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Subfornical Organ: Site of Drinking Elicitation by Angiotensin II

Abstract. *Angiotensin II applied directly to the subfornical organ in a dose as small as 0.1 nanogram elicited short-latency drinking behavior in water-sated rats. Lesions in the body of this structure blocked drinking induced by angiotensin II applied to the basal telencephalon (including preoptic area). These results call attention to the subfornical organ as an important central nervous structure involved in the conrol of drinking behavior.*

The renin-angiotensin system, known for its pressor effects mediated through angiotensin II, has been implicated in processes of body fluid regulation. Hypovolemia, a decrease in the absolute volume of the body fluids, is one of several stimuli now thought to increase renin secretion from the juxtaglomerular cells of the kidney (*1*). Circulating renin catalyzes the conversion of the serum α_2 globulin peptide angiotensinogen into angiotensin I, and this product is transformed by circulating angiotensin-converting enzymes into the biologically active angiotensin II (*2*). A renal dipsogenic factor (that is, a factor eliciting drinking) thought to be renin has been identified (*1*), and direct intracranial injection of angiotensin II produces drinking in water-sated animals, a result suggesting a central nervous system site of action of angiotensin II (*3*).

Autoradiographic analysis of the penetration of angiotensin II into the brain has shown that the octapeptide reaches equilibrium slowly with brain interstitial fluid (*4*). It therefore seemed plausible that a central dipsogenic receptor for angiotensin II would lie outside the blood-brain barrier. Evidence from our laboratory had implicated the subfornical organ (SFO) as a major site of action of the dipsogenic cholinomimetic, carbachol, and this structure may have a significant role in body fluid regulation processes (*5*, *6*). Anatomical and histochemical evidence also suggested that this structure lies outside the blood-brain barrier (*7*). We therefore tested the dipsogenic effect of angiotensin II applied directly to the SFO. Because intracranially injected chemicals can also spread via the ventricles (*8*) or the vasculature (*9*), we also studied the consequences of SFO lesions on drinking produced by application of angiotensin II to the preoptic area, which Epstein *et al.* (*10*) reported to be the most effective central locus in eliciting drinking behavior.

In the first experiment, we performed dose-response and time course analyses of the drinking behavior elicited by angiotensin II applied directly to the SFO. The methods have been described (*6*). Adult male albino rats, weighing 300 to 400 g, were each implanted with a single 27-gauge guide cannula aimed at the SFO by stereotaxic methods (*11*). Animals were permitted to recover from surgery for 3 days with free access to food and water. On days 4 to 7 after surgery, baseline measurements of food and water intake were taken once daily for a 0.5-hour period. Mean food and water intakes during these 4 days were defined as baselines, and these values were subtracted from those after intracranial chemical injections to give the net intake values shown in Fig. 1. On day 8 each animal received a single 0.5-μl intracranial injection of angiotensin II solution (Hypertensin, Ciba) delivered in an isosmotic saline vehicle (*12*) at 0.1 μl/sec. Food and water intakes were monitored for the 0.5-hour period after the injection as they had been during the preceding 4 days of baseline measurements. Within 6 hours after completion of testing, animals were killed with an overdose of pentobarbital; brain tissue was fixed in situ by intracardiac aldehyde perfusion. Alternating 30-μm frozen sections through the cannula tract in each brain were stained for myelin (Weil method) or for Nissl substance (thionin method).

The first experiment demonstrated that the SFO was remarkably sensitive to the dipsogenic effect of intracranially administered angiotensin II. As shown in Fig. 1a, angiotensin II applied to the SFO in doses ranging from 0.1 to 100 ng elicited a drinking response. As little as 0.1 ng of angiotensin II in the SFO elicited a drinking response in a group of five animals; their water intake for the first 15 minutes after injection was more than the mean baseline intake for a similar period ($t = 3.061$, d.f. $= 4$, $P < .05$).

