ANGIOTENSIN IMMUNOREACTIVITY IN VASOPRESSIN CELLS IN RAT HYPOTHALAMUS AND ITS RELATIVE DEFICIENCY IN HOMOZYGOUS BRATTLEBORO RATS *

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INTRODUCTION

It has been twenty years since a role for angiotensin II (AII) in the regulation of blood pressure by the central nervous system was suggested by cross-circulation experiments in dogs.[1] Administration of small doses of AII into the brain revealed additional central actions including thirst, vasopressin (VP) and adrenocorticotropin (ACTH) secretion, sodium appetite, and suppression of renal renin.[2] Although an endogenous brain renin-angiotensin system was proposed ten years ago,[3, 4] its existence is still debated.[2, 5, 6] One issue now apparently resolved concerned the presence of a renin enzyme in brain distinct from cathepsin D.[7] Another has been the inability of most laboratories to extract significant quantities of endogenous AII from brain.[2] Therefore, it is still not known whether brain renin is actually involved in the production of angiotensin II, and whether this might occur within specific neuronal systems. Immunoreactive renin has been demonstrated in neurons in the supraoptic (SON), paraventricular (PVN) and suprachiasmatic (SCN) nuclei of hypothalamus, as well as other brain regions of the mouse.[8] In addition, the possibility that a single cell can contain the entire renin-angiotensin system was recently demonstrated in a neuroblastoma-glioma X cell line in tissue culture.[9]

In recent years AII-like immunoreactivity has been reported in nerve fibers in a number of regions of the brain and spinal cord by immunocytochemical methods.[6, 10-16] The neuronal perikarya most consistently labeled within the cytoplasm were found in the magnocellular nuclei of the hypothalamus: SON and PVN.[13-16] We previously reported that the "AII-like" reactivity in the SON and PVN was localized in vasopressin-containing neurons and behaved like vasopressin: it increased in fibers to the zona externa (ZE) of the median eminence (ME) in response to adrenalectomy, and was deficient in homozygous Brattleboro rats (DI rats), which also lack vasopressin.[15, 16] We also suggested that the antiserum had some preference for 5-valine AII compared to 5-isoleucine AII.[16] In this communication we report the results of further experiments with DI rats, and additional absorption studies on normal rat hypothala-

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mus. We also report the use of a more specific antiserum to VP than the one used previously on normal and Brattleboro rats.[17]

METHODS

Normal Sprague-Dawley, Long-Evans, and homozygous Brattleboro rats (Blue Spruce Farms, Altamont, New York) were used. Five Brattleboro rats were treated with daily injections of 100 mU VP tannate in oil for two weeks. Both types of normal rats and DI rats were subjected to bilateral adrenalectomy two weeks prior to sacrifice. Others were given 60 μg colchicine into the cerebral ventricles 48 hours prior to sacrifice. Brains were fixed by perfusion with 10% formalin or by immersion in Bouin's solution, blocked and embedded in paraffin. Serial sections 3 or 6 μm, on glass slides were deparaffinized, rehydrated, and incubated with one of four primary rabbit antisera overnight as the first step in the peroxidase antiperoxidase (PAP) technique: [17-19] antiserum to VP (N_1-F, 1:1,000), to oxytocin (OT) (N-3, 1:1,000), to rat neurophysins (NPS) (Robinson #4),[17] 1:4,000), and to AII (Kilcoyne 10 A-6, 1:500). Antisera to synthetic arginine VP (AVP) and synthetic OT were obtained by immunizing rabbits with synthetic peptide (M. Manning) conjugated by the carbodiimide technique to bovine thyroglobulin. Antiserum to AII was prepared by immunizing a rabbit with 5-valine AII amide (Hypertensin®) coupled to rabbit serum albumin by the carbodiimide method.

Specificity of antisera was tested by overnight preincubation of 1 ml of diluted antiserum with synthetic antigen at 4° C prior to use: antiserum to VP or OT with 1 μg AVP or 1 μg OT; antiserum to AII with 1, 10, or 100 μg 5-valine AII or 5-isoleucine AII, or 100 μg AI, AIII (Peninsula Laboratories Inc.), 100 μg AVP and OT, or 600 μg rat or bovine serum albumin.

