Cellular and Molecular Neurobiology, Vol. 5, No. 3, 1985

Review and Commentary

Quantitative Autoradiographic Characterization of Receptors for Angiotensin II and Other Neuropeptides in Individual Brain Nuclei and Peripheral Tissues from Single Rats

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Received December 3, 1984; revised April 19, 1985; accepted April 19, 1985

KEY WORDS: quantitative autoradiography; receptor autoradiography; neuropeptide receptors; angiotensin II receptors; receptor quantitation in brain nuclei.

SUMMARY

1. Autoradiographic techniques coupled with computerized microdensitometry and comparison with ^{125}I standards were used to characterize and quantitate receptors for neuropeptides in rat brain and adrenal and pituitary glands.

2. These techniques are rapidly performed, anatomically precise, and more sensitive than membrane binding techniques. They permit the determination of complete saturation curves and Scatchard analysis in discrete nuclei of the rat brain and in single rat pituitary and adrenal glands.

3. Angiotensin II (AII) receptors were quantitated after incubation of 16-μm tissue sections with the AII agonist ^{125}I-[Sar^1]-AII.

4. High-affinity, high-density AII receptors were present in the organon subfornicalis, organon vasculosum laminae terminalis and nuclei triangularis septalis, suprachiasmatis, and paraventricularis of the rat and in rat adrenal capsule-zona glomerulosa area, adrenal medulla, and anterior pituitary.

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0272-4340/85/0900-0211$04.50/0

5. These techniques could be used for precise localization and quantitation of other neuropeptide receptors in single rat brain nuclei, after optimizing the assay conditions and provided that suitable ^{125}I ligands are available.

INTRODUCTION

Precise quantitation is essential for studies on the kinetic properties of receptors. In addition, discrete anatomical localization is necessary in order to study the physiological regulation of brain receptors in specific neuroregulatory systems. In recent years, techniques have been developed for the precise localization of neurotransmitter and neuropeptide receptors in brain. These methods involve the incubation of tissue sections with appropriate ^{3}H or ^{125}I ligands, autoradiography with exposure to ^{3}H-sensitive film and, in some cases, microdensitometry for quantitation of receptor concentrations in specific areas of the brain (Scott Young and Kuhar, 1979; Palacios *et al.*, 1981; Rainbow *et al.*, 1982, 1984; Unnerstall *et al.*, 1982).

We have adapted the existing technology to the study of brain neuropeptide receptors. With the use of ^{125}I ligands, autoradiography coupled to computerized microdensitometry, comparison with ^{125}I standards, and determination of optimum exposure times for the ^{3}H-sensitive film, we can quantitate and completely characterize neuropeptide receptors in discrete rat brain nuclei from single animals. This method combines the advantages of precise anatomical localization, high sensitivity, and short film exposure times, and final results can be obtained 1 to 3 days after the experiment (Israel *et al.*, 1984a).

As an example, we present a quantitative study of central and peripheral angiotensin II (AII) receptors in the rat. Identical techniques can be used for the characterization of many other neuropeptide receptors provided that a suitable ^{125}I ligand is available.

METHODS

Materials

Subbed glass slides were prepared as follows. The slides were soaked in Alconox solution, removed from the solution and boiled gently in deionized water for 1 hr, washed three times with water, and soaked in 80% alcohol overnight. The slides were then soaked overnight in deionized water and dipped in a freshly made gelatin subbing solution (2.5 g gelatin powder and 250 mg chromium potassium sulfate in 500 ml distilled water), dried in a 37°C oven overnight, and stored in dust-free slide boxes.

Angiotensin II (AII) (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.01 *N* acetic acid and kept in aliquots at −70°C until the day of the experiment. [Sar^{1}]-AII was a gift from Dr. M. Khosla, Cleveland Clinic, Cleveland, Ohio. Aliquots of [Sar^{1}]-AII, dissolved in 0.01 *N* acetic acid, were kept frozen until iodination by a modified cloramine-T method (Meloy Laboratories, Inc., Springfield, Va.). The specific activity of the ^{125}I-[Sar^{1}]-AII was 1666 Ci/mmol. ^{125}I-AII (sp act, 1280 Ci/mmol) was obtained from New England Nuclear, Boston, Mass.

Preparation of ^{125}I Standards

Sets of ^{125}I standards were prepared as originally described for ^{3}H standards (Israel *et al.*, 1984a). Known amounts of increasing concentrations of ^{125}I-AII were thoroughly mixed with rat brain tissue aliquots previously ground to a paste and degassed by repeated mixing under vacuum. The aliquots were placed as blocks of tissue on microtome specimen holders and frozen on dry ice. Tissue sections, 16 μm thick, were cut in a cryostat at -14°C and thaw-mounted onto subbed glass slides. Parallel sets of standards obtained from consecutive sections were used for determination of protein concentrations (Lowry *et al.*, 1951) and radioactivity.