Epstein *et al.* (*10*), using similar intracranial injection methods but not injecting into the SFO, reported that the brain area most responsive to the dipsogenic effect of angiotensin II was the medial preoptic area, and that 5 ng was the lowest effective dose. Our effective dose of 0.1 ng is more than 0.5 log unit less than that reported by Epstein *et al.* Our data suggest, therefore, that the SFO is a more effective site than the medial preoptic area for eliciting angiotensin-induced drinking. In addition, we believe this is the lowest reported dose of a chemical applied to the brain which elicits a behavioral response.

Analysis of the time course of the elicited water intake (Fig. 1b) supported the view that the SFO may be an important site of action of angiotensin II since, at all doses, drinking behavior began rapidly, usually in less than 30 seconds. The duration of the elicited drinking behavior varied with dose: larger doses of angiotensin II (10 to 100 ng) elicited bursts of intense drinking behavior lasting 10 to 15 minutes, whereas lower doses (0.1 to 0.5 ng) elicited 1- to 3-minute drinking bursts. The rapid onset of drinking observed with doses of angiotensin II applied to the SFO is consistent with the view that the dipsogen is acting at this central nervous system site.

A second experiment was designed to determine whether drinking induced by angiotensin II applied to the preoptic area, initially reported by Epstein *et al.* (*10*), would be affected by lesions of the SFO. Since the SFO is highly sensitive to angiotensin II, we thought it possible, on the basis of earlier work on spread of neurochemicals (*13*), that diffusion of angiotensin II from the medial preoptic area to the SFO may have occurred in the Epstein *et al.* study. If this were true, then lesions of the SFO would eliminate the drinking induced by angiotensin II applied to the preoptic area.

The methods for intracranial injection combined with lesions have been described [experiment 3 in (*6*)]. Animals were implanted with a single 27-gauge intracranial cannula localized either in the preoptic area or in the adjacent ventral section of the diagonal band of Broca. Baseline intake was measured for each animal (0.5 hour daily for 4 days), followed by intracerebral injection of angiotensin II solution on the next day. This intracranial injection was followed by two additional days of baseline measurements; on the third day, a second intracranial injection was administered to each animal. The injections were 0.5 μl in volume and contained either 0.1 or 0.5 μg of angiotensin II. Only animals with net water intake greater than 5.0 ml for 0.5 hour after injection of angiotensin II solution were studied in the next phase of this experiment.

On the day after the second injection, each animal received either an operative control procedure involving anesthesia, incision, and production of an aperture in the skull, but with no electrode penetration into the brain; or a brain lesion procedure in which SFO tissue or adjacent brain tissue was fulgurated (*14*). The animals were permitted 3 days of recovery after surgery, and baseline measurements of food and water intake were made for 4 days. On the fifth day, each animal received a single intracranial injection of the same dose of angiotensin II solution as it had earlier received. Subjects were killed promptly after the experiment, and the brains were prepared for microscopic examination.

A total of 27 animals completed both phases of this experiment. The tips of the intracranial cannulas were localized in the medial or lateral preoptic area or in adjacent basal portions of the diagonal band (Fig. 1d). Each animal in this study had an intracranial can-

