bly because of the more indirect methodology used in the earlier experiments, but is consistent with clinical reports showing lowered concentrations of NA metabolites in the cerebrospinal fluid of patients being treated with tricyclics (*15*). Neurons in chronically treated rats showed a marked resistance to further inhibition by IMI or by subsequent injections of clonidine (Fig. 2) (n = 11). The resistance to clonidine was less marked in chronically treated rats that were not given IMI immediately before clonidine (the last IMI injection was 12 hours earlier). Also, in these rats the response to microiontophoretic application of the α-agonist (44.0 ± 5.5 percent inhibition; n = 9) was significantly reduced compared to that in saline-treated controls (74.0 ± 5.5 percent inhibition, n = 10) ($P < .005$, Student's t-test). The mechanism could conceivably involve reduced sensitivity of the presynaptic receptor, but in view of the apparent dependence on dose of IMI, a receptor-blocking action of the tricyclics involved may also be implicated. Some biochemical data support the latter contention (*16*).

In any case, the reduced response of the presynaptic α-receptor may be significant in the antagonism by tricyclics of the antihypertensive effect of clonidine (*11*). The result also raises the possibility that with chronic antidepressant treatment, not only is NA reuptake inhibited but the NA system is also stabilized against changes, particularly reduction in firing rate.

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Angiotensin Regulates Release and Synthesis of Serotonin in Brain

Abstract. *Angiotensin II released serotonin from neuron terminals and accelerated synthesis of the serotonin. This increase in synthesis depended on the activation of tryptophan hydroxylase. A biphasic effect was observed: at high doses the stimulatory effect depended on conversion of angiotensin II to angiotensin III. At low doses an inhibitory effect was found, possibly dependent on an angiotensin II metabolite. These actions represent a subtle regulation of the open-loop serotonin system.*

Angiotensins I and II have been found in the nervous tissue (*1-4*). The distribution of angiotensin II in mammalian brain has been mapped (*5*). The occurrence of this polypeptide in certain neurons, fibers, and terminals of the central nervous system (*6-8*) facilitates the study of its possible participation in neurotransmission.

One of the questions we have studied is whether pressor response of angiotensin II, when injected into cerebral ventricles, is a direct action or one mediated by a particular neurotransmitter. The results of Vollmer and Buckley (*9*), showing that the intraventricular administration of phentolamine—an adrenergic receptor antagonist—potentiates the central hypertensive effect of angiotensin II, suggested that the central adrenergic system could modify the angiotensin response. Moreover, angiotensin II activates certain neurons, causing an enhanced sympathetic outflow (*10*), and can increase the biosynthesis of norepinephrine from tyrosine (*11*). We have also demonstrated that serotonin is selectively released from mast cells by angiotensin II (*12*), and others have found that serotonin plays some role in the regulation of arterial blood pressure (*13*). Since a good correlation was demonstrated between the concentration of angiotensin in cerebrospinal fluid and systolic blood pressure in patients with essential hypertension, we postulated the existence of an angiotensin-serotonin axis in the central nervous system (*14*, *15*). Thus, in the experiments reported here, we studied the pressor responses to angiotensin II injected either into the third ventricle or the cisterna magna in rats depleted of serotonin by treatment with *p*-chlorophenylalanine (*p*-CPA), a tryptophan hydroxylase inhibitor (*16*). For these experiments we used white Wistar rats weighing 300 g. The rats were anesthetized with embutal and a 21-gauge BD needle was inserted through the nose just into the third ventricle. Cisternal punctures were performed through the first vertebral space, and a polyethylene cannula was placed in the cisterna and fixed to the skin. At the end of each experiment the site of injection was verified with a color dye. The volume injected was usually 20 μl and always less than 100 μl. Arterial blood pressure was monitored throughout the experiment.

The injection of angiotensin II (from 2 to 200 ng) into the third ventricle of the brain produced significant increases in blood pressure. Both systolic and diastolic pressure increased 20 ± 3 (standard error) and 15 ± 4 mm-Hg, respectively, after the injection of 50 ng of angiotensin II. Intraventricular injection of saline (100 μl) did not elicit any significant

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change in blood pressure. A demonstration of the peripheral sympathetic mediated pressor effect of angiotensin II was obtained by blocking the increase in blood pressure with a subcutaneous injection of 10 mg of pentolinium per kilogram of body weight. The injection of serotonin (from 5 to 500 ng) into the cerebral third ventricle increased the rat blood pressure. With an injection of 50 ng of serotonin, systolic pressure increased 35 ± 3 mm-Hg and diastolic pressure 20 ± 4 mm-Hg. When the same dose was injected into the cisterna, the systolic pressure decreased 35 ± 4 mm-Hg and the diastolic pressure decreased 30 ± 3 mm-Hg. Cisternal injection of angiotensin II had no effect on blood pressure.