RESULTS

Analysis of serial 3-μm sections of colchicine-treated normal brains revealed that oxytocin and vasopressin were localized in totally different cells in the SON and PVN, all of which appeared neurophysin positive (antiserum visualizes both rat neurophysins).[17] AII-reactivity was associated with VP- and not at all with OT-reactive perikarya. Although one can not always clearly identify the same cell in serial adjacent sections, AII and VP immunoreactivity was always found in the same cell. There was also an excellent correlation in non-colchicine-treated animals. There was more perikaryal reactivity after colchicine and some reduction in fibers. Dorsomedial cells in the SCN were reactive for AII in the same pattern as for VP and NP, but AII staining was weaker and the cells too small for serial analysis.

Like VP and OT, AII reactivity was found in axonal projections to ME (FIGURE 1A) and posterior pituitary. Only a rare fiber was found outside the hypothalamus, for example in the amygdala, the nucleus solitarius of the medulla and the spinal cord. In the ME of normal rats some AII and VP fibers projected to the ZE, and after adrenalectomy there was a marked build up of both types of reactivity in ZE (FIGURE 1B). In fact AII reactivity was stronger than vasopressin after adrenalectomy. DI rat ME did not react with antiserum to AII whether adrenalectomized (FIGURE 1C) or not.

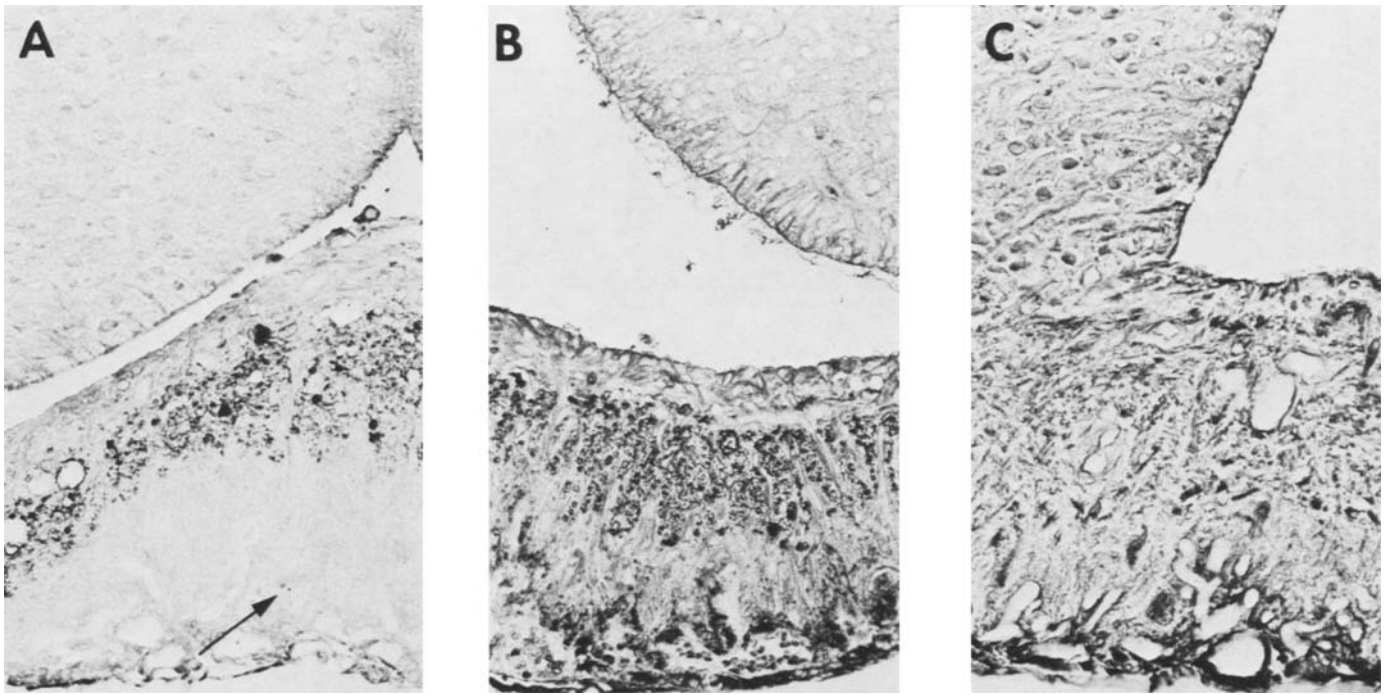

FIGURE 1. Photomicrographs of 6 μm coronal sections of the hypothalamus at the middle level of the median eminence of normal (A) and adrenalectomized Long-Evans (B) rats, and homozygous Brattleboro (C) rats. All reacted with antiserum to angiotensin II and immunoperoxidase technique. Note very few reactive fibers to the zona externa (arrow) of normal rat (A), which increases with adrenalectomy in normal (B) rat, but not in Brattleboro rat (C). $\times$113.