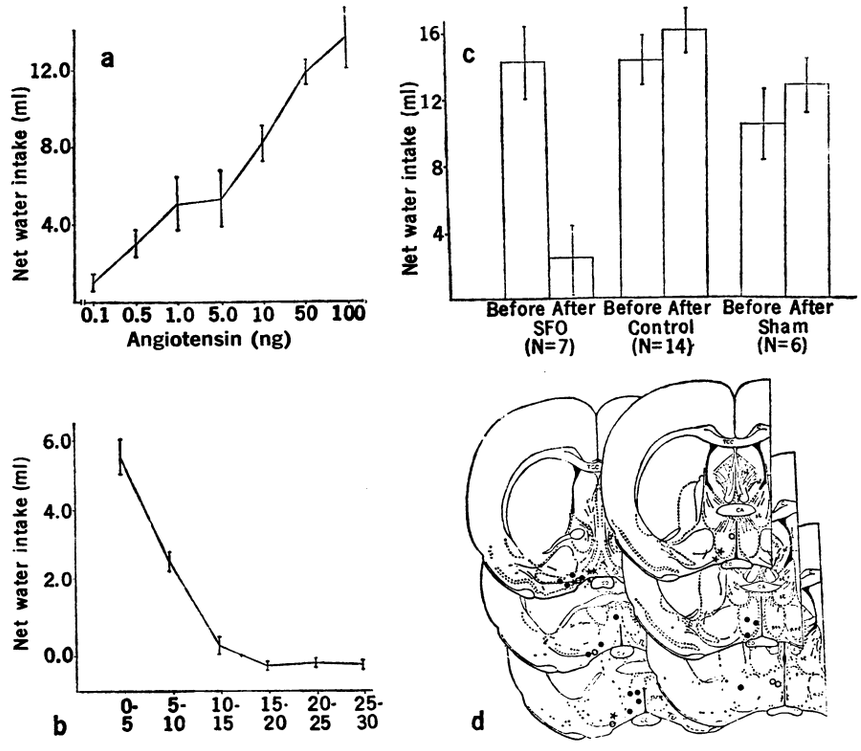

Fig. 1. (a to c) Net water intakes, mean ± standard error of mean. All values are based on a least five animals. (a) Net water intake for animals injected in the SFO as a function of angiotensin II dose. (b) Net water intake as a function of successive 5-minute epochs for all doses of angiotensin II applied to the SFO. The 0.0 point on the ordinate is the mean water intake for 5-minute epochs during baseline measurement periods. (c) Net water intake for animals with angiotensin II applied to basal telencephalon before and after the surgical lesion procedure. All animals with lesions including the body of the SFO are reported on this graph (*16*). (d) Schematic representation from the atlas of König and Klippel (*21*) of the location of the tips of intracranial cannulas. Symbols: star, angiotensin-induced drinking disrupted by lesions in the body of the SFO; closed circle, control lesion group, without disruption of angiotensin-induced drinking; and open circle, sham lesion control group.

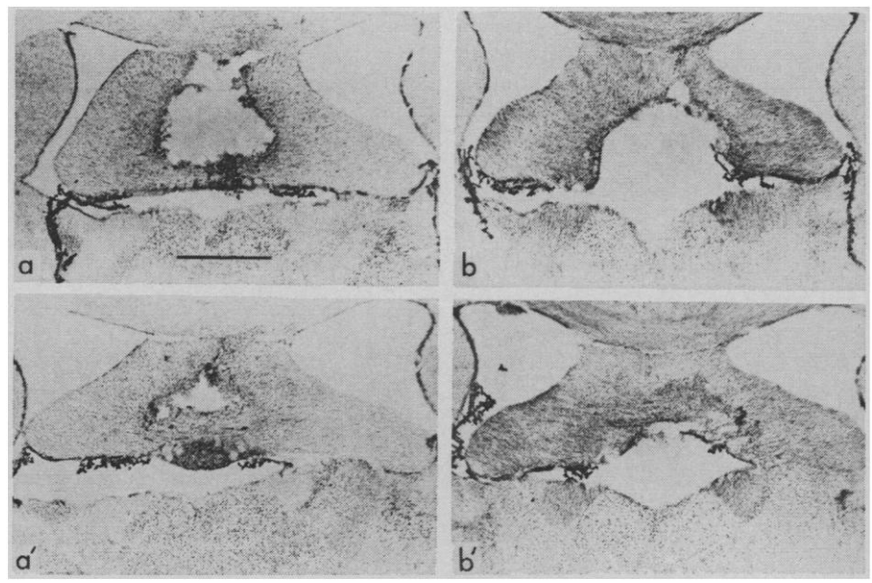

Fig. 2. Unretouched photomicrographs of thionin sections indicating the extent of lesions in two brains. Calibration line in (a) is 1.0 mm and applies to all photomicrographs. (a and a') Control lesion in the ventral fornical commissure and the dorsal portion of the triangular septal nucleus. The intact, densely basophilic cells in the body of the SFO are seen in (a'). (b and b') Lesion destroying the body of the SFO and damaging the overlying ventral fornical commissure. This lesion was effective in blocking intracranial angiotensin-induced drinking.

nula in an effective site for application of angiotensin II. Six animals received the sham lesion procedure, and the remaining 21 were subjected to the experimental lesion procedure.