When rats received *p*-CPA (330 mg/kg, intraperitoneally) 72 hours before the experiments, intraventricular angiotensin II always caused a sustained decrease in systolic and diastolic blood pressure. Conversely, treatment with morphine (in a single intraperitoneal dose of 10 mg/kg) which activates serotonin synthesis (*17*, *18*), produced a significantly increased pressor response to intraventricular angiotensin II. Since changes in the brain concentration of serotonin induced by *p*-CPA and morphine elicited reciprocal variations in the effect of angiotensin II, it could be anticipated that blockade of serotoninergic receptor should abolish the pressor response produced by the polypeptide. Thus, when 50 μg of methysergide, a serotoninergic receptor antagonist was injected into the cerebroventricular system of the rats prior to the experiments, 50 ng of angiotensin II produced a brief hypotensive response and arterial blood pressure decreased 48 ± 7 mm-Hg. A subsequent intraventricular injection of 50 μg of phentolamine abolished any pressor or depressor effect of angiotensin II.

These results suggested the possibility of two neuronal sites of serotonin action: (i) a probable hypothalamic excitatory center and (ii) a bulbospinal inhibitory region. When the serotoninergic pathways are blocked, angiotensin II could act mainly on the hypothalamic α-adrenergic inhibitory loop. Angiotensin II may have a predominantly excitatory action on the hypothalamus mediated by serotonin. However, direct measurements of the release of serotonin by angiotensin in the central nervous system had not previously been made. We therefore decided to investigate the effect of the injection of angiotensin II into the cerebral ventricles on the serotonin content in hypothalamus and brainstem.

A single dose of 100 ng of angiotensin II was injected by way of the nose into the third ventricle of rats under light ether anesthesia. Control rats were injected with the same volume (100 μl) of saline. After 15 minutes the rats were decapitated, and the hypothalamus and brainstem were separated from the brain. Serotonin concentration was determined by a radioenzymatic procedure (*19*). In ten control rats, the serotonin concentration was 0.62 ± 0.03 μg/g in the brainstem and 1.18 ± 0.02 μg/g in the hypothalamus. The corresponding values in 12 rats injected with angiotensin II were 0.90 ± 0.06 μg/g and 0.76 ± 0.01 μg/g, respectively. Thus the intraventricular injection of angiotensin II significantly reduced ($P < .01$) the hypothalamic serotonin content by 36 percent, while increasing its concentration in the brainstem by 31.8 percent. Because the brainstem contains serotoninergic neuronal bodies with synaptic nerve terminals which reach the hypothalamus, these results indicated that angiotensin II might release serotonin