In addition to producing reaction products in totally different cells, the antisera to OT and VP were shown to be specific by total absorption with homologous and not at all with heterologous antigen. Furthermore antiserum to VP did not react with DI rat hypothalamus. Antiserum to AII absorbed with 100 μg (FIGURE 2A,B) and 10 μg 5-valine or 5-isoleucine AII, and about 50% with 1 μg. AI and AIII absorbed partially, and VP, OT, NP, and albumin not at all.

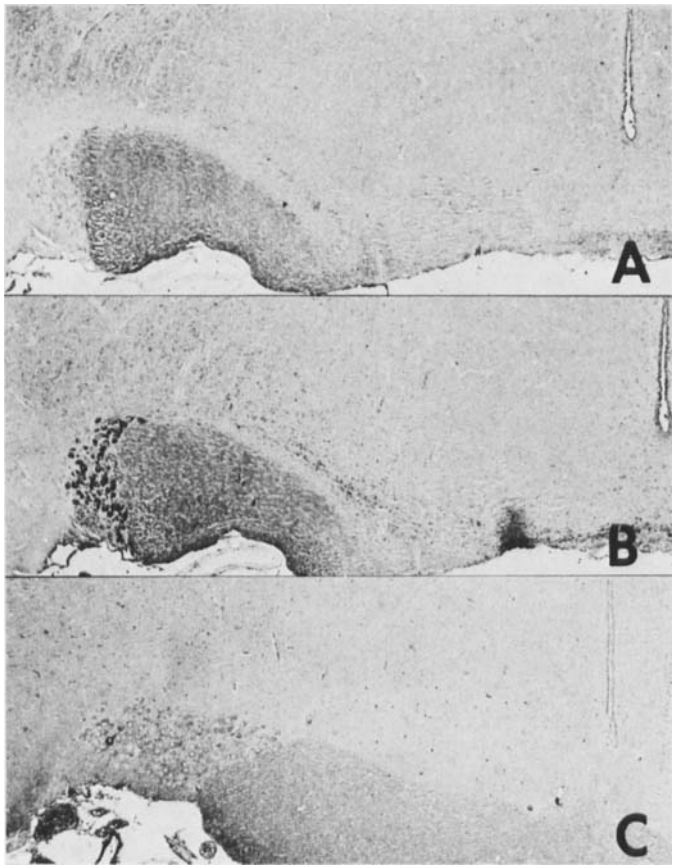

FIGURE 2. Coronal sections of rat brains at the level of the supraoptic nucleus reacted for angiotensin II. (A, B) normal rat, (C) Brattleboro rat. In (A) preabsorption with synthetic angiotensin II eliminated the reactivity in supraoptic neurons seen in (B, unabsorbed). Only a single reactive neuron is found in this homozygous Brattleboro rat section. $\times 80$.

Examination of sections of the hypothalamus of the DI rats with the antiserum to AII revealed that only two of the 17 rats tested had any positive staining. In those two, only one or two reactive perikarya were found in the SON or PVN in some sections (FIGURES 2C, 3). No fibers reacted. We confirmed that these were homozygous Brattleboro rats by lack of reactivity to VP, and the absence of NP staining in half of the SON-PVN cells.[17] The VP-treated and adrenalectomized DI rats were totally unreactive for AII or VP.