Tissue Preparation

Male Sprague–Dawley rats (250 g) were obtained from Zivic Miller, Allison Park, Pa. Animals were given tap water and rat chow ad libitum and housed in groups of four at a constant temperature of 24°C, with lights on from 0600 to 1800hr.

Rats were sacrificed by decapitation between 0900 and 1100hr. Tissues were immediately removed and frozen by immersion in isopentane at -30°C. Frozen, 16-μm-thick sections were cut in a cryostat at -14°C, less than 24hr after decapitation, thaw-mounted onto subbed glass slides, desiccated under vacuum at 4°C for 2hr, and kept at -20°C until incubation (Israel *et al.*, 1984a) (Fig. 1).

Labeling of AII Binding Sites by Incubation of Tissue Sections

Within 48hr of section preparation, AII binding sites were labeled *in vitro* by incubation with ^{125}I-[Sar^{1}]-AII (Mendelsohn *et al.*, 1984). Tissue sections were preincubated for 15 min at 20°C in 5 ml of 10 m*M* sodium phosphate buffer, pH 7.4, containing NaCl (120 m*M*), Na_2 EDTA (5 m*M*), bacitracin (0.1 m*M*), and bovine serum albumin (0.2%), and then incubated for 60 min in fresh buffer with concentrations of ^{125}I-[Sar^{1}]-AII ranging from 25 p*M* to 10 n*M*. Nonspecific binding was determined in the presence of 0.125 to 50 μ*M* unlabeled AII (Israel *et al.*, 1984a).

After incubation, the slides were washed four times (60 sec each) with ice-cold 50 m*M* Tris–HCl buffer, pH 7.56, and dried under a cold stream of air (Fig. 1).

^{125}I Autoradiography

After incubation, tissue sections were placed in cassettes (CGR Medical Corp., Baltimore, Md.) along with ^{125}I standards and opposed against [^{3}H]Ultrofilm (LKB Industries, Rockville, Md.) at room temperature for 1 day (concentrations from 0.625 to 10 n*M*) or 2 to 3 days (concentrations from 25 to 300 p*M*).

The films were developed at 20°C for 4 min with undiluted D19 Kodak developer. Optical densities (OD) from the Ultrofilm standards and specific areas of the tissue sections were quantitated by computerized microdensitometry (Israel *et al.*, 1984a) (Fig. 1).

Data Analysis

A complete set of ^{125}I standards was processed with every cassette and developed with every film. Optical densities were measured by computerized microdensitometry

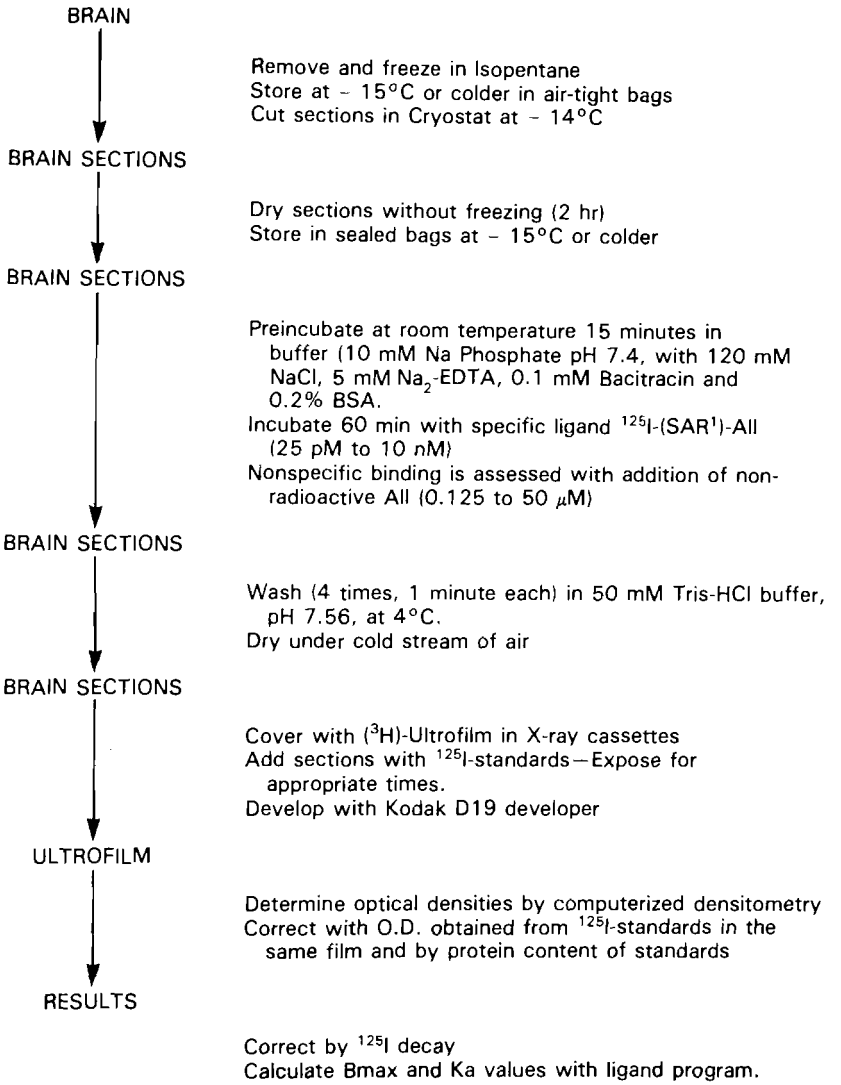


Fig. 1. Flow chart for AII receptor characterization by autoradiography.

