

QUANTITATIVE MEASUREMENT OF ANGIOTENSIN II (A II) RECEPTORS IN  
DISCRETE REGIONS OF RAT BRAIN, PITUITARY AND ADRENAL GLAND BY  
AUTORADIOGRAPHY.

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INTRODUCTION

All components of the angiotensin (AII) system are present in the central nervous system (1). AII receptors have been characterized in membrane fractions of mammalian brain (2).

Binding sites for drugs and neurotransmitters can be localized and quantitated in brain by autoradiography using [<sup>3</sup>H]Ultrafilm and computerized densitometry after in vitro incubation with appropriate ligands (3,4). The AII agonist <sup>125</sup>I-[Sar<sup>1</sup>]-AII is a suitable ligand for AII receptor studies since it shows complete cross displacement with AII (5). These receptors have been recently localized in discrete rat brain nuclei (6). Precise quantitation, however, is essential for studies on the physiological regulation of receptors.

We report the quantitative determination of AII receptors in rat brain, pituitary and adrenal gland after incubation with <sup>125</sup>I-[Sar<sup>1</sup>]-AII. This method involves the use of <sup>3</sup>H-sensitive film for autoradiography (4), selection of appropriate film exposure times, quantitation of optical densities by computerized microdensitometry (3) and comparison to <sup>125</sup>I-standards prepared from brain tissue (7).

METHODS.

Rats were killed by decapitation, tissues immediately removed and frozen in isopentane at -30°C, and 8 μm sections were cut in a cryostat at -14°C and thaw-mounted onto subbed glass slides (4).

Tissue sections were preincubated for 15 minutes at 20°C in 10 mM sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na<sub>2</sub> EDTA, 0.1 mM bacitracin and 0.2% bovine serum albumin, and then incubated for 60 minutes in fresh buffer with different concentrations of <sup>125</sup>I-[Sar<sup>1</sup>]-AII (specific activity 1666 Ci/mmol, from 50 pM to 5 nM). Non-specific binding was determined in the presence of 1 μM unlabelled AII. After incubation, the slides were

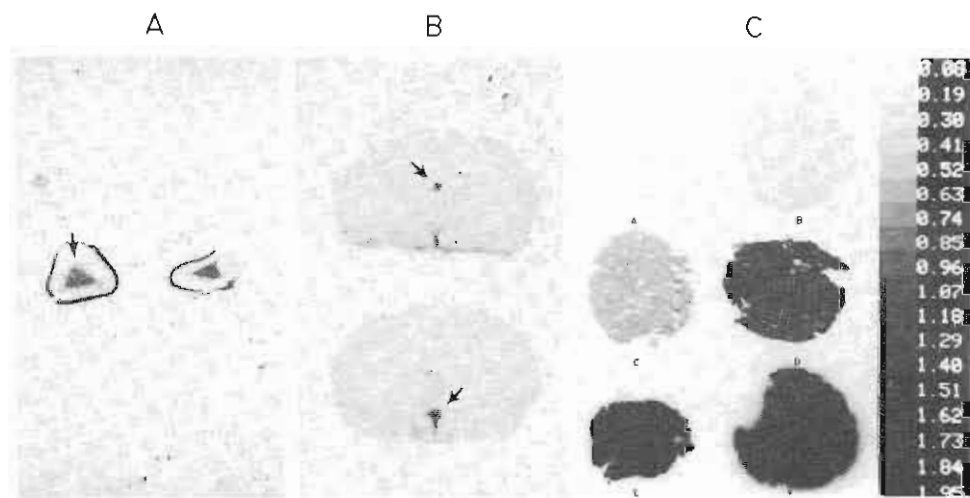


FIGURE 1. AUTORADIOGRAPHIC IMAGES WITH COMPUTERIZED DENSITOMETRY OF AII BINDING SITES AND  $^{125}\text{I}$ -STANDARDS. A. Adrenal gland. B. Fore-brain. C.  $^{125}\text{I}$ -standards. Each section contains a different amount of radioactivity per mg of protein. In d.p.m.: A: 9.7; B: 20.8; C: 72.8; D: 200.5; E: 536.4; F: 1008.0. Arrows point to adrenal medulla (A) and to subfornical organ (B, upper panel) and paraventricular nucleus (B, lower panel).

washed four times, one minute each, with ice-cold 50 mM Tris-HCl buffer, pH 7.56, and dried under a stream of air.

Sets of  $^{125}\text{I}$ -standards were prepared as described (4). Known amounts of increasing concentrations of  $^{125}\text{I}$ -AII (specific activity 1280 Ci/mmol) were mixed with brain tissue aliquots previously ground to a paste, placed as blocks of tissue on microtome specimen holders, and frozen on dry ice. Tissue sections, 8  $\mu\text{m}$  thick, were cut in a cryostat and thaw-mounted onto subbed glass slides (4). Parallel sets of standards were used for determination of protein concentrations (8) and radioactivity (7).

Tissue sections after incubation and  $^{125}\text{I}$ -standards were placed in cassettes and opposed against [ $^3\text{H}$ ] Ultrafilm (LKB Industries), for varied periods. The films were developed with Kodak D19 developer and optical densities were quantitated by computerized densitometry (3,4). The optical densities in tissues were compared with the standard curves generated by processing sets of standards with each of the autoradiograms (4,7).

