin</td>
<td>icv</td>
<td>Increased c-Fos in SFO, MnPO, OVLT, PVN, SFO</td>
<td>icv Losartan</td>
<td>113</td>
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<td>Increased c-fos in AT1R and AVP expressing SON and PVN</td>
<td>icv Captopril; icv ghrelin or TRPV4 agonist (blocks polydipsia)</td>
<td>38, 49, 79, 92, 95, 107, 116, 123, 141, 154</td>
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<td>c-Fos in SFO, SON, MnPO after repetitive icv ANG II</td>
<td>iev Ad-DN-Rac1 or Ad-DN-Nox2</td>
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<td>c-Fos in SFO, SON, MnPO</td>
<td>hypothalamic disconnect acutely but not chronic</td>
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<td>PKC-a activity CAMKII in septum and hypothalamus</td>
<td>chronically decreases polydipsia induced by</td>
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<td>Increased c-fos in SFO, MnPO, OVLT, PVN, SON</td>
<td>icv AnalR −/− or AT2R −/−</td>
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<td>icv Losartan</td>
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Extracellular dehydration and sodium deficiency can be induced by the combined actions of furosemide (a diuretic/natriuretic agent). Rats with water and sodium deficit do not increase their water and sodium intakes if the SFO is lesioned (99, 129). If the cell bodies within, but not the fibers passing by the MnPO (a main efferent target of the SFO) are ablated, the increase in saline intake is reduced but water intake is not altered (26). Thus the SFO may also act as a sensor for intracellular dehydration by signaling to other nuclei, such as the OVLT and/or MnPO in the process of generating a sodium appetite.

It was reported that in rats, fluid intake induced by subcutaneous hypertonic saline is attenuated by a high dose of PD123319 (administered centrally), an AT2R antagonist, but not by an AT1R antagonist (112). Similarly, intracerebroventricular injection of an AT2R antagonist attenuates increased
drinking after 24-h fluid restriction or intravenous hypertonic saline. It has been proposed that AT2R may be acting as a mechanosensor of cellular swelling in response to intracellular dehydration rather than being activated by angiotensin peptides. This is supported by data suggesting that angiotensin peptides were not needed for intracellular dehydration-induced polydipsia in AGT−/− mice (90). Thus fluid intake can occur centrally through different ANG II receptors. This may occur through the SFO since it is important in mediating fluid intake due to peripheral stimuli, such as ANG II and osmosality.

The SFO also responds to conditions in the CSF, such as osmolarity and ANG II. The brain senses a change in osmolarity since blood-borne hypertonic saline or mannitol induce water intake at doses only effective as an intracarotid rather than intravenous injection (120). Increasing the osmolarity of the CSF increases the neural activity of the SFO which can stimulate the intake of water, release AVP, and elevate blood pressure through an AT1R-dependent mechanism (6, 55, 87, 111, 138). Adding NaCl to a hypertonic aCSF injection was a more potent dipson in than if sucrose was used, so both osmosensitive and sodium-sensitive receptors may mediate polydipsia (87). Furthermore, ablation of the lamina terminalis, which includes the SFO, abolishes the induction of drinking and release of AVP that occurs upon intravenous hypertonic saline (88, 89). These studies demonstrate that an elevation in osmolarity can induce water intake through central mechanisms, which may in part involve the SFO.

In addition to sodium in the CSF, the SFO can increase fluid intake due to ANG II within the brain. It has been known for decades that a central injection of ANG II is a potent dipson (124), but we will focus on the fluid balance effects of physiological and genetic elevation of the brain-RAS within areas of endogenous ANG II synthesis and action. First, ANG II can be elevated within the brain in response to acute application of the diuretic furosemide (furo) concomitant with a low dose of the ACE inhibitor captopril (cap), a model referred to as furo/cap (106). In rats, furo/cap increases fluid intake and sodium appetite within 2 h, which can be further enhanced by subsequent injections over a period of weeks. Furo/cap increases the activity of cells (as assayed by the number of c-Fos immunoreactive cells) within the SFO, OVLT, MnPO, both magnocellular and parvocellular PVN, PB, area postrema (AP), and NTS (105, 113, 140). Interestingly, a high dose of captopril blocks this increase in c-fos activity (140). Presumably this occurs because captopril passes into the brain where it blocks the de novo production of ANG II. Indeed, furo/cap induces ANG II production within the SFO, OVLT, MnPO, and PVN, and intracerebroventricular losartan attenuates the increase in fluid intake and sodium appetite due to furo/cap suggesting it is mediated by AT1R (83).