In 6 of the 21 animals with lesions, drinking induced by angiotensin II applied to the preoptic area or diagonal band was eliminated 8 days after the lesion (Fig. 1c). In all of these animals, the salient common characteristic of the lesions was the destruction of cells in the body but not necessarily the stalks (*15*) of the SFO. A photomicrograph of this type of lesion is presented in Fig. 2, b and b′. Also, induced drinking behavior was not disrupted in the 14 animals with lesions in adjacent tissue but with the body of the SFO intact. In the six animals with lesions of the SFO, mean net angiotensin-induced water intake decreased by 15.4 ml and was indistinguishable from baseline. Thus, an elimination of the angiotensin-induced drinking was observed only if there was damage to the body of the SFO (*16*).

The 14 remaining animals had lesions located in tissue adjacent to but not including the body of the SFO. Combinations of tissue destruction in any of the following loci were observed: triangular septal nucleus, septal fimbrial nucleus, ventral fornical commissure, fornix, caudal extents of medial and lateral septal areas, dorsal thalamus adjacent to the third ventricle, and anterior stalk of the SFO. A photomicrograph of this type of lesion is presented in Fig. 2, a and a′. In animals with lesions in combinations of these areas, no reduction from the mean net intake before lesions (14.1 ml) to that after lesions (16.2 ml) was observed ($t = 1.841$, d.f. $= 13$, $P > .05$). Animals receiving the sham lesion manipulation likewise showed no alteration between the mean net intake before the sham procedure (10.3 ml) and that afterward (12.8 ml) ($t = 0.786$, d.f. $= 5$, $P > .05$). Thus, lesions including tissue in the area of the SFO were ineffective in reducing the efficacy of intracranial angiotensin II if these lesions did not include the body of the SFO. In summary, drinking induced by angiotensin II in the basal telencephalon can be disrupted by brain lesions that include cells in the body of the SFO. Destruction of adjacent tissue is without effect.

The present results demonstrate that the SFO is the most angiotensin-sensitive area of the central nervous system described to date. A reliable drinking response after injection of 0.1 ng of angiotensin II was observed, substantially lower than that of previous reports (*10*). Our working hypothesis is that, at physiological conditions, elevated blood concentrations of angiotensin II act at the SFO to provoke drinking behavior. The route by which angiotensin II is normally delivered to the SFO and the anatomical pathways and physiological mechanism by which drinking is initiated remain unknown. Because of the rich innervation of the SFO by the vasculature (*17*) and the lack of a blood-brain barrier in this structure (*7*), the major route of delivery of angiotensin II may be via the three major arterial systems whose fields of irrigation overlap extensively within the SFO (*17*).

The decrease in water intake in animals with SFO lesions can be interpreted in at least two different ways. (i) Angiotensin II acts directly on both the medial preoptic area and the SFO, and both structures form part of a neural circuit in which the SFO is closer to the final common path. (ii) Angiotensin II acts only on the SFO and spreads either through the ventricle (*8*) or the vasculature (*9*) from the preoptic area or diagonal band to receptor sites in cells of the SFO. The time required to move from the preoptic site of application through brain tissue and the ventricle to the SFO is probably greater than the latency of preoptic angiotensin-induced drinking (*10*). Flow through blood vessels from the preoptic area to the SFO (*18*) would be a more direct and quicker route.