(Goochee *et al.*, 1980) in both ^{125}I standards and specific areas of tissue sections from each film.

After determination of the standard curve [ln optical densities × 100 vs ln disintegrations per minute (dpm) of standards], the optical densities of the tissue areas studied were interpolated in the straight line to obtain the corresponding dpm bound to the tissue (Israel *et al.*, 1984a). Results were corrected for the decay of ^{125}I.

Calculations of the molar quantities of ligand bound to the tissue, saturation curves, and Scatchard analysis were performed by the use of the LIGAND computer program (Munson, 1983), followed by correction for the protein content of the standards (Fig. 1).

RESULTS

^{125}I Standard Curve

A typical autoradiographic image of standards with computerized densitometry after 2 days of exposure to [^{3}H]Ultrofilm is shown in Fig. 2 (top). There was a linear relationship between the ln of the OD and the ln of the concentration of radioactivity in the standards at any exposure of [^{3}H]Ultrofilm [Fig. 2 (bottom)].

Determination of the "Characteristic Curve" of the Film

The characteristic curve of the film was determined from the optical densities obtained with different exposure times of the same set of ^{125}I standards. The curve was generated by the semilog plot of the optical densities read from the film vs the product of the amount of radioactivity present in each standard and the exposure time. For ^{125}I standards, the relationship was similar to that established for ^{3}H standards (Israel *et al.*, 1984a) (Fig. 3).

The slope of the characteristic curve gave a measure of the photographic contrast. At an OD of 0.2 unit or less, the changes in the slope of the curve as a function of the exposure time were very small, and discrimination among different values was difficult. At an OD greater than 1.6 unit, the film rapidly saturated and no useful data could be obtained. A linear relationship ($r = 0.839$) existed between OD and dpm per microgram of protein $\times$ time of exposure for values between 0.3 and 1.6 OD units. In this case, the contrast among different OD values was high, making it easy to discriminate among small differences in the concentration of radioactivity, that is, among different concentrations of binding sites for a particular structure (Figs. 4 and 6). With this information, the exposure time which produces the optimal contrast could be estimated for a particular tissue section.

AII-Receptor Localization in Rat Forebrain

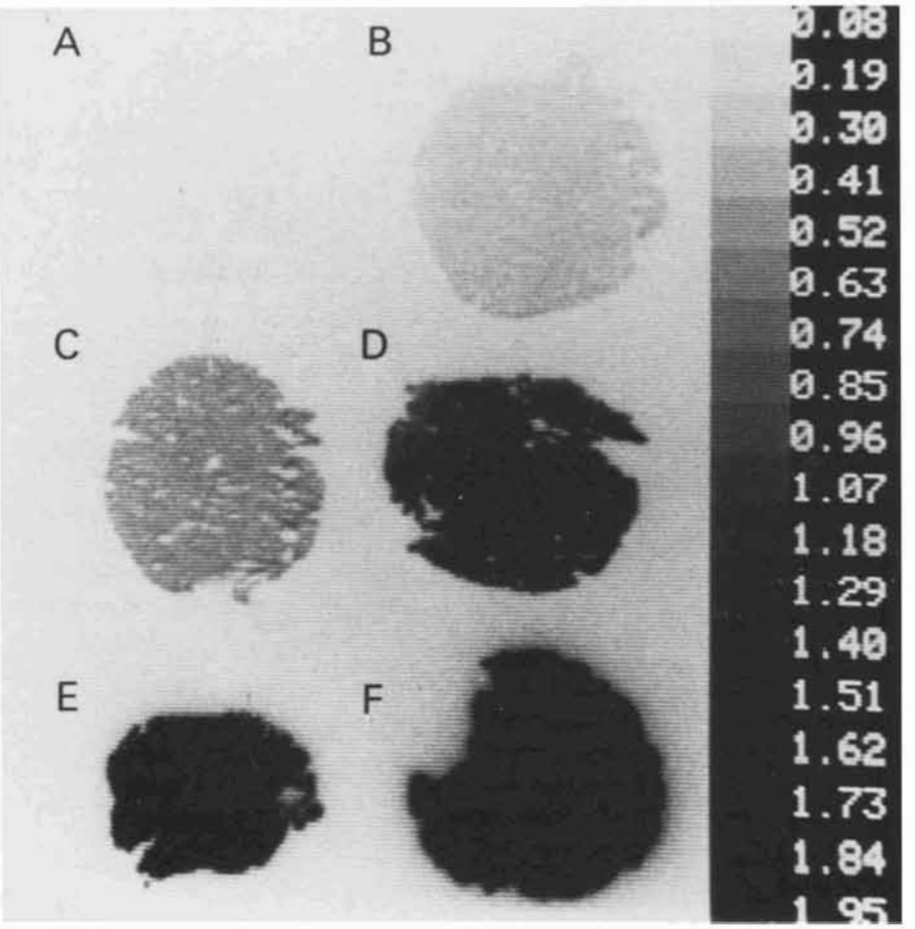
AII receptors were discretely localized to specific areas of the rat forebrain. High densities of saturable AII receptors were found in the organon subfornicalis, organon vasculosum laminae terminalis and nuclei triangularis septalis, paraventricularis, and suprachiasmatis (Fig. 4 and Table I). The AII receptors in the organon subfornicalis, nucleus paraventricularis, and organon vasculosum laminae terminalis had a high affinity for the [Sar^{1}]-AII ligand (Table I and Fig. 5).