Second, DOCA-salt and aldosterone elevates brain RAS activity within endogenous areas, resulting in increased blood pressure and fluid intake through central activation of AT1R (44, 47, 60, 104, 148). DOCA-salt increases c-Fos expression within the OVLT, BNST, MnPO, PVN, SON, amygdala, and preoptic area in rodents (85, 140). DOCA-salt or aldosterone increases the expression of RAS genes (102, 149). Autoradiography for radiolabeled Sar1ANG II after DOCA treatment alone (i.e., without high salt fluid) demonstrates that AT1R binding activity increases within the MnPO, SFO, PVN, but not the SCN, whereas high salt alone increases binding selectively within the MnPO and SFO (27, 47). Treatment with both DOCA and salt further enhances expression of AT1R within the MnPO, SFO, PVN, NTS, and AP compared with either treatment alone. Cotreatment of DOCA-salt with aldosterone in rodents further increases AT1R expression only within the SFO (27). This DOCA-salt mediated increase of AT1R in areas of the brain important for cardiovascular regulation increases blood pressure, fluid intake, and sodium appetite through an ANG II and AT1R-dependent mechanism since both central (and region specific) losartan and captopril can attenuate these phenotypes (27, 60, 71, 104). The AV3V region and the anterior hypothalamic area (AHA) have been shown to be important for the DOCA-salt elevation of fluid intake, sodium appetite, and blood pressure (8, 71). Lesion of the AV3V region, which is highly interconnected with the AHA, indicates its importance in mediating the hypertensive effects of DOCA-salt. While lesion of just the SFO does not affect the DOCA-salt increase in blood pressure, fluid intake, or sodium appetite, genetic ablation of AT1AR from the SFO attenuates DOCA-salt hypertension and polydipsia (52, 103). Thus these and other data support a functional synergism between ANG II and aldosterone (42, 148, 149). These studies also highlight the importance of the SFO and its target nuclei to mediate central ANG II effects through AT1R.

Transgenic methods have also been used to specifically and supraphysiologically increase the RAS within endogenous areas of the brain. Transgenic mice expressing human renin in all neurons driven by the synapsin promoter (termed sR mice) have been bred to transgenic mice expressing human angiotensinogen driven by 1.5 kb of its endogenous promoter (termed A mice) (45, 97). This double transgenic model, termed the sRA mouse, has many properties in common with DOCA-salt mice. Moreover, expression of human renin in sR mice mimics endogenous expression, because renin is primarily expressed in neurons (73). Since AGT is driven by its endogenous promoter, and ANG II production can only occur in sites where AGT is made, the sRA model selectively increases the brain RAS in those areas where endogenous ANG II is produced (72). We have shown that ANG II production is upregulated particularly in the AV3V region and hypothalamus in sR mice (45). This causes the same physiological effects, such as polydipsia, seen in models of elevated brain RAS activity, such as DOCA-salt. Plasma aldosterone is also increased in sR mice. Peripheral treatment with spironolactone (an antagonist for the aldosterone receptor) attenuates the fluid and sodium phenotypes of sR mice, whereas adrenalectomy completely corrects them, further highlighting the synergism between central ANG II and aldosterone.

Just as AT1AR in the SFO are necessary for the polydipsia and hypertension induced by DOCA-salt (52), we have also shown that de novo production of ANG II in the SFO is both necessary and sufficient for central ANG II-mediated fluid intake (20, 117, 126). We have shown this by using a unique extension of the sRA model termed sRAFloxi and by using a conditionally and spatially inducible model of ANG II production called sRARed (Fig. 2). sRAFloxi are functionally identical to sRA mice except that the hAGT transgene contains loxp sites surrounding exon-II, providing a substrate for the selective deletion of AGT upon Cre-mediated recombination. An injection of AdCRE directly into the SFO of double transgenic sRAFloxi mice selectively turns off the brain RAS within the...
SFO, which attenuates their polydipsia (117). Similarly, intracerebroventricular injection of AdCRE into single transgenic hAGT\textsuperscript{Flox} mice selectively removes hAGT from the SFO, which blocks the pressor response to an intracerebroventricular injection of purified human renin (126). Like sRA mice, sRA\textsuperscript{Red} mice express human renin in all neurons; however, a stop sequence surrounded by loxP sites prohibits the expression of human angiotensinogen unless it is removed by Cre-mediated recombination. In this model, inducing the production of ANG II in the SFO in sRA\textsuperscript{Red} mice increases total fluid intake and increases the preference for a nonaversive concentration of NaCl (20). Surprisingly, the induction of ANG II in the SFO in sRA\textsuperscript{Red} mice was not sufficient to increase blood pressure or metabolism. This led us to suggest the possibility that the mechanisms controlling ANG II-dependent drinking and blood pressure in the SFO may differ in its dose response or the cellular specificity of where ANG II acts. ANG II may act separately on neurons projecting to nuclei controlling water and electrolyte homeostasis or to nuclei controlling the preganglionic neurons of the sympathetic nervous system, and this may be favored toward the former in the sRA\textsuperscript{Red} model.