It is tempting to speculate that the central dipsogenic receptors for circulating angiotensin II are within the SFO. If this is the case, then the central nervous receptors for two major known stimuli of primary drinking behavior (*1*) are identified. A lateral preoptic osmosensitive zone for the cellular dehydration stimulus of drinking behavior has been demonstrated by Peck and Novin (*19*) in the rabbit and by Blass and Epstein (*20*) in the rat. The central receptors for the extracellular dehydration stimulus have not been identified, although this type of thirst appears to be at least partially dependent upon the renin-angiotensin system (*1*). It would be interesting to explore whether the SFO is involved in processes of extracellular thirst, whether as a central dipsogenic receptor for angiotensin II or, in addition, as the direct receptor for the hypovolemia stimulus.

In conclusion, our data indicate that the SFO may contain the dipsogenic receptors for one of the known physiological stimuli of drinking, the renin-angiotensin system. These results call attention again (*5, 6, 9*) to the SFO as an important central nervous system substrate involved with body fluid regulation.

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11. A total of 87 rats were implanted with intracranial cannulas aimed at the SFO. Histological investigation revealed that 38 of these had cannulas in the SFO; data for these animals are reported here. Stereotaxic coordinates for this surgery were 0.3 mm posterior to bregma, 5.3 mm ventral to the surface of the skull, and 1.3 mm lateral to bregma with an angulation of 11° directed medially. The cannula was inserted perpendicularly in the anterior-posterior plane. There is difficulty in accurately manipulating the SFO with stereotaxic methods for the following reasons: (i) its small size and flat, irregular shape; (ii) the resistance of the overlying ventral fornical commissure to penetration by a probe; and (iii) its apparently variable location in relation to bregma.
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14. Because of the difficulty in stereotaxic manipulation of the SFO (*11*), the following procedure was used in attempted lesions of this structure. Three electrode penetrations were made into the brain of each animal. These penetrations were separated by 0.2 mm in the anterior-posterior plane. Each lesion was produced by a 1.0-ma anodal current for 20 seconds through a 127-μm tungsten electrode.
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16. Seven animals with a lesion including the body of the SFO were observed in the present study. As a group, the decrease in angiotensin-induced drinking was significant ($t = 2.954$, d.f. $= 6$, $P < .05$). One animal with a lesion in the body of the SFO did not show a decrease in net water intake after the lesion. In this animal, a considerable number of cells in the SFO posterior stalk were present and nonchromatolytic. Perhaps some form of recovery of function took place in this animal and accounted for the angiotensin-induced drinking.
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Wilson's Disease: Identification of an Abnormal Copper-Binding Protein

Abstract. *The metal-binding protein metallothionein was isolated from the livers of Wilson's disease patients and control subjects. The metals were removed from the native protein to produce the apoprotein, and copperthionein was prepared by equilibrium dialysis. Copperthionein from Wilson's disease patients had a copper-binding constant four times as great as that of the protein from control subjects. These results suggest that the alterations in copper homeostasis in Wilson's disease result from the synthesis of an abnormal metal-binding protein with an increased affinity for copper.*

Wilson's disease (hepatolenticular degeneration) is an inborn error of metabolism that affects copper homeostasis. Hepatolenticular degeneration was first described in 1912 (*1*), and the pattern of inheritance, clinical features, and pathological changes associated with the disease have been well characterized (*2*).

The genetic abnormality in Wilson's disease leads to an excessive accumulation of copper in the liver, brain, kidneys, and cornea. Other abnormalities in the symptomatic patient include low serum ceruloplasmin concentrations, elevation of serum copper not bound to ceruloplasmin, increased urinary copper, and decreased fecal copper. Although many theories have been suggested to explain the genetic defect in Wilson's disease, none has been proved.

Uzman *et al.* (*3*) first suggested that the primary defect in Wilson's disease is the synthesis of an abnormal protein with a high affinity for copper. In adult mammals, the major portion of the total hepatic copper is located in the cytosol (*4*), where the metal is bound to the enzyme superoxide dismutase and a low molecular weight protein similar to metallothionein (*5*). The experiments reported here demonstrate that hepatic metallothionein from patients with Wilson's disease has a higher copper-binding constant than does that from control patients.