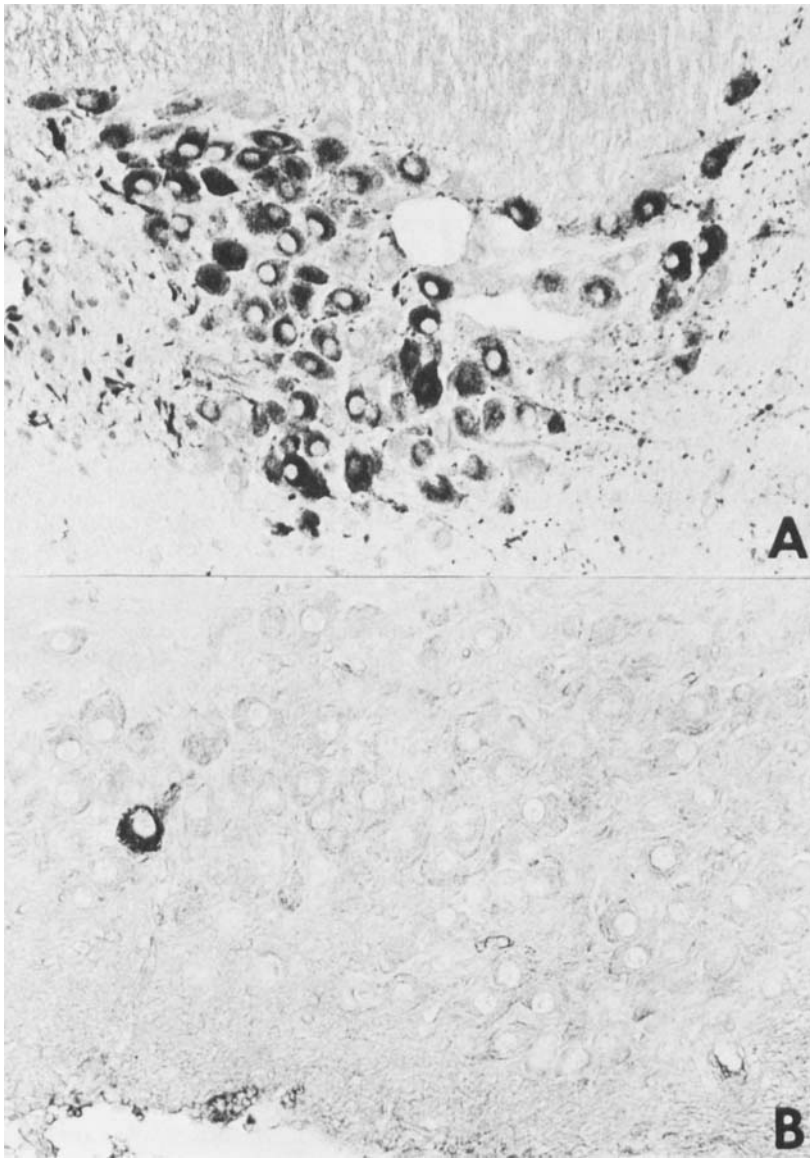

FIGURE 3. Higher magnifications of angiotensin reactivity in normal rat (A) from FIGURE 2B (rotated 45° and reversed), and the reactive cell in Brattleboro rat (B) from FIGURE 2C. ×250.

Summary and Conclusions

These results suggest that vasopressin neurons contain a substance immunologically related to AII. Absorptions controls indicate that its antigenic determinants are more like AII than AI or AIII, and that the antiserum has equal affinity for 5-isoleucine AII and 5-valine AII. The presence of AII material in the perikaryon and its build up after colchicine suggests that it is produced in these cells. Failure to find it in most homozygous DI Brattleboro rats that have high circulating concentrations of AII [20] is additional evidence that it does not come from the general circulation.

The true chemical nature of this AII material cannot be determined by this immunological method. The absorptions suggest that it is not VP, yet it appears to be related. It increases along with VP and VP-NP in fibers to ZE in response to adrenalectomy in normal rats.[19] In addition it is absent, or nearly so, from DI rats that cannot produce VP, VP-NP, or its precursor.[21] Attempts to react radiolabeled precursor (propressophysin) with this antiserum failed (E. A. Zimmerman, H. Gainer, and M. J. Brownstein, unpublished data). Attempts to reduce the possibly rapid turnover of AII reactivity by VP treatment of DI rats were not successful.

At the moment we do not know how to interpret these findings, or those of others who have also used immunocytochemistry. Extrahypothalamic fibers containing AII reactivity could come from PVN like those containing VP,[22] but this remains to be proven. Increases in ZE AII with adrenalectomy support the idea that AII which has effects on corticotropin release [23] may be mediated by the same PVN-ME system as VP.[19] Regarding vasopressin release by AII,[24] one would have expected to see AII in nerve terminals on VP neurons rather than AII in them. It is conceivable that AII-producing neurons lie elsewhere in the brain and the material found here in VP neurons represents cross-reactivity with some other peptide, or alternatively, that there are several AII systems in the brain. It is hoped that these issues may be resolved in future studies by chemical analysis, and by application of monoclonal antibodies to different parts of the renin-angiotensin system.

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