AII Receptors in Rat Adrenal Gland and Pituitary Gland

Very high concentrations of high-affinity AII receptors occurred in the anterior pituitary, the adrenal gland capsule-zona glomerulosa, and the adrenal medulla (Figs. 6 and 7 and Table I). Conversely, the AII-receptor density in the posterior pituitary and in the rest of the adrenal cortex was very low (Fig. 6 and Table I).

DISCUSSION

Autoradiographic techniques coupled to computerized microdensitometry and comparison with ^{125}I standards allow the quantitative determination and complete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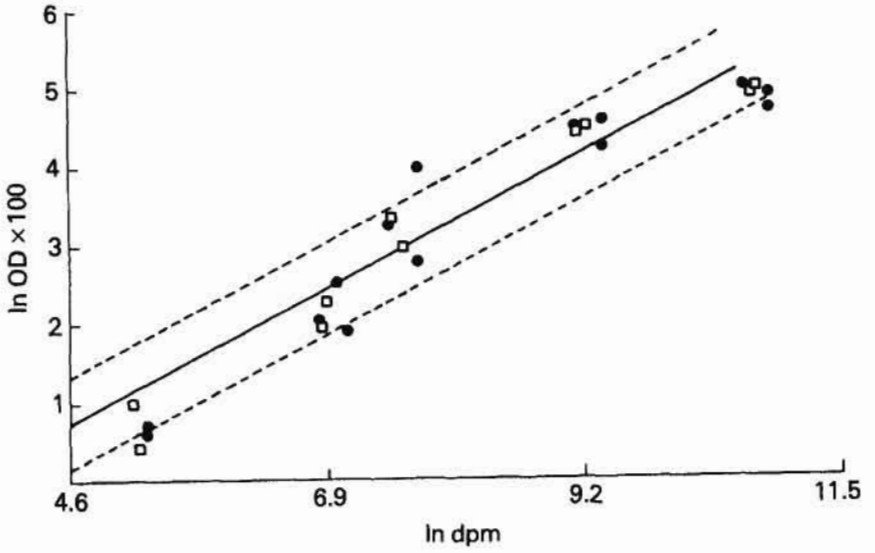
ln OD x 100
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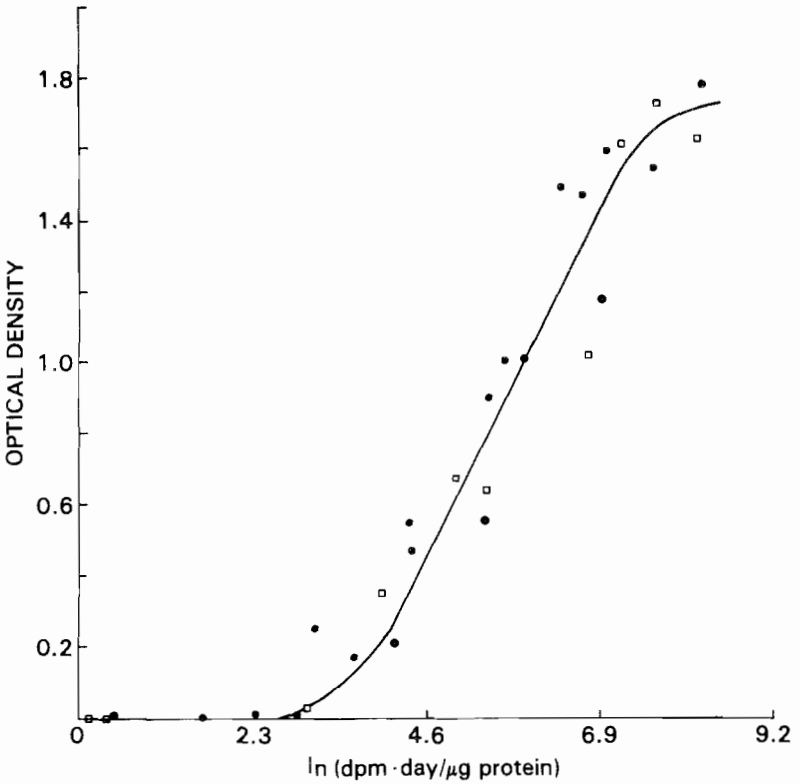