Liver samples were obtained from two teenage symptomatic Wilson's disease patients (*6*) and two teenage patients with elevated hepatic copper due to biliary cirrhosis (*7*). Each sample was homogenized in 0.025*M* phosphate buffer, *p*H 7.4; 1.0 μc of ^{64}Cu was added to each homogenate; and the contents were stirred for 1 hour. The ^{64}Cu-labeled metallothionein was isolated by the method of Pulido *et al.* (*8*). Briefly, the procedure consisted of extraction in phosphate buffer followed by precipitation with ethanol and chloroform. After precipitation, the supernatants were dialyzed against distilled water and concentrated by freeze-drying. The concentrated samples were then purified by gel filtration chromatography on Sephadex G-50 (*9*), Sephadex G-75, and Bio-Gel P-10 (in that order). The [^{64}Cu]metallothionein was detected during purification by monitoring radioactivity in a gamma-well counter. Protein concentration was determined with biuret reagent.

After isolation and purification of metallothionein, the apoprotein, thionein, was prepared by dissolving 1.0 mg of metallothionein in a 1.0-ml solution that had been adjusted to *p*H 2 with 0.1*N* HCl. The acidified protein solution was chromatographed on a 2.5 by 40 cm column packed with Sephadex G-25 that had been equilibrated with 0.01*N* HCl. The purity of the apoproteins was assayed by electrophoresis in 5 percent polyacrylamide gels as described by Davis (*10*). Thionein prepared from both Wilson's disease patients and control patients migrated as a single band at *p*H 8.6 and at *p*H 6.3, which indicated a high degree of purity in the preparations.

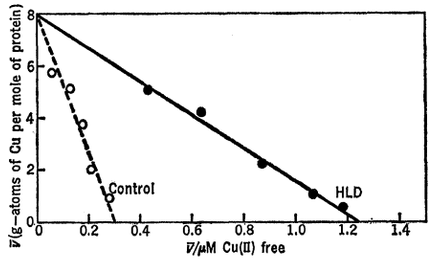

Fig. 1. Scatchard plots of equilibrium dialysis data for thionein and Cu(II). Thionein was dialyzed against various concentrations of $CuSO_4$. Least-squares regression lines are drawn through the plotted average data for controls and subjects with hepatolenticular degeneration (*HLD*).

Copperthionein was prepared from thionein by equilibrium dialysis. The apoprotein (1.0 mg) was dissolved in 10.0 ml of a solution containing 0.1*M* $KHPO_4$ (*p*H 7.4) and $CuSO_4$. The sample was placed in Visking Nojax tubing (24/32) and dialyzed against 10.0 ml of the same solution. The same procedure was repeated with several different concentrations of $CuSO_4$, and the solutions were dialyzed with continuous stirring for 48 hours at 4°C. After dialysis, the copper concentration of the solution was determined by atomic absorption spectrophotometry (*11*). The number of Cu(II) binding sites on the protein and the binding constants were determined by the method of Scatchard (*12*).

Although thionein from both groups of subjects contained the same number of copper-binding sites, the copper-binding constant for the protein from Wilson's disease patients was approximately four times as great as that from control subjects (Fig. 1). If a molecular weight of 10,000 is assumed for thionein, the copper-binding constant of the protein from Wilson's disease patients was $1.25 \times 10^6 M^{-1}$ whereas that from the control subjects was $3.0 \times 10^5 M^{-1}$; the number of copper-binding sites on the protein from both groups of subjects was calculated to be 7.9 g-atom/mole, which agrees with the number of metal-binding sites calculated in previous studies (*8*).

An abnormal protein with increased affinity for copper would explain many of the defects in copper homeostasis in hepatolenticular degeneration, especially in asymptomatic patients. In normal mammalian liver, copper enters the hepatocyte and becomes distributed throughout the cytosol and subcellular organelles where the metal is stored, incorporated into enzymes, or excreted. However, the increased binding affinity of the temporary storage protein in the hepatocyte of Wilson's disease patients probably shifts the normal equilibrium of the hepatic copper pool which results in both depressed biliary copper excretion and decreased incorporation of copper into ceruloplasmin. As the disease progresses, the binding sites on the storage protein