## RESULTS

There was a linear relationship between the  $\ln$  of optical densities and the  $\ln$  of the concentration of radioactivity for the  $^{125}\text{I}$ -standards at any exposure time of [ $^3\text{H}$ ] Ultrafilm to the

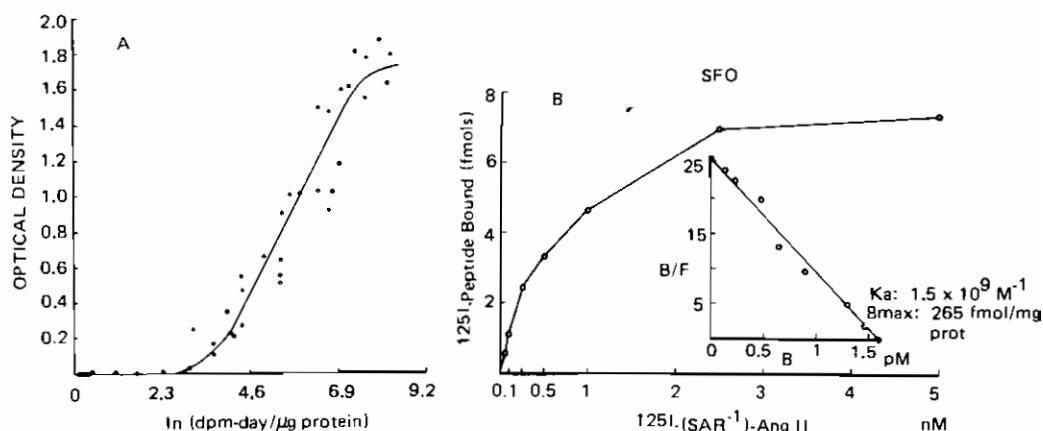


FIGURE 2. A. CHARACTERISTIC CURVE OF [<sup>3</sup>H] ULTROFILM WITH <sup>125</sup>I-STANDARDS. Optical densities are plotted as a function of  $\ln$  of d.p.m./ $\mu\text{g}$  protein  $\times$  exposure time. Exposure times were  $\circ$  0.3,  $\square$  1,  $\diamond$  3 and  $\triangle$  4 days. B. SATURATION CURVE OF SPECIFIC <sup>125</sup>I[SAR<sup>-1</sup>]-AII BINDING. Insert: Scatchard plot of saturation of specific <sup>125</sup>I-[Sar<sup>-1</sup>]-AII binding.

standards (7). A typical autoradiographic image of standards, with computerized densitometry, is shown in Figure 1-C. Autoradiographic images of rat brain coronal sections and adrenal gland are shown in Figure 1-(A-B). The molar quantities of ligand bound per mg of protein for each particular structure were determined by interpolating the optical densities in the straight line obtained from the  $\ln/\ln$  standard curve.

The slope of the "characteristic" curve of the film gives a measure of the photographic contrast and was determined by the semi-log plot of the optical densities read in <sup>125</sup>I-standards from the film vs the exposure time and amount of radioactivity present in the standards (Figure 2-A). With this information, the exposure time which produces the optimal contrast can be estimated for a particular tissue.

The distribution of AII receptors was studied in rat brain, adrenal and pituitary gland. Some of the values obtained, using a 5 nM ligand concentration, were, in fmoles/mg protein: anterior pituitary:  $435 \pm 66$ ; posterior pituitary:  $68 \pm 6$ ; subfornical organ:  $365 \pm 33$ ; paraventricular nucleus:  $289 \pm 24$ ; organon vasculosum laminae terminalis:  $440 \pm 56$ ; adrenal cortex (zona glomerulosa):  $675 \pm 6$ ; adrenal medulla:  $1390 \pm 42$ .

Saturation curves and Scatchard analysis were performed in single rat brain nuclei. The subfornical organ had a single, high affinity AII receptor type, with a  $K_a$  of  $1.5 \times 10^9 \text{ M}^{-1}$  and a  $B_{\text{max}}$  of 265 fmol/mg protein (Figure 2-B).

## DISCUSSION

Our studies demonstrate a very discrete localization of AII receptors in brain. High number of AII binding sites occurred in specific nuclei of the hypothalamus, circumventricular organs, anterior pituitary, adrenal medulla and zona glomerulosa of adrenal cortex. Both circumventricular organs (subfornical organ and organon vasculosum laminae terminalis) lack blood brain barrier and can bind blood-borne AII. Receptors in other areas, such as the paraventricular nuclei, do not have access to peripheral AII, but probably may represent binding sites for centrally formed AII. AII receptors in anterior pituitary and adrenal medulla indicate local effects of peripheral AII on pituitary hormone and catecholamine release, respectively.

The quantitative autoradiographic technique for AII receptors allows the precise estimation of affinity constants and receptor number in single rat brain nuclei. With this method, it is now possible to study the physiological regulation of AII receptors in each specific area of the rat brain. This technique can be applied to the quantitative study of receptors for a large number of neuropeptides and may become the method of choice for the analysis of receptor binding data in single, discrete areas of the brain.

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