**Downstream Mediators of Central Angiotensin Action: Role of Protein Kinase C and Mitogen-Activated Protein Kinases**

\(\text{AT}_{\text{I}}\text{R} \) is a G protein-coupled receptor (GPCR) linked to a Gq/11 \(\alpha\)-subunit, which can increase diacylglycerol (DAG) and inositol trisphosphate (IP3) through activation of phospholipase C (PLC) (reviewed in Ref. 132). Classically, protein kinase C (PKC) can then be activated directly by DAG or indirectly through IP3 release of calcium. PKC isoforms are categorized according to their activators (101). Conventional PKCs (\(\alpha, \beta, \gamma\)) are activated by DAG, calcium, and phospholipids; novel PKCs (\(\delta, \xi, \eta, \theta\)) are activated by DAG, but not calcium; and atypical PKCs (\(\iota, \zeta, N1, N2, N3\)) require neither DAG nor calcium for activation. Intracerebroventricular injection of ANG II in rodents increases PKC-\(\alpha\) activity in the MnPO, SFO, and PVN, whereas direct microinjection of ANG II into the RVLM increases the activation of all three conventional PKC isoforms (16, 38). Phorbol 12-myrstiate 13-acetate (PMA), an activator of PKC that bypasses receptor activation, induced PKC-\(\alpha\) activity in primary cultures of cells derived from the rat SFO (21). PMA increased c-Fos expression and firing rate more in primary cultures of the AHA, brain stem, or hypothalamus from newborn spontaneously hypertensive rats (SHR) compared with similar cultures from Wistar-Kyoto rats (3, 70). The enhanced c-Fos expression in SHR is mediated by PKC-\(\beta\), since knockdown of PKC-\(\beta\) prevented the induction (3). Moreover, pharmacologically blocking PKC with H-7 in ANG II-sensitive AHA neurons from SHR attenuates the enhanced c-Fos expression and neuronal firing rate more than control cultures (3, 70). Central injection of chelerythrine (a general inhibitor of all PKC isoforms) in rats blocks the increase in water intake due to intracerebroventricular ANG II (22, 38). Intracerebroventricular administration of
Gö-6976, an inhibitor of the catalytic domains of PKC-α and PKC-β attenuated ANG II but not carbachol-induced water intake (38). Specifically decreasing PKC-α activity via intracerebroventricular injection of a dominant negative PKC-α attenuates the elevated water and 0.9% saline intake of sRA mice (21). Thus an elevation of the brain RAS activates PKC, and conventional isoforms of PKC mediate the diposogenic effect of central ANG II.

Mitogen-activated protein kinase (MAPK) pathway has also been implicated to mediate the effects of central ANG II (23). This pathway may lie downstream of PKC because antisense inhibition of either PKC-α or PKC-β attenuates ANG II-induced phosphorylation of p38 MAPK and ERK1/2 in the RVLM of rats (16). Other data suggest that ERK1/2 activation in response to AT1R activation may occur independently of PKC through a β-arrestin pathway, although it remains unclear if this pathway is active in the brain (143). This AT1R-β-arrestin pathway may also be activated in response to membrane stretch (135). Inducing the brain RAS by furo/caf increases phosphorylation of ERK1/2 within the OVLT, SFO, SON, and PVN via AT1R; and its activation mediates the increase in sodium appetite (but not water intake) that occurs in furo/caf-treated rats (34). Activation of ERK1/2 has also been shown to regulate AT1R expression because blocking ERK1/2 inhibits the increase in AT1R expression within the SFO and PVN due to ANG II treatment (144, 152). These studies suggest that ANG II can activate ERK1/2, which upregulates AT1R, elevates blood pressure, and enhances sodium appetite, but not water intake.