Fig. 3. Characteristic curve of [^{3}H]Ultrofilm with ^{125}I standards. Optical densities are plotted as a function of ln of dpm/μg protein × exposure time. Each point represents the average of three optical density readings from autoradiograms generated from triplicate standard curves. Exposure times were 1 (□) and 2 (●) days.

characterization of neuropeptide receptors in specific brain nuclei from individual rats and in single rat adrenal and pituitary glands. These techniques have advantages over receptor binding assays with partially purified membrane preparations, including precise anatomical localization and a severalfold increase in sensitivity. The use of ^{125}I ligands requires very short film exposure times, 1 to 3 days instead of several weeks for ^{3}H ligands. Final results can be obtained within a week of the binding experiments, allowing for rapid data collection.

Quantitation is achieved by optimizing the film exposure time, which requires prior determination of the characteristic curve of the film (Unnerstall *et al.*, 1982) with

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Fig. 2. (top) Autoradiographic image with computerized densitometry of a set of ^{125}I standards after 2 days of exposure to [^{3}H]Ultrofilm. Each section contains a different amount of radioactivity per microgram of protein. (A) 8.3 dpm; (B) 57.9 dpm; (C) 74.9 dpm; (D) 368 dpm; (E) 1739 dpm; (F) 4617 dpm. (bottom) A ln–ln plot of OD vs radioactivity (dpm/μg protein) in standards. Exposure times were 1 (□) and 2 (●) days. Each point represents the average of three optical density readings from autoradiographs generated from triplicate standard curves and prepared for each film utilized for the quantitation of AII receptors. Dashed lines represent the standard errors of estimates of X from Y. The general equation of the straight line was as follows, for 24 pairs of data: $Y = 0.757X - 2.76$; $r = 0.84$; $F = 68.4$ ($P < 0.001$).

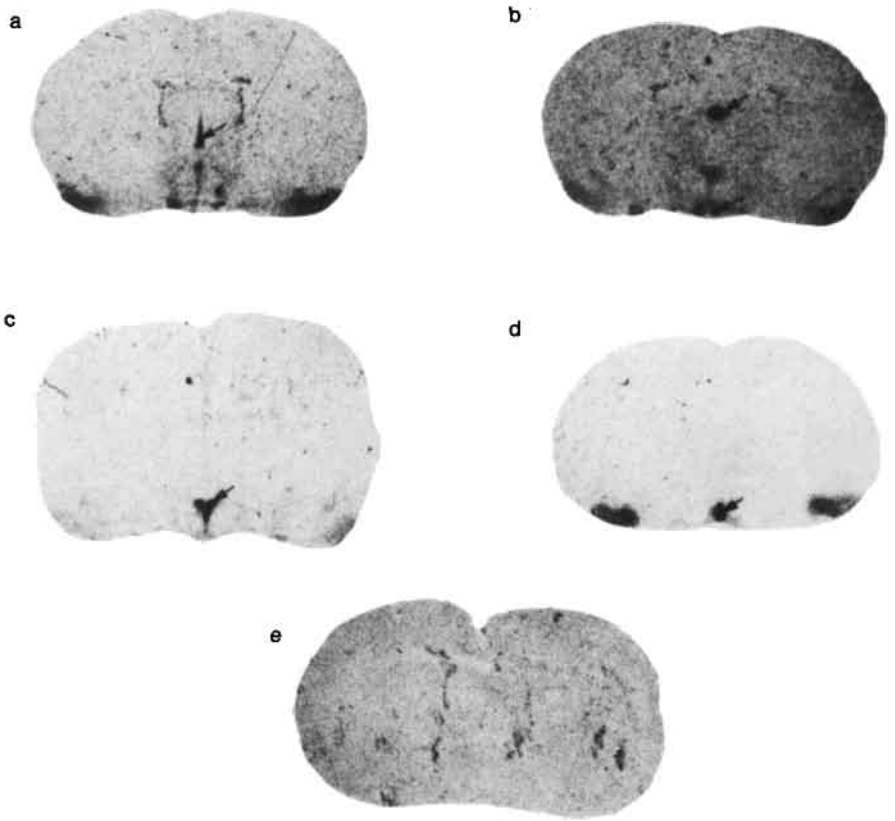

Fig. 4. Autoradiographic image with computerized microdensitometry of AII binding sites in rat forebrain. Arrows point to the nucleus septalis triangularis (a), organon subfornicalis and nucleus suprachiasmatis (b), nucleus paraventricularis (c), and organon vasculosum laminae terminalis (d). Sections were incubated with 5 nM ^{125}I-[Sar^1]-AII. Nonspecific binding for a section consecutive to a is shown in e. The exposure time of Ultrofilm was 2 days.

Table I. Maximal Binding Capacity and Affinity of AII Receptors in Specific Rat Brain Nuclei, Adrenal Gland, and Pituitary Gland[a]

	Maximal binding capacity (B_{max}; fmol/mg protein)	Affinity constant (K_a; 10^9 M^{-1})
Brain area		
Organon subfornicalis	450 ± 7	1.47 ± 0.26
Organon vasculosum laminae terminalis	2653 ± 373	0.40 ± 0.18
Nucleus paraventricularis	1028 ± 98	1.23 ± 0.30
Peripheral tissue		
Anterior pituitary	2667 ± 214	0.51 ± 0.03
Adrenal medulla	3880 ± 598	0.84 ± 0.02
Adrenal gland, capsule zona glomerulosa	7063 ± 724	0.80 ± 0.06

[a] Complete analysis (seven points, in duplicates) was performed for each individual rat. Results represent means ± SE for a group of six rats.

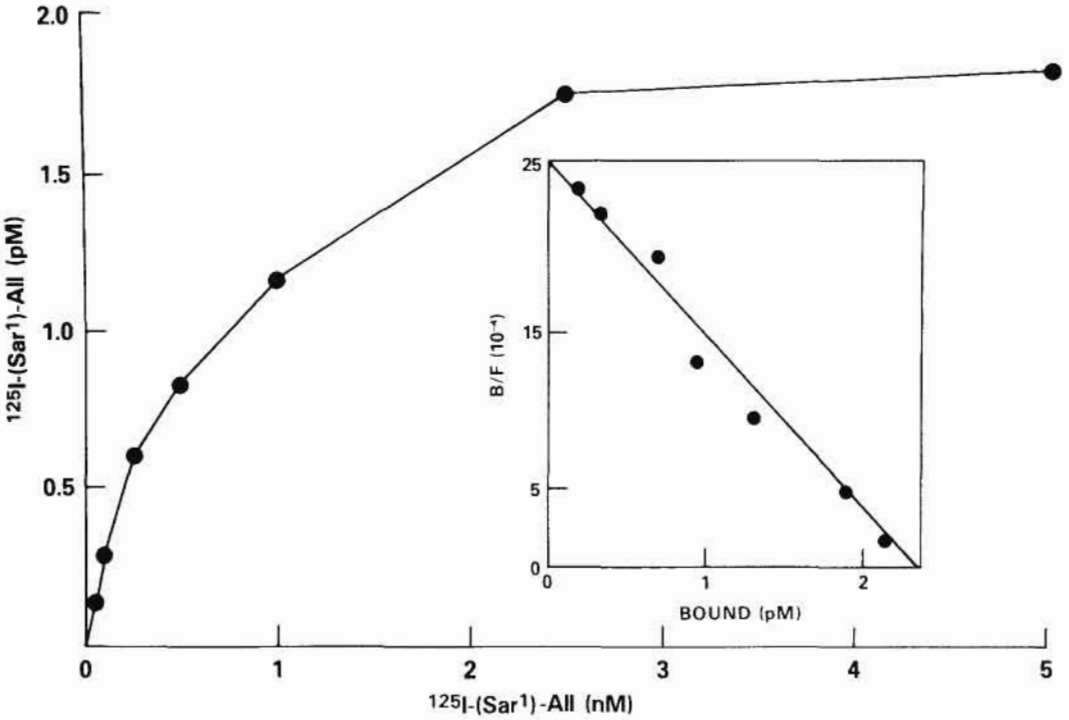

Fig. 5. Saturation curve and Scatchard analysis of specific ^{125}I-[Sar^1]-AII binding to rat organon subfornicalis. Tissue sections were incubated for 60 min with ^{125}I-AII at concentrations from 25 p*M* to 10 n*M*. Inset: Scatchard analysis of binding data.