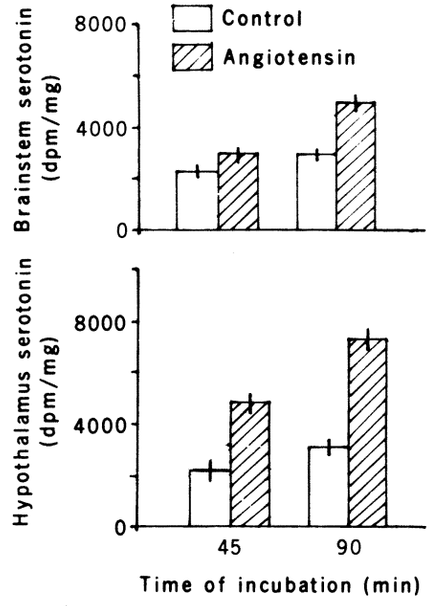

Fig. 1. Acceleration of serotonin synthesis by angiotensin II in synaptosomes. Hypothalamus and brainstem synaptosomes were isolated from the brains of Wistar rats. Tissue was homogenized in ice-cold 0.32*M* sucrose (1:10, weight to volume) and centrifugated at 1000*g* for 10 minutes. Supernatants were centrifuged again at 10,000*g* for 30 minutes. Pellets were resuspended and placed on a sucrose density gradient (0.8*M* and 1.2*M* sucrose in equal volumes) and centrifuged at 60,000*g* for 90 minutes. The fraction at the 0.8 and 1.2*M* interphase was collected and incubated in a Ringer-Krebs solution containing L-tryptophan (0.15 m*M*), pargyline (0.5 m*M*), and ascorbic acid (0.056 m*M*), diluted to a final concentration of 2 mg of protein per milliliter. Serotonin concentration was determined by a radioenzymatic procedure (*19*). Results are the mean of eight experiments: the values for synaptosomes incubated with angiotensin II were significantly greater than the corresponding control values (paired samples, Student's *t*-test, $P < .05$ for brainstem at 45 minutes and $P < .01$ at 90 minutes, $P < .001$ for hypothalamus at 45 and 90 minutes).

Table 1. The effect of angiotensin II on the activity of tryptophan hydroxylase in the supernatant obtained by centrifugation (10,000*g* for 20 minutes) of brainstem and hypothalamus of rats. Brainstem and hypothalamus were homogenized in 10 volumes of 0.26*M* tris-acetate buffer, *p*H 7.5, containing 0.05*M* 2-mercaptoethanol. Portions (0.6 ml) containing 12 mg of protein per milliliter were incubated with 0.2 ml of 0.4*M* tris-acetate, *p*H 7.4; 6,7-dimethyl-5,6,7,8-tetrahydropterine, 0.856 m*M*; mercaptoethanol, 0.05*M*; carbidopa, 0.1 m*M*; L-tryptophan 0.29 m*M*; and catalase, 180 U/ml. The reaction was stopped by the addition of 70 μl of perchloric acid (70 percent). After centrifugation of the mixture at 6000*g*, 0.18 ml of concentrated HCl was added to 0.6 ml of the supernatant. Tryptophan hydroxylase activity was measured as 5-hydroxytryptophan and was expressed as picomoles per milligram of protein. The fluorescence of the 5-hydroxytryptophan was measured at an excitation wavelength of 295 nm and emission wavelength of 598 nm.

Concentration of angiotensin II (g/mg protein)	Incubation period			
	45 minutes		90 minutes	
	Activity	*P*	Activity	*P*
	Low dose of angiotensin II (N = 7)			
0	265 ± 5		525 ± 15	
10^{-10}	290 ± 20	NS*	510 ± 10	NS
5×10^{-10}	110 ± 20	↓ < .01	435 ± 35	↓ < .05
10^{-9}	170 ± 20	↓ < .01	345 ± 5	↓ < .01
5×10^{-9}	150 ± 60	↓ < .05	325 ± 75	↓ < .05
10^{-8}	230 ± 10	NS	510 ± 20	NS
10^{-7}	580 ± 70	↑ < .01	725 ± 35	↑ < .01
	High dose of angiotensin II (N = 5)			
0	317 ± 5		453 ± 9	
10^{-7}	485 ± 22	↑ < .01	522 ± 10	↑ < .01
2.5×10^{-7}	522 ± 19	↑ < .01	427 ± 5	↑ < .05
7.5×10^{-7}	447 ± 24	↑ < .01	415 ± 20	NS
10^{-6}	377 ± 5	↑ < .01	493 ± 1	↑ < .01

*Not significant.

from nerve terminals and increase the neurotransmitter synthesis within the neuronal bodies. We therefore conducted a series of experiments in vitro, using synaptosomes obtained from hypothalamus and brainstem. Synaptosomes were prepared as described (*20*). Angiotensin II (5×10^{-8} g per milligram of protein) increased serotonin synthesis in hypothalamic synaptosomes by 32.8 and 110.0 percent over controls after 45 and 90 minutes of incubation, respectively. In brainstem synaptosomes, a similar response was obtained (Fig. 1). This acceleration of serotonin synthesis by angiotensin II suggested two possibilities, which we explored: (i) angiotensin II could increase substrate L-tryptophan incorporation into synaptosomes and (ii) angiotensin II could activate the first enzyme of the serotonin biosynthetic pathway, namely, tryptophan hydroxylase. As Fig. 2 shows, both angiotensins II and III decreased the incorporation of ^{3}H-labeled L-tryptophan into synaptosomes within 3 to 6 minutes of incubation; this process was dose-dependent. The data also show that angiotensins and phenylalanine had additive effects. These results make it less probable that the stimulation of serotonin biosynthesis by angiotensin is dependent on tryptophan incorporation.