**Downstream Mediators of Central Angiotensin Action: Role of Reactive Oxygen Species and Endoplasmic Reticulum Stress**

Peripheral infusion of ANG II increases reactive oxygen species (ROS) production within the SFO, and ANG II elevates ROS through AT1R in cultured cells (including the SFO) from the central nervous system (153, 155). Prior transfection of the SFO with either the intracellular or mitochondrial form of superoxide dismutase prevents polydipsia and hypertension induced by an intracerebroventricular injection of ANG II. However, a non-ANG II-dependent dipsojen and pressor agent carbachol is still able to increase fluid intake and blood pressure. Thus the production of ROS either in the cytosol or mitochondria of the SFO mediates the physiological effects of central ANG II action.

The source of ROS that mediates the effects of ANG II may occur through PKC-mediated activation of NADPH oxidase (NOX), specifically NOX2 and NOX4 (reviewed in Ref. 59). In the forebrain of mice, which includes the SFO, NOX2 is the main isoform expressed, though NOX1 and NOX4 are also expressed. In the midbrain, which includes the hypothalamus, NOX4 is the main isoform expressed, though the other isoforms are also expressed. In the hindbrain, which includes the NTS, RVLM, and AP, both NOX2 and NOX4 are highly expressed with much lower expression for NOX1. GF109203X (a pharmacological inhibitor of PKC-α or PKC-β) attenuates ANG II induction of ROS in NTS neurons, and NOX2-deficient NTS neurons do not increase ROS production in response to ANG II (142). Furthermore, chelerythrine blocks the phosphorylation of NOX2 in response to microinjection of ANG II into the RVLM (16). Downstream of PKC, pharmacologically blocking ROS in the RVLM prevents the phosphorylation of p38 MAPK due to microinjection of ANG II into the RVLM, and it prevents the phosphorylation of ERK1/2 due to chronic activation of AT1R (15). Thus central ANG II can increase the activity of PKC, production of ROS, and activation of MAPK pathways through NADPH oxidase-dependent mechanisms.

The importance of NADPH oxidase in mediating the physiological effects of central ANG II was demonstrated by blocking Rac-1 (part of the Rho family of GTPases) with adenoviral transfection of a dominant negative Rac-1 (AdDN-Rac1) in primary cultures derived from the lamina terminalis (154). Dominant negative Rac-1 virtually abolished NADPH oxidase activity, and when targeted to the SFO, AdDN-Rac1 abolished the increase in blood pressure and significantly attenuated the increase in fluid intake in response to central ANG II. Moreover, targeting NOX2 and NOX4 with RNA silencing in the SFO revealed that NOX2 mediates polydipsia, whereas either NOX2 or NOX4 can mediate the pressor effect of central ANG II (107). Neither had an effect on baseline fluid intake or blood pressure.

Endoplasmic reticulum (ER) stress has recently emerged as another mediator of the effects of ANG II. Injection of thapsigargin, a noncompetitive inhibitor of the ER calcium ATPase pump that blocks the uptake of calcium from the cytosol into the ER, centrally increases markers of ER stress such as phosphorylated PERK and phosphorylated IRE-1α (108). Thapsigargin-induced ER stress in the brain was recently shown to increase blood pressure and renal sympathetic nerve activity (151). ANG II treatment induced the expression or activity of several ER stress biomarkers (e.g., p58IPK, GRP78, and phosphorylated PERK) within the SFO. Central injection of tauroursodeoxycholic acid (TUDCA), a chemical chaperone, and adenoviral overexpression of GRP78, a molecular chaperone, both of which decrease ER stress, prevented the increase in blood pressure in response to peripheral ANG II. Blocking ER stress in the SFO also attenuated the ANG II-induced increase in ROS production. Thus ANG II can increase ER stress within the SFO to increase the production of ROS resulting in ANG II-induced hypertension.

**Perspectives and Significance**

The SFO is anatomically situated to respond to and integrate signals from both the periphery and CNS, and blood-borne or CSF ANG II can activate the SFO to increase fluid intake. Activation of AT1R within the SFO increases fluid intake through a PKC/ROS pathway and also mediates the increase in blood pressure due to ANG II through a PKC/ROS/ER Stress/ERK1/2 pathway.

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