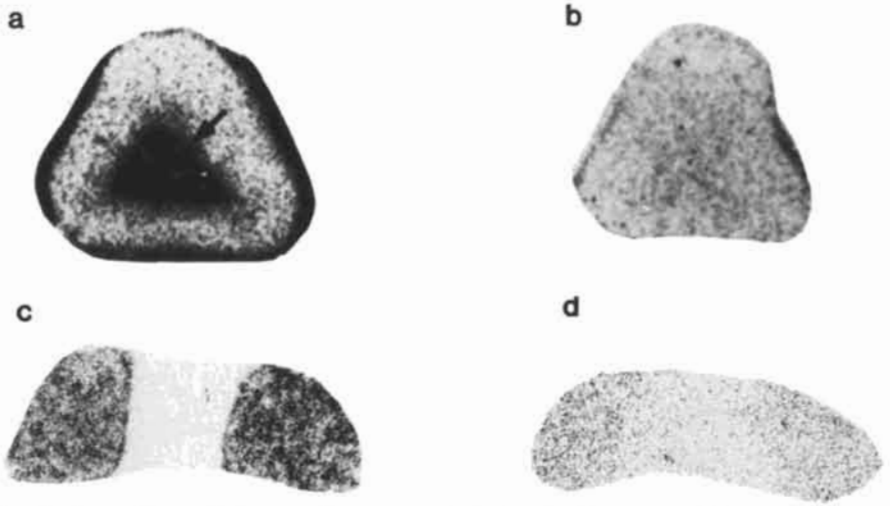

Fig. 6. Autoradiographic image with computerized microdensitometry of AII receptors in rat pituitary and adrenal glands. (a) Rat adrenal gland incubated with 5 n*M* ^{125}I-[Sar^1]-AII. Exposure time of Ultrofilm was 1 day. Arrow points to adrenal medulla. (b) Adjacent section incubated with 5 n*M* ^{125}I-[Sar^1]-AII and 25 μ*M* unlabeled AII. (c) Rat pituitary gland incubated with 5 n*M* ^{125}I-[Sar^1]-AII. Exposure time of Ultrofilm was 1 day. (d) Adjacent section incubated with 5 n*M* ^{125}I-[Sar^1]-AII and 25 μ*M* unlabeled AII.