Brain tryptophan hydroxylase was studied both in the particulate fraction obtained by differential centrifugation (the supernatant obtained at 10,000*g*) and in the nonparticulate fraction (the supernatant obtained at 70,000*g*). In the particulate fraction, angiotensin II produced a biphasic effect on tryptophan hydroxylase depending on the concentration. At concentrations of 3×10^{-10} to 3×10^{-9} g per milligram of protein, angiotensin II significantly inhibited the enzyme, whereas at higher concentrations (above 5×10^{-8} g/mg) it produced a significant increase of enzymatic activity (Table 1). Angiotensin II lacked any effect on the particle-free fraction at any concentration. When the nonparticulate fraction was incubated in the presence of angiotensin III, a significant activation of tryptophan hydroxylase occurred with 10^{-10} g per milligram of the heptapeptide. Activation of the enzyme was observed with an angiotensin III concentration as low as 10^{-11} g per milligram of protein (Table 2). The inability of angiotensin II to activate the enzyme in the particle-free fraction and the efficiency of the heptapeptide angiotensin III to increase tryptophan hydroxylase activity in this particular fraction suggest that (i) conversion of angiotensin II to angiotensin III is a prerequisite for enzyme activation and (ii) this process occurs in the particulate fraction. Since the decay rate of angiotensin II is supposed to be about 10^{-8} g per milligram of protein per hour (*21*), the biphasic effect could be explained as follows. (i) When angiotensin II inactivation is total, the inhibitory effect of the polypeptide should depend on a metabolite of angiotensin II different from angiotensin III. (ii) When the amount of angiotensin II present in the preparation is above the level of degradation, a certain amount of angiotensin III is formed which predominates over the inhibitor metabolite and produces enzymatic activation of tryptophan hydroxylase. These results seem to indicate that control of the activity of the tryptophan hydroxylase, which regulates the biosynthetic pathway of serotonin, involves an interaction between the activation effects of angiotensin III and the inhibition produced by another metabolite.

Table 2. Effect of angiotensin III in supernatant obtained after high-speed centrifugation. Incubation time, 45 minutes. The results are the mean of five experiments.

Concentration of angiotensin III (g/mg protein)	5-Hydroxytryptophan (pmole/mg protein)	
	Activity	*P*
0	240 ± 30	
10^{-12}	280 ± 11	NS*
10^{-11}	311 ± 82	NS
10^{-10}	370 ± 5	< .05
5×10^{-10}	444 ± 58	< .05
10^{-9}	423 ± 20	< .01
10^{-8}	350 ± 57	NS

*Not significant.

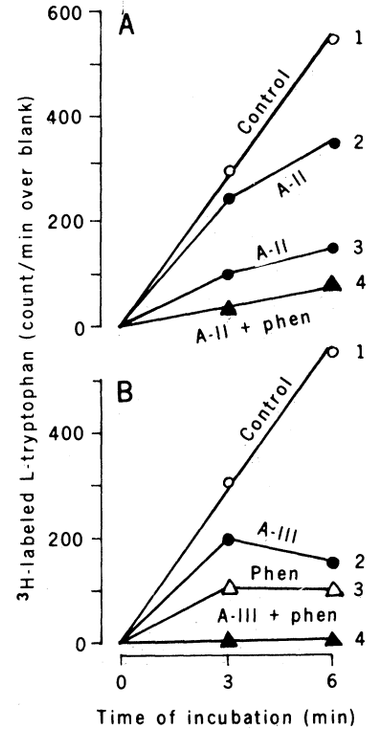

Fig. 2. Uptake of L-[^{3}H]tryptophan by synaptosomes from hypothalamus and brainstem. After incubation, portions were filtered in a Millipore filter (0.45 μm) apparatus. The filters were dried, scintillation fluid added, and the samples counted. Results are the mean of four experiments. (A) Curve 1, control; curve 2, angiotensin II (1 ng); curve 3, angiotensin II (100 ng); curve 4, angiotensin II (100 ng) plus phenylalanine. (B) Curve 1, control; curve 2, angiotensin III (1 ng); curve 3, phenylalanine; curve 4, angiotensin III (1 ng) plus phenylalanine.

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