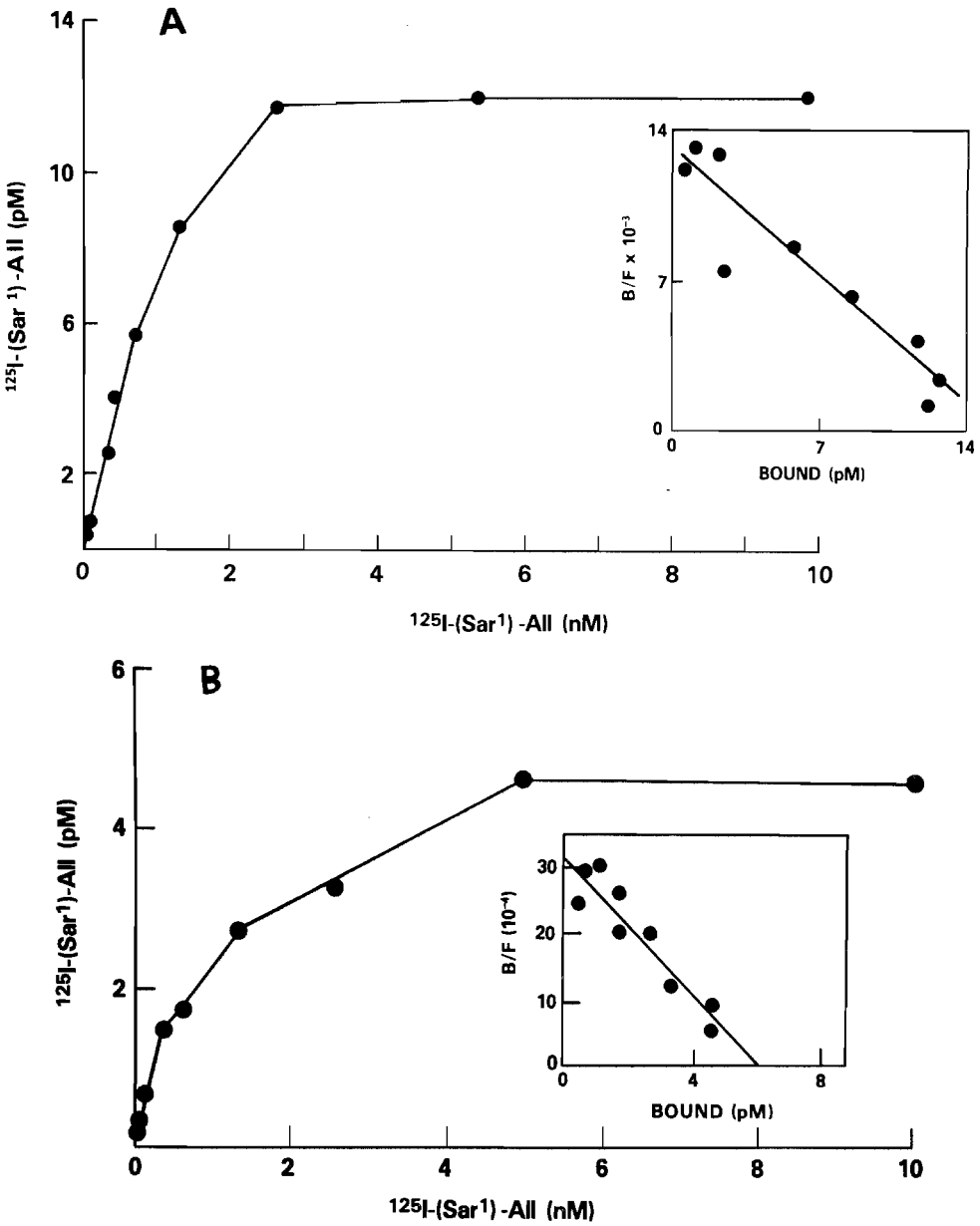

Fig. 7. Saturation curves and Scatchard analysis of specific ^{125}I-[Sar^{1}]-AII binding to rat adrenal medulla (A) and anterior pituitary glands (B). Tissue sections were incubated for 60 min with ^{125}I-[Sar^{1}]-AII at concentrations from 25 p*M* to 10 n*M*. Inset: Scatchard analysis of binding data.

the use of the ^{125}I standards. The slope of the curve gives a measure of the photographic contrast (Fig. 3). The optimum film exposure time depends on the receptor density in a particular area and on the concentration and specific activity of the ligand. For tissues with high receptor densities or when high ligand concentrations are used, the time of exposure of the [^{3}H]Ultrofilm should be relatively short, to prevent saturation of the

film (Fig. 3). It is desirable, in preliminary experiments, to expose the labeled tissue sections for varying lengths of time to determine the conditions for optimum contrast.

We have utilized the autoradiographic method to localize (Mendelsohn *et al.*, 1984) and to characterize (Israel *et al.*, 1984a) AII receptors in rat brain and peripheral tissues. AII has well-defined actions on smooth muscle, adrenal gland, and brain, resulting in increased blood pressure, aldosterone secretion, and stimulation of drinking and salt appetite (Fitzsimons, 1980; Phillips, 1978). In addition, AII has been reported to increase the release of the anterior pituitary hormones prolactin and ACTH (Dufy-Barbe *et al.*, 1982; Steele *et al.*, 1981).

Brain, anterior pituitary, and adrenal cortical AII receptors have been previously characterized *in vitro* by incubation of ^{125}I-AII or ^{125}I-[Sar^1]-AII with partially purified membrane preparations (Sirett *et al.*, 1977; Mukherjee *et al.*, 1982; Hauger *et al.*, 1982; Mendelsohn *et al.*, 1983). With the use of the autoradiographic technique reported here, we have recently reported the quantitation of AII receptors by autoradiography in specific rat brain nuclei, adrenal zona glomerulosa, adrenal medulla, and anterior pituitary (Israel *et al.*, 1984b).

The data reported here concerning AII binding in discrete brain nuclei are the first reports of complete Scatchard analysis in individual nuclei of single animals (Table I and Fig. 5). Kinetic analysis is an important part of quantitative receptor binding studies, for it allows one to determine not only the maximum binding capacity (B_{max}), a measure of receptor density, but also the affinity constant (K_a) for the ligand–binding site interaction. Once ^{125}I standard comparison provides a value for the amount of radioactive ligand bound to a particular tissue, a Scatchard plot of bound/free vs bound ligand provides the basis for calculation of B_{max} and K_a. Our results show that each brain nucleus examined has a characteristic K_a and B_{max} value for ^{125}I-[Sar^1]-AII binding (Table I). Thus, comparisons can now be made among different populations of animals. Alterations in AII binding site kinetics could be determined and related to the physiological state of the animal or to the manipulations performed prior to the *in vitro* binding assay.

The use of the present technology is not restricted to the study of AII receptors. Other neuropeptide receptors as well as neurotransmitter receptors could be quantitated with the same degree of sensitivity and anatomical localization, provided that a suitable ligand is available.

Table II shows a number of localization studies for receptors for neuropeptides and neurotransmitters recently published. With the exception of one study on adrenoceptors (Rainbow *et al.*, 1984), these reports offer a semiquantitative analysis, not suitable for determination of receptor kinetics in discrete brain areas. By the use of

Table II. Autoradiographic Localization of Neuropeptide Receptors in Brain Using ^{125}I Ligands

Neuropeptide	Ligand	Reference
Angiotensin II	^{125}I-[Sar^1]-AII	Mendelsohn *et al.* (1984); Israel *et al.* (1984a)
Cholecystokinin	^{125}I-CCK-33	Zarbin *et al.* (1983
Corticotropin-releasing factor	[Nle^{21}, ^{125}I-Try^{32}]-CRF	De Souza *et al.* (1984)
Somatostatin-14	^{125}I-Somatostatin-14	Tran *et al.* (1984)
Substance P	^{125}I-Bolton-Hunter SP	Helke *et al.* (1984)

^{125}I standards and by optimizing the assay conditions, a complete characterization of these and other receptors would be possible. These autoradiographic techniques could be considered the method of choice for the study of the regulation of discrete groups of receptors for neuropeptides or for neurotransmitters in the mammalian